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High-performance chromatographic methods for determination of proteins

THESIS

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Science without religion is lame, religion without science is blind.

Albert Einstein (1879-1955)

I declare that all the results presented in this Thesis are based on my experimental work. All the other information used is properly acknowledged.

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List of Abbreviations

ACN	Acetonitrile
Ala	Alanine
ALA	α-lactalbumin
ALC	Affinity chromatography
AQN	Protein of boar seminal plasma (according to N-terminal amino acid sequence Ala-Gln-Asn)
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
AWN	Protein of boar seminal plasma (according to N-terminal amino acid sequence Ala-Trp-Asn)
BLG	β-lactoglobulin
BSA	Bovine serum albumin
BSP	Proteins of bull seminal plasma
CE	Capillary electrophoresis
CN	Casein
Con A	Concanavalin A
Cys	Cysteine
CZE	Capillary zone electrophoresis
DEAE	Diethylaminoethyl
DQH	Protein of boar seminal plasma (according to N-terminal amino acid sequence Asp-Gln-His)
DTT	Dithiothreitol
E	Epoxy
EDTA	Ethylenedinitrilotetraacetic acid
EtOH	Ethanol
EVAL	Poly(ethylene vinyl alcohol)
Gal	Galactose
GalNAc	N-acetyl-D-galactosamine
Gdn.HCl	Guanidinium chloride
GFC	Gel filtration chromatography

Glc	Glucose
GlcNAc	N-acetyl-D-glucosamine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
H+	Heparin binding fraction of seminal plasma
H-	Heparin non-binding fraction of seminal plasma
HEMA	Hydroxyethyl methacrylate
HIC	Hydrophobic interaction chromatography
His	Histidine
IEC	Ion-exchange chromatography
IgE	Immunoglobulin E
IMAC	Immobilized-metal affinity chromatography
Leu	Leucine
LSD	Light scattering detector
MALLS	Multi-angle laser light scattering detector
Man	Mannose
MetOH	Methanol
MW	Molecular weight
MS	Mass spectrometry
NANA	N-acetylneuraminic acid
NPLC	Normal phase chromatography
OA	Ovalbumin
P+	Phosphorylcholine binding proteins
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer
PC	Phosphorylcholine
PDC-109	Bull seminal plasma proteins which can interact with phosphorylcholine
PE	Polyethylene
PET	Poly(ethylene terephalate)
PP	Polypropylene
Pro	Proline
PSP	Protein of boar seminal plasma of H- fraction
PSA	Prostate-specific antigen

RID	Refractive index detector
Rnase B	Ribonuclease B
RPLC	Reversed-phase chromatography
SDS	Sodium dodecyl sulphate
SEC	Size-exclusion chromatography
Ser	Serine
Sg	Semenogelin (human seminal plasma protein)
Thr	Threonine
Tris	Tris(hydroxymethyl)amino-methane
Tris.HCl	Tris(hydroxymethyl)amino-methane hydrochloride
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
VS	Vinyl sulphone

Table of Contents

1.

Introduction

Proteins. Nowadays almost a magical word. Everybody is interested in this topic. Time is going too fast for us so some people already think about healthy life-style and about what they eat and drink as well as how to keep their body health. It is possible to buy a lot of peptides and proteins in a pharmacy as nutrition supplements. On the other hand, it is well known that a lot of proteins and glycoproteins can cause allergy. Food sensitivity (e.g., allergy to milk proteins) or pollen allergy (e.g., allergy to plant pollen) are two typical examples. Allergy to milk proteins is defined as any adverse reaction incurred by immunological mechanisms to one or several milk proteins. It is manifested by three major proteins found in milk: α-lactalbumin, β-lactoglobulin and caseins. The first two proteins are dealt with in this Thesis, in the part concerned with development of a method for determination of the β-lactoglobulin/α-lactalbumin ratio in whey protein aggregates. The possibilities of analysis of glycoproteins and allergens were studied in another part of this Thesis.

Cancer. Its occurrence rapidly increases in civilized world. According to the data of the Czech Statistical Office, Czech Republic ranks among countries with the highest increase in incidence of cancer of the large intestine. Patients with a suspicion of gastric cancer or other gastric disorders have to undergo unpleasant, expensive and invasive examinations. Moreover, early stages of gastric cancer need not necessarily be detected. Therefore, it is essential to find a fast, cheap and reliable method for determination of pepsin and its non-active precursor pepsinogen, as it is known that a certain ratio of pepsinogens can indicate some gastric diseases. A study of interactions of pepsin and possibilities of its determination forms another chapter of this Thesis.

All these aspects conform with the contemporary science, mainly with proteomics. Proteomics is one of the most dynamic scientific fields at present. Its development is made possible by the great progress in analytical separation methods. The complexity of biological systems places great demands on the separation methods used, primarily on high separation efficiency and selectivity. The affinity methods based on molecular recognition represent very good choice for this purpose.

Proteomics is a branch of science that extends over many fields, e.g., analytical chemistry, biochemistry, biology and medicine. These fields have their own points of view and provide different kinds of information. From these data, the whole picture of a given protein can be constructed, mainly its structure and the sequence of amino acids, as well as the character of its interactions with other proteins and other substances. The genetic coding and posttranslational modifications are further very important data. The abundance

of a protein may not reflect the abundance of the corresponding transcript because not all the mRNA in the cells are translated.

It is clear that one branch of science cannot cover all these aspects of proteomics and thus it is essential to collaborate with other scientists and scientific disciplines. High-performance methods play an important role in all proteomics studies.

2.

Chromatographic methods used in protein analysis

Currently, six different modes of high-performance liquid chromatography (HPLC) are commonly employed for the separation of proteins. There are ion-exchange chromatography (IEC), size-exclusion chromatography (SEC), affinity chromatography (ALC), hydrophobic interaction chromatography (HIC), reversed-phase chromatography (RPLC) and normal-phase chromatography (NPLC). The basic principles of these methods are summarized in Table 2.1. The choice of the chromatographic mode has a great impact on the separation and resolution of the proteins of interest [1].

Tab. 2.1: An overview of chromatographic methods employed for separation of proteins.

Name of chromatography	**Separation principle**
Ion-exchange	According to net charge of molecules
Size-exclusion	According to size and shape of molecules
Affinity	Biospecific adsorption
Hydrophobic interaction	Hydrophobic complex formation
Reversed-phase	Distribution of molecules between m.p. and s.p.
Normal-phase	Distribution of molecules between m.p. and s.p.

m.p. = mobile phase, s.p. = stationary phase

It is often impossible to find an ideal medium suited for protein chromatography and compromises must be sought. The chemical nature of proteins determines the surface properties of the separation media (the stationary phases), while the large size of proteins determines their physical properties. First of all, a large surface is required to get a high binding capacity. A large surface can be obtained with a highly porous material, containing numerous small pores, but small pores would prevent diffusion into them. Thus, a compromise must be found between the surface area and the pore size. Common media for protein chromatography have a pore size of 30 nm. Most proteins have a diameter below 3 nm. In order to allow a reversible adsorption, the chromatography material must be very hydrophilic. In order to improve the pressure stability, media have been cross-linked. Introduction of cross-linkers leads to a more hydrophobic medium and the proportion of generally unspecific adsorption increases. The ideal properties of a medium for protein chromatography are: (i) high selectivity, (ii) high binding capacity, (iii) high mass transfer, (iv) low unspecific adsorption, (v) incompressibility, (vi) chemically stable immobilization of ligands, (vii) non-toxic leachables, (viii) high number of working cycles (re-usability), (ix) low cost [2].

2.1 Ion-exchange Chromatography

One of the common methods for fractionation of biological substances, ion exchange chromatography (IEC), separates biomolecules on the basis of differences in their anionic or cationic charge characteristics. The method is based on the interaction of proteins with strongly or weakly charged ion-exchange groups of the stationary phase. IEC is generally used as a batch step in initial purification or a selective step in the latter stages of a purification scheme [3].

Ion exchange chromatography separates molecules according to their net charge. Matrix-bound charged groups reversibly adsorb oppositely charged sample molecules like proteins, peptides and nucleotides (Figure 2.1). The main way how to influence selectivity in IEC is consequently by varying the running buffer pH [3].

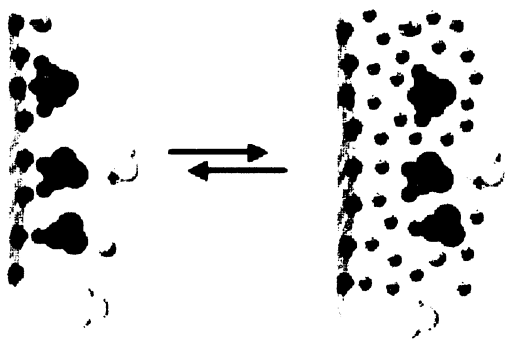

Fig. 2.1: Charged sample molecules adsorb on ion exchangers of the opposite sign. The interaction is a dynamic equilibrium that can be influenced by the pH or the salt concentration [3].

Proteins solubilized in a liquid act as ampholytes. It means that both positive and negative charges are present on their molecules. The resulting charge of the whole molecule is given by the sum of all the partial charges. In a solution with a pH < 7.0, amino acid side chains are protonated and the overall charge is shifted toward positive values. On the contrary, in a solution with pH>7.0, some amino acids carry a negative charge and the overall charge of the protein is negative. The most common functional groups of ion-exchangers are given in Table 2.2.

Tab. 2.2: The most common functional groups of exchangers.

Functional group of exchanger	Exchanger
carboxylic group (-COOH)	weak cation
sulpho-group ($-SO_3H$)	strong cation
amino-group (NH_2)	weak anion
quaternary amine ($N^+(R)_3$)	strong anion

2.2 Size exclusion chromatography

Size exclusion chromatography (SEC) is also known under the name, gel filtration chromatography (GFC). It is a simple and reliable chromatographic method for separating molecules according to their size and shape. Its versatility makes it generally applicable to the purification of all classes of biological substances, including macromolecules, not readily fractionated by other techniques, and it is widely employed for the separation of molecules in the oligomer and polymer ranges, especially those of proteins. Biological molecules vary greatly in size and SEC techniques need to be adapted accordingly [3].

In SEC, the column is filled with the material having precisely controlled pore size. Larger molecules are eluted first; smaller molecules penetrate into the pores of the packing particles and elute later. Under ideal conditions, only the size and shape of the molecules play role in the separation and their molecular mass can be estimated after calibration with standards [4]. The principle of SEC is given in Figure 2.2.

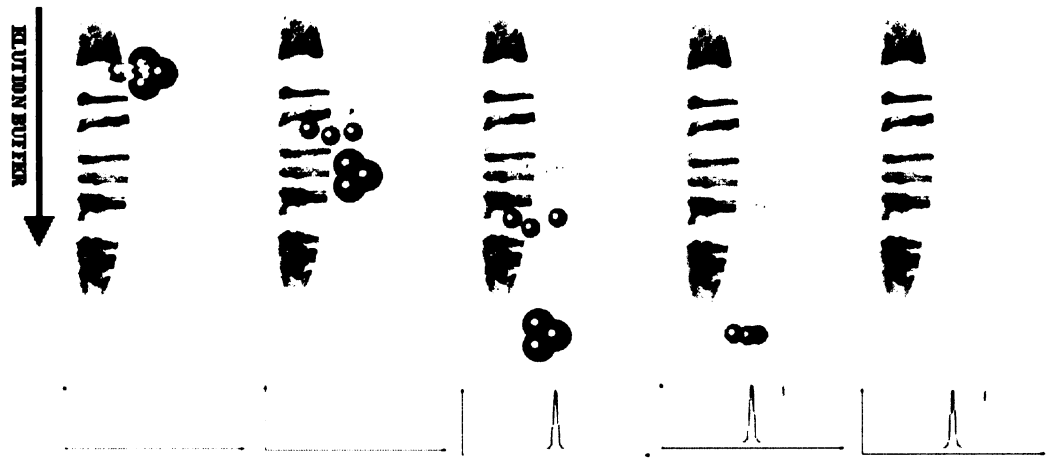

Fig. 2.2: The principle of SEC. The sample mixture is separated according to the size and shape of the molecules. The biggest molecules leave the column first, the smallest molecules penetrate into the pores of the stationary phase, are retained and eluted later [3].

In a column, all the sample molecules have access to the liquid between the stationary phase beads. This volume is called the void volume and in gel filtration it equals ~30% of the column geometric volume. The overall volume of the pores is called the pore volume. The non-porous part of the beads is called the backbone and is inaccessible to the sample molecules. In the size exclusion column, sample molecules are partitioned between the eluent (the mobile phase) and the accessible pores of the gel filtration beads (the stationary phase). This partitioning tends to establishing of a dynamic equilibrium of the sample molecules between the mobile and the stationary phases and is driven exclusively by diffusion [3].

2.3 Affinity chromatography

Affinity chromatography is a type of adsorption chromatography which is based on the exceptional ability of biologically active compounds to bind specifically and reversibly complementary structures. These are generally called ligands or affinity ligands. The complexes of active or regulatory sites of enzymes with their inhibitors, substrates and cofactors, antibodies with antigens or haptens, lectins with saccharides, hormones and toxins with receptors may be mentioned as examples. The formation of biologically functioning complexes involves the participation of common molecular forces and interactions systemized under the terms of ionic bonds, hydrogen bonds, hydrophobic interactions, London's dispersion forces and coulombic interactions [5]. This method enables purification of biomolecules with respect to their biological function or individual chemical structures [6].

A single enzyme E can interact with a ligand L and form a complex E...L. The equilibrium between the attached affinity ligand and the isolated enzyme is given by the equation (1) and equilibrium constant K [5].

$$E + L \leftrightarrow E...L \qquad (1)$$

$$K = \frac{[E...L]}{[E]\cdot[L]}$$

A value of this constant should be in the range of 10^{-4}-10^{-6}. K_D values > 10^{-4} provide weak binding and the target molecule may "leak" as a diluted broad zone during the sample application and washing. If a ligand binds too strongly, it will be difficult to elute the target molecule without introducing harsh conditions. Under such conditions there is always a risk of abolishing the biological activity of the target molecule or finding it "irreversibly" adsorbed to the affinity medium [3].

Each affinity analysis consists of three basic steps. 1) Equilibration: The column is conditioned to promote adsorption of the target molecule by equilibrating it with a starting mobile phase (binding buffer). This step ensures a stable baseline. 2) Sample application and washing: The sample is applied under the binding conditions. The target molecule binds specifically to the affinity ligands, while all the other sample components are washed through. 3) Elution: The target molecule is desorbed and eluted by switching to an elution mobile phase (elution buffer) [3]. The elution can be done by a change in the physical properties of the elution mobile phase (e.g., pH, ionic strength or temperature) or by addition of substances to a mobile phase that form stronger complexes with the affinity ligand. The procedure is schematically depicted in Figure 2.3.

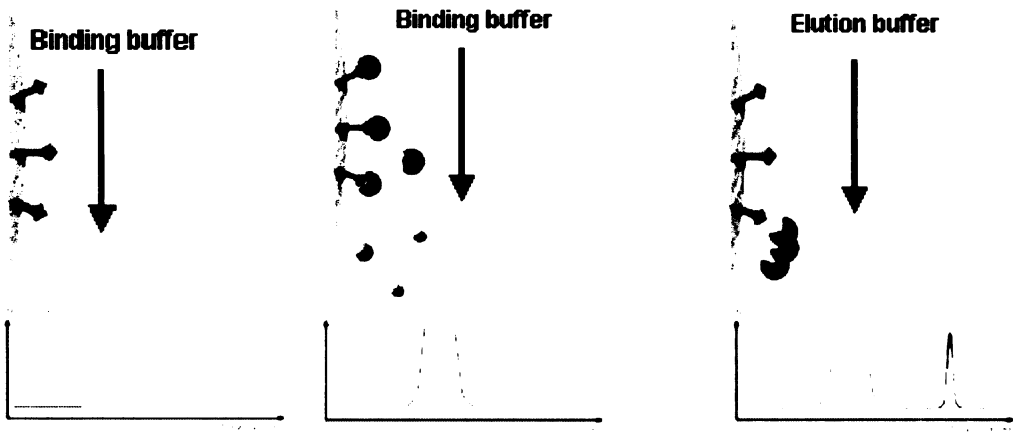

Fig. 2.3: A scheme of the course of affinity chromatography [3].

Two types of ligands are utilized in affinity chromatography. Ligands used for mono-specific affinity chromatography are structurally and biologically closely related to the target molecule to be purified. This makes the selection of the ligand specific for each case. This also makes it commercially difficult to produce affinity media for mono-specific separations. With small ligands (<1 000) there is a risk of steric interference, like binding

between the matrix and the target molecule, or a low accessibility of the ligand. Introducing a spacer arm will minimize these risks. Ligands used for group-specific affinity chromatography have a much wider applicability and affinity media for this purpose are consequently commercially available [3]. Table 2.3 lists examples of commonly used group-specific ligands.

Tab. 2.3: Examples of group specific ligands (adapted from ref. [3]).

Group-specific ligands	**Specificity**
Concanavalin A	Man, Gal, Glc
Lysine	Ribosomal RNA
Arginine	Serine proteases
Heparin	Lipoproteins, hormones, nucleic-acid binding enzymes

As follows from the above text, commercial stationary phases are not commonly available; usually they are prepared in laboratories. The procedure (Figure 2.4) consists of the following steps:

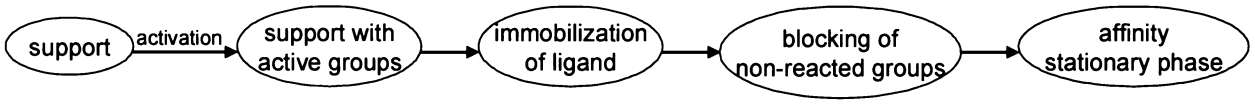

Fig. 2.4: A scheme of a procedure for preparation of an affinity stationary phase.

The properties of supports have to be taken into account. Affinity matrixes have to possess some important properties: minimum nonspecific sorption, chemical, mechanical, pH, and thermal stability, sufficient porosity (affinity fractionation of large biomolecules) and sufficient amount of groups for covalent attachment of affinity ligands. It is always necessary to respect the properties of both the ligand and the protein and make a compromise between various requirements. An overview of commonly used supports for preparations of affinity stationary phases is given in Table 2.4. The most important sorbents, their physico-chemical properties, advantages and drawbacks, as well as their practical applications are covered in the review [7] (see appendix III).

Tab. 2.4: Properties of commonly used supports for affinity chromatography (adapted from ref. [7]).

Support	**Properties**
Agarose gel	Linear polysaccharide consisting of altering D-galactose and 3,6-anhydro-L-galactose connected by β-(1,4)-O-glycosidic bound. High chemical stability, hydrophilicity, high exclusion limit and low tendency to non-specific interactions. Differences in chemical and mechanical stability because of different level of cross-linking. Commercially available as Sepharose. Drawback – sensitivity to microbial attack.
Dextrans	Branched-chain glucose polymer (more than 90% of α-1,6-glucosidic bonds, branched by 1,2-, 1,3- and 1,4-glucosidic bonds). It affords three-dimensional gel when cross-linked with 1-chloro-2,3-epoxypropane in alkaline solution. pH stability 2-10. Commercially available under trade names Sephadex and Superdex. Drawback – sensitivity to microbial attack.
Methacrylate polymers	Hydrophilic hydroxyalkyl methacrylate copolymers. Particle size and pores are controlled over a wide range. High pressure and microbial attack resistance, pH stability (2-12) and compatibility with most organic solvents. Gel contains a large amount of hydroxyalkyl groups for immobilization of affinity ligands. High protein recovery and high protein activity of the isolated proteins. Commercially available as HEMA and Toyopearl.
Acrylamide derivatives	Synthetic polymers, chemically stable (salts, urea, Gdn.HCl, detergents). Support has many groups for easy modification. Polymer is very hydrophilic and suitable for separation of biological macromolecules. Stable at pH 2-10. They do not support microbial growth or leach carbohydrates (in contrast to dextrans or agarose gel). Commercially available as Bio-Gel. Drawback – low degree of bed porosity.
Silica	Various forms, colloidal particles with a 3-D aggregates = silica hydrogel, dried out porous silica. Mechanically stable, regular particle shape. Porous silica can be modified to obtain particles with the required porosity. A higher tendency to nonspecific interactions. Countless bulk silica commercially available. Drawback - pH stability only 2-8.

Tab. 2.4: Continued.

Support	**Properties**
Monoliths	"Continuous polymers beds". Provide a better performance than conventional particle-based chromatographic media because of enhanced mass transport. Easy to fabricate, can be used in small-scale chromatography. Some are commercially available but usually home-made polymerization.
Affinity membranes	Improved mass-transfer efficiency, higher throughput values due to the presence of macropores that enable convective flow. Analogous preparation – selection of suitable membrane, surface activation followed by coupling of affinity ligands. Membranes based on cellulose, polyamide, polymethacrylate, PE, PET, PP, EVAL, glass fiber, TiO_2 ion-exchange.
Magnetic particles	Magnetic carrier bearing an immobilized ligand is mixed with a sample followed by an incubation period when the target compounds bind to magnetic particles. The whole complex is easily and rapidly removed from the sample using a magnetic separator. After washing out the contaminants, the isolated target compound can be eluted and used for further work.

HEMA = hydroxyethyl methacrylate, Gdn.HCl = guanidinium hydrochloride, PE = polyethylene, PET = poly(ethylene terephthalate), PP = polypropylene, EVAL = poly(ethylene vinyl alcohol)

The branch of affinity chromatography which employs immobilized antigens or antibodies for the purification of biologically active materials is known as immunoaffinity chromatography. The introduction of sensitive and specific methods in clinical chemistry increased the demand for various biologically active compounds, e.g., the use of immunoassay for the determination of steadily increasing number of various clinically significant compounds increased the demand for the preparation of various immunosorbents [5].

Immobilized-metal affinity chromatography (IMAC) is a separation technique that uses a covalently bound chelating compounds on solid chromatographic supports to entrap metal ions, which serve as affinity ligands for various proteins, making use of coordinative binding of some amino acids residues exposed on the surface. It is appropriate for purification of proteins with natural surface-exposed histidine residues and for recombinant proteins with engineered histidine tags or histidine cluster [8]

2.4 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is widely used for separation and purification of proteins. During a HIC separation, proteins are induced to bind to a weakly hydrophobic stationary phase using a buffered mobile phase of a high ionic strength and then selectively desorbed during a decreasing salt concentration gradient. Proteins are usually separated in HIC according to their degree of hydrophobicity similarly to reversed-phase chromatography (RPLC). Unlike RPLC, hydrophobic ligands covalently attached to the solid support are formed mainly by short-chain aliphatic or aryl chains. Because of gentler nature of the separation mechanism, there is a greater probability that proteins will elute with their conformational structure (biological activity) intact [6].

HIC media contain ligands that are compatible with hydrophobic surfaces of proteins. In pure water, the hydrophobic effect is too weak to induce interaction either between the ligand and the proteins, or between the proteins themselves. However, certain salts enhance hydrophobic interactions and their presence supports adsorption to HIC media. The following salts strengthen hydrophobic interaction in the order indicated: $Na_2SO_4 > K_2SO_4 > (NH_4)_2SO_4 > NaCl > NH_4Cl > NaBr > NaSCN$. Ammonium sulphate is commonly used to control adsorption in HIC. The sample is applied and adsorbed at high salt concentrations (~ 1M). To facilitate selective desorption of analytes, the salt

concentration is gradually decreased and the sample components are eluted in the order of their hydrophobicity [3].

The protein chromatographic behavior and prediction of their retention in HIC has already been reviewed [9, 10]. According to retention model, the most important parameter for retention in HIC is the salt molality concentration. An increase in this parameter results in an improvement in the surface tension and consequently in an increased retention of proteins. Unfortunately, this model is not valid for a low salt concentration. More information can be found in refs. [9, 10].

HIC has found wide use in the purification of proteins, e.g., membrane proteins, serum proteins, nuclear proteins, polynucleotides, receptors and recombinant proteins in research and industrial processes. The potential of HIC has also been demonstrated for other biomolecules, such as nucleic acids [9].

2.5 Reversed-phase chromatography

Based on the hydrophobic interactions between solute molecules and immobilized, matrix-bound ligands, reversed phase chromatography (RPLC) has found favor in a wide variety of preparative applications including micropurification of protein fragments for sequencing and process-scale purification of recombinant protein products. RPLC relies on a partitioning mechanism among the sample molecules, those of the mobile phase and the functional groups of the stationary phase in order to achieve separation. The retention of molecules of interest can be controlled by manipulating the properties of the mobile phase, and molecules with only small differences in their hydrophobicity can be separated. RPLC also offers the benefit of exceptional flexibility in separation conditions so that either molecules of interest can be bound while contaminants pass through, or contaminants can be bound while the molecule of interest passes through [3]. Neutral and ionic solutes can be separated simultaneously and the rapid equilibration of the stationary phase with changes in the mobile phase composition allows gradient elution techniques to be used routinely [6]. All these aspects cause that this method is very popular and versatile.

The main separation mechanism is a distribution of the sample components between the stationary and mobile phases. The distribution depends on their respective solubility characteristics and the polar properties of the phases. The solubility "rule of

thumb" says: "equal dissolves equal", e.g., non-polar substances dissolve preferably in non-polar solvents, while polar ones dissolve in polar solvents. By varying the polar properties of one of the phases one can change the distribution of the components to be purified as well as the contaminants [3]. The differences between reversed-phase and normal-phase chromatography are summarized in Table 2.5.

Tab. 2.5: A comparison of reversed- and normal-phase chromatography.

	RPLC	**NPLC**
Stationary phase	Hydrophobic (ligands: C_4, C_8, C_{18})	Hydrophilic (ligands: phenyl-, cyano-group)
Mobile phase	Polar/non-polar (water/organic solvents such as EtOH, MetOH, ACN)	Non-polar (organic solvents – hexane, methylene chloride)
Elution order	Polar first, then non-polar	Non-polar first, then polar
Effect of increasing solvent polarity	Increases retention time	Decreases retention time

EtOH = ethanol, MetOH = methanol, ACN = acetonitrile

2.6 Normal-phase chromatography

Normal phase chromatography (NPLC) was the first kind of HPLC. Retention of the analytes depends on their polarity. NPLC is a mode of chromatography commonly using two types of packing materials, in which the solvent or mobile phase is less polar than the stationary phase or packing material of the column [11]. Contrary to RPLC, in this mode the stationary phase is polar (hydrophilic) and the mobile phase is non-polar. The sample molecules are distributed between the stationary and mobile phase according to their hydrophilicity. This method is used when the analyte of interest has a polar nature. The polar analyte associates with and is retained by the polar stationary phase. NPLC has fallen out of favor recently with the development of reversed phase HPLC.

2.7 References

1. J. M. Rodriguez-Nogales, M. Concepción Garcia, M.L. Marina: *J. Sep. Sci.* **29** (2006) 197-210.

2. A. Jungbauer: *J. Chromatogr. A* **1065** (2005) 3-12.

3. http://www5.amershambiosciences.com (11-10-2006).

4. J. M. Rodriguez-Nogales, M. Concepción Garcia, M.L. Marina: *J. Sep. Sci.* **29** (2006) 197-210.

5. J. Turková: *Bioaffinity chromatography*. 2nd edition, Journal of Chromatography Library – vol. 55, Elsevier, Amsterdam 1993.

6. C.F. Poole, S.K. Poole: *Chromatography today*. Amsterdam, Elsevier 1991.

7. T. Varilova, M. Madera, V. Pacakova, K. Stulik: *Curr. Proteomics* **3** (2006) 33-54.

8. V. Gaberc-Porekar, V. Menart: *J. Biochem. Biophys. Meth.* **49** (2001) 335-360.

9. M.E. Lienqueo, A. Mahn: *Chem. Eng. Technol.* **28** (2005) 1326-1334.

10. A. Mahn, A.J. Asenjo: *Biotechnol. Adv.* **23** (2005) 359-368.

11. www.foodtechsource.com (12-10-2006).

3.

Aims of this Thesis

The main goal of this work was a study of interactions of various proteins in high-performance chromatographic methods. This is in agreement with contemporary trends in proteomics.

The partial goals were:

1. Pepsin

Preparation of stationary phases with immobilized 3,5-diiodo-L-tyrosine for affinity chromatography of pepsin was the first objective of this part of the Thesis. The second objective was to test the stationary phases prepared on a model sample of porcine pepsin A and apply them to human pepsin.

The third objective was to use RPLC for characterization of peptide fragments of pepsin obtained after α-chymotrypsin digestion.

2. Seminal plasma proteins

The first objective was to study the interactions of boar, bull and human seminal plasma proteins and phosphorylcholine by affinity chromatography using heparin immobilized on a Toyopearl support. The second objective was to evaluate the amount of heparin-binding and phosphorylcholine-binding proteins.

3. Glycoproteins

The first objective was to prepare stationary phases with immobilized Concanavalin A for affinity chromatography of glycoproteins. The second objective was to test two phases by standard derivatives of saccharides. The third objective was to utilize the stationary phases prepared for analysis of selected glycoproteins. And the last objective was to verify a possibility to analyze some allergens.

4. Whey proteins

The main aim of this project was the development of analytical methods of two-dimensional size exclusion chromatography for study of the aggregation behavior of whey proteins upon heat treatment at a neutral pH. The objective of the work was to determine the ratio β-lactoglobulin/α-lactalbumin (BLG/ALA) in mixed aggregates as a function of the particle size.

This part of the Thesis was prepared at Unilever, Research & Development, Vlaardingen, the Netherlands and was supported by a Marie Curie grant (EU).

4.

Pepsin

4.1 Pepsin

The gastric juice of vertebrates contains aspartic proteases that are responsible for the digestion of dietary protein; these are classified into the following four groups, based on enzymatic and immunochemical properties: pepsin A (EC 3.4.23.1, pepsin), pepsin B (EC 3.4.23.2), pepsin C (EC 3.4.23.3, gastricsin) and chymosin (EC 3.4.23.4). Pepsin A, pepsin B and gastricsin are found in gastric juice of adult vertebrates, while chymosin is found exclusively in neonates. As members of the aspartic protease class of enzymes, these gastric proteases exhibit optimal activity at acidic pH, are inhibited by pepstatin, and possess two active-site aspartic residues that are required for catalysis. In order to protect host tissue from damage and to prevent self-autocatalysis, the gastric proteases are synthesized as inactive precursors known as zymogens. These zymogen form of pepsin A, pepsin B, gastricsin and chymosin are referred to as pepsinogen A (PGA), pepsinogen B (PGB), pepsinogen C (PGC, known as well as progastricsin) and prochymosin respectively. Relative to the active enzymes, these zymogens have additional residues at N-terminus (called prosegment) [1].

The gastric zymogens are synthesized and stored in the chief cells of the gastric mucosa. Upon ingestion of food, the zymogens are secreted into the lumen of the stomach and are converted into their active forms in the acidic gastric juice of the lumen. Conversion requires proteolytic removal of the prosegment and is carried out by the enzyme itself [1]. A scheme of the activation of human pepsin is depicted in Figure 4.1.

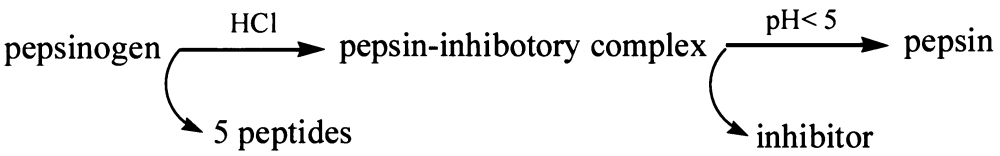

Fig. 4.1: A scheme of activation of human pepsin in stomach (according to ref. [2]).

The presence and relative concentrations of pepsin A and pepsin C vary in vertebrates in dependence on the species and genetic variation; in humans they also vary during some stomach diseases [3, 4]. The ratio among individual forms of human pepsins and their zymogens constitute important diagnostic tools, e.g., low concentration of pepsinogen A in serum was found as a marker of gastric cancer, similar to a low ratio of PGA to PGC. The change in the level of individual isoforms of both the proteases and

zymogens is important for early diagnosis of gastric diseases. Serum pepsinogen A and pepsinogen C levels can be used as subclinical markers of gastric cancer [3, 5, 6].

The most important pepsins in human stomach are pepsin A and pepsin C. They differ in their biochemical and immunological properties. Their molecular masses are around 35 000, or higher with pepsinogens (around 42 000) [7, 8]. A comparison of the properties of human and porcine pepsins is given in Table 4.1.

Tab. 4.1: A comparison of the human and porcine pepsin properties [7].

Pepsin	Theoretical MW	Theoretical pI
Human pepsin A	34 628	3.36
Human pepsin C	35 461	3.59
Porcine pepsin A	34 622	3.24

Porcine pepsin A (Figure 4.2) belongs to the most widely studied aspartic proteases because it is very similar to human pepsin [9]. It consists of 327 amino acid residues (Table 4.2) and contains one chain and three disulphide bridges. Two residues of aspartic acid (Asp_{32} and Asp_{215}) are in the active site of the molecule [1]. Pepsin A exhibits a high affinity toward 3,5-diiodo-L-tyrosine (DIT) [5, 10].

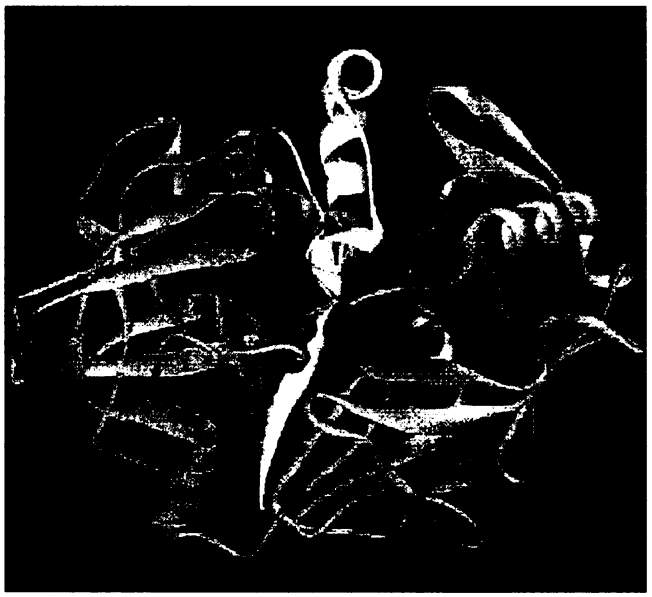

Fig. 4.2: Tertiary structure of porcine pepsinogen A [1]. The active enzyme moiety is shown in blue, the prosegment in yellow and the two active-sites of aspartic acid in red.

Tab. 4.2: Amino acid composition of porcine pepsin A [11].

Amino acid	**Number of residues in molecule**
Lysine	1
Histidine	1
Arginine	2
Aspartic acid	30
Asparagine	12
Threonine	27
Serine	43
Phosphoserine	1
Glutamic acid	13
Glutamine	13
Proline	15
Glycine	35
Alanine	16
Cysteine	6
Valine	22
Methionine	4
Isoleucine	25
Leucine	27
Tyrosine	15
Phenylalanine	14
Tryptophan	5
Total	*327*

Affinity chromatography represents not only a method for specific separations of macromolecular substances, but also a valuable tool for distinguishing individual isoforms of proteins differing only slightly in their binding interactions. The search for ligands having such properties is very useful for many studies. Affinity chromatography of immobilized ligands can differentiate between individual pepsinogens and is a method useful for this purpose [12].

There exist only a limited number of ligand types described for the affinity chromatography of aspartic proteases and their zymogens; to these belong poly-lysine, antibodies, and inhibitors such as pepstatin or peptides [6, 12]. Pepsin is an endopeptidase with a broad specificity. Pepsin hydrolyzes N- and C-terminally blocked synthetic dipeptides like Glu-Tyr or Phe-Phe but with a poor efficiency [3]. Aromatic amino acids and their derivatives were used as affinity ligands for pepsin. L-tyrosine, 3-iodo-L-tyrosine, 3,5-diiodo-L-tyrosine (DIT), L-phenylalanine, p-iodo-L-phenylalanine and N-acetyl-L-phenylalanine were used and coupled directly to divinylsulphone-activated Sepharose [5]. An advantage of iodinated L-tyrosine as an affinity ligand is its ability to differentiate between pepsin A and pepsin C [5, 6].

RPLC was used for the comparison of peptide maps of pepsin after its digestions by different forms of soluble and immobilized α-chymotrypsin. The immobilized enzyme was prepared by its coupling to different copolymers or gels. The result was that modified α-chymotrypsin was not suitable for efficient pepsin cleavage [13, 14]. A combination of RPLC and CZE (capillary zone electrophoresis) was also used for the characterization of peptide maps. Peptide maps obtained by the two methods were compared and five selected chromatographic peaks were identified on an electropherogram [15].

Bank and co-workers [16] published a combination of conventional DEAE (diethylaminoethyl) chromatography with HPLC on Sephacryl and Mono-Q columns for isolation and fractionation of human pepsinogen isozymogens from urine. This simple method allows obtaining pepsinogen in a native non-denatured conformation.

Commercial pepsin from porcine stomach mucosa was characterized by capillary electrophoresis in a poly(ethylene glycol)-coated capillary with a photodiode-array detector [17]. Gilges [18] published acceptable separations of acidic proteins (including pepsin) in poly(vinyl alcoho)l-coated capillaries. The basic pH of buffer used caused the negative charge of proteins. The proteins were separated using a negative-polarity voltage. Pepsin was also analyzed in a bare capillary with putrescine as a buffer additive with a positive voltage applied [19].

The main goal of this part of the Thesis was to develop a method for a quick and easy determination of pepsin. For affinity chromatography, a preparation and testing of stationary phases with coupled 3,5-diiodo-L-tyrosine was the main goal. The results of this work were published [20]. For reversed-phase, a cleavage and analysis of pepsin fragments was the main objective.

4.2 Experimental

4.2.1 Samples and chemicals

Porcine pepsin A, DIT, ovalbumin and lysozyme were purchased from Sigma Chemicals (St. Louis, USA), a sample of human pepsinogen and the Placer buffer were provided by the Institute of Pathophysiology, First Faculty of Medicine, Charles University. The stationary phases Separon HEMA BIO 1000 E and Separon HEMA BIO 1000 VS were obtained from Tessek (Prague, Czech Republic) and the phase AF epoxy Toyopearl 650 M from Tossoh (Tokyo, Japan). Dried milk was a product of Nutricia (Opočno, Czech Republic), glycine was supplied by Reanal (Budapest, Hungary). All the other chemicals were products of Pliva-Lachema (Brno, Czech Republic). All the chemicals were used as received. All buffers and mobile phases were filtered through a membrane filter type HA, pore size 0.45 μm (Millipore Corporation, Bedford, USA) prior to use.

4.2.2 Affinity chromatography

Liquid chromatographic measurements were carried out using a DIONEX instrument (Westmont, USA) provided with a gradient pump DIONEX P580 and a UV/VIS detector 170S/340S. The samples were injected through a RHEODYNE valve (Cotati, USA) with a 10 and a 100 μL loops. The data were handled by the Chromeleon 6.01 software (DIONEX).

A sample of porcine pepsin A was dissolved in a phosphate buffer of pH 3.5 (starting mobile phase). The column was first washed with the starting mobile phase. The sample was then injected into the column and non-adsorbed substances were eluted. Adsorbed pepsin was desorbed and eluted by changing the mobile phase pH to a value of 5.6. The protein was detected at 214 nm [17].

The pepsin activity was checked by a turbidimetric milk-clotting assay [21]. The determination of chymase activity is based on determination of the sum of proteases using milk curdling. Fractions of 1 mL from affinity chromatography were collected and the time of the beginning of milk curdling was measured. The activity was plotted as the

dependence of the reciprocal of curdling time of the given elute fraction against the time of the fraction collection.

The homogenate of human pepsin sample was filtered through cotton wool. A 9 x 20 mm DEAE-Sepharon column with a volume of 1 mL was washed with 0.05 M Tris.HCl buffer of pH 7.3, allowed to swell for 1 hour and then washed again with 30 mL of the same buffer. A 1 mL volume of the homogenate sample was applied to the column, three 10 mL portions of the above buffer were passed through the column and pepsinogen was then eluted with 10 mL of a 0.1 M acetate buffer of pH 5.6 containing 0.5 M NaCl. The first 5 mL of the eluate were dialyzed overnight against the 0.1 M acetate buffer of pH 5.6. A new portion of the buffer was used after 2 hours of dialysis. After dialysis, the sample was concentrated at a decreased temperature (refrigerator) from a volume of 5 mL to 1 mL in a Vivapore Concentrator vessel with a 7500 MWCO PES membrane. Finally, the pepsinogen was activated for approximately 45 min. to yield pepsin using 1 M HCl, to attain a pH of ca. 3.5. The sample was then injected onto the affinity column.

4.2.3 Preparation of affinity stationary phases

The procedure is based on ref. [6] and our previous experience with the preparation of affinity stationary phases: an amount of 3.5 g of a support containing either vinylsulphonic or epoxy active groups was rinsed with distilled water and mixed with the affinity ligand, 3,5-diiodo-L-tyrosine (500 mg of DIT suspended in 10 mL of a 0.2 M carbonate buffer, pH 10.7). The suspension was shaken overnight at ambient temperature, washed 3 times with distilled water, once with a 0.2 M carbonate buffer of pH 9.0 and once with an acetate buffer of pH 4.0. A small sample was collected for elemental analysis for iodine. A glycine solution was added to the suspension (100 mg in 10 mL a 0.2 M carbonate buffer of pH 9.0) to block the residual active groups of the support. The mixture was shaken overnight at ambient temperature, washed 3 times with distilled water, once with an acetate buffer of pH 4.0 and finally once with distilled water. The stationary phase was packed into an 80 x 8 cm stainless steel column in the Watrex Company, Prague.

The reaction between the vinylsulphonic active groups and 3,5-diiodo-L-tyrosine or glycine corresponds to the scheme given in Fig. 4.3. DIT is linked to a support via amino group. This was proved by ^{1}H-NMR analysis [6].

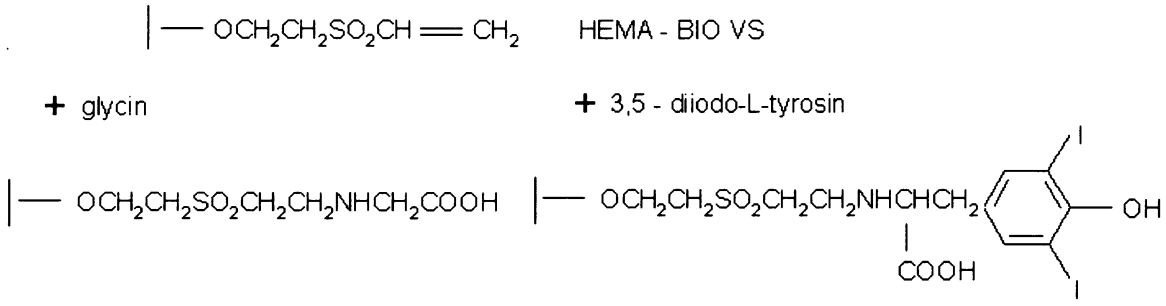

Fig. 4.3: The reaction of HEMA VS with either glycine or DIT.

Three stationary phases were prepared, containing 3,5-diiodo-L-tyrosine bound to supports HEMA VS, HEMA E and Toyopearl E. The amount of iodine was determined by elemental analysis and the amount of DIT was calculated from it (see Table 4.3). It can be seen from this table that DIT amount bound to epoxy activated support is greater, by about 10 mg per g of support, than that bound to the vinylsulphone activated support. These differences can follow from different numbers of the active functional groups.

Tab. 4.3: Amount of DIT bound to supports.

Affinity stationary phase	**Amount of DIT (mg per g of support)**
HEMA BIO VS-DIT	50
HEMA BIO E-DIT	61
Toyopearl epoxy-DIT	64

It was not possible to compare the results on the amount of the affinity ligand coupled with the results by other authors [3, 5]. In this work, the ligand content was calculated as grams of DIT coupled into 1 g of solid support. Kučerová *et al.* [3, 5] stated the ligand amount immobilized as μmol per 1 mL of Sepharose gel VS-activated.

4.2.4 Reversed-phase chromatography

Liquid chromatographic measurements were carried out using a DIONEX instrument (Westmont, USA) provided with a gradient pump DIONEX P580 and a UV/VIS detector 170S/340S. The samples were injected through a RHEODYNE valve (Cotati, USA) with a 100 μL loop. The data were handled by the Chromeleon 6.01 software (DIONEX). Samples of fragments of porcine pepsin A were injected into a pC_{18} BioSuite column (Waters, Czech Republic).

Digestion of porcine pepsin A was carried out according to [14]: 2 mg of pepsin was dissolved in 2 mL of 0.4 M NH_4HCO_3 containing 8 M urea. This solution was incubated with 200 μL of 0.045 M dithiothreitol (DTT) solution at 50 °C for 15 min. After cooling to laboratory temperature, the solution of 200 μL of 0.1 M iodoacetic acid (IAA) was added and the mixture was incubated at 25 °C for 10 min. A volume of 5.6 mL of deionized water and 1 mg of α-chymotrypsin were added. The mixture with final pH 8.2 was incubated at 37 °C for 24 hrs. The reaction was stopped by acidification to pH 4.5 using 50 % acetic acid.

4.3 Results and discussion

4.3.1 Affinity chromatography

Three different types of affinity stationary phases with immobilized 3,5-diiodo-L-tyrosine were prepared. Vinyl sulphone HEMA, epoxy HEMA and epoxy Toyopearl were chosen as solid supports. The highest amount of DIT coupled was introduced into an epoxy-Toyopearl. The affinity ligand was bound via amino groups.

The stationary phases prepared were tested using porcine pepsin A. The course of chromatography is given in Figure 4.4. The enzyme was applied onto a column at 0.05 M acetate buffer pH 3.5 (starting mobile phase). Substances non-interacting with DIT were eluted first (peak No. 1 in Fig. 4.4) and probably correspond to impurities present in commercial porcine pepsin A sample, or are system peaks. Pepsin (peak no. 3) interacted with DIT and was eluted by increasing the pH of the mobile phase to a value of 5.6 (eluting mobile phase). This peak exhibited a chymase activity (Figure 4.5) which confirms

that this peak corresponds to pepsin. Similar chromatograms were obtained for all the affinity stationary phases prepared.

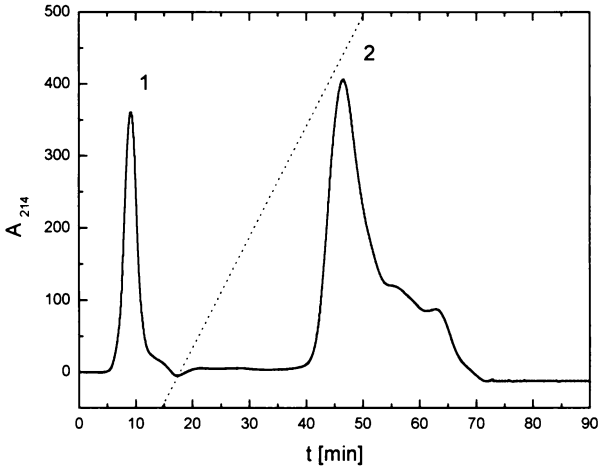

Fig. 4.4: Affinity chromatography of porcine pepsin A on epoxy Toyopearl-DIT.
Starting mobile phase, 0.05 M acetate buffer, pH 3.5, eluting mobile phase, the same buffer, pH 5.6; pH gradient from the 15th to 50th min (dot line).
(1) impurities, (2) porcine pepsin A.

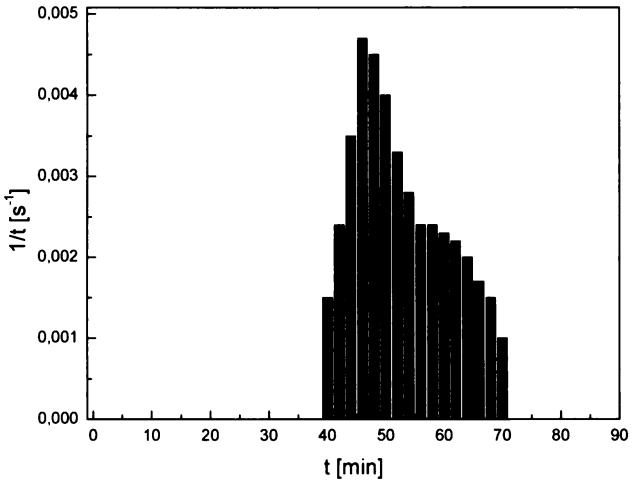

Fig. 4.5: The profile of chymase activity of porcine pepsin A.
The fractions were obtained from column epoxy Toyopearl-DIT.

Affinity ligand 3,5-diiodo-L-tyrosine is specific for aspartate proteases. This property was verified by an injection either of only ovalbumin or a mixture of pepsin, ovalbumin and lysozyme. This mixture was injected into all the columns prepared. The course of affinity chromatography of ovalbumin on epoxy activated HEMA is given in Figure 4.6. It is clear from this figure that ovalbumin was not retained.

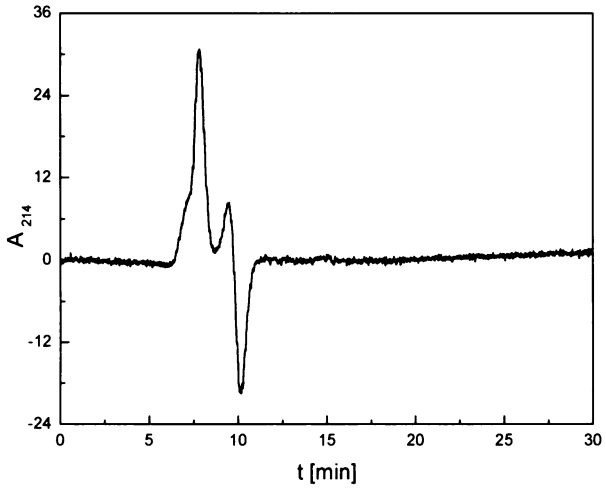

Fig. 4.6: Affinity chromatography of ovalbumin on epoxy HEMA-DIT.
Starting mobile phase, 0.05 M acetate buffer, pH 3.5, eluting mobile phase, the same buffer, pH 5.6; pH gradient from the 15th to 30th min.

The dependence of the peak area of porcine pepsin A on its concentration was determined for all the stationary phases prepared. The peak area increases linearly up to a concentration of 3 mg/mL. Above this concentration, the curve has a parabolic profile. It can be explained by the fact that the binding sites are already saturated. The results are summarized in Table 4.4

Tab. 4.4: Dependence of the porcine pepsin A peak area on its concentration for all the columns prepared. Experimental conditions: porcine pepsin A 0.5-5 mg/mL, pH gradient 3.5-5.6, λ=214 nm.

Stationary phase						
Toyopearl E						
c_{pepsin} (mg/mL)	0.5	1	2	3	4	5
A (mAU*min)	1620	2135	2783	3255	3443	3506
HEMA VS						
c_{pepsin} (mg/mL)	0.5	1	2	3	4	5
A (mAU*min)	566	809	1238	1652	1896	2005
HEMA E						
c_{pepsin} (mg/mL)	0.5	1	2	3	4	5
A (mAU*min)	788	1009	1480	1921	2192	2326

The reproducibility of measurements was examined for all the stationary phases prepared. A sample of porcine pepsin A was injected ten times into a column and the changes in the peak area were evaluated (Table 4.5). The measurements were repeated after two months (Table 4.6). From the coefficients of variation given in Table 4.7 it can be concluded that the measurements are reproducible.

Tab. 4.5: Repeatability of measurements of porcine pepsin A for all the columns.
Experimental conditions: porcine pepsin A 0.5 mg/mL (Toyopearl) or 1mg/mL (HEMA), pH gradient 3.5-5.6, λ=214 nm.

Stationary phase										
Toyopearl E										
Measurement	1	2	3	4	5	6	7	8	9	10
A (mAU*min)	1615	1628	1629	1622	1631	1612	1624	1629	1610	1617
HEMA VS										
Measurement	1	2	3	4	5	6	7	8	9	10
A (mAU*min)	815	806	823	819	808	800	802	809	814	808
HEMA E										
Measurement	1	2	3	4	5	6	7	8	9	10
A (mAU*min)	1012	1009	1018	1010	1001	1007	1012	1009	1013	1017

Tab. 4.6: Repeatability of measurements of porcine pepsin A measured after two months. Experimental conditions: porcine pepsin A 0.5 mg/mL (Toyopearl) or 1mg/mL (HEMA), pH gradient 3.5-5.6, λ=214 nm.

Stationary phase										
Toyopearl E										
Measurement	1	2	3	4	5	6	7	8	9	10
A (mAU*min)	1601	1605	1620	1632	1625	1629	1614	1608	1619	1647
HEMA VS										
Measurement	1	2	3	4	5	6	7	8	9	10
A (mAU*min)	805	836	813	809	814	838	800	843	822	814
HEMA E										
Measurement	1	2	3	4	5	6	7	8	9	10
A (mAU*min)	1022	998	1017	1004	1011	1026	1042	1029	1051	1015

Tab. 4.7: Variation coefficients for all the stationary phases prepared.

	First set of measurements	Second set of measurements
Toyopearl E	0.42 %	0.92 %
HEMA VS	0.92 %	1.72 %
HEMA E	1.22 %	1.69 %

The course of affinity chromatography of human pepsin (for sample preparation see section 4.2.2) on the epoxy-Toyopearl-DIT is given in Figure 4.7. Peaks Nos. 1 and 2 represent impurities present. Peak No. 3 exhibits chymase activity and corresponds to pepsin. The behavior of the model porcine pepsin A and human pepsin is similar, even if the human pepsin eluted earlier (its retention time was 26 min whereas porcine pepsin 47 min).

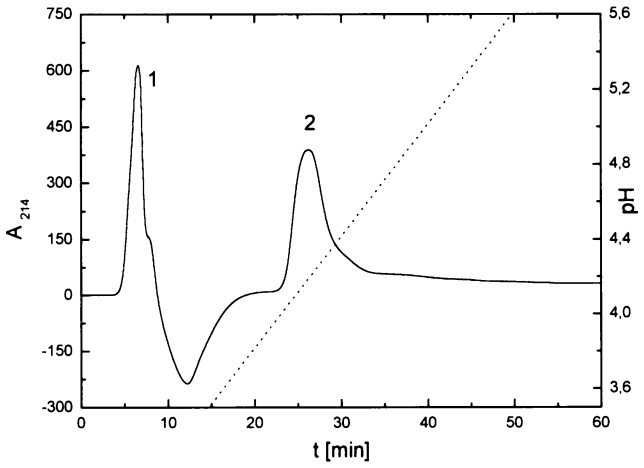

Fig. 4.7: Affinity chromatography of human pepsin on epoxy Toyopearl-DIT.
Starting mobile phase, 0.05 M acetate buffer, pH 3.5, eluting mobile phase, the same buffer, pH 5.6; pH gradient from the 15th to 50th min (dot line).
(1) impurities, (2) human pepsin.

4.3.2 Reversed-phase chromatography

The column pC18 BioSuite was a gift from Waters. This column was tested by the fragments of porcine pepsin A. This enzyme was cleaved by a soluble α-chymotrypsin (for greater details about the procedure see section 4.2.4). The course of liquid chromatography is given in Figure 4.8. The peaks eluted at the beginning (first ten minutes) correspond to buffer components (urea, DTT and IAA). Hynek *et al.* [15] published that peaks eluted in time interval between 14th and 20th min are related to α-chymotrypsin and its self-cleavage fragments. However, in our experiments no peak was eluted at this time. It can probably be explained by a different activity of α-chymotrypsin used. Peptide fragments were separated in the time interval from 40th to 70th min. The peptides were eluted by a gradient of acetonitrile (ACN).

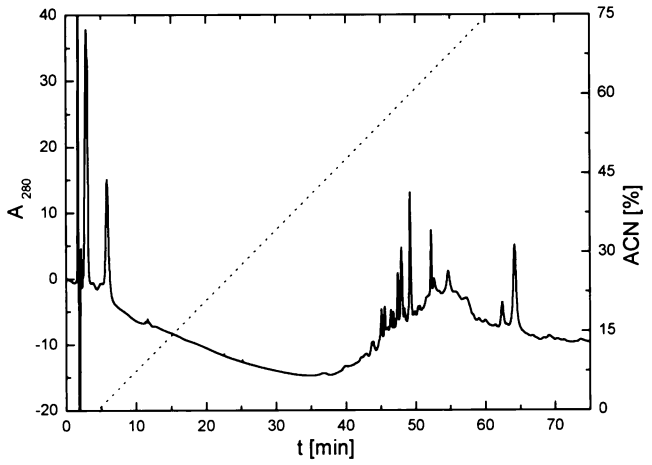

Fig. 4.8: RPLC of fragments of porcine pepsin A after hydrolysis by soluble α-chymotrypsin. Mobile phase: 0-5 min H_20 with 0.1% TFA, 5-60 min 0-75% H_2O/ACN (40/60) with 0.1% TFA (....gradient of ACN).

4.4 Conclusion

The results obtained demonstrate that the preparation of affinity stationary phases with immobilized 3,5-diiodo-L-tyrosine is reproducible and quite easy. Stationary phases exhibited sufficient selectivity, proved to be useful for analysis of porcine pepsin A and are applicable to practical samples of human pepsin. Reversed-phase C_{18} column is suitable for analysis of pepsin fragments obtained after α-chymotrypsin digestion.

A study of interaction of various forms of pepsin and pepsinogen are essential because of their high importance as clinical diagnostic markers and as stomach enzymes. Different methods can be employed for this purpose, primarily affinity chromatography, reversed-phase chromatography and capillary electrophoresis (CE). It was expected that CE would be part of this Thesis but instrumental problems made it impossible.

4.5 References

1. C. Richter, T. Tanaka, R.Y. Yada: *Biochem J.* **335** (1998) 481-490.

2. A. Jindra: *Biochémia – molekularnobiologické a farmaceutické aspekty.* Bratislava, Osveta 1985.

3. J. Frýdlová, Z. Kučerová, M. Tichá: *J. Chromatogr. B* **800** (2004) 109-114.

4. I.M. Samloff: *Gastroenterology* **96** (1989) 586-595.

5. Z. Kučerová, M. Tichá: *J. Chromatogr. B* **770** (2002) 121-128.

6. H. Arnoštová, Z. Kučerová, I. Tišlerová, T. Trnka, M. Tichá: *J. Chromatogr. A 911* (2001) 211-216.

7. http://ca.expasy.org/cgi-bin/pi_tool (15-02-2003).

8. I. Gritti, G. Banfi, G.S. Roi: *Pharm. Res.* **41** (2000) 265-281.

9. X.-L. Liu, R.N.S. Wong, J. Tang: *J. Biol. Chem.* **264** (1989) 4482-4489.

10. R.A. da Silva Gomes, R.P. Batista, A.C. de Almeida, D.N. de Fonseca, L. Juliano, V. Hial: *Anal. Biochem.* **316** (2003) 11-14.

11. J. Tang, P. Sepulveda, J. Marciniszyn, C.S. Chen, W.-Y. Huang, N. Tao, D. Liu J.P. Lanier: *Proc. Nat. Acad. Sci.* **70** (1973) 3437-3499.

12. Z. Kučerová, M. Tichá: *J. Sep. Sci.* **26** (2003) 669-673.

13. H. Vaňková, Z. Kučerová, J. Turková: *J. Chromatogr. B* **753** (2001) 37-43.

14. H. Vaňková, Z. Kučerová, M. Tichá: *Collect. Czech. Chem. Commun.* **64** (1999) 710-716.

15. R. Hynek, V. Kašička, Z. Kučerová, J. Káš: *J. Chromatogr. B* **681** (1996) 37-45.

16. R.A. Bank, A.W. Eriksson, G. Pals: *J. Chromatogr. B* **571** (1991) 47-59.

17. J. Haginaka, Y. Miyano, Y. Saizen, C. Seyama, T. Murashima: *J. Chromatogr. A* **708** (1995) 161-168.

18. M. Gilges, M.H. Kleemiss, G. schomburg: *Anal. Chem.* **66** (1994) 2038-2046.

19. J.P. Landers, R.P. Oda, B.J. Madden, T.C. Spelsberg: *Anal. Biochem.* **205** (1992) 115-124.

20. T. Vařilová, A. Vránková, V. Pacáková, M. Tichá, K. Štulík: *J. Chromatogr. A* **1084** (2005) 207-213.

21. P. Mc Phie: *Anal. Biochem.* **73** (1976) 258-261.

5.

Seminal plasma proteins

5.1 Seminal plasma proteins

Mammalian fertilization is a unique event in which morphologically disparate gamettes recognize and bind each other and fuse. This event includes highly regulated biochemical interactions: binding of seminal plasma proteins to the sperm surface during ejaculation, interaction of sperm surface proteins with oviduct epithelial cells, sperm capacitation, gamette recognition, primary and secondary binding of the sperm to the ovum, acrosome reaction of sperm, penetration of the sperm through the zona pellucida of the ovum and fusion of sperm and egg [1-4]. Sperm-binding proteins of the seminal plasma play important roles in these events.

Seminal plasma is a very complex fluid containing a wide variety of both inorganic and organic constituents; its high-molecular-weight part is mostly represented by proteins. Protein composition, as well as protein amounts secreted by individual accessory glands, varies from species to species. It is evident that seminal plasma proteins affect sperm in its functions and participate in the fertilization process [5].

Boar seminal plasma contains various types of proteins. The first type, spermadhesins (AQN, AWN, PSP-porcine seminal plasma), belong to the most abundant boar seminal plasma proteins. It's a group of 12 000 – 16 000 polypeptides which belong to one protein family. The N-terminal amino acid sequence of all the members of AQN and AWN proteins begins with either Ala-Gln-Asn (AQN) or Ala-Trp-Asn (AWN). They are characterized by 40-60% amino acid sequence identity and contain two conserved disulphide bridges. They are multifunctional proteins showing a range of ligand-binding abilities, e.g., saccharides (e.g., heparin and other sulfated polysaccharides, hyaluronic acid, simple saccharides and their derivatives), phospholipids and protease inhibitors, suggesting that they may be involved in different steps of fertilization [6].

DQH (according to its N-terminal amino acid sequence Asp-Gln-His) is another type of protein isolated from boar seminal plasma. It differs in its structure from boar spermadhesin [6, 7]. Binding of biotinylated DQH protein to the acrosome of boar epididymal spermatozoa indicates that this protein belongs to the sperm surface proteins. The protein is a polypeptide of 105 amino acids with four disulfide bridges in the molecule and consists of an N-neutral O-glycosylated peptide followed by two fibronectin repeats. The DQH sperm surface protein is a member of the large family of cell and matrix adhesion proteins [6].

The major proteins found in bull seminal plasma are called BSP proteins (bovine seminal plasma proteins [8-10]. BSP-A1, BSP-A2, BSP-A3 and BSP-30kDa are acidic proteins secreted by the seminal vesicle epithelium [11-13]. These proteins are present in seminal plasma at very high concentrations [8, 9]. BSP-A1, -A2 and A3 have apparent molecular masses ranging from 15 500 to 16 500, whereas the BSP-30kDa protein has a molecular mass of 28 000-30 000 [12]. All members of this family are glycoproteins with the exception of BSP-A3 [11]. The BSP-A1 and BSP-A2 proteins turned out to be molecular variants of bovine seminal plasma peptide known as PDC-109. These two proteins differ in their degree of glycosylation, but otherwise contain identical polypeptide backbones [12]. BSP-A3 and BSP-30kDa proteins each have an amino acid composition different from BSP-A1 and BSP-A2 [13, 14].

The binding sites of the four BSP proteins on the sperm surface appear to be lipids: BSP-A1, BSP-A2 and BSP-A3 bind specifically to phospholipids which contain phosphorylcholine (PC) group. BSP-30kDa, however, displays a much broader binding specificity. It preferentially binds to choline phospholipids but also interacts with phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and other compounds. The BSP proteins bind also heparin [15].

Quite different proteins are present in human seminal plasma. Major protein constituents of human seminal plasma are semenogelin I (Sg I) and semenogelin II (Sg II). They participate in the formation of sperm-entrapping gel immediately after ejaculation [16-18]. Sg I is a non-glycosylated protein of 439 amino acid residues with a molecular mass of 50 000. The Sg II molecule with a MW of 63 000 consists of 559 amino acid residues with a primary structure that is from 78% similar to that of Sg I. It has one potential site for N-linked glycosylation and around half of the molecules in seminal plasma are glycosylated [17]. Immediately after ejaculation, human semen forms a coagulum. This is mainly due to interactions between semenogelin I and semenogelin II. The coagulum can be dissolved using urea and a reducing agent, indicating that the interactions are mainly of non-covalent nature, via disulfide bridges. The gel structure also dissolves spontaneously within minutes after ejaculation as the result of proteolytic degradation of the semenogelin molecules by prostate-specific antigen (PSA) [17, 18].

A common feature of many seminal plasma proteins is their ability to interact with different types of substances. Some of them are bound to the sperm surface during ejaculation and thus they affect binding interactions of sperm. The following interactions of sperm that are connected with seminal plasma proteins belong to the most studied ones:

(i) an interaction with different types of glycoconjugates, (ii) an interaction with membrane phospholipids, (iii) mutual interactions between proteins [6].

Preparative (low-pressure) affinity chromatography belongs to one of the methods used for the isolation and separation of seminal plasma proteins. Among the ligands used, immobilized heparin is often used to separate heparin binding (H+) and heparin non-binding (H-) fractions of proteins [19-22]. Affinity techniques are useful not only for the protein isolation but they represent a valuable and essential tool for the study and analysis of binding properties of protein components that participate in the fertilization process.

Liberda *et al.* [19] published an immobilization of mannan linked to divinyl sulfone-activated Sepharose. A phosphate buffer saline solution of lyophilized bull seminal plasma was applied onto a column pre-equilibrated with the same buffer. The non-adsorbed proteins were washed with the same buffer until the absorbance at 280 nm decreased to the baseline. The adsorbed proteins were either eluted directly with 3M NaCl or first eluted with 2% D-mannose solution and then with 3M NaCl.

Another paper [21] introduced an affinity sorbent containing immobilized L-glyceryl phosphorylcholine for affinity chromatography of phosphorylcholine binding proteins (P+). Seminal plasma proteins dissolved in Tris.HCl buffer containing NaCl were applied onto a column and the adsorbed proteins were specifically eluted with phosphorylcholine solution.

Heparin, owing to its unique structure and surface charge distribution, is capable of interacting strongly with several proteins. This was utilized for preparation of polyacrylamide-based carriers with immobilized heparin for affinity chromatography of heparin-binding proteins from boar seminal plasma [23, 24].

The main aim of this project was to study the interactions of various seminal plasma proteins with heparin and phosphorylcholine using affinity chromatography at an elevated pressure and, based on the results obtained, to evaluate the contents of H+ and P+ proteins in different species.

5.2 Experimetal

5.2.1 Samples and chemicals

Boar and bull ejaculates were obtained from the Veterinary Research Institute, Brno, Czech Republic. Human ejaculates from the Institute of Sexology were kindly provided by Dr. Jonáková (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague). Ejaculates were centrifuged (600 g, 20 min, 5° C) to separate plasma and sperms and seminal plasma was lyophilized directly (for a greater detail, see ref. [21]).

Tris.HCl and phosphorylcholine were purchased from Sigma Chemicals (St. Louis, USA). Sodium dihydrogen phosphate, di-sodium hydrogen phosphate dihydrate and sodium chloride were from Pliva-Lachema (Brno, Czech Republic). All the chemicals were used as received.

All buffers and mobile phases were filtered through a membrane filter type HA, pore size 0.45 μm (Millipore Corporation, Bedford, USA) prior to use. An overview of buffers used are given in Table 5.1.

Tab. 5.1: Composition of buffers.

Buffer composition	**pH**
0.02 M Tris.HCl + 0.15 M NaCl	7.5
0.02 M Tris.HCl + 1.50 M NaCl	7.5
0.02 M Tris.HCl + 0.15 M NaCl + 0.05 M phosphorylcholine	7.5

5.2.2 Affinity chromatography

Liquid chromatographic measurements were carried out using a DIONEX instrument (Westmont, USA) connected with a gradient pump P580 and a UV/VIS detector UVD 170S/340S. All samples were detected at 280 nm. The flow rate used was 0.4 mL/min. The samples were injected through a RHEODYNE valve (Cotati, USA) with 10 μL sampling loops. The data were handled by Chromeleon software version 6.01 (DIONEX).

The TOYOPEARL–heparin stationary phase (Tosoh Bioscience) was packed into a steel column with dimensions of 80 mm x 8 mm (Watrex, Czech Republic). The column parametres: particle size 65 μm, polymer based resin pH range, 5.5 – 9.0, binding capacity, 5 mg antithrombin III/mL (according to the information of Tosoh Bioscience).

Samples of seminal plasma proteins were dissolved in a buffer of 0.02 M Tris.HCl containing 0.15 M NaCl pH 7.5 (2 mg/mL). Non-adsorbed proteins were eluted with the same buffer (starting buffer). For the elution of heparin-binding seminal plasma proteins, a step gradient elution was used with increasing ionic strength of the mobile phase. For the elution of phosphorylcholine-binding fraction of seminal plasma proteins, a two-step gradient was used. Non-adsorbed proteins were eluted with a mobile phase Tris.HCl containing 0.15 M NaCl (starting buffer). The adsorbed proteins were first eluted using phosphorylcholine in the starting buffer and then using a 1.50 M NaCl in the starting buffer. After the analysis, the stationary phase was equilibrated with the starting mobile phase. An overview of the mobile phases and the gradient used is given in Table 5.2.

Tab. 5.2: An overview of mobile phases and gradient used.

H+		**P+**	
0-20 min	0.02 M Tris.HCl + 0.15 M NaCl	0-20 min	0.02 M Tris.HCl + 0.15 M NaCl
20-30 min	0.02 M Tris.HCl + 0.15-1.50 M NaCl	20-40 min	0.02 M Tris.HCl + 0.15 M NaCl + 0.05 M phosphorylcholine
from 30. min	0.02 M Tris.HCl + 1.50 M NaCl	from 40. min	0.02 M Tris.HCl + 1.50 M NaCl

5.3 Results and discussion

The studies of binding interactions of seminal plasma proteins and their analysis are very important in understanding their role in the fertilization process. There exist several types of methods that could be applied to this purpose and affinity chromatography represents one of those that could yield reliable results. Systems used for isolation (classical low-pressure afiinity chromatography with immobilized heparin [19, 20]) are

time- and material consuming in analytical applications and thus affinity chromatography at an elevated pressure was used.

Many seminal plasma proteins can interact with sulphated polysaccharides such as heparin. This property was utilized for analysis of these proteins. In this study, the commerially available affinity stationary phase Toyopearl with immobilized heparin was used.

Separation of full boar, bull and human seminal plasma was optimized using affinity stationary phase with immobilized heparin. Different mobile phases were tested. The best results were obtained with a step salt gradient from 0.15 to 1.50 M NaCl in a Tris.HCl buffer at pH 7.5. Examples of affinity chromatography of boar, bull and human seminal plasma proteins are given in Figures 5.1 A, B and C. Peak No.1 represents proteins non-interacting with heparin while peak No. 2 contains proteins binding to heparin. The content of the proteins bound to heparin (H+) in seminal plasma of different species was evaluated and the results are shown in Table 5.3. It follows from this table that the amounts of proteins bound to heparin differ in different species: the highest ratio (55 %) of H+ to total proteins was found in human plasma and the lowest ratio (15 %) was found in bull plasma.

Tab. 5.3: The contents of heparin binding fraction (H+) and phosphorylcholine binding fraction (P+) in seminal plasma of different species.

Seminal plasma	**Content of H+ proteins in seminal plasma* [%]**	**Content of P+ proteins in seminal plasma* [%]**
Bull	15	4
Boar	35	3
Human	55	0

* expressed in % of the total amount of proteins in seminal plasma, determined on the basis of A_{280} nm measurement

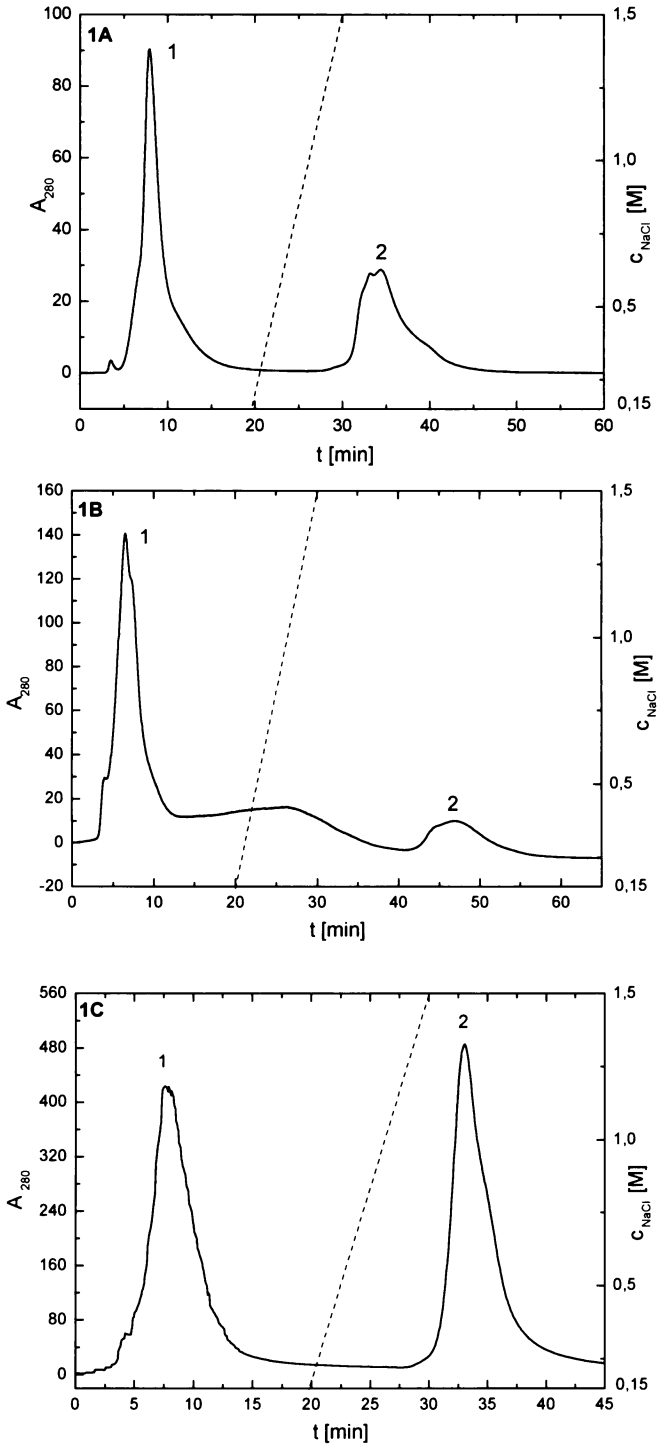

Fig 5.1: Affinity chromatography of full seminal plasma on a heparin-Toyopearl column.

A: boar; **B**: bull; **C**: human.

Mobile phases – gradient of NaCl in 0.02 M Tris.HCl, pH 7.5: 0-20 min 0.15 M NaCl, 20-30 min 0.15-1.50 M NaCl (--- gradient of NaCl), from 30. min 1.50 M NaCl.

(1) proteins unbinding heparin; (2) proteins binding to heparin.

Seminal plasma of some species contains proteins which have an ability to interact with phosphorylcholine. This property participates in the binding of seminal plasma proteins to sperm membrane and thus protein sperm coating layers change the binding properties of this gamette [5]. For the isolation of P+ proteins from seminal plasma, affinity chromatography on immobilized heparin was combined with subsequent elution of the adsorbed proteins with a phosphorylcholine solution [17, 18]. We used this approach in this study.

Using the Toyopearl-heparin column, elution of P+ proteins was attained with a mobile phase consisting of 0.02 M Tris.HCl, 0.15 M NaCl with the addition of 0.05 M phosphorylcholine, pH 7.5. Chromatograms of boar, bull and human seminal plasma proteins are given in Figures 5.2 A, B and C.

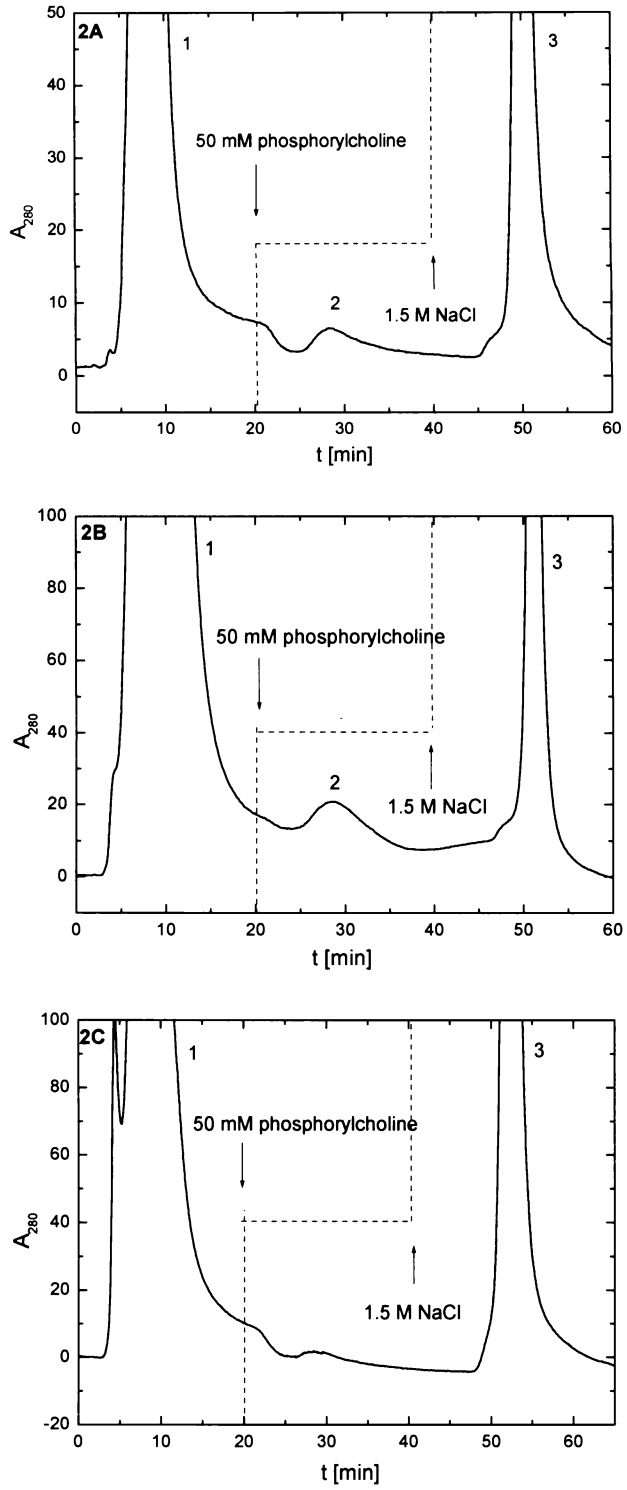

Fig. 5.2: Affinity chromatography of the phosphorylcholine binding fraction of full seminal plasma proteins on a heparin-Toyopearl column.

A: boar; **B**: bull; **C**: human

Mobile phases: 0-20 min 0.02 M Tris.HCl + 0.15 M NaCl, pH 7.5; 20-40 min 0.02 M Tris.HCl + 0.15 M NaCl + 0.05 M phosphorylcholine pH 7.5; from 40. min 0.02 M Tris.HCl + 1.50 M NaCl, pH 7.5; (1) proteins unbinding heparin; (2) proteins eluted by 0.05 M phosphorylcholine; (3) proteins eluted by 1.50 M NaCl.

Proteins non-interacting with heparin are present in peak No. 1 while peaks No. 2 and No. 3 correspond to proteins interacting with heparin. Peak No. 2 contains proteins eluted by phosphorylcholine solution and corresponds to phosphorylcholine-binding proteins (P+). Quantitative evaluation of the P+ proteins is given in Table 5.3. It follows from Figs. 5.2 A, B, C and Tab. 5.3 that the highest amount of phosphoryl-bound proteins was found in bull seminal plasma (4 %). No proteins eluted by phosphorylcholine were found in human plasma.

Proteins BSP-A1 and BSP-A2 from bull seminal plasma can interact with phosphorylcholine (they are indicated together as PDC-109) [19]. Only a small part of proteins from boar seminal plasma can interact with phosphorylcholine (probably DQH and AQN-1) [20]. These results are in agreement with the results obtained using classical (low-pressure) affinity chromatography with immobilized L-glyceryl-phosphorylcholine [19].

5.4 Conclusion

Seminal plasma proteins participate in the fertilization process which is one the fundamental process in a nature. The mechanism of fertilization of the egg with sperm is determined by mutual recognition of both gamettes and their binding. Although the interaction mechanism has not yet been fully elucidated, much of the data shows that the system of complementary molecules situated on the surface of both gamettes plays a key role in this interaction. Damage to the surface proteins of both gamettes makes the fertilization impossible or strongly disturbed [6]. Studies of the structure of seminal plasma proteins, their characterization and recognition of all their functions will significantly help to understand a complex process of fertizilization. The results of this work contribute to achieve this global task.

Affinity chromatography with heparin immobilized to Toyopearl support proved to be useful for the analysis of the binding properties of boar, bull and human seminal plasma proteins. The elution of heparin-binding proteins was attained using a salt gradient while the seminal proteins interacting with phosphorylcholine were eluted with 0.05 M phosphorylcholine added to the mobile phase. Different interactions with heparin and phosphorylcholine in different species were confirmed. The highest ratio of

heparin-binding proteins was found in human full seminal plasma. On the other hand, this plasma does not contain phosphorylcholine-binding proteins. Bull seminal plasma contains the highest amount of phosphorylcholine-binding proteins.

5.5 References

1. J.P. Evans, G.S. Kopf: *Andrologia* **30** (1998) 297-307.

2. S. Jansen, M. Ekhlasi-Hundrieser, E. Töpfer-Petersen: *Cell Tissues Organs* **168** (2001) 82-92.

3. S.S. Suarez: *Cell Tissues Organs* **168** (2001) 105-112.

4. P.M. Wassarman, L. Jovine, E.S. Litscher: *Nat. Cell Biol.* **3** (2001) E59-E64.

5. P. Maňásková, H. Ryšlavá, M. Tichá, V. Jonáková: *Am. J. Reprod. Immunol.* **48** (2002) 283-290.

6. V. Jonáková, M. Tichá: *Collect. Czech. Chem. Commun.* **69** (2004) 461-475.

7. P. Maňásková, J. Liberda, M. Tichá, V. Jonáková: *Folia Biologica (Prague)* **46** (2000) 143-151.

8. P. Jelínková, H. Ryšlavá, J. Liberda, V. Jonáková, M. Tichá: *Collect. Czech. Chem. Commun.* **69** (2004) 616-630.

9. M. Gasset, J.L. Saiz, J. Laynez, L. Sanz, E. Töpfer-Petersen, J.J. Calvette: *Eur. J. Biochem.* **250** (1997) 735-744.

10. J.J. Calvete, M. Raida, M. Gentzel, C. Urbanke, L. Sanz, E. Töpfer-Petersen: *FEBS Lett.* **407** (1997) 201-206.

11. L. Chandonnet, K.D. Roberts, A. Chapdelaine, P. Manjunath: *Mol. Reprod. Dev.* **26** (1990) 313-318.

12. P. Manjunath, I. Thérien: *J. Reprod. Immunol.* **53** (2002) 109-119.

13. L. Desnoyers, P. Manjunath: *Arch. Biochem. Biophys.* **305** (1993) 341-49.

14. N.G. Seidah, P. Manjunath, J. Rochemont, M.R. Sairam, M. Chrétien: *Biochem. J.* **243** (1987) 195-203.

15. J.J. Calvete, K. Mann, L. Sanz, M. Raida, E. Töpfer-Petersen: *FEBS Lett.* **399** (1996) 147-152.

16. J. Malm, J. Hellman, H. Magmersson, C.-B. Laurell, H. Lilja: *Eur. J. Biochem.* **238** (1996) 48-53.

17. Å. Lundwall, A. Bjartell, A.Y. Olsson, J. Malm: *Molec. Hum. Reprod.* **8** (2002) 805-810.

18. A. Peter, H. Lilja, Å. Lundwall, J. Malm: *Eur. J. Biochem.* **252** (1998) 216-221.

19. J. Liberda, H. Ryšlavá, P. Jelínková, V. Jonáková, M. Tichá: *J. Chromatogr. B* **780** (2002) 231-39.

20. P. Maňásková, J. Liberda, M. Tichá, V. Jonáková: *J. Chromatogr. B* **770** (2002) 137-143.

21. J. Liberda, P. Maňásková, M. Švesták, V. Jonáková, M. Tichá: *J. Chromatogr. B* **770** (2002) 101-110.

22. M. Kraus: *J. Reprod. Immunol.* **51** (2001) 131-44.

23. M. Tichá, B. Železná, V. Jonáková, K. Filka: *J. Chromatogr. B* **656** (1994) 423-426.

24. V. Jonáková, M. Kraus, L. Veselský, D. Čechová, K. Bezouška, M. Tichá: *J. Reprod. Fertil.* **114** (1998) 25-34.

6.

Glycoproteins

6.1 Glycoproteins

Glycoproteins are protein molecules that have carbohydrate units attached to side chains of certain amino acid residues [1]. Protein chemists considered glycoproteins to be a nuisance that complicated protein "purification". In fact, most eukaryotic proteins are glycoproteins. They vary in their carbohydrate contents from <1% to >90% by weight. They occur in all forms of life and have functions that span the entire spectrum of protein activities, including those of enzymes, transport proteins, receptors, hormones, and structural proteins. The polypeptide chains of glycoproteins are synthesized under genetic control. Their carbohydrate chains, in contrast, are enzymatically generated and covalently linked to polypeptide without the rigid guidance of nucleic acid templates. The processing enzymes are generally not available in sufficient quantities to ensure the synthesis of uniform products. Glycoproteins thus have variable carbohydrate compositions, a phenomenon known as microheterogenity, that causes difficulties in their purification and characterization [2].

Protein glycosylation is more abundant than all the other types of posttranslational modifications. Oligosaccharides form two types of direct attachments to these proteins: N-linked and O-linked. In N-glycosidic attachments, an N-acetylglucosamine (NAG) is invariably β-linked to the amide nitrogen of asparagine. The oligosaccharides in these linkages usually have a distinctive core whose peripheral mannose residues are linked to either mannose or NAG residues. The most common O-glycosidic attachment involves the disaccharide core β-galactosyl-(1→3)-α-N-acetylgalactosamine α-linked to the OH group of either serine or threonine [2].

A characteristic property of N-linked glycoproteins is the existence of numerous glycoforms. Cells tend to synthesize a large repertoire of a given N-linked glycoprotein, in which each variant species (glycoform) differs somewhat in the sequences, locations, and numbers of its covalently attached oligosaccharides. An example of N-linked glycoprotein is ribonuclease B (Rnase B). N-linked oligosaccharides are given in Figure 6.1.

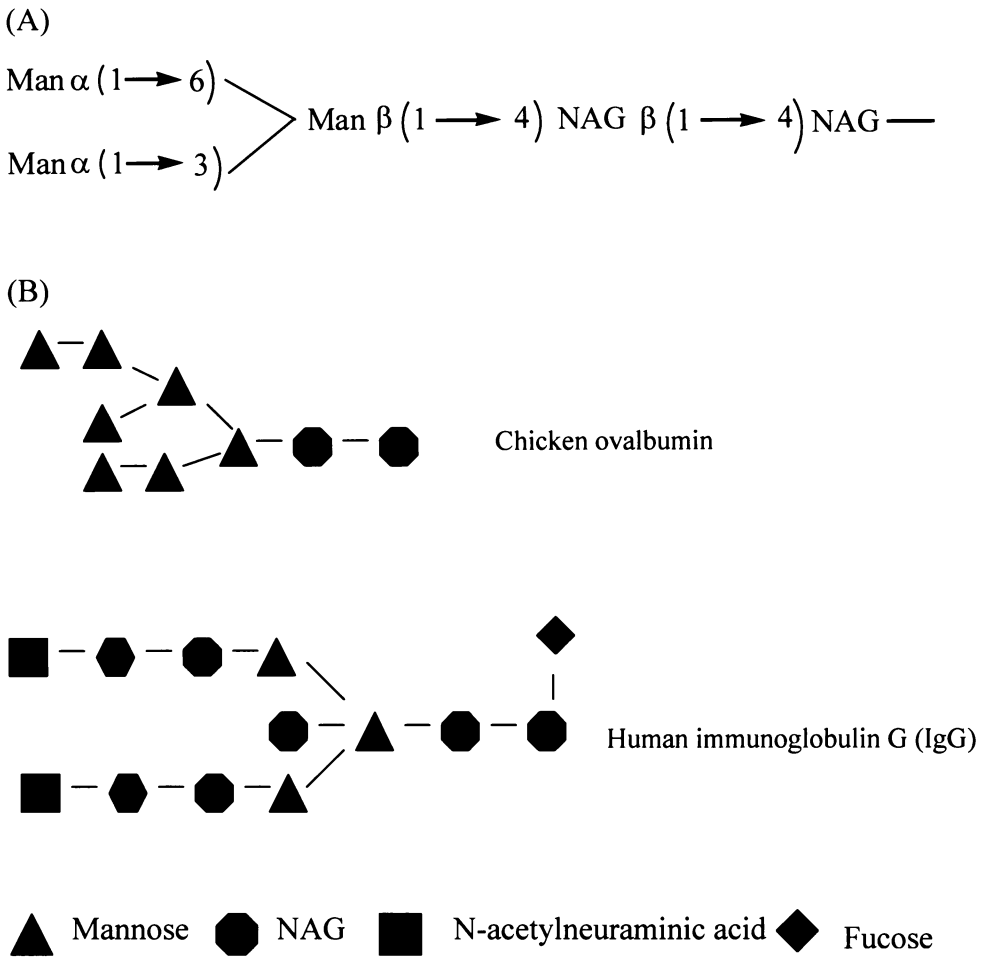

Fig. 6.1: N-linked oligosaccharides (adapted from ref. [2]).

A: N-linked oligosaccharides usually have the branched $(mannose)_3(NAG)_2$ core shown.

B: Two examples of N-linked oligosaccharides.

O-linked polysaccharides tend not to be uniformly distributed along polypeptide chains. They are rather clustered into heavily glycosylated (65-85 % carbohydrate by weight) segment in which glycosylated Ser and Thr residues comprise 25 to 40% of the sequence. An example of O-linked glycoprotein is mucin [2]. An O-glycosidic attachment of oligosaccharide to glycoprotein is given in Figure 6.2.

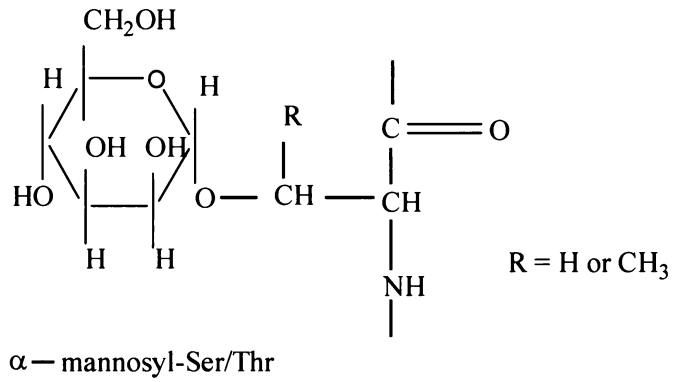

Fig. 6.2: O-glycosidic attachment of oligosaccharide to glycoprotein (adapted from ref. [2]).

Glycoproteins have a lot of important and positive functions in organisms. On the other hand, a lot of glycoproteins belong among allergens. They can be present in animal products as well as in plants.

Chicken ovalbumin (OA) is one of the major egg white allergens which cause IgE-mediated food hypersensivity [3]. It was one of the first glycoproteins prepared in a crystalline form. Its molecular weight is around 45 kDa. The molecule consists of a polypeptide with up to two phosphate groups per mole and a side chain of mannose and glucosamine residues [4]. OA is composed of 385 amino acid residues. The sugar content is around 3.5 % of the total molecular weight [5].

Pollen allergens are water-soluble proteins or glycoproteins of molecular weight between 10 and 70 kDa. Many pollen allergens are resistant to pH changes and high temperatures, even up to 100 °C. Their nomenclature has been unified on the basis of recommendations published by World Health Organization in 1994. The designations are derived from the first letter of the species. The Arabic numeral accompanying the letters denotes the sequence of identification and description of a given allergen [6].

Allergens of grass pollen are the most common cause of allergic inflammation of nasal mucosa and conjunctivitis. The allergy-inducing proteins from grass pollen have been comprehensively studied and, on the basis of the structural similarities, have been divided into 7 groups [6].

Phleum pratense L, timothy, is a relatively short-lived, cool-season perennial that grows in stools or clumps and has a shallow, compact, and fibrous root system [7]. Timothy grass pollen allergens belong to a family of pectin-degrading enzymes. The

corresponding allergen Phl p 13 represents glycoproteins with molecular weight around 42 kDa [8]. It prefers a pH of 5.5 to 7.0 [7].

6.2 Lectins

Lectins comprise a group of proteins with unique affinities toward carbohydrate structures. They have long been used in biomolecular isolation, carbohydrate chemistry and histochemistry, and more recently as the mimicking agents in intercellular interactions studies [9]. Lectins are defined as proteins or glycoproteins of nonimmunoglobulin nature capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrates without altering covalent structure of any recognized glycosyl ligands [10].

Glycosylation is the key step in a number of processes at the cellular level. Oligosaccharide-mediated recognition plays a very important role in a variety of biological processes such as fertilization, immune defense, viral replication, parasitic infection, cell-matrix interaction, cell-cell adhesion and enzymatic reactivity. Lectins have been implicated in most, if not in all these recognition events. Carbohydrates can interact with lectins via hydrogen bonds, metal coordination bonds and van der Waals and hydrophobic interactions [11].

Lectins can be found in all kingdoms of life ranging from viruses through bacteria and plants to animals [12]. Plant lectins, a unique group of proteins and glycoproteins with potent biological activity, occur in foods like wheat, corn, tomato, peanut, kidney bean, banana, pea, lentil, soybean, mushroom, rice and potato. Many lectins resist digestion, survive gut passage, and bind to gastrointestinal cells and/or enter to circulation intact, maintaining full biological activity. Several lectins have been found to possess anticancer properties in vitro, in vivo and in human case studies; they are used as therapeutic agents, preferentially binding to cancer cell membranes or their receptors [13].

Concanavalin A (Con A, Figure 6.4) isolated from jack bean (*Canavalia ensiformis* L) was the first lectin whose structure became known. The protein consists of 237 amino acids (MW 26 500) and has two metal binding sites. It exists as a dimer at pH<6 while at pH>7 it forms a tetramer which posseses the highest ability to form complexes with saccharides [14]. The structural features of the amino acid chain are two antiparallel beta sheets. There are only minor helical sections [12].

Concanavalin A

Fig. 6.4: Structure of Concanavalin A [12].

In its native state, one molecule of Con A binds two metal atoms: one Ca^{2+} and one ion of a transition metal, usually Mn^{2+} (these ions are necessary to form a complex of Con A-saccharides). The metal atoms are bound by amino acids in a loop that points away from the seven-strand pleated sheet. The ligands for the transition metal are Glu_8, Asp_{10}, Asp_{19} und His_{24}, the calcium ion is bound by Asp_{10}, Tyr_{12}, Asn_{14} and Asp_{19}. The binding site for the sugar (specificity for α-D-Glc, α-D-Man [15]) is adjacent to the metal atoms. Nitrogen atoms from Asn_{14}, Leu_{99}, Tyr_{100}, Asp_{208} und Arg_{228} are involved in fixing the saccharide. In its active state Con A forms aggregates. Joining two six-stranded sheets to an extended twelve-stranded one results in a dimeric molecule. Two dimers form a functional complex by layering side to side the twelve-stranded sheets. The resulting complex now contains four spatially well separated binding sites for oligosaccharides. Thereby it is well apt for building aggregates of, e.g., erythrocytes [146.

The principal function of animal lectins is to act as recognition molecules within the immune system. This may be subdivided into four categories: direct defense (antibody- and/or complement-like), recognition and trafficking within the immune system, immune regulation and prevention of autoimmunity. Perhaps the best-established functions outside the immune system are lysozomal enzyme transport by the phosphomannosyl receptors and the molecular chaperone role of calreticulin in the endoplasmic reticulum [17].

Table 6.1 gives a short overview of some lectins and their specificity.

Tab. 6.1: List of selected lectins from various sources and their sugar specificity.

Lectin: source - name	**Recognition structure**
Bacteria	
Escherichia coli	Man
Salmonella typhimurium	Man
Bacillus anthracis	α-D-Gal
Plants	
Con A (*Canavalia ensiformis* L)	α-D-Man, α-D-Glc
Jacalin lectin (*Artocarpus integrifolia* L)	Gal, Man, NANA
Ricin (*Ricinus communis L*)	GalNAc
Animals	
Cobra venom cardiotoxin	Heparin
Anti-A lectin in snail (*Helix pomatia*)	GalNAc
Ficolin amphibian (*Xenopus laevis*)	GalNAc, GlcNAc

NANA: N-acetylneuraminic acid, GalNAc: N-acetyl-D-galactosamine,
GlcNAc: N-acetyl-D-glucosamine

Lectin affinity chromatography is based on a reversible and highly biospecific interaction of glycoproteins with lectins covalently attached to the surface of a solid support. In particular, sample in a buffered solution is loaded onto a lectin affinity column, where glycoproteins having affinity toward immobilized lectin are bound while other proteins are washed out. Bound glycoproteins are later displaced from the column with an elution buffer usually containing hapten sugar which inhibits the binding. If required, the affinity column can be re-equilibrated with the starting buffer allowing the repeated purification of other samples [18].

A number of authors have utilized the advantages of connecting lectin affinity chromatography to mass-spectrometry (MS). It is possible to fractionate and/or preconcentrate glycoproteins or glycopeptides in a column with bound lectin(s) and then to identify them by highly sensitive MS, a trend in a contemporary glycoproteomics. Although glycoproteins can be fractionated using lectins with different specificity, in most cases lectin affinity chromatography has been employed to isolate the whole glycoconjugates rather than specific structures [19].

The main aim of this project was to optimize a high-performance affinity separation method using concanavalin A as the ligand coupled to two Toyopearl supports, AF-epoxy and AF-tresyl. Commercially available columns are expensive and thus it is important to find an easy and cheap way how to prepare home-made affinity stationary phases.

6.3 Experimental

6.3.1 Samples and chemicals

Samples of p-nitrophenylderivatives of α-D-saccharides (glucoside Glc, galactoside Gal and mannoside Man) and ovalbumin were purchased from Sigma (St. Louis, USA). Lyophilized samples of allergens were provided by Sevac a.s. (Kostelec nad Černými Lesy, Czech Republic). Concanavalin A was obtained from Lectinola, a.s. (Prague, Czech Republic).

The sorbents Toyopearl AF-epoxy 650 M and AF-tresyl 650 M provided Tosoh Bioscience (Tokyo, Japan). Sodium hydrogen carbobate, sodium chloride, ethanolaminehydrochloride, sodium dihydrogen phosphate, di-sodium hydrogen phosphate dihydrate, magnesium dichloride, manganese dichloride and hydrochloric acid were from Pliva-Lachema (Brno, Czech Republic). Tris(hydroxymethyl)amino-methane hydrochloride (Tris.HCl), tris(hydroxymethyl)amino-methane (Tris) were from Merck (Darmstadt, Germany). Calcium dichloride was from International Enzymes (Berkshire, UK). Methyl-α-D-mannopyranoside was from Sigma (St. Louis, USA). All the chemicals were used as received. All buffers and mobile phases were filtered through a membrane filter type HA, pore size 0.45 μm (Millipore Corporation, Bedford, USA) prior to use.

6.3.2 Affinity Chromatography

Liquid chromatographic measurements were carried out using a DIONEX instrument (Westmont, USA) connected with a gradient pump P580 and a UV/VIS detector UVD 170S/340S. All the samples were detected at 280 nm. The flow rate used was in a range of 0.4-1.0 mL/min. The samples were injected through a RHEODYNE

valve (Cotati, USA) with 10 μL sampling loops. The data were handled by Chromeleon software version 6.01 (DIONEX).

Samples of selected saccharides and glycoproteins were dissolved in 0.01 M Tris.HCl buffer, pH 7.5. For p-nitrophenylderivatives, the column was equilibrated with the Tris.HCl buffer pH 7.5 containing 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ until the baseline was stabilized. Samples were then injected and eluted by the same mobile phase. For glycoproteins, the column was equilibrated with the Tris.HCl buffer pH 7.5 containing 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (starting mobile phase) until the baseline was stabilized. Samples were then injected and eluted by the starting mobile phase with an additoin of 0.5 methyl-α-D-mannopyranoside (elution mobile phase). The presence of the salts in a mobile phase is essential for a complex-formation (Con A-saccharide).

All the samples were detected at 280 nm. 4-nitrogroup enables an easy detection of saccharides in the UV range. Glycoproteins and allergens exhibit two absorbing maxima, at 215 nm and 280 nm. The wavelength of 215 nm was not chosen because buffer components also adsorb at this area. A wavenlength of 280 nm is typical for proteins containing aromatic amino acids. A spectrum of ovalbumin is given in Figure 6.3.

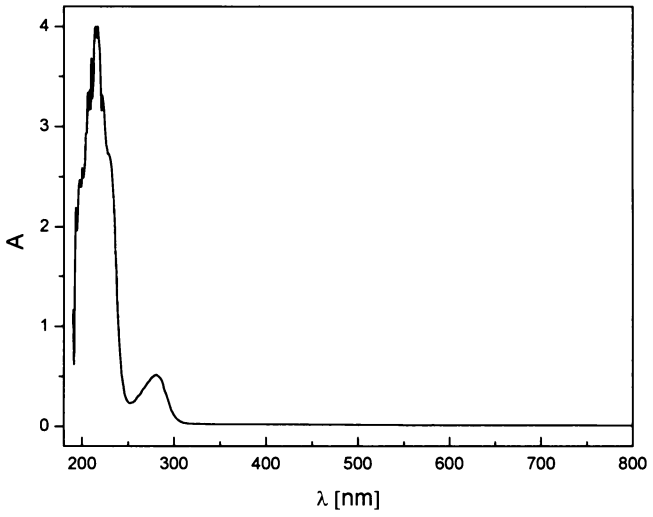

Fig. 6.3: Spectrum of ovalbumine obtained by diode-array detector.

6.3.3 Preparation of stationary phases

The preparation of the stationary phases was based partially on ref. [18] and partially on our previous experience: 80.9 mg of Con A (epoxy) or 80.5 mg of Con A (tresyl) was dissolved in 15 mL of 0.1 M $NaHCO_3$ pH 8.0. The suspension was shaken for 24 hours at 4 °C, washed three times with 50 mL of distilled water and dried on a frit. A small amount of suspension was taken for amino acids analysis. The rest of suspenison was shaken at room temperature in 0.5 M etaholamine hydrochloride pH 8.0 for 3 hours (epoxy) or in 0.01 M Tris.HCl pH 8.0 for 1 hour (tresyl) to block residual active groups of the supports. The mixture was washed three times with 50 mL of distilled water. The stationary phases were packed into a stainless steel columns (80 x 8 mm) in the Watrex company (Prague, Czech Republic).

Based on the results of amino acid analysis, Toyopearl epoxy-Con A stationary phase contained 10.3 mg Con A/g support, while the other phase, Toyopearl tresyl-Con A, 13.2 mg/g. All samples eluted earlier on epoxy activated Toyopearl due to a lower content of Con A.

6.4 Results and discussion

A great variety of HPLC columns for reversed-phase, size exclusion or ion-exchange chromatography is commercially accessible, while the situation in affinity chromatography is different. Only a few commercial affinity stationary phases are available, so they are commonly prepared home-made. A number of chromatographic supports for preparation of affinity phases are available. However, supports applicable to high-performance affinity columns have to maintain a constant volume at higher pressures. Another important condition lies in minimizing non-specific interactions. All these aspects were taken into account when Toyopearl supports were chosen. A plant lectin Concanavalin A was immobilized onto an epoxy- and tresyl-activated support.

The selection of the optimum immobilization pH is of great importance. As follows from Maděra´s results [20], the maximum amount of Con A binds to support HEMA at pH 4.0. However, at this pH Con A is not present in the form of the tetramer which is preferred for its interaction with saccharide moieties. For this reason, pH 8.0 was used in

the immobilization experiments. The amounts of Con A bound to the two supports are higher than in the case of HEMA supports (about 8 mg/g, [20]).

The stationary phases prepared were tested by p-nitrophenyl derivatives of α-D-galactoside, glucoside and mannoside. The retention times and peak areas are summarized in Table 6.2.

Tab. 6.2: Retention times and peak areas of selected p-nitrophenyl derivatives of saccharides on two affinity stationary phases. Experimental conditions: concentration of each sample, 2 mg/mL, injected volume, 10 μL, flow rate, 1.0 mL/min, mobile phase, 0.01 M Tris.HCl buffer, pH 7.5 containing 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, detection at 280 nm.

epoxy Toyopearl-Con A

p-nitrophenyl derivative of	Retention time (min)	Area (mAU*min)
α-D-galactoside	5.895	884
α-D-glucoside	6.594	900
α-D-mannoside	8.436	205

tresyl Toyopearl-Con A

p-nitrophenyl derivative of	Retention time (min)	Area (mAU*min)
α-D-galactoside	6.640	418
α-D-glucoside	8.617	336
α-D-mannoside	15.460	160

As expected, all the selected saccharides eluted earlier on the epoxy Toyopearl-Con A stationary phase. This can be explained by the lower amount of Con A bound to the epoxy Toyopearl support.

A mixture of all the three samples was injected into both the columns. The results are shown in Figure 6.4.

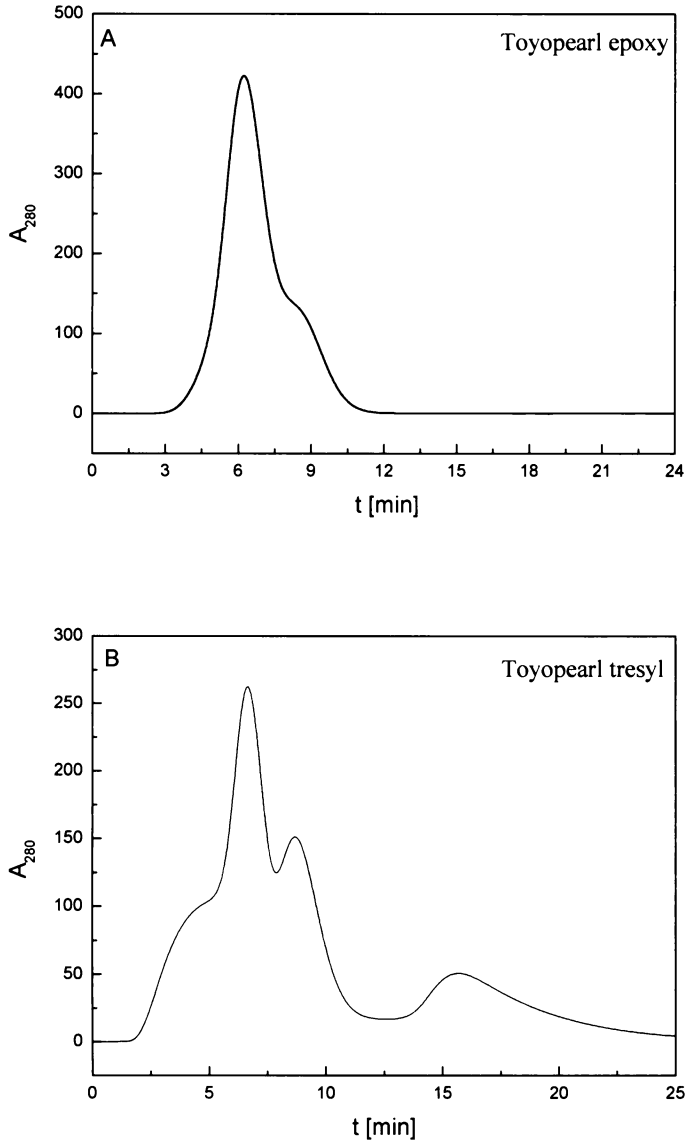

Fig. 6.4: Affinity chromatography of a mixture of Gal, Glc and Man.

A: epoxy Toyopearl-Con A; **B**: tresyl Toyopearl-Con A.

Experimental conditions: concentration of each samples, 2 mg/mL, injected volume, 10 μL, flow rate, 1.0 mL/min, mobile phase 0.01 M Tris.HCl buffer, pH 7.5 containing 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

It is evident from Fig. 6.4 that the epoxy column does not separate p-nitrophenyl derivatives of saccharides. The tresyl column partially separates α-D-galactoside, α-D-glucoside and α-D-mannoside, but the separation efficiency is low. The elution times correspond to affinity of Con A to sugar moiety, their order is α-D-galactoside < α-D-glucoside < α-D-mannoside. Similar results were obtained in ref. [20] using Con A immobilized on HEMA-based supports.

Testing of stationary phases prepared for ovalbumin was the second step in this project. In this case, it was necessary to elute glycoprotein by a gradient of 0.5 M methyl-α-D-mannopyranoside which was added to the starting mobile phase. Different gradient slopes were tested; obtaining the best results by a step change. The course of affinity chromatography of ovalbumin is given in Figure 6.5.

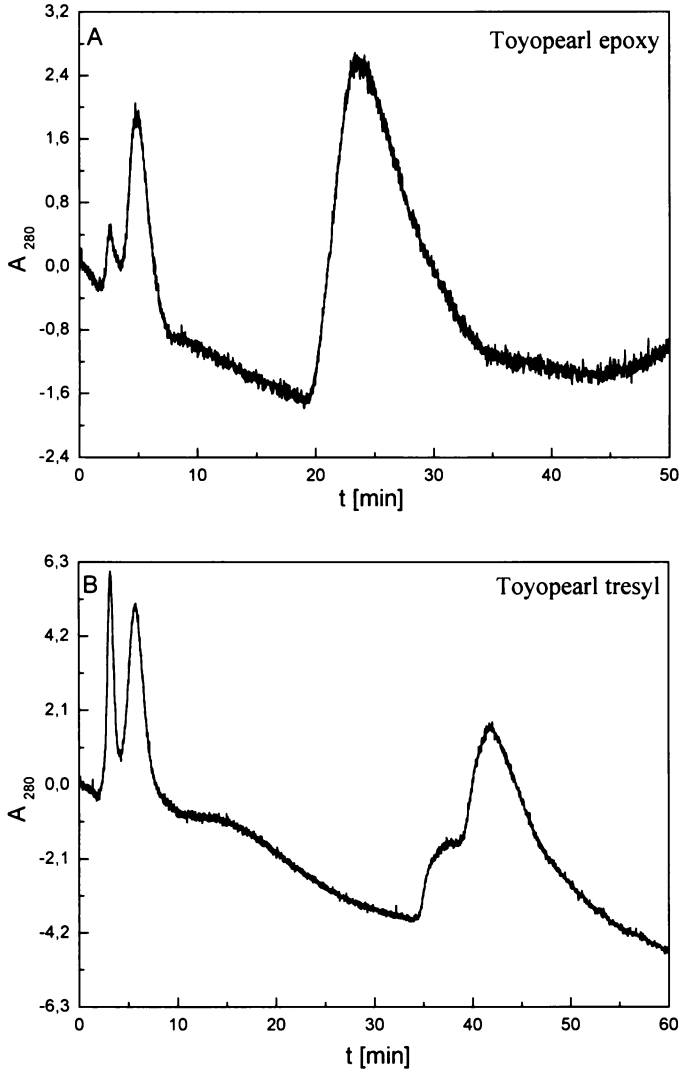

Fig. 6.5: Affinity chromatography of ovalbumin.

A: epoxy Toyopearl-Con A; **B**: tresyl Toyopearl-Con A.

Experimental conditions: concentration of OA, 0.3 mg/mL, injected volume, 100 μL, flow rate, 0.5 mL/min, starting mobile phase, 0.01 M Tris.HCl buffer, pH 7.5 containing 0.15 M NaCl, 1 mMMnCl$_2$, 1 mM CaCl$_2$ and 1 mM MgCl$_2$, elution after 15th min by an addition of 0.5 M methyl-α-D-mannopyranoside.

As expected, ovalbumin eluted earlier on epoxy activated Toyopearl (t_R=23 min - epoxy and t_R=42minut – tresyl). The peaks eluted before 10 min do not interact with Con A whereas the peaks after 10 min represent glycoproteins interacting with Con A.

The stationary phases prepared were tested by selected allergens using optimized experimental conditions. The allergens were eluted by addition of 0.5 M methyl-α-D-mannopyranoside to the starting mobile phase, similar to ovalbumin. The course of pollen allergen *Phleum pratense* separation on the epoxy column is depicted in Figure 6.6.

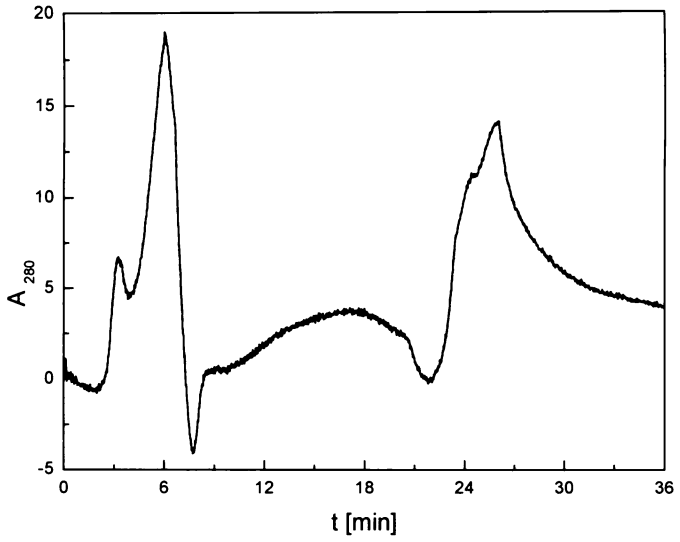

Fig. 6.6: Affinity chromatography of *Phleum pratense* on epoxy Toyopearl-Con A.
Experimental conditions: saturated solution of allergen, injected volume, 100 μL, flow rate, 0.5 mL/min, starting mobile phase, 0.01 M Tris.HCl buffer, pH 7.5 containing 0.15 M NaCl, 1 mMMnCl_2, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, elution after 15th min by an addition of 0.5 M methyl-α-D-mannopyranoside.

The results obtained in this study were compared with those obtained by Maděra [20] who bound Con A on support HEMA GMB 1000 E. The results are similar: a broad peak eluted by the elution mobile phase which can represents the overall curve of glycoforms presented in a sample of allergen.

A similar chromatogram was obtained using a tresyl column. Other allergens were analyzed as well, including, e.g., dog and cat allergens (the data are not shown). Peaks corresponding to glycoproteins were broad and represented unseparated glyforms that cannot be distinguished by this method.

6.5 Conclusion

Glycoproteomics is in the centre of interest of contemporary scientists. Glycosylated proteins are ubiquitous components of eukaryote cellular surfaces, where the glycan moieties are an important part of cell-cell recognition events. Nowadays it becomes clearer that a lot of proteins in mammalian organisms are glycosylated. A wide variety of biological functions, spanning over all possible kinds of protein activities (e.g., hormonal control, immunological protection, enzymatic catalysis, blood clotting, cell adhesion) is one of the reasons responsible for enormous interest in structural and functional analysis of glycoproteins. Changes in glycosylation in some glycoproteins are connected with certain diseases, e.g., with a cancer.

The main goal of this work was to prepare affinity stationary phases with a bound lectin, concavalin A. Con A is well-known plant lectin with a high affinity toward α-D-mannoside and α-D-glucoside residues. This property was utilized for analysis of selected proteins and pollen allergens. This method is useful for this reason. On the other hand, the simple arrangement of affinity chromatography is not satisfactory for a complete fractionation of allergens. It is therefore expedient to combine lectin affinity chromatography with high-sensitivity mass-spectrometry. Using this hyphenated technique, it is, e.g., possible to identify traces of glycoproteins in a small volume of human serum [21].

6.6 References

1. H.F. Holtzclaw Jr., W.H. Robinson: *College Chemistry with Qualitative Analysis.* 8th edition, Lexington, D.C. Heath and Company 2004.

2. D. Voet, J.G. Voet: *Biochemistry.* 3rd edition, Danvers, John Wiley&sons 2004.

3. P. Rupa, Y. Mine: *Biotechnol. Letters* **25** (2003) 1917-1924.

4. http://www.worthington-biochem.com/OA/cat.html (23-8-2006).

5. A.D. Nisbet, R.H. Saundry, A.J. Moir, L.A. Fothergill, J.E. Fothergill: *Europ. J. Biochem.* **115** (1981) 335-345.

6. M. Puc: *Ann. Agric. Environ. Med.* **10** (2003) 143-149.

7. http://plants.usda.gov/factsheet/doc/fs_phpr3.doc (18-9-2006).

8. I. Swoboda, M. Grote, P. Verdino, W. Keller, M.B. Singh, N. de Weerd, W.R. Sperr, P. Valent, N. Balic, R. Reichelt, R. Suck, H. Fiebig, R. Valenta, S. Spitzauer: *J. Immunol.* **172** (2004) 6490-6500.

9. M. Madera, Y. Mechref, M. Novotny: *Anal. Chem.* **77** (2005) 4081-4090.

10. J. Kocourek, V. Horejsi: *Lectins: Biology, Biochemistry and Clinical Biochemistry*, T.C. Bog-Hansen, G.A. Spengler (Eds.), Berlin, Walter de Gruyter 1983.

11. S.S. Komath, M. Kavitha, M.J. Swamy: *Org. Biomol. Chem.* **4** (2006) 973-988.

12. R. Loris: *Biochim. Biophys. Acta* **1572** (2002) 198-208.

13. E.G. de Mejía, V.I. Prisecaru: *Crit. Rev. Food Sci.* **45** (2005) 425-445.

14. A. Kobata, T. Endo: *J. Chromatogr.* **597** (1992) 111-122.

15. B.B.L. Agrawal, I.J. Goldstein: *Biochim. Biophys. Acta - Prot. Str.* **133** (1967) 376-379.

16. http://www.life.uiuc.edu/crofts/bioph354/bergman/1cvn/e1cvnm.htm (4-10-2006).

17. D.C. Kilpatrick: *Biochim. Biophys. Acta* **1572** (2002) 187-197.

18. M. Maděra: *Thesis*. Department of Analytical Chemistry, Charles University in Prague 2006.

19. Y. Mechref, L. Zidek, M.V. Novotny: *Glycobiology* **10** (2000) 231-235.

20. M. Maděra: *Diplomová práce*. Department of Analytical Chemistry, Charles University in Prague 2000.

21. M. Madera. Y. Mechref, I. Klouckova, M.V. Novotny: *J. Proteome Res.* **5** (2006) 2348-2363.

7.

Whey proteins

7.1 Whey proteins

In the development of foods with improved structures and structure stability, a better understanding of how ingredient composition and product processing affect the ultimate properties of the food product is crucial. Because food structure is largely the result of proteins and polysaccharides and their (mixed) complexes, understanding the properties of these compound classes is vital.

Whey proteins are particularly relevant. They belong to the most widely employed proteins in food formulations [1-4] because of their nutritional value and functionality [5]. Whey proteins are commonly used in food products to increase viscosity, to form gels, to stabilize emulsions or foams or to act as water holding agents. Highly significant is their ability to form gels after heat-induced denaturing and aggregation [5]. Milk and dairy products are generally submitted to thermal treatments in order to remove undesirable microorganisms. The most commonly used is pasteurization/sterilization [6]. The milk allergy is connected with three major proteins found in milk: α-lactalbumin (ALA), β-lactoglobulin (BLG) and caseins (CN). However, the heat treatment of milk proteins considerably reduces their allergenicity [7].

Bovine α-lactalbumin (Figure 7.1) is a small compact globular protein with a molecular mass of 14.2 kDa. It is an acidic (pI 4-5), Ca^{2+}-binding whey protein, which consists of 123 amino acid residues [8]. The molecule is stabilized by four disulphide bonds and does not contain free thiol groups. Its content in milk is around 20 %.

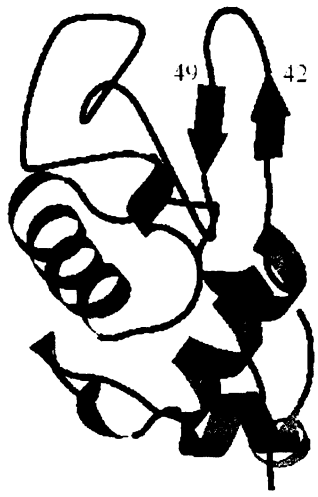

Fig. 7.1: α-lactalbumin (allergenic site Val 42-Glu 49 is indicated in red color) [7].

This protein exists in a number of environment-dependent conformations, including the holo (native, calcium-bound) form, which is the major form under the physiological conditions. Holo form can be converted to the apo (metal-ion-free) form by a low pH (1.7) dialysis or adding an EDTA to a solution of protein [4]. ALA forms high-molecular mass-disulphide oligomers at high pressure (10 kbar, 1 000 MPa) in the presence of low-molecular-mass thiol reducers such as cysteine, mercaptoethanol and dithiothreitol (DTT). These compounds act as initiators of the thiol ↔ disulfide exchange reaction among unfolded molecules of this protein [8]. ALA has been studied as a model for protein folding [9, 10].

Bovine β-lactoglobulin (Figure 7.2) is a globular protein with a molecular mass of 18.3 kDa. Its polypeptide chain is composed of 162 amino acid residues including two disulphide bonds (Cys 66-Cys 160 and Cys 106-Cys 119) and one free cysteine 121 [8]. Its content in milk is around 50 % [3]. Common A and B variants of bovine BLG differ at positions 64 and 118, where Asp and Val in the A variant are substituted by Gly and Ala in the B variant [4]. Molecule of BLG is constituted by nine anti-parallel β-strands and one α-helix. BLG shows a remarkable stability at a low pH, resisting to denaturation even at pH 2, but denatures easily in alkaline pH and aggregates at intermediate pH values undergoing multiple conformational changes [8].

Fig. 7.2: β-lactoglobulin (the allergenic loop Pro 48-Glu 55 is indicated in red color) [7].

Soluble aggregates of polymerized whey proteins are formed during the heat-induced gelation. The subsequent polymerization results in the formation of a rigid gel network which is highly relevant for food structuring. Composition of the aggregates changes during the heat treatment [1]. Clearly the composition and structure of the aggregates can influence the actual properties of dairy products. The SDS-PAGE method was used to assess the BLG/ALA ratio in the aggregates [1, 2]. However, this method has many disadvantages: it is slow, laborious and does not allow studying the composition as a function of aggregate size.

Heat-induced aggregation of whey proteins has been studied [1-5]. Some of the proteins are able to self-aggregate (e.g., BLG; it contains a free thiol group), whereas others are not (e.g., ALA). ALA is devoid of the free cysteinyl residues needed to initiate and propagate the aggregation reaction (see below). However, in the presence of proteins with free thiol groups such as BLG or bovine serum albumin (BSA), ALA may participate in aggregation [6, 11, 12].

BLG plays a crucial role in network formation. It aggregates and is subjected to conformation changes at intermediate pH. At pH 7.5, a reversible transition takes place increasing the reactivity of the free thiol group at Cys 121. Thermal denaturation of BLG occurs in the range of 50-90 °C with different conformational changes. At neutral pH, three steps have been described [6]: (i) Initiation: reversible reactions which lead to monomeric BLG; followed by an irreversible modification of the structure unveiling the free thiol group directed towards the exterior of the molecule. (ii) Propagation: the reactive free thiol group is involved in chemical exchange with one of the two disulphide bridges of another non-reactive molecule of BLG. An intermolecular bridge is formed and a new free thiol group becomes reactive on the second molecule and etc. (iii) End of the reaction: reactive intermediates form a dimer or polymer without reactive free thiol groups. The scheme of this reaction is given in Figure 7.3.

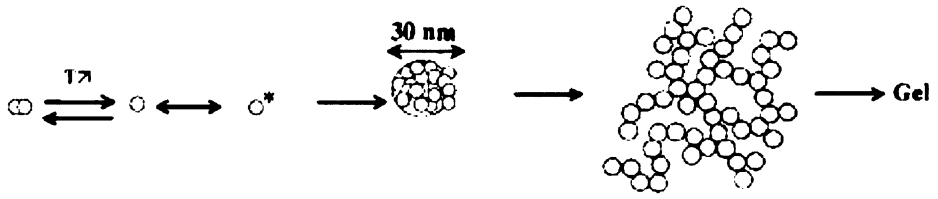

Fig. 7.3: Schematic representation of the aggregation process of BLG at pH 7.0 and 0.1 M salt [13].

In whey and milk systems, the aggregation rate of ALA is slower than that of BLG, and BLG B aggregates slightly faster than BLG A. Almost all BLG is incorporated into the aggregates via disulphide bonds, whereas ALA is bond via disulphide bonds only partly, but to a considerable extent via hydrophobic interactions. A heated mixture of ALA and BLG contains large aggregates that are held together by disulphide bonds and intermediate-sized aggregates that are held together mainly by disulphide bonds and to a lesser extent by non-covalent bonding. In the early stages of heating, aggregates containing relatively more BLG than ALA are formed, whereas, in the later stages, they contain equal amounts of both proteins [4].

ALA exhibits a complex unfolding behavior. Three conformational states are populated under different conditions: the fully folded, native conformation; the fully unfolded state; and a poorly defined compact intermediate state, generally known as the "molten globule" [14]. BLG shows a unique characteristic in its folding. Secondary structure predictions indicated that BLG has a high α-helical propensity (in spite of its predominant β-sheets) determined by local interactions. During the refolding of BLG, intermediates with non-native α-helices accumulate because of the strong local α-helical preference. However, non-local interactions and the overall free energy favor the native β-structure and slow α-helix-to-β-sheet transition produces the native BLG [15].

For the analysis of peptides and proteins in biological samples, one-dimensional separation methods often do not have sufficient selectivity. An increase in selectivity of a multi-dimensional system can be obtained when the dimensions are based on different separation mechanisms. The second dimension of a multi-dimensional system should not destroy the resolution achieved by the previous one. Most separation modes can easily be combined if their mobile phases are compatible. The method of size exclusion chromatography (SEC) is often combined with other chromatographic modes, especially with RPLC. This connection is very useful and comprehensive, mainly with on-line mass-detection [17].

SEC is a commonly used for quantitative assessment of aggregate size and content. However, it has several limitations. First, since an aggregated proteins sample prior to injection is usually filtered to remove any particulates, SEC method is limited for the determination of soluble aggregates. Second, the size of a protein or its aggregates can be overestimated by this method if protein is not spherical (such as highly coiled proteins) and its Stokes radius is greater than that of a globular protein. Third, if a protein contains carbohydrates or interacts with the stationary and mobile phase (non-ideal behavior), the

elution profile of the protein may change, leading to an erroneous estimation of its molecular weight. To solve these issues, SEC can be coupled with detectors (light scattering (LSD) or refractive index detectors (RID)), which enable the absolute determination of the size of a protein and its aggregates [18].

Almost all authors dealing with whey proteins employed the SEC for studies of their aggregation upon heat treatment. Different composition of protein solutions (ALA, BLG A, BLG B, BLG, CN, BSA) and various heating time were studied by SEC with a different detection mode (either multi-angle laser light scattering MALLS [2, 3, 5] or UV detector [1, 5, 8]).

The main aim of this project was the development of two-dimensional size exclusion chromatography for study of the aggregation behavior of whey proteins upon heat treatment at neutral pH. The objective of the work was to determine the ratio BLG/ALA in mixed aggregates as a function of particles size.

The first dimension is used to separate aggregates from single protein molecules based on differences in the size of the "particles". In the second dimension a separation according to the ratio BLG/ALA is performed. This main goal is schematically depicted in Figure 7.4.

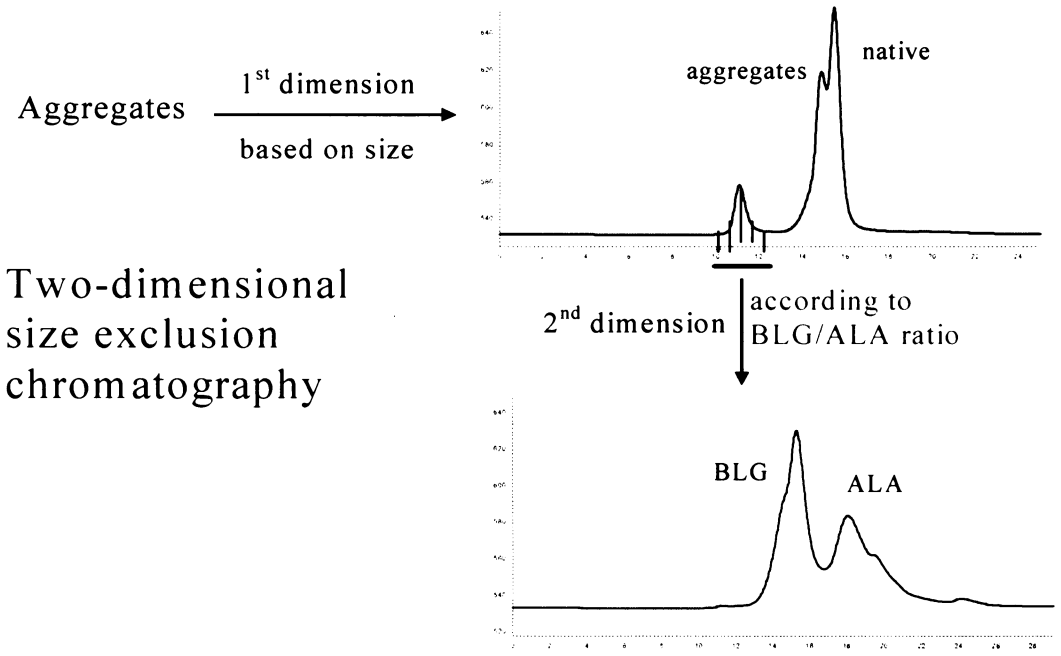

Fig. 7.4: A scheme of the two-dimensional SEC for the determination of BLG/ALA ratios.

For assessing the ratio BLG/ALA, two different methods were investigated: 1) the conversion of aggregates back into the native proteins and 2) the full denaturation of whey proteins. The scheme of the overall procedure is shown in Figure 7.5.

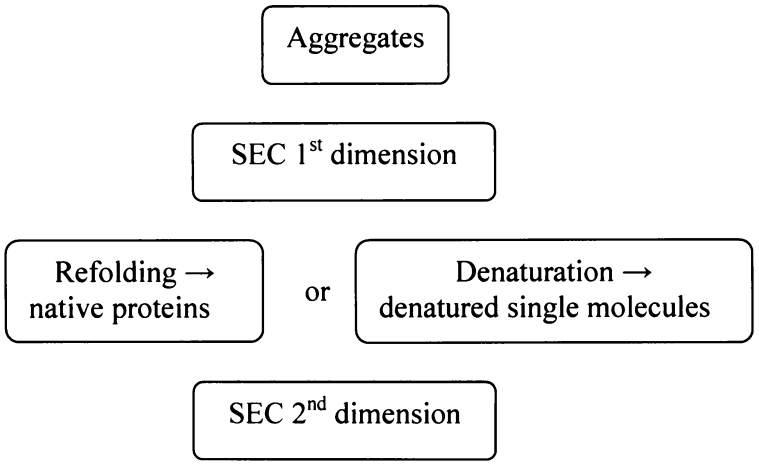

Fig. 7.5: Overall procedure for two-dimensional size exclusion chromatography.

7.2 Experimental

7.2.1 Samples, chemicals, mobile phases and buffers

Samples and chemicals
All samples of whey proteins are listed in Table 7.1.

Tab. 7.1: List of samples used.

Samples	**Origin**	**Specification**	**Supplier**
α-lactalbumin	bovine milk	type III, ≥85 % (PAGE)	Sigma[1] (L6010)
β-lactoglobulin A	bovine milk	min. 90% (PAGE)	Sigma (L7880)
β-lactoglobulin B	bovine milk	min. 90% (PAGE)	Sigma (L8005)
β-lactoglobulin A and B	bovine milk	min. 90% (PAGE)	Sigma (L2506)

[1] Sigma-Aldrich, Steinheim, Germany

DL-dithiothreitol (DTT), α-mercaptoethanol and urea were purchased from Sigma-Aldrich (Steinheim, Germany). Tris(hydroxymethyl)amino-methane hydrochloride (Tris.HCl), tris(hydroxymethyl)amino-methane (Tris), ethylenedinitrilotetraacetic acid (titriplex II, EDTA), L-cysteine, L-cystine, calcium chloride dihydrate, guanidinium chloride (Gdn.HCl), sodium dodecyl sulphate (SDS), methanol, acetone, hydrochloric acid, sodium hydroxide, sodium azide, sodium dihydrogen phosphate, disodium hydrogen phosphate dihydrate and sodium chloride were from Merck (Darmstadt, Germany). All the chemicals were used as received.

Mobile phases and buffers

Various solutions and mobile phases were tested in experiments. The composition of solutions used is given in Table 7.2. All buffers and mobile phases were filtered through a membrane filter type HA, pore size 0.45 μm (Millipore Corporation, Bedford, USA) prior to use.

Tab. 7.2: Composition of the mobile phases and buffers.

Buffer	**Composition**	**pH**
50 mM NaCl + 20 mM NaN_3	50 mM NaCl, 20 mM NaN_3	7.0
30 mM phosphate buffer	30 mM NaH_2PO_4, 30 mM $Na_2HPO_4.2H_2O$	6.7
50 mM phosphate buffer	50 mM NaH_2PO_4, 50 mM $Na_2HPO_4.2H_2O$	7.0
50 mM NaCl	50 mM NaCl	7.0
20 mM NaN_3	20 mM NaN_3	7.0
Refolding buffer	0.1 M Tris.HCl, 50 mM NaCl, 10 mM $CaCl_2$, 2 mM cysteine and 2 mM cystine	7.0
Denaturation buffer	0.1 M Tris.HCl, 6 M Gdn.HCl, 10 mM DTT, 1 mM EDTA	7.0
SDS buffer I	0.1 M Tris.HCl, 10 mM SDS, 5% mercaptoethanol	6.8
SDS buffer II	0.1 M Tris.HCl, 20% SDS, 5% mercaptoethanol	6.8
Tris.HCl	0.1 M Tris.HCl	6.8

7.2.2 Size exclusion chromatography

A Shimadzu Liquid chromatograph LC-10Ai (Kyoto, Japan) was used for all HPLC experiments. All mobile phases were degassed through a Shimadzu DGU-14 A degasser. A Shimadzu SIL-10AD VP autoinjector was used for sample injection. The injection volume was in the range of 5 to 20 µL. UV detection was performed with a Shimadzu dual wavelength UV/VIS detector operated at 215 and 280 nm. Different columns were used (see Table 7.3; all purchased from Tosoh Bioscience, Tokyo, Japan). The columns were thermostated at 20 °C using a MISTRA Thermostat (Spark Holland, Emmen, The Netherlands). The flow rate was 0.5 mL/min. A Series 900 Interface (PE Nelson, PerkinElmer Inc., Wellesley, USA) was used for data collection. All data were recorded and analyzed using TotalChrom, Version 6.2.1 for Windows 2000 (PerkinElmer).

Tab. 7.3: Overview of chromatographic columns [19].

Column	**TSKgel G1000PW**	**TSKgel G2500PWXL**	**TSKgel G3000PWXL**	**TSKgel G5000PWXL**
Dimension	7.8 mm ID 30.0 cm L	7.8 mm ID 30.0 cm L	7.8 mm ID 30.0 cm L	7.8 mm ID 30.0 cm L
Particle size (µm)	6.0	6.0	6.0	10.0
Pore size (nm)	10	<20	20	100

7.2.3 Sample preparation and instrumental procedure

Aggregate formation

In the initial experiments, aggregates were prepared following the procedure described by Schokker *et al.* [3] and de la Fuente *et al.* [5]. In our experiments it turned out that occasionally none, or only a very low amount of aggregates was found when using these procedures. Therefore, the procedure was adapted slightly as described below.

Samples of whey proteins (ALA and BLG) were dissolved (separately or as mixtures in different ratios) in water or phosphate buffer. Solutions were heated in Eppendorf vials at different temperatures in a water bath. The heating time was varied

from 10 to 60 min. After that samples were cooled to room temperature. Samples were stored at 4 °C maximally for one week.

Study of the composition of aggregates

A. Refolding

For refolding, i.e., converting aggregates into single native molecules, heat-treated samples were dissolved in a refolding buffer (0.1 M Tris.HCl etc.) and injected onto a TSKgelG5000PWXL column (100 nm) using the same refolding buffer as the mobile phase.

B. Denaturation

Three different denaturation solutions were tested: denaturation buffer, SDS buffer I and SDS buffer II (Tab. 7.2).

Denaturation buffer

Samples were directly dissolved in the denaturation solution and injected into a SEC system operated with the same denaturation buffer as the mobile phase.

SDS buffers

Samples containing aggregates were diluted by SDS buffer in a ratio 1:1 and heated for different times in boiling water (0, 5, 10 and 20 minutes). After that the samples were filtered through a 0.2 μm filter (DynaGard filter mixed ester, surface area 2.5 cm^2, Omnilabo International B.V., Breda, the Netherlands). Samples were either analyzed directly after filtration or kept at room temperature overnight prior to analysis.

7.3 Results and discussion

7.3.1 Isolation of the aggregates from single molecules

As outlined in Fig. 7.2, the first step in the overall procedure is the isolation of the aggregates from the single, native and denatured protein molecules. This task is accomplished in the first dimension of the two dimensional SEC system. The main idea of this procedure is depicted in Figure 7.6.

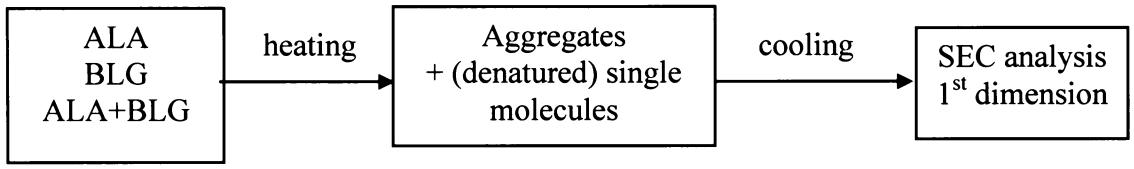

Fig. 7.6: Schematic representation of the preparation of aggregates.

For the isolation of aggregates from single molecules of ALA and BLG, two different columns with a dissimilar pore size (20 nm and 100 nm) were tested in the first dimension. They were selected on the basis of the molecular weights of the proteins studied (according to Tosoh Biosciences guide [19]). Table 7.4 gives a summary of the mobile phases employed in these experiments [2, 4].

Tab. 7.4: Mobile phases tested for the separation of aggregates from (single) BLG and ALA.

Mobile phases
50 mM NaCl + 20 mM NaN_3
30 mM phosphate buffer
50 mM phosphate buffer
50 mM NaCl
20 mM NaN_3

Aggregates were prepared using the method as described in experimental (see section 7.2.3). ALA and BLG at 2 mg/mL, each in a PBS buffer, were heated to 75 °C for increasing times to obtain different levels of aggregation. The separations of aggregates from ALA and BLG on the two SEC columns (20 nm and 100 nm) are shown in Figure 7.7. The best results were obtained with a 50 mM phosphate buffer pH 7.0 as the mobile phase.

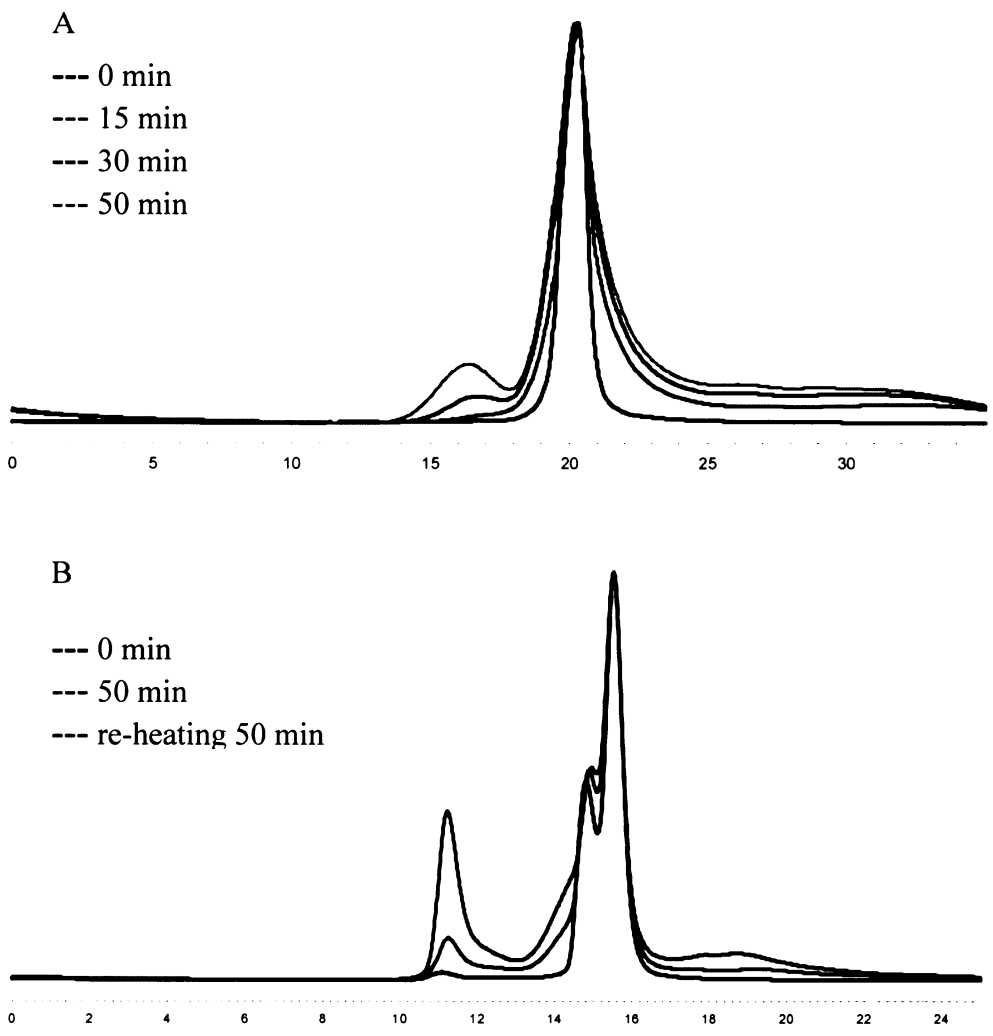

Fig. 7.7: Separation of mixed ALA/BLG aggregates from remaining single molecules.

A: 100 nm column; ALA and BLG 2 mg/mL in PBS; heated various times at 75 °C; mobile phase 50 mM PBS, pH 7.0; for other conditions see Experimental. First peak ~ aggregates, second peak ~ remaining single molecules.

B: 20 nm column; other conditions as in Fig. 7.7 A.

As follows from Fig. 7.7 A and B, good separations of the aggregates from single proteins were obtained on both the columns. For all further experiments, 100 nm column was used for the first dimension separation.

To illustrate that the proposed SEC system can be used for studying whey protein aggregation behavior upon heat treatment, the method was investigated in detail. As follows from Fig. 7.7, aggregate formation is dependent on heating times. With increasing heating time the peak of the aggregates increases. BLG A shows less aggregate formation compared to BLG B. Aggregate formation is more pronounced in a buffer solution compared to water (data not shown).

During the experiments it turned out that aggregate formation was poorly reproducible. In general, the data in the literature regarding aggregation treatment are also not in agreement [3, 5, 6]. According to our experiments, the following procedure leads to reproducible aggregate formation: 1) dissolve proteins in PBS buffer in a range of 1 to 10 mg/mL and 2) apply heat treatment for 45-50 min at 75 °C.

7.3.2 Methods for the studying composition of the aggregates

Determination of the molecular composition of the aggregates requires conversion of the aggregates back into single ALA or BLG molecules followed by their separation and quantification. A problem with the separation of single protein molecules lies in the fact that their elution behavior depends not only on the type of molecule (e.g., ALA *vs.* BLG), but also on its conformation. A good separation is obtained if all molecules of one type are in the same conformation.

In studies of the composition of the aggregates, two different conversion strategies were investigated. The first approach was based on the conversion of the aggregates back into the single fully refolded native molecules (refolding), the second on full denaturation of ALA and BLG (denaturation).

A. Refolding

Refolding was carried out by treating the aggregates with a refolding buffer. The composition of the refolding buffer was taken from work by Yagi *et al.*: [15; similar refolding buffers were also used by other authors for other proteins 16-18]. The refolding buffer proposed by these authors consisted of 0.1 M Tris.HCl, 50 mM NaCl, 10 mM $CaCl_2$, 2 mM cysteine and 2 mM cystine, pH 7.0.

Refolding was studied in two set-ups:

1. Inject the aggregates into a SEC system using the refolding buffer as the mobile phase ⇒ **SEC refolding**.
2. Dilute the aggregates with the refolding buffer and allow a certain time for the reaction ⇒ **dilution refolding**.

A schematic representation of the overall procedure is given in Figure 7.8. Chromatograms of SEC and dilution refolding are showed in Figure 7.9.

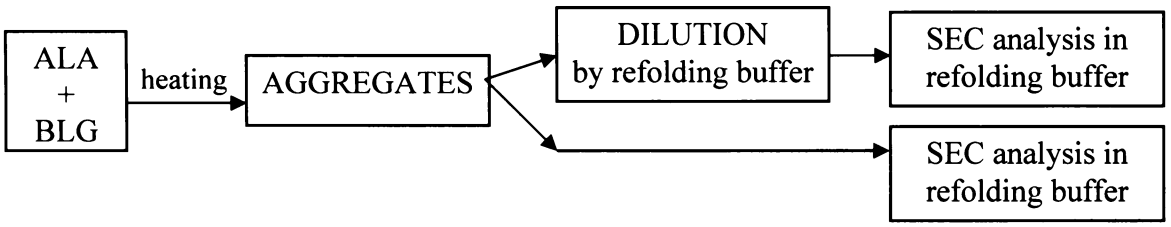

Fig. 7.8: Scheme of refolding and compositional analysis of whey protein aggregates.

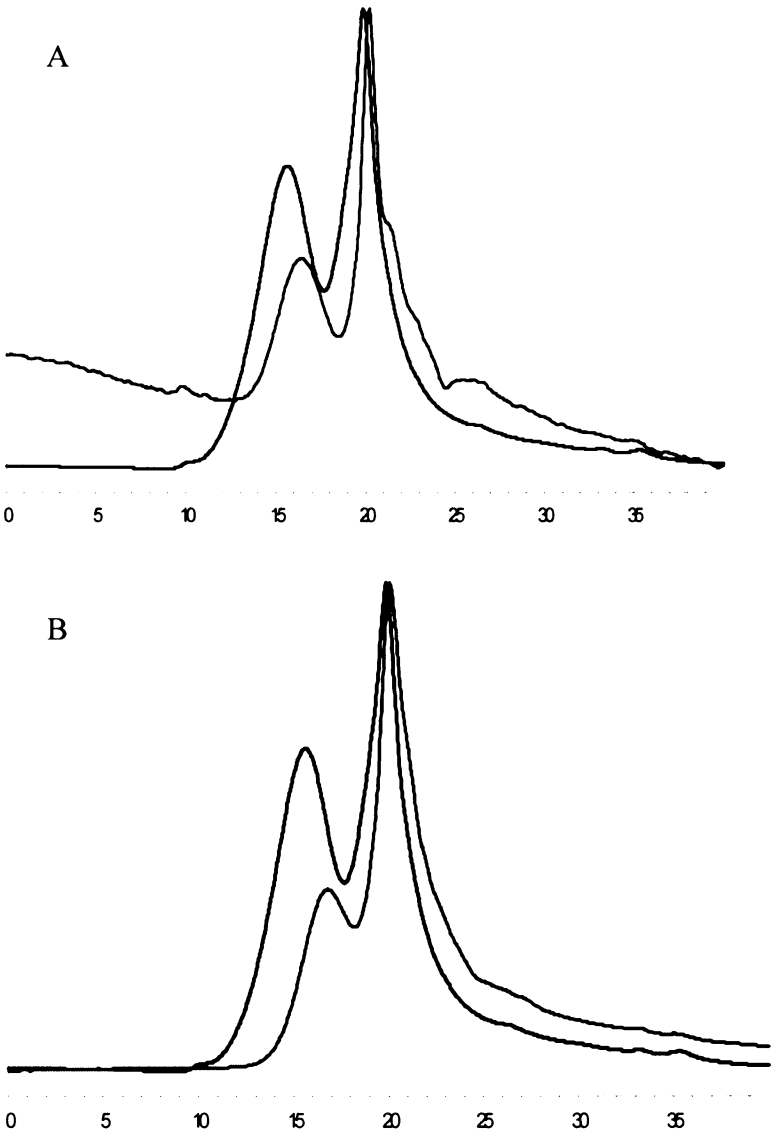

Fig. 7.9: Refolding of ALA and BLG.

A: SEC refolding **B**: dilution refolding; ALA + BLG 5 mg/mL PBS, pH 7.0, heated 50 min at 75 °C; mobile phase --- PBS pH 7.0 ---//--- refolding buffer pH 7.0; TSKgelG3000PWXL.

The chromatograms in Fig. 7.9 show that refolding is far from complete, both in SEC refolding as well as in dilution refolding (first peak corresponds to aggregates, second peak to native molecules). Comparing the peak areas of aggregates measured in PBS buffer (black line in Fig. 7.9 A) and refolding buffer (red line in Fig. 7.9 B), it was found that only approximately 48% of the aggregates were refolded (SEC refolding). A small time shift indicative of a size reduction can also be observed. This can be explained by the fact

that refolding of proteins is a very complex process and the ultimate protein form is probably not exactly the same as at the beginning. Conformational changes may occur during refolding treatment.

To study the influence of refolding time in dilution refolding SEC, a series of runs was performed with increased refolding times (Figure 7.10). It follows from the results that the only important difference is between the first and second analysis. No further reduction of the peak of the aggregates was observed after 90 minutes.

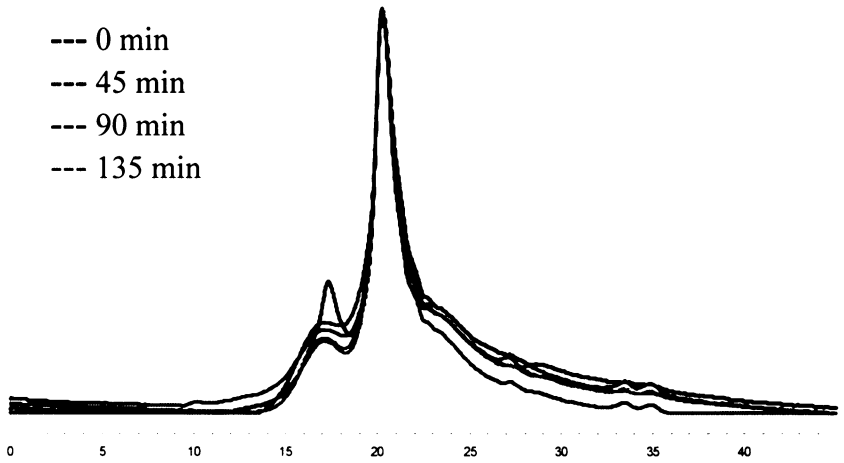

Fig. 7.10: Influence of the allowed refolding time on the aggregate level.

ALA and BLG 5 mg/mL PBS pH 7.0; heated 50 min 75 °C (aggregation); mobile phase refolding buffer, pH 7.0.

These results illustrate that only partial refolding occurs. Apparently the aggregates are very stable and resist refolding for prolonged periods. It can be concluded that refolding yielding identical molecules cannot be used for our purpose. Conversion to the fully refolded native proteins is far from complete. Therefore, we tested the second approach called denaturation.

B. Denaturation

Full denaturation of the whey protein aggregates to give fully denatured single molecules is the second option for studying the aggregate composition. The principle of this approach is straightforward. Aggregates isolated from heated solutions are dissolved in a buffer that destroys all inter- and intramolecular bonds. Three different denaturation solutions were tested. The compositions of these buffers were taken from the literature:

denaturation buffer pH 7.0 [9, 15, 20], SDS buffers I and II [1, 2]. SDS buffers are commonly used in SDS-PAGE separations of proteins. More details on the composition of these buffers are given in Tab. 7.2.

Denaturation buffer

Denaturation experiments were carried out using the denaturation buffer as the mobile phase. Aggregates were again prepared by heating solutions of ALA and BLG in PBS buffer (5 mg/mL each) for 50 min at 75 ° C and their solutions were injected into a SEC system. Chromatograms of the aggregates obtained on two different columns are shown in Figure 7.11.

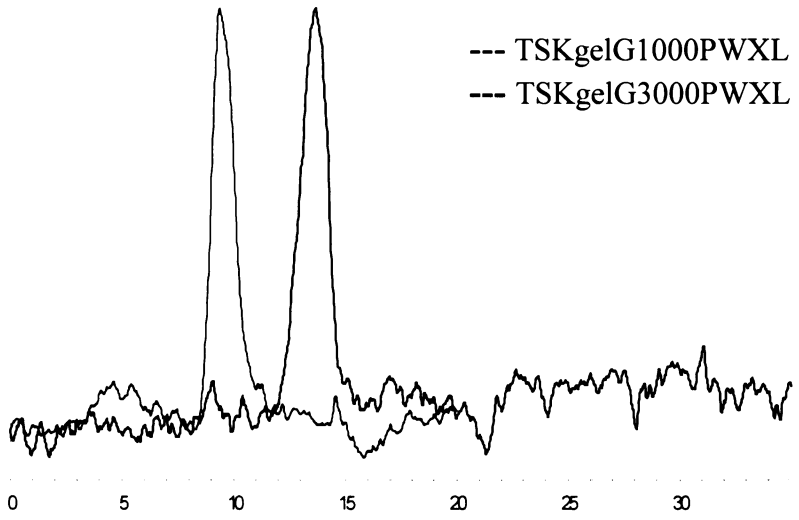

Fig. 7.11: Denaturation of ALA/BLG aggregates in a denaturation buffer using two columns.

ALA and BLG 5 mg/mL PBS pH 7.0; heated 50 min at 75 °C (aggregation); mobile phase denaturation buffer, pH 7.0.

On both the columns (20 nm, 100 nm), only one peak is seen. It is not certain, whether this peak still contains aggregates. However, no separation between ALA and BLG is obtained. Therefore, ALA and BLG were injected separately, but they were not separated as well. It can be concluded that this denaturation buffer might possibly give full conversion of aggregates into single denatured molecules, however, this cannot be proved as the buffer does not allow their separation. Therefore, it is useless for establishing BLG/ALA ratios. For this reason SDS buffers were tested.

SDS buffers

Two SDS buffers containing different concentrations of SDS were tested (for detailed information see Tab. 7.3.) [1, 2]. The protocol for sample treatment in these buffers is schematically depicted in Figure 7.12.

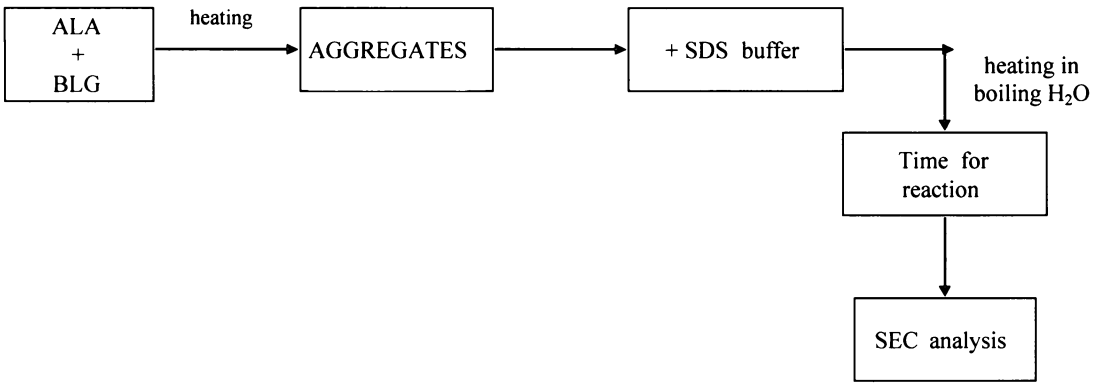

Fig. 7.12: Schematic procedure for denaturation and analysis of whey proteins in SDS buffers.

SDS buffer I

The concentration of SDS was 10 mM. Chromatograms of ALA, BLG and aggregates in this buffer are shown in Figure 7.13. Single proteins or their aggregates were dissolved in the SDS buffer I (at 15 mg/mL), heated in boiling water for 5 minutes and injected into the SEC system using the same buffer as the mobile phase.

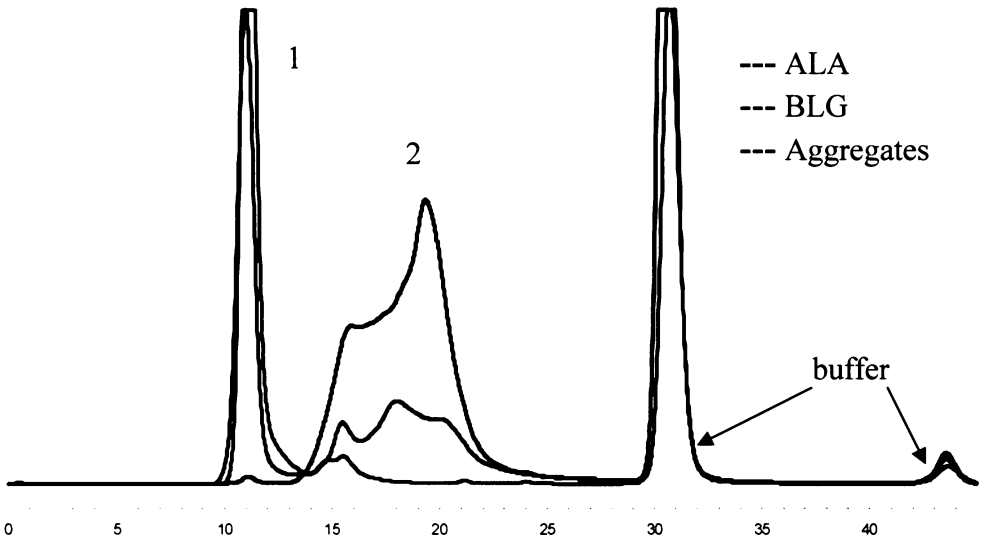

Fig. 7.13: Denaturation of whey proteins in SDS buffer I. Aggregates heated 50 min at 75 ° C; added 1 mL SDS buffer I; all samples denatured in boiling water for 5 min. prior to injection; mobile phase Tris.HCl, pH 6.8; TSKgelG3000PWXL.

From comparison of the chromatograms in Fig. 7.12 it follows that there is some separation between ALA and BLG (peak No. 2) but the resolution is clearly insufficient (second peaks). Moreover, the peak corresponding to ALA (black line) is very broad (more than 5 min.) and has a distorted shape. Increased denaturing times (in boiling water) were attempted (tested for pure ALA, pure BLG, a mixture of ALA and BLG and for aggregates). Chromatogram is shown in Figure 7.14.

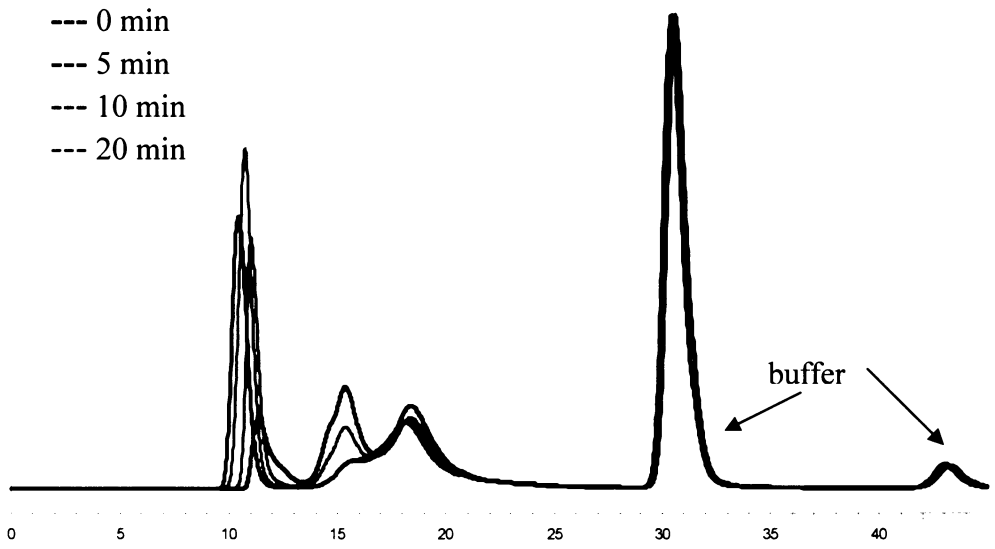

Fig. 7.14: Denaturation of aggregates in SDS buffer I – influence of denaturing times.

ALA + BLG 5 mg/mL PBS; heated 50 min at 75 ° C (aggregation); added 1 mL SDS buffer I; different heating time in boiling H_2O; mobile phase Tris.HCl, pH 6.8; TSKgelG3000PWXL. Aggregates preparation: 1 mL of the aggregate solution was mixed with 1 ml of SDS buffer I, heated for different times in boiling water and kept over night at room temperature for stabilization before analysis.

SDS buffer II

Because the resolution of ALA and BLG using SDS buffer I as the mobile phase was not satisfactory, higher concentrations of SDS were tested. The concentration of SDS in this buffer was 20%. Chromatograms of ALA, BLG and their mixed aggregates in this buffer are shown in Figure 7.15. Sample pre-treatment was the same as described for SDS buffer I.

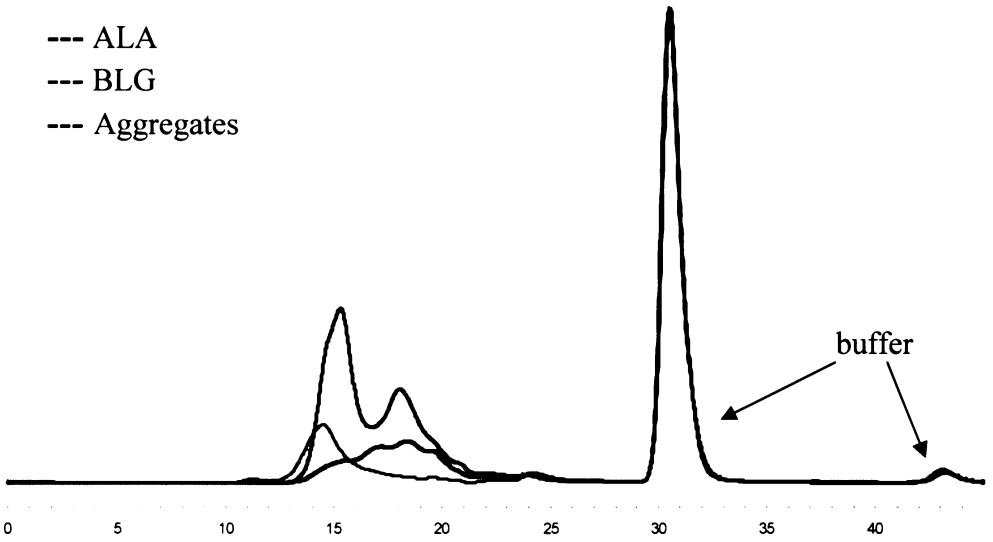

Fig. 7.15: Denaturation of whey proteins in SDS buffer II. Aggregates heated 50 min at 75 °C; added 1 mL SDS buffer II; all samples denatured in boiling water for 5 min. prior to injection; mobile phase Tris.HCl, pH 6.8; TSKgelG3000PWXL.

A series of experiments with various heating times was performed in SDS buffer II for pure ALA, pure BLG, a (non-heated) mixture of ALA and BLG and for aggregates. For the mixed aggregates only the un-heated samples was different. All heated samples of aggregates were separated into two denatured individual proteins (data are not shown). The chromatograms obtained for the samples are independent of the applied denaturation time (so for next experiments the denaturation time of 5 min. was used).

From the results obtained with SDS buffer II it can be concluded that this method gives the desired information. Aggregates after denaturation yield single peaks for ALA and BLG.

7.5.3 Composition of the aggregates

Quantification of ALA and BLG was necessary for determination of the aggregate composition. Calibration curves for ALA and BLG were constructed by dissolving different amounts of the pure components in SDS buffer II, heat the sample for 5 minutes in boiling water, wait overnight and analyze the denatured samples by SEC using Tris.HCl pH 6.8 as the eluent. The calibration curves are shown in Figure 7.16 A and B.

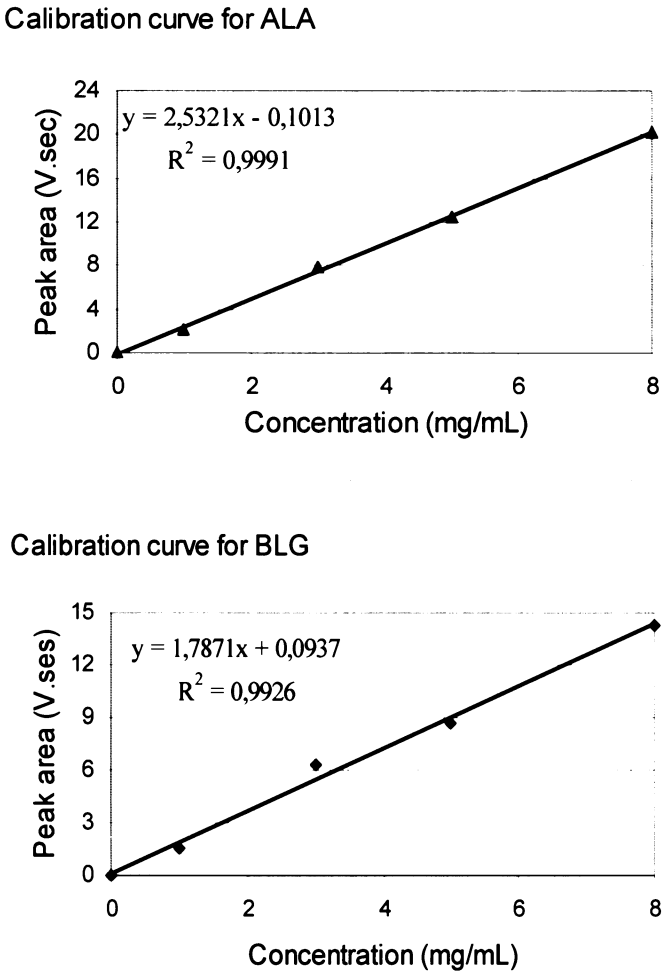

Fig. 7.16: Calibration curves for ALA and BLG.

A: ALA; **B**: BLG; conditions for samples see Fig. 7.15.

The determined concentrations of ALA and BLG were 2.17 mg/mL and 2.80 mg/mL, respectively, and their ratio, BLG/ALA, approximately 1.3 (their original ratio before aggregation was 1.00). This value is in good agreement with the literature: Kazmierski *et al.* [2] published a value of 1.69 for aggregates prepared at 75 °C with a heating time of 30 min. Dalgleish *et al.* [1] published the value close to the original ratio.

7.4 Conclusion

The understanding of whey protein aggregation is important in the food industry as they are widely used for their useful properties, e.g., form gels, stabilize emulsions or act as a waterholding agent. In future, it will be desirable to develop new milk products with a higher nutritional value and lower undesirable properties.

For the study of the composition of mixed aggregates of whey proteins, two different options were studied: conversion of the aggregates back into the native protein molecules (refolding), or full denaturation of the whey proteins followed by quantification in the denatured state (denaturation). The denaturation approach led to the establishment of the ratio BLG/ALA. Buffers containing SDS and mercaptoethanol were found appropriate for this purpose. Using this method the ratio BLG/ALA of a mixed aggregate was established. A value of 1.29 was found.

It can be concluded the two-dimensional size exclusion chromatography method combined with a denaturation approach is suitable for an estimation of BLG/ALA ratio in mixed-heated aggregates.

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7.5 References

1. D.G. Dalgleish, V. Senaratne, S. Francois: *J. Agric. Food Chem.* **45** (1997) 3459-3464.
2. M. Kazmierski, M. Corredig: *Food Hydrocolloid* **17** (2003) 685-692.
3. E.P. Schokker, H. Singh, L.K. Creamer: *Int. Dairy J.* **10** (2000) 843-853.
4. Y.-H. Hong, L.K. Creamer: *Int. Dairy J.* **12** (2002) 345-359.
5. M.A. de la Fuente, Y. Hemar, H. Singh: *Food Chem.* **86** (2004) 1-9.
6. C. Bertrand-Harb, A. Baday, M. Dalgalarrondo, J.-M. Chobert, T. Haertlé: *Nahrung/Food* **46** (2002) 283-289.
7. S. Sharma, P. Kumar, C. Betzel, T.P. Singh: *J. Chromatogr. B* **756** (2001) 183-187.
8. K. El-Zahar, M. Sitohy, M. Dalgalarrondo, Y. Choiset, F. Métro, T. Haertlé, J.-M. Chobert: *Nahrung/Food* **48** (2004) 177-183.
9. R. Schlegl, G. Iberer, C. Machold, R. Necina, A. Jungbauer: *J. Chromatogr. A* **1009** (2003) 119-132.
10. R. Wijesinha-Bettoni, C.M. Dobson, C. Redfield: *J. Mol. Biol.* **312** (2001) 261-273.
11. J.I. Boye, I. Alli: *Food Res. Int.* **33** (2000) 673-682.
12. J. Gezimati, L.K. Creamer, H. Singh: *J. Agric. Food Chem.* **45** (1997) 1130-1136.
13. P. Croguennoc, T. Nicolai, D. Durand: *Langmuir* **17** (2001) 4380-4385.
14. J.J. Ewbank, T.E. Creighton: *Biochemistry* **32** (1993) 3677-3693.
15. X. Yagi, K. Sakurai, C. Kalidas, C.A. Batt, Y. Goto: *J. Biol. Chem.* **278** (2003) 47009-47015.
16. J. M. Rodriguez-Nogales, M. Concepción Garcia, M.L. Marina: *J. Sep. Sci.* **29** (2006) 197-210.
17. T. Stroink, M.C. Ortiz, A. Bult, H. Lingeman, G.J. de Jong, W.J.M. Underberg: *J. Chromatogr. B* **817** (2005) 49-66.
18. W. Wang: *Int. J. Pharm.* **289** (2005) 1-30.
19. http://www.tosohbiosep.de/contents.htm (10-11-2004).
20. B. Batas, J.B. Chaudhuri: *Biotechnol. Bioeng.* **50** (1996) 16-23.

8.

Conclusion

Substances studied in this work exhibit various physico-chemical properties as well as various chromatographic behavior. However, they all belong among the group of biologically active compound - proteins. They have enormous importance for life and thus are in the centre of attention of scientists. The dynamic and fast growing field of proteomics covers all aspect of study of proteins including decoding of gene expression.

This Thesis describes several chromatographic methods for protein analysis. It does not cover any hyphenated technique. In my opinion, in near future it will be impossible to make fundamental discoveries without using modern high-performance hyphenated techniques, such as LC-MS, LC-MS/MS. For a very small amount of proteins it is necessary to use high-sensitivity MS and capillary liquid chromatography (LC) or even liquid chromatography on a chip. All these instruments are commercially available but their cost is very high. However, their possibilities are really great. It is possible to analyze extremely small amounts of proteins, peptides and their glycosylated forms in complex samples (e.g., protein level at pg/mL in serum sample). It is rewarding for biomarker discoveries. It can bring wonderful progress in medicine because it can provide for easy and non-invasive analyses of proteins/glycoproteins in blood samples of patients with cancer or other dangerous diseases.

9.

Appendix

List of papers enclosed.

Paper I:

Preparation and testing of stationary phases and modified capillaries for affinity chromatography and affinity capillary electrophoresis of pepsin.

Paper II:

Affinity liquid chromatography and capillary electrophoresis of seminal plasma proteins.

Paper III:

Separation media in affinity chromatography of proteins – a critical review.

The print quality of this paper is low because Bentham Science Publishers Ltd. did not provide the final manuscript in pdf format. For this reason it was necessary to scan the original paper and print it out.

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Preparation and testing of stationary phases and modified capillaries for affinity chromatography and affinity capillary electrophoresis of pepsin

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Abstract

Three stationary phases have been prepared for affinity liquid chromatography isolation and separation of porcine and human pepsin. The phases contain 3,5-diiodo-L-tyrosine (DIT) bound to the supports HEMA BIO VS, HEMA BIO E and EPOXY TOYOPEARL. These phases have been tested on a model sample of porcine pepsin A and applied to human pepsin. Fractions have been collected and the chymase activity determined in selected analyses. For affinity CE, capillaries have been prepared by modifying the wall with 3-aminopropyltriethoxysilane, followed either by direct binding of DIT, or by binding L-tyrosine that was subsequently iodated. The dissociation constant K_d has been determined for the pepsin–DIT complex from the changes in the electrophoretic mobilities.

Keywords: 3,5-Diiodo-L-tyrosine; Pepsin; Affinity liquid chromatography; Affinity capillary electrophoresis

1. Introduction

Pepsins are enzymes that belong among aspartate proteases; as most of them exhibit optimum activities in acidic media, they are also called acidic proteases. Pepsin is produced in vertebrates by stomach mucous membrane in the form of zymogene (pepsinogen) that is converted into pepsin at a low pH [1–4]. The presence and relative concentrations of pepsin A (EC 3.4.23.1, pepsin) and pepsin C (EC 3.4.23.3, gastricsin) vary in vertebrates in dependence on the species and genetic variation; in humans, they also vary during some stomach diseases [2]. The ratios among individual forms of human aspartate proteases and their zymogens constitute important diagnostic tools [2,4].

Pepsins A and C differ in their biochemical and immunological properties. Their molecular masses are around 35,000, or higher with pepsinogens (around 42,000) [5,6]. Pepsin A exhibits a high affinity toward 3,5-diiodo-L-tyrosine (DIT) [7,8]. The properties of porcine pepsin A are very similar to those of human pepsin, as can be seen in Table 1 [5,9].

Affinity chromatographic and electrophoretic techniques, based on a high selectivity of biospecific interactions and a high separation efficiency, play an important role in isolation of pepsins and pepsinogens and substantially extend the possibilities over other existing chromatographic methods used so far for pepsin isolation and analysis (see, e.g. references [10–13]). An increased affinity of pepsin toward substrates containing iodine-substituted tyrosine residues has already been observed [7,14,15]. This property has been used in preparations of affinity supports that permit selective adsorption of pepsin. However, low-pressure chromatography has been used. Capillary electrophoresis has been shown to be promising for porcine pepsin analysis [16–18]. This paper broadens the possibilities of pepsin isolation and characterization by describing the preparation of stationary phases for HPLC and of modified capillaries for affinity capillary electrophoresis.

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Table 1
A comparison of the human and porcine pepsin properties [5,9]

	Theoretical molecular mass	Theoretical p*I*	Affinity to DIT	Affinity to *N*-acetyl-L-phe-D-phe-3,5-DIT	Affinity to ε-aminocaproyl L-phe-D-phe-OCH_3
Human pepsin A	34628	3.36	++	++	–
Human pepsin C	35461	3.59	++	+	–
Porcine pepsin A	34622	3.24	++	++	++

(++) strong interaction; (+) weak interaction; (–) not measurement.

2. Experimental

2.1. Chemicals

Porcine pepsin A (3100 units/mg; from porcine stomach mucosa), DIT (crystalline), ovalbumin and lysozyme (lyophilized powder, 50 000 units/mg protein) were purchased from Sigma Chemicals (St. Louis, USA); a sample of human pepsinogen and the Placer buffer (4 mL 0.1 mol/L $CaCl_2$ + 40 mL 0.2 mol/L CH_3COONa, pH 5.3) were provided by the Institute of Pathophysiology, First Faculty of Medicine, Charles University. The stationary phases Separon HEMA BIO 1000 E and Separon HEMA BIO 1000 VS were obtained from Tessek (Prague, Czech Republic) and the phase AF epoxy Toyopearl 650 M from Tossoh (Tokyo, Japan). Dried milk was a product of Nutricia (Opočno, Czech Republic); glycine was supplied by Reanal (Budapest, Hungary). All the other chemicals were products of Lachema (Brno, Czech Republic).

2.2. Apparatus

Liquid chromatographic measurements were carried out using a DIONEX instrument (Westmont, USA) provided with a gradient pump DIONEX P580 and a UV/VIS detector 170S/340S. The samples were injected through a RHEODYNE valve (Cotati, USA) with 10 and 100 μL loops. The data were handled by the Chromeleon 6.01 software (DIONEX).

A CRYSTAL CE SYSTEM ATI UNICAM (Cambridge, UK), Model 310, was used for capillary electrophoresis. The Chromatography Data Handling System Unicam 4880, version 1.1, was employed. The fused-silica capillary parameters: unmodified fused silica capillary from CACO (Bratislava, Slovak Republic), 75 μm i.d.; total length, L_T, 63 cm; length to the detector, L_D, 47 cm; and the same capillary modified by DIT, L_T 61 cm, L_D 47 cm. The capillary modified by a hydrophilic film of polyvinylalcohol (PVA) was obtained from Agilent Technologies (Palo Alto, USA) and its parameters were 75 μm i.d.; L_T, 87 cm; L_D, 75 cm.

2.3. Preparation of the stationary phase—binding of DIT

The procedure is based on references [4,19]: an amount of 3.5 g of a support containing either vinylsulphonic or epoxy active groups was rinsed with distilled water and mixed with the affinity ligand, 3,5-diiodo-L-tyrosine (500 mg of DIT suspended in 10 mL of a 0.2 mol/L carbonate buffer, pH 10.7). The suspension was shaken overnight at ambient temperature, washed three times with 50 mL distilled water, once with 50 mL of 0.2 mol/L carbonate buffer of pH 9.0 and once with 50 mL of 0.1 mol/L acetate buffer of pH 4.0. A small sample was collected for elemental analysis for iodine. An amount of 100 mg of glycine was dissolved in 10 mL of 0.2 mol/L carbonate buffer, pH 9.0, and added to the suspension to block the residual active groups of the support. The mixture was shaken overnight at room temperature, washed three times with 50 mL of distilled water, once with 50 mL of 0.1 mol/L acetate buffer of pH 4.0 and finally, once with 50 mL of distilled water. The stationary phase was packed into an 80 cm × 8 cm stainless steel column in the Watrex Company, Prague.

Reaction between the vinylsulphonic active groups and 3,5-diiodo-L-tyrosine corresponds to the scheme given in Fig. 1.

Three stationary phases were prepared, containing 3,5-diiodo-L-tyrosine bound to three different supports HEMA BIO VS (column no. 1 and 2; prepared twice), HEMA BIO E (column no. 3) and EPOXY TOYOPEARL (column no. 4). The amount of iodine was determined by elemental analysis and the amount of 3,5-diiodo-L-tyrosine was calculated from it (see Table 2). It can be seen from this table that the DIT amount bound to the epoxy activated supports (no. 3 and 4) is greater, by about 10 mg per g of support, than that bound to the divinylsulphone activated supports (no. 1 and 2). These differences follow from different numbers of the active functional groups.

2.4. Chromatographic conditions

The column was first washed with the starting mobile phase (0.05 mol/L acetate or Britton–Robinson buffer, both of pH 3.5) until the baseline stabilized (35–45 min). Sample was

Table 2
Amounts of DIT bound to the individual supports

Column	Affinity stationary phase	Amount of DIT (mg per g of support)
No. 1	HEMA BIO VS-DIT	48
No. 2	HEMA BIO VS-DIT	51
No. 3	HEMA BIO E-DIT	61
No. 4	EPOXY TOYOPEARL-DIT	64

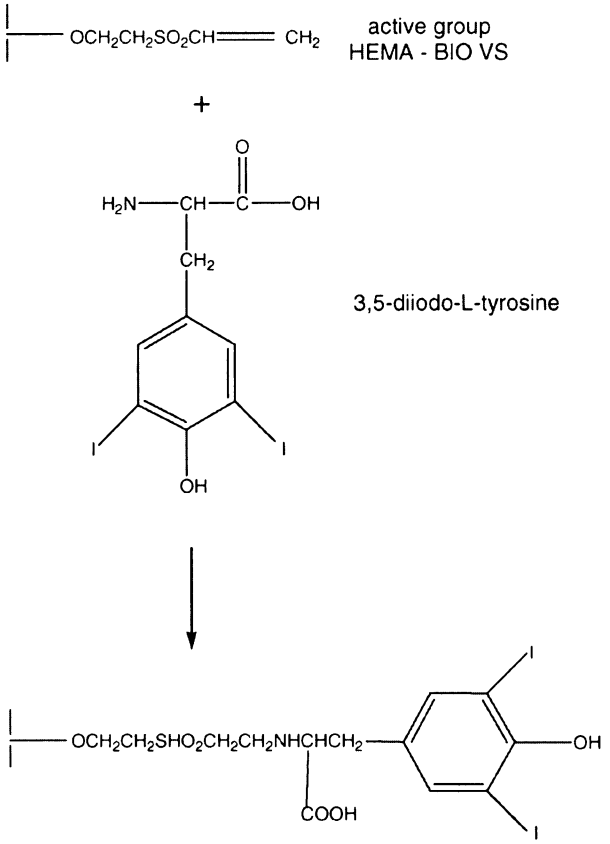

Fig. 1. Scheme of the vinylsulphone group reaction with 3,5-diiodo-L-tyrosine.

then injected and the starting mobile phase was further passed to elute non-adsorbed substances. Adsorbed substances were then eluted with the elution mobile phase (0.05 mol/L acetate buffer of pH 5.6, a phosphate buffer of pH 6.8 or a Britton–Robinson buffer of pH 6.8). A pH gradient elution was used with various gradient slopes, from 100% starting buffer to 100% elution buffer. The column was washed with the starting buffer after each analysis for baseline stabilization (25–35 min). Flow rate used was 0.5 mL min^{-1} and the detection wavelengths were 214 nm [16] and 280 nm [4].

The chymase activity (determination of the sum of proteases using milk curdling) was determined in some analyses [20]. Fractions of 1 mL were collected, the time of the beginning of milk curdling was measured and the reciprocal of the curdling time for 70 μL of the given elute fraction was plotted against the time of the fraction collection.

2.5. Human pepsin sample preparation

Homogenate was filtered through cotton wool. A 9 mm × 20 mm DEAE-Separon column with a volume of 1 mL was washed with 50 mL 0.05 mol/L Tris–HCl buffer of pH 7.3, allowed to swell for 1 h and then washed again with 30 mL of the same buffer. A 1 mL volume of the homogenate sample was applied to the column; three 10 mL portions of the above buffer were passed through the column and pepsinogen was then eluted with 10 mL of a 0.1 mol/L acetate buffer of pH 5.6 containing 0.5 mol/L NaCl. The first 5 mL of the eluate was dialyzed overnight against the 0.1 mol/L acetate buffer of pH 5.6. A new portion of the buffer was used after 2 h of dialysis. After dialysis, the sample was concentrated at a decreased temperature (refrigerator) from a volume of 5 to 1 mL in a Vivapore Concentrator vessel with a 7500 MWCO PES membrane. Finally, the pepsinogen was activated for about 45 min to yield pepsin using 1 mol/L HCl to attain a pH about 3.5. Sample was then injected onto the affinity column.

2.6. Preparation of silica capillary modified with 3,5-diiodo-L-tyrosine

The silica capillary was first washed at 1000 mbar for 10 min with 1 mol/L NaOH, for 10 min with deionised water, for 5 min with acetone and then air was passed for 5 min. Capillary was then activated by filling it with a solution of 3-aminopropyltriethoxy silane for 20 min, washing it with methanol, passing air through it and then heating it to 45 °C for 12 h. Two procedures were used to bind DIT to the amino groups thus formed.

The first procedure involved binding of L-tyrosine followed by its iodation. A 5% solution of BOC-L-tyrosine hydoxysuccinimide ester in dimethylformamide (DMF) was aspirated into the capillary and left there for 12 h at ambient temperature. Capillary was then washed with DMF and dichloromethane. It was washed with anhydrous trifluoroacetic acid (TFA) for 1 h at a pressure of 1000 mbar and ambient temperature to remove BOC, washed with tetrahydrofuran (THF) and with deionised water. The bound tyrosine was iodated by filling the capillary with a solution of 6.6 g of potassium iodide and 2.2 g of chloramine B in 10 mL of phosphate-buffered saline (PBS). Reaction was stopped after 5 min by washing the capillary with a solution of 50 mg of sodium pyrosulphite in 1.1 mL of PBS and finally with water. The other procedure was direct binding of DIT to the activated capillary wall: a solution containing 0.5 mL of DMF, 20 mg of BOC-diiodotyrosine and 15.5 mg of dicyclohexylcarbodiimide (DCC) was introduced into the capillary and left for 12 h at ambient temperature. Capillary was then washed with DMF, dichloromethane and was finally treated with a 50% solution of TFA in dichloromethane at ambient temperature for 1 h at a pressure of 1000 mbar. Afterwards, capillary was washed with dichloromethane, acetone and air was then passed through it.

Hydrophilically modified capillary was maintained in a reproducible state by washing with deionised water (1000 mbar), 10 mmol/L phosphoric acid and methanol. Prior to a several-day break in the measurements, the capillary was washed with deionised water for 20 min, with methanol for 5 min, and then dried by air. The diiodotyrosine-modified capillary was only washed with deionised water.

2.7. Electrophoretic conditions

The capillary was washed (1000 mbar) with deionised water and the separation buffer (0.025 or 0.05 mol/L phosphate buffer, pH 3.0, 6.8 or 8.5) at the beginning of measurement and washed with the separation buffer between runs. The detection wavelength was 214 nm [16].

2.8. Determination of the dissociation constant K_d

The dissociation constant of the porcine pepsin complex with DIT was determined from the mobility changes in a bare silica capillary [21–23]. DIT (ligand) plus thiourea (marker) were injected into the running buffer of 0.25 mol/L phosphate buffer, pH 3.0, containing porcine pepsin at increasing concentrations. The porcine pepsin concentration ranged from 0 to 14.3 μmol/L. UV detection at 214 nm was used.

3. Results and discussion

3.1. Stationary phases with immobilized DIT

Support activated by the manufacturer was used for preparation of stationary phases. The affinity ligand, 3,5-diiodo-L-tyrosine, was bound to the active vinylsulphone- and epoxy groups via amino group by the reaction given in Fig. 1. The ligand amount was calculated from the iodine content (see Table 2).

The stationary phases prepared were tested using porcine pepsin A samples. The effects of the buffer compositions and of the slope of the elution pH gradients were studied. Two UV detection wavelengths were tested, 214 and 280 nm. An optimum separation was attained for the 0.05 mol/L acetate buffer of pH 3.5 (starting) and pH 5.6 (eluting); detection at 214 nm was more sensitive. A pH gradient from 0 to 100% of the eluting buffer within 30 or 50 min was used.

The course of the affinity chromatography of porcine pepsin A in an acetate buffer on HEMA BIO VS-DIT (column no. 1) can be seen in Fig. 2. The enzyme was adsorbed at pH 3.5 and eluted by changing the pH to a value of 5.6. The course of the chymase activity is given in Fig. 3. It can be seen that the peaks eluted at the dead time (no. 1 and 2) did not exhibit chymase activity; therefore, these peaks probably corresponded to impurities present in the commercial porcine pepsin A preparation, or they were system peaks. Peak at time $t = 28$ min (no. 3) exhibited a chymase activity and thus corresponded to pepsin.

Dependence of the area of the porcine pepsin A peak on the pepsin concentration was obtained for all the stationary phases. It was observed that the peak area linearly increases with increasing pepsin concentration only to a concentration of 3.0 mg/L; the binding sites are then saturated and the area increases more slowly (Fig. 4).

The repeatability of measurement was found for HEMA BIO E-DIT (column no. 3) from a comparison of the peak areas for a series of six measurements performed with a new column and for another series of six measurements with the column after two months of its use. The coefficients of variation 0.9 and 6.2% demonstrate a limited life-time of the affinity stationary phase, as the repeatability substantially deteriorated after two months of use of the column.

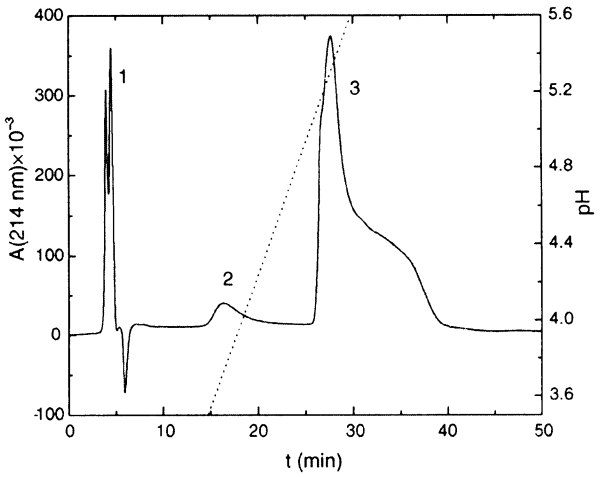

Fig. 2. Affinity HPLC of porcine pepsin on HEMA BIO VS-DIT (column no. 1)—protein elution: (1, 2) impurity; (3) porcine pepsin. Starting mobile phase, 0.05 mol/L acetate buffer, pH 3.5; elution mobile phase, 0.05 mol/L acetate buffer, pH 5.6; pH gradient elution from the 15th to 30th min (dashed line); flow rate 0.5 mL/min; sample concentration 1.0 mg/mL.

To verify specificity of the affinity binding of pepsin to 3,5-diiodo-L-tyrosine, a mixture of pepsin, ovalbumin and lysozyme was injected onto the column (Fig. 5). It can be seen that only pepsin interacts with DIT, because ovalbumin and lysozyme are not retained.

The course of the affinity chromatography of human pepsin on the EPOXY TOYOPEARL-DIT (column no. 4) is

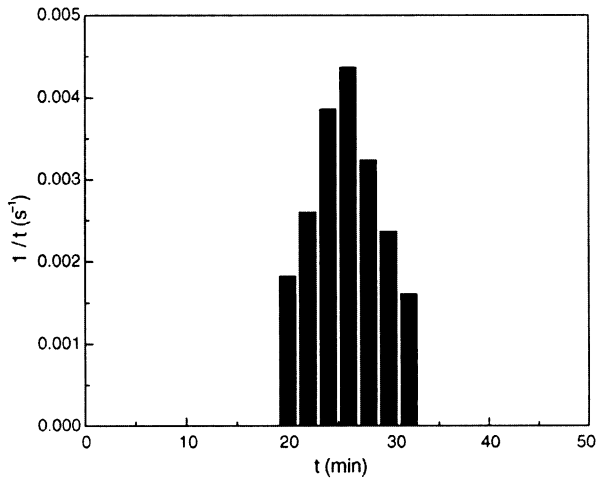

Fig. 3. Profile of chymase activity of porcine pepsin (samples taken during analysis on HEMA BIO VS-DIT (column no. 1)): x-axis, time of analysis; y-axis, reciprocal time corresponding to precipitation milk casein.

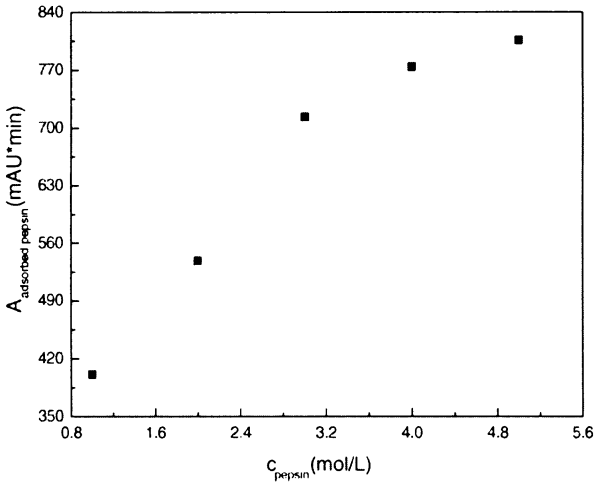

Fig. 4. Dependence of amount of adsorbed porcine pepsin A on its concentration on applied HEMA BIO VS-DIT (column no. 1). Amount of adsorbed enzyme expressed in the value of area (mAU* min) of pepsin peak. Experimental conditions as in Fig. 2.

shown in Fig. 6. It is evident that the behaviour of the model porcine pepsin A and of human pepsin is analogous (human pepsin elutes faster: its retention time is 26.2 min, whereas that of porcine pepsin A is 46.6 min). The chymase activity was measured for the fractions collected during the whole analysis. The peak located at the dead time (no. 1) exhibits no chymase activity and apparently corresponds to non-sorbing substances present in the human pepsin sample. The elution peak (no. 2) exhibits chymase activity whose maximum corresponds to the peak apex, i.e., it corresponds to pepsin. For

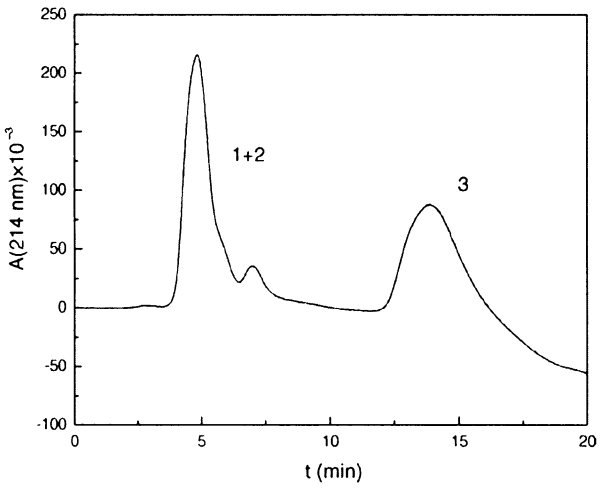

Fig. 5. Affinity chromatography of porcine pepsin, ovalbumin and lysozyme on HEMA BIO E-DIT (column no. 3): (1, 2) ovalbumin and lysozyme; (3) porcine pepsin. Starting mobile phase, 0.05 mol/L acetate buffer, pH 3.5; elution mobile phase, 0.05 mol/L acetate buffer, pH 5.6; pH gradient elution from the 15th to 30th min; flow rate 0.5 mL/min; sample concentration: ovalbumin 0.5 mg/mL, lysozyme 0.5 mg/mL, pepsin 0.5 mg/mL.

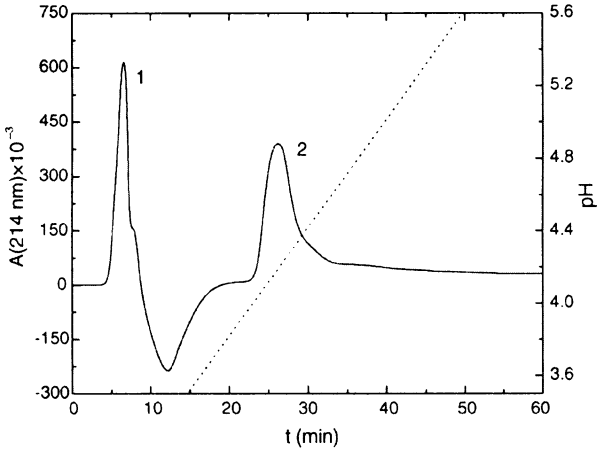

Fig. 6. Affinity chromatography of human pepsin on EPOXY TOYOPEARL-DIT (column no. 4): (1) non-sorbing material; (2) pepsin. Experimental conditions as in Fig. 2 except for the pH gradient: elution from 15th to 50th min.

the column EPOXY TOYOPEARL-DIT (column no. 4), the time of the elution pH gradient from pH 3.5 to pH 5.6 had to be prolonged (from 15th to 50th min).

3.2. *Affinity capillary electrophoresis*

Affinity capillary electrophoresis has become a popular method for studying protein–ligand interactions [24,25]. An interaction of pepsin with DIT has been studied by ACE in this paper.

Commercial capillary hydrophilically modified with PVA has been recommended for separation of peptides and proteins [17]. An electropherogram of porcine pepsin in this capillary is shown in Fig. 7. A partial separation of porcine pepsin A can be seen in Fig. 7. It was reported that separated peaks correspond to pepsin glycoforms [17,18] or that they reflect the autodigestion of porcine pepsin [16].

We prepared DIT-modified capillaries for ACE, using the procedure described in Section 2. DIT was bound to the capillary by two different procedures and the capillaries were tested with porcine pepsin. We found that reproducible results were only obtained with the capillary prepared by binding L-tyrosine to the wall and subsequent iodation. A CE analysis of porcine pepsin A in this capillary is given in Fig. 8. Peak of porcine pepsin A is separated into several isoforms. A comparison with Fig. 7 reveals that the capillary with immobilized DIT yielded a better separation and a shorter migration time.

3.3. *Determination of the dissociation constant of the DIT–porcine pepsin complex*

Dynamic equilibrium affinity electrophoresis was used in this paper for determining the dissociation constant [22].

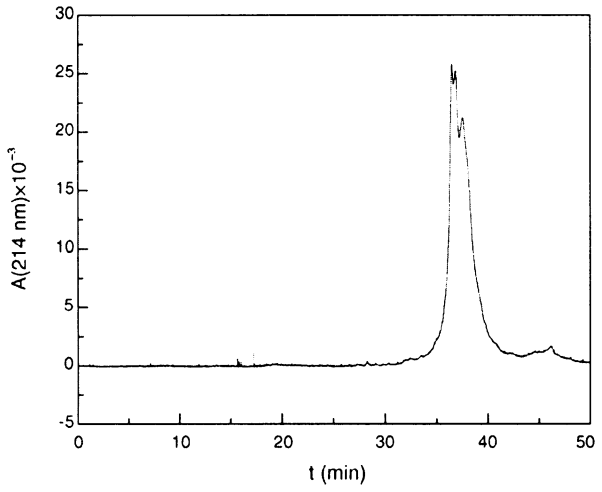

Fig. 7. Affinity capillary electrophoresis of porcine pepsin A on capillary modified PVA. Separation buffer, 0.025 mol/L phosphate buffer, pH 6.8; voltage, 13 kV; pneumatic sampling, 10 mbar for 20 s; sample concentration, 57.1 μmol/L.

Receptor peak migration shifting as a function of the concentration of the ligand in the separation buffer was used. Eq. (1) links mobility shifts ($\Delta\mu$) with ligand concentration [L] and dissociation constant K_d:

$$\Delta\mu = \Delta\mu_{max} - K_d\left(\frac{\Delta\mu}{[L]}\right) \quad (1)$$

Since $\mu = L_D/Et$, where L_D is the migration distance to the detector, E the field intensity, and t is the migration time, Eq. (1) can be transformed into a practical form employing the difference in the migration times, Eq. (4), provided that the values of E and L_D are the same in all the experiments, and correcting t-values by t_m-values obtained from non-interacting marker molecule [22]:

$$\Delta\mu = \frac{I}{E}\left[\left(\frac{1}{t} - \frac{1}{t_m}\right) - \left(\frac{1}{t_0} - \frac{1}{t_{m0}}\right)\right] \quad (2)$$

$$\Delta\mu = \frac{I}{E}\left[\Delta\left(\frac{1}{t}\right)\right] \quad (3)$$

$$\Delta\left(\frac{1}{t}\right) = \Delta\left(\frac{1}{t}\right)_{max} - K_d\left(\frac{\Delta(1/t)}{[L]}\right) \quad (4)$$

where t_{m0} and t_0 are the migration times of a marker and a receptor for an electrolyte without ligand ($[L]=0$) and t_m and t correspond to the migration times of a marker and a receptor in an electrolyte with a ligand concentration ($[L]\neq 0$).

In our approach, 3,5-diiodo-L-tyrosine and thiourea (the eof marker) were injected as the sample while porcine pepsin A was dissolved in the phosphate buffer. The concentration of 3,5-diiodo-L-tyrosine was 2.3 mmol/L, that of thiourea was 13.1 mmol/L and the porcine pepsin concentration varied from 0 to 14.3 μmol/L. An electropherogram of DIT and thiourea in a bare silica capillary is shown in Fig. 9. The DIT peaks change in dependence on the addition of porcine pepsin A (Fig. 10). The dissociation constant is then estimated from the linearized plot with ($-K_d$) as the slope [22]. The slope of the straight line, $y = -6.10x + 203.1$, corresponds to the $-K_d$ which amounts to 6.1 μmol/L. No value has so far been published for K_d of pepsin–DIT. However, the results are similar to those obtained in our measurement of K_d of complexes of concanavalin A with other glycoproteins [26]. The values of 8.1 μmol/L for ovalbumin, 9.5 μmol/L for fetuin and

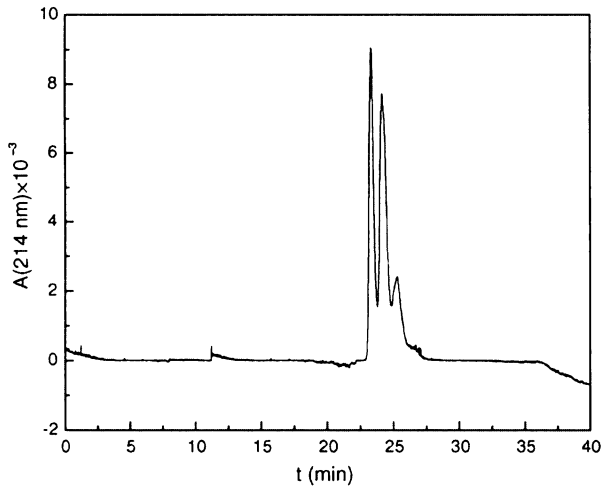

Fig. 8. Affinity capillary electrophoresis of porcine pepsin A on capillary modified with L-tyrosine with subsequential iodation. Separation buffer, 0.025 mol/L phosphate buffer, pH 6.8; voltage, 13 kV; pneumatic sampling, 10 mbar for 20 s; sample concentration, 57.1 μmol/L.

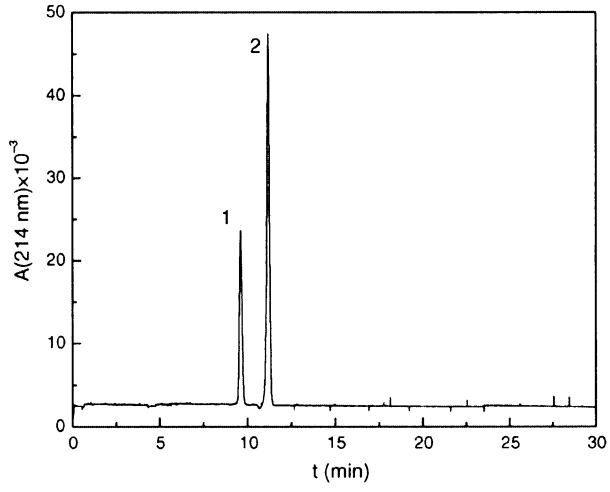

Fig. 9. Capillary electrophoresis of DIT and thiourea (sample) in present porcine pepsin A (dissolved in buffer) on silica capillary: (1) DIT; (2) thiourea. Separation buffer, 0.025 mol/L phosphate buffer, pH 3.0; voltage, 28 kV; pneumatic sampling, 10 mbar for 20 s; sample concentration: DIT 2.3 mmol/L, thiourea 13.1 mmol/L.

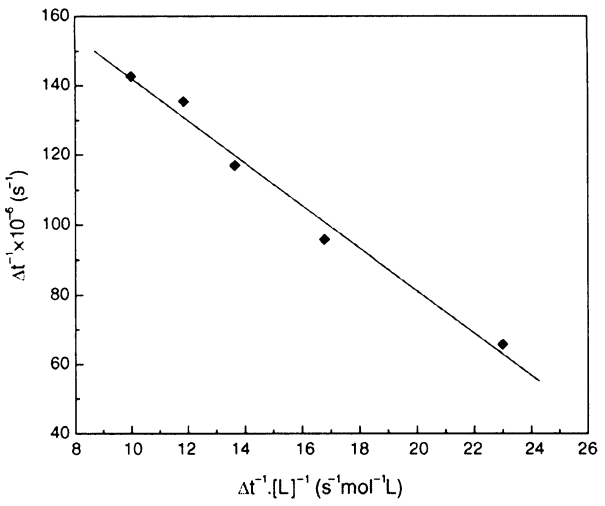

Fig. 10. Graphic dependence for determination of dissociation constant K_d of complex porcine pepsin A-DIT. Dependence of Δt on $K_d\Delta(\mu)/[L]$.

9.5 μmol/L for acid-α-glycoprotein were obtained, respectively.

4. Conclusions

The results obtained demonstrate that the preparation of affinity stationary phases with 3,5-diiodo-L-tyrosine as the affinity ligand is reproducible, that these phases exhibit sufficient selectivity for pepsin and are applicable to practical samples, such as those of human or porcine pepsin. Their lifetime is limited to roughly two months; this is not particularly long but their use in practice is not prevented.

Of the two procedures tested for the preparation of CE capillaries modified with DIT, only the two-step synthesis involving the binding of L-tyrosine followed by its iodation produced usable capillaries. We compared analyses for porcine pepsin A on PVA modified capillary (commercially available) and DIT modified capillary (prepared by us); the capillary with immobilized DIT permitted a better separation and a shorter migration time.

The dissociation constant $K_d = 6.1$ μmol/L has been determined for the pepsin-3,5-diiodo-L-tyrosine complex using dynamic equilibrium ACE approach.

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References

[1] B.C. Bryksa, T. Tanaka, R.Y. Yada, Biochemistry 42 (2003) 13331.
[2] J. Frýdlová, Z. Kučerová, M. Tichá, J. Chromatogr. B 800 (2004) 109.
[3] M. Wells, B. Brown, J. Hall, Cell. Phys. Biochem. 13 (2003) 301.
[4] H. Arnoštová, Z. Kučerová, I. Tišlerová, T. Trnka, M. Tichá, J. Chromatogr. A 911 (2001) 211.
[5] Internet address, http://ca.expasy.org./cgi-bin/pi_tool (15 February 2003).
[6] I. Gritti, G. Banfi, G.S. Roi, Pharm. Res. 41 (2000) 265.
[7] Z. Kučerová, M. Tichá, J. Chromatogr. B 770 (2002) 121.
[8] R.A. da Silva Gomes, R.P. Batista, A.C. de Almeida, D.N. da Fonseca, L. Juliano, V. Hial, Anal. Biochem. 316 (2003) 11.
[9] J. Turková, Bioaffinity Chromatography, second ed., Elsevier, Amsterdam, 1993 (completely revised edition).
[10] J.Y. Fu, S.M. Wu, Ch.T. Chang, H.Y. Sung, J. Agric. Food Chem. 51 (2003) 1042.
[11] K. Peek, N.B. Roberts, W.H. Taylor, J. Chromatogr. 476 (1989) 491.
[12] J.S. Fruton, Q. Rev. Biol. 77 (2002) 127.
[13] A.T. Jones, K.K. Balan, S.A. Jenkins, R. Sutton, M. Critchley, N.B. Roberts, J. Clin. Pathol. 46 (1993) 254.
[14] E. Tonková, M. Tichá, Z. Kučerová, IJBC 4 (1998) 35.
[15] Z. Kučerová, M. Tichá, J. Sep. Sci. 26 (2003) 669.
[16] J. Haginaka, Y. Miyano, Y. Saizen, Ch. Seyama, T. Murashima, J. Chromatogr. A 708 (1995) 161.
[17] M. Gliges, M.H. Kleemiss, G. Schomburg, Anal. Chem. 66 (1994) 2038.
[18] J.P. Landers, R.P. Oda, B.J. Madden, T.C. Spelberg, Anal. Biochem. 205 (1992) 115.
[19] Affinity Chromatography—Principles and Methods, Pharmacia Fine Chemicals (bulletin), Uppsala, Sweden, Epoxy-activated Sepharose 6B.
[20] P. Mc Phie, Anal. Biochem. 73 (1976) 258.
[21] K. Shimura, K. Kasai, Anal. Biochem. 251 (1997) 1.
[22] N.H.H. Heegaard, S. Nilsson, N.A. Guzman, J. Chromatogr. B 715 (1998) 29.
[23] J. McKeon, L.A. Holland, Electrophoresis 25 (2004) 1243.
[24] Y.H. Chu, L.Z. Avila, J. Gao, G.M. Whitesides, Acc. Chem. Res. 28 (1995) 461.
[25] H. Kajiwara, Anal. Chim. Acta 383 (1999) 61.
[26] S. Hubená, Characterization of glycoproteins by capillary electrophoresis, Diploma thesis, Charles University, Prague, 1999.

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Original Paper

Affinity liquid chromatography and capillary electrophoresis of seminal plasma proteins

Interactions of boar, bull, and human seminal plasma proteins with heparin and phosphorylcholine were studied by affinity LC using heparin immobilized to a Toyopearl support. A step gradient elution from 0.15 to 1.50 M NaCl was employed to elute the seminal plasma proteins. Relative amounts of the heparin-binding fraction of seminal plasma proteins (H+) in seminal plasma of three species were determined. Further on, the fraction of seminal plasma proteins interacting with phosphorylcholine-binding proteins (P+) was evaluated. P+ proteins were not found in human seminal plasma and their highest amount was present in bull seminal plasma. A CE method was developed for separation of seminal plasma proteins. Various capillaries and separation conditions were tested; the best resolution was obtained in a bare-silica capillary, with a micellar system consisting of a 0.02 M borate buffer and 0.05 M SDS pH 10.0. The optimized conditions were applied to the identification of the components in boar plasma.

Keywords: Affinity chromatography / Heparin / Micellar capillary electrophoresis / Phosphorylcholine / Seminal plasma proteins

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1 Introduction

Mammalian fertilization is a unique event in which morphologically disparate gametes recognize and bind each other and fuse. This event includes highly regulated biochemical interactions: binding of seminal plasma proteins to the sperm surface during ejaculation, interaction of sperm surface proteins with oviduct epithelial cells, sperm capacitation, gamete recognition, primary and secondary binding of the sperm to the ovum, acrosome reaction of sperm, penetration of the sperm through the zona pellucida of the ovum, and fusion of sperm and egg [1–4]. Sperm-binding proteins of seminal plasma play important roles in these events.

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Abbreviations: **AQN**, protein of boar seminal plasma (according to N-terminal amino acid sequence Ala-Gln-Asn); **AWN**, protein of boar seminal plasma (according to N-terminal amino acid sequence Ala-Trp-Asn); **BSP**, proteins of bull seminal plasma; **DQH**, protein of boar seminal plasma (according to N-terminal amino acid sequence Asp-Gln-His); **H+**, heparin-binding fraction of seminal plasma; **H−**, heparin-nonbinding fraction of seminal plasma; **P+**, phosphorylcholine-binding proteins; **PSP**, protein of boar seminal plasma of H− fraction; **Tris-HCl**, Tris-hydrochloride

Proteins of seminal plasma, as well as their amounts, greatly differ from species to species. Boar seminal plasma contains different types of proteins. The first type, spermadhesins (protein of boar seminal plasma (according to N-terminal amino acid sequence Ala-Gln-Asn) (AQN), protein of boar seminal plasma (according to N-terminal amino acid sequence Ala-Trp-Asn) (AWN), and protein of boar seminal plasma of H− fraction (PSP)-porcine seminal plasma), belong to the most abundant boar seminal plasma proteins. The N-terminal amino acid sequence of all the members of AQN and AWN proteins begins with either Ala-Gln-Asn (AQN) or Ala-Trp-Asn (AWN). They are characterized by 40–60% amino acid sequence identity and belong to one protein family. Protein of boar seminal plasma (according to N-terminal amino acid sequence Asp-Gln-His) (DQH) is another type of protein isolated from boar seminal plasma. It differs in its structure from boar spermadhesin [5, 6].

The major proteins found in bull seminal plasma are called BSP proteins (bovine seminal plasma proteins: BSP-A1, BSP-A2, BSP-A3, and BSP-30 kDa). These proteins are present in seminal plasma at very high concentrations [7, 8]. They have apparent molecular masses ranging from 15500 to 16500, whereas the BSP-30 kDa protein has a molecular mass of 28000–30000 [9].

Quite different proteins are present in human seminal plasma. Major protein constituents of human seminal

plasma are semenogelin I (Sg I) and semenogelin II (Sg II) with M_rs of 50 000 and 63 000, respectively. They participate in the formation of sperm-entrapping gel immediately after ejaculation [10–12].

A common feature of many seminal plasma proteins is their ability to interact with different types of substances. Some of them are bound to the sperm surface during ejaculation and thus they affect binding interactions of sperm. The following interactions of sperm that are coated with seminal plasma proteins belong to the most studied ones: (i) an interaction with different types of glycoconjugates, (ii) an interaction with membrane phospholipids, and (iii) mutual interactions between proteins [5].

Preparative (low-pressure) affinity chromatography belongs to one of the methods used for the isolation and separation of seminal plasma proteins. Among the ligands used, immobilized heparin is often applied to separate H+ and heparin-nonbinding fraction of seminal plasma (H−) proteins [7, 13, 14]. Affinity techniques not only are useful for the protein isolation but also represent a valuable and essential tool for the study and analysis of binding properties of protein components that participate in the fertilization process.

In the present study, we have employed affinity chromatography at an elevated pressure, using heparin immobilized on a Toyopearl support, to investigate the heparin- and phosphorylcholine-binding properties of boar, bull, and human seminal plasma proteins.

CE is also favored in protein analysis because it is highly efficient, fast, relatively cheap, and requires only small amounts of samples [15, 16]. Because it has not yet been applied to seminal plasma proteins, a CE procedure has been developed for separation and identification of boar seminal plasma components.

2 Experimental

2.1 Chemicals

Tris-hydrochloride (Tris-HCl), phosphorylcholine, triethylamine, methanol, ACN, 1,6-diaminohexane, and SDS were purchased from Sigma Chemicals (St. Louis, USA), and ethanol from Merck (Darmstadt, Germany). All the other chemicals were from Pliva-Lachema (Brno, Czech Republic). All the chemicals were used as received.

2.2 Samples

Boar and bull ejaculates were obtained from the Veterinary Research Institute, Brno, Czech Republic. Human ejaculates from the Institute of Sexology were kindly provided by Dr. Jonáková (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague). Ejaculates were centrifuged (600 × g, 20 min, 5°C) to separate plasma and sperms, and seminal plasma was lyophilized directly (for a greater detail, see [17]).

H+ and nonheparin-binding proteins of boar seminal plasma were isolated by preparative (low-pressure) affinity chromatography on heparin immobilized in polyacrylamide gels [13]. These fractions were subjected to CE. Individual protein components of these fractions were obtained using RP-HPLC separation as described previously [14]. These individual proteins were used as standards for CE.

2.3 Affinity chromatography

Liquid chromatographic measurements were carried out using a Dionex instrument (Westmont, USA) connected with a gradient pump P580 and a UV/VIS detector UVD 170S/340S. All samples were detected at 280 nm. The flow rate used was 0.4 mL/min. The samples were injected through a Rheodyne valve (Cotati, USA) with 10 µL sampling loops. The data were handled by Chromeleon software version 6.01.

The Toyopearl–heparin stationary phase (Tosoh Bioscience) was packed into a steel column with dimensions 80 mm × 8 mm (Watrex, Prague, Czech Republic). The column parameters: particle size 65 µm, polymer-based resin pH range, 5.5–9.0, binding capacity, 5 mg antithrombin III/mL.

Samples of seminal plasma proteins were dissolved in a buffer of 0.02 M Tris-HCl containing 0.15 M NaCl (2 mg/mL). Nonadsorbed proteins were eluted with the same buffer (starting buffer). For the elution of seminal plasma proteins, a step gradient elution was used with increasing ionic strength (0.15–1.50 M NaCl in 0.02 M Tris-HCl, pH 7.5) of the mobile phase. For the elution of the P+ fraction of seminal plasma proteins, a two-step gradient was used. Nonadsorbed proteins were eluted with a mobile phase containing 0.02 M Tris-HCl with 0.15 M NaCl (starting buffer). The adsorbed proteins were first eluted using 0.05 M phosphorylcholine in the starting buffer and then using an NaCl gradient (0.15–1.50 M NaCl in 0.02 M Tris-HCl pH 7.5).

After the analysis, the stationary phase was equilibrated with the starting mobile phase. Under the conditions of affinity chromatography used, no nonspecific interactions were observed. The yield of the proteins eluted was evaluated from the absorbance measured at 280 nm. The affinity column could be used repeatedly, without changes in its binding capacity.

2.4 CE

Electrophoretic measurements were carried out using a Crystal CE System ATI-Unicam (Cambridge, UK), model 310 with a UV detector (280 nm). The Chromatography Data Handling System Unicam 4225, version 1.1, was

employed. The unmodified fused-silica capillary from CACO (Bratislava, Slovak Republic) had these parameters: 50 μm id, total length, L_T, 80 cm; length to detector, L_D, 64.8 cm. The parameters of the capillary modified by a hydrophilic layer of polyvinylalcohol (PVA) from Agilent Technologies (Palo Alto, USA) were 75 μm id, L_T 73.1 cm; L_D 57.6 cm.

The samples were dissolved in either 0.025 M phosphate buffer or 1 mM HCl to enhance their solubility. A 0.025 M phosphate buffer, pH 3.0, was used for the CE system containing an unmodified bare-silica capillary. Later on, a 0.125 M phosphate buffer was tested either alone, or with the addition of 20% ACN or 30% methanol. A micellar system consisting of a 0.02 M borate buffer with 0.05 M SDS, pH 10.0, was used. This method was also used for the identification of individual proteins of boar seminal plasma. Proteins AQN 1, AQN 2, AQN 3, AWN 1, AWN 2, and DQH were added to the original sample at a ratio of 1:1 (200 μL of 1 mg/mL H+ fraction and 200 μL of 1 mg/mL of each protein). For the CE system containing the hydrophilically modified capillary, a 0.025 M phosphate buffer, pH 3.0, and this buffer with an addition of 6 M urea was tested.

The capillaries were maintained in a reproducible state by washing with deionized water and the separation buffer. At the end of the measurements, the unmodified capillary was washed with deionized water, 0.1 M HCl, deionized water, 0.1 M NaOH, deionized water, and then dried by passage of air. Each step took 5 min, and the pressure was 1000 mbar. The hydrophilically modified capillary was washed with deionized water for 25 min, with methanol for 5 min, and then dried by passage of air for 3 min.

3 Results and discussion

3.1 Affinity chromatography on immobilized heparin

The detailed study of binding interactions of seminal plasma proteins and their analysis are very important in understanding the role of the proteins in the fertilization process. There exist several types of methods that could be applied for this purpose and affinity chromatography represents one of those that could yield reliable results. Systems used for isolation (classical low-pressure affinity chromatography with immobilized heparin [13]) are time- and material-consuming in analytical applications and thus we used affinity chromatography at an elevated pressure.

Separation of full boar, bull, and human seminal plasma was optimized using an affinity stationary phase with immobilized heparin. Different mobile phases were tested. The best results were obtained with a step salt gradient from 0.15 to 1.50 M NaCl in Tris-HCl buffer at pH 7.5. Examples of affinity chromatography of boar, bull, and human seminal plasma proteins are given in Fig. 1A–C. Peak no. 1 represents proteins noninteracting with heparin while peak no. 2 contains proteins binding to heparin. The content of the proteins bound to heparin

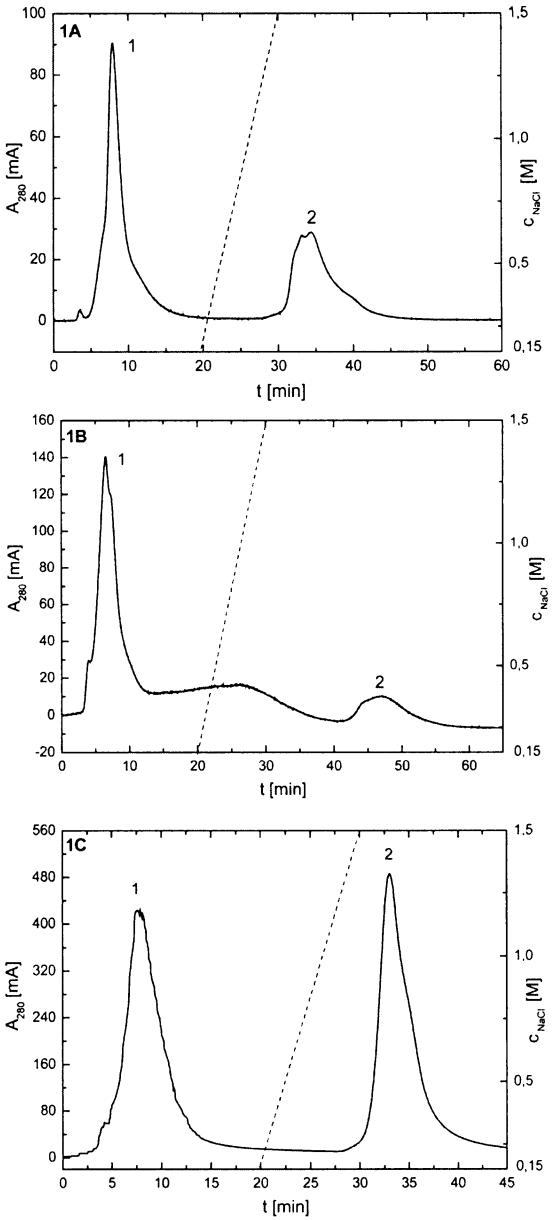

Figure 1. Affinity chromatography of full seminal plasma on a heparin–Toyopearl column. (A) boar; (B) bull; (C) human; mobile phases – gradient of NaCl in 0.02 M Tris-HCl pH 7.5: 0–20 min 0.15 M NaCl, 20–30 min 0.15–1.50 M NaCl (– step gradient of NaCl), from 30 min 1.50 M NaCl; (1) proteins not binding to heparin; (2) proteins binding to heparin.

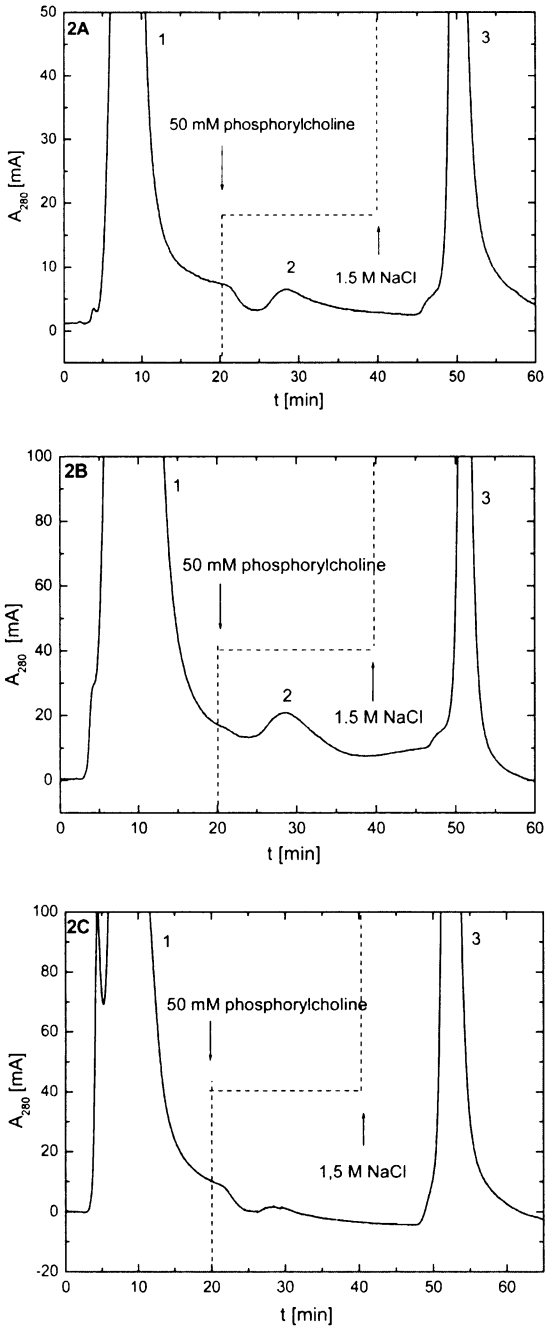

Figure 2. Affinity chromatography of the phosphorylcholine-binding fraction of full seminal plasma proteins on a heparin–Toyopearl column. (A) boar; (B) bull; (C) human; mobile phases: 0–20 min 0.02 M Tris-HCl + 0.15 M NaCl pH 7.5, 20–40 min 0.02 M Tris-HCl + 0.15 M NaCl + 0.05 M phosphorylcholine pH 7.5, from 40 min 0.02 M Tris-HCl + 1.50 M NaCl pH 7.5; (1) proteins not binding to heparin; (2) proteins eluted by 0.05 M phosphorylcholine; (3) proteins eluted by 1.50 M NaCl.

Table 1. Contents of H+ and P+ in seminal plasma of different species

Seminal plasma	Content of H+ proteins in seminal plasma[a] (%)	Content of P+ proteins in seminal plasma[a] (%)
Bull	15	4
Boar	35	3
human	55	0

[a] Expressed in % of the total amount of proteins in seminal plasma, determined on the basis of $A_{280\ nm}$ measurement.

(H+) in seminal plasma of different species was evaluated, and the results are shown in Table 1. It follows from Table 1 that the amounts of proteins bound to heparin differ in different species: the highest ratio of H+ to total proteins was found in human plasma and the lowest ratio was found in bull plasma.

Seminal plasma of some species contains proteins that have an ability to interact with phosphorylcholine. This property participates in the binding of seminal plasma proteins to the sperm membrane and thus protein sperm coating layers change the binding properties of this gamete [5]. For the isolation of P+ proteins from seminal plasma, affinity chromatography on immobilized heparin was combined with subsequent elution of the adsorbed proteins with a phosphorylcholine solution [18, 19]. We used this approach for our own analytical study.

Using the Toyopearl-heparin column, elution of P+ proteins was attained with a mobile phase consisting of 0.02 M Tris-HCl, 0.15 M NaCl with the addition of 0.05 M phosphorylcholine, pH 7.5. Chromatograms of boar, bull, and human seminal plasma proteins are given in Fig. 2A–C. Proteins noninteracting with heparin are present in peak no. 1; peak nos. 2 and 3 correspond to proteins interacting with heparin; peak no. 2 contains proteins eluted by phosphorylcholine solution and corresponds to P+. Quantitative evaluation of the P+ proteins is given in Table 1. It follows from Fig. 2A–C and Table 1 that the highest amount of phosphoryl-binded proteins was found in bull seminal plasma. No proteins eluted by phosphorylcholine were found in human plasma. Proteins BSP-A1 and BSP-A2 from bull seminal plasma can interact with phosphorylcholine (they are indicated together as proteins from bull seminal plasma which can interact with phosphorylcholine (PDC-109)) [17]. Only a small part of proteins from boar seminal plasma can interact with phosphorylcholine (probably DQH and AQN-1) [20]. These results are in agreement with the results obtained using classical (low-pressure) affinity chromatography with immobilized L-glyceryl-phosphorylcholine [17].

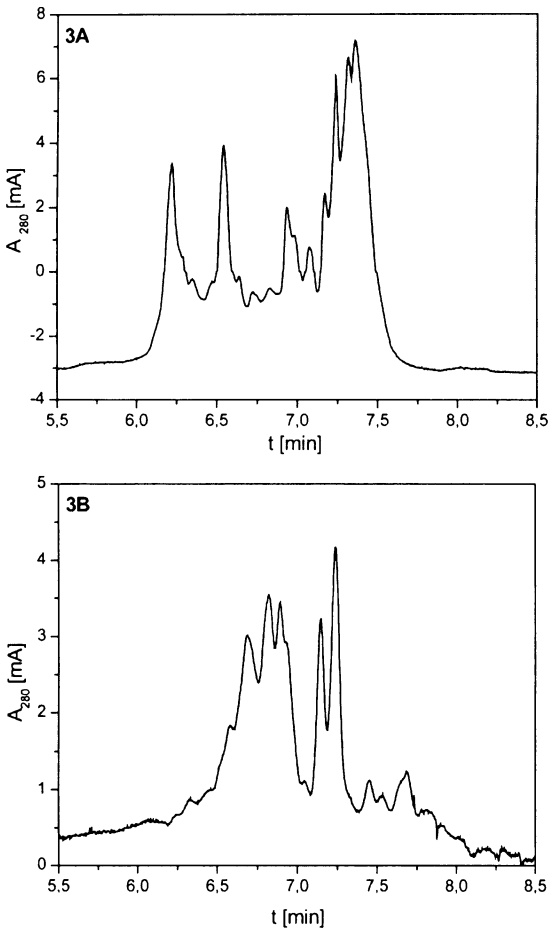

Figure 3. Separation of H+ and H− fraction of boar seminal plasma by CE (unmodified fused-silica capillary – a micellar system). (A) H+ fraction; (B) H− fraction; mobile phase, 0.02 M borate buffer with 0.05 M SDS, pH 10.0.

3.2 CE

No CE procedure has so far been published for the analysis of seminal plasma proteins, apparently for these reasons: the solubility of seminal plasma proteins in aqueous buffers is very poor [6]; proteins easily aggregate and can be strongly adsorbed on the capillary walls [21]. Therefore, we tested several approaches to increasing the solubility of the proteins studied, suppressing their sorption, and breaking their aggregates.

The CE system containing a bare-silica capillary and a 0.025 M phosphate buffer of pH 3.0 did not work for bull and boar seminal plasma proteins. An increase in the concentration (to 0.125 M) of the phosphate buffer and an addition of ACN (20%) or methanol (30%) did not suppress the protein sorption on the capillary walls. Modification of capillary walls by 1,6-diaminohexane (DAH,

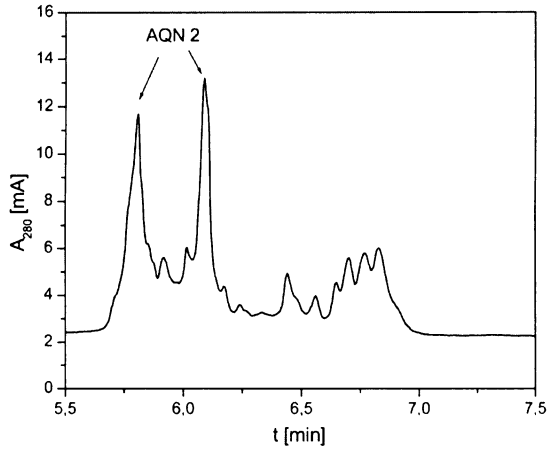

Figure 4. An electropherogram of the H+ fraction of boar seminal plasma with an addition of AQN 2 protein (1 : 1) – two isoforms (unmodified fused-silica capillary – a micellar system). Two isoforms of AQN 2 protein are marked directly in the electropherogram; mobile phase, 0.02 M borate buffer with 0.05 M SDS, pH 10.0.

1 mM) [22] or by hydroxypropylcellulose (HPC, 0.1%) enabled the migration of H+ and H− boar and H− of bull seminal plasma proteins but the peaks were broad and the separation was unsatisfactory.

A hydrophillically modified capillary recommended for separations of proteins was tested in combination with a 0.025 M phosphate buffer at pH 3.0, but seminal proteins did not migrate in this system. To increase the solubility of proteins and suppress their aggregation, 6 M urea was added to the running buffer. In this medium the separations of H+ and H− of boar and the H+ fraction of bull seminal plasma proteins were possible but, as previously, the peaks were broad and the separation efficiency was very poor.

A micellar system consisting of a 0.02 M borate buffer and 0.05 M SDS at pH 10.0 has proved to be the best solution. Electropherograms of boar H+ and H− are given in Fig. 3A and B. As follows from the electropherograms, seminal plasma proteins are complicated mixtures of several components. The proteins detected in the H− fraction most probably represent differently glycosylated isoforms of two main proteins of the H− fraction of boar seminal plasma: PSP-I and PSP-II [5]. The proteins present in the H+ fraction of boar seminal plasma (AQN 1, AQN 2, AQN 3, AWN 1, AWN 2, and DQH) were identified after adding the isolated proteins to the H+ fraction of boar seminal plasma. The individual proteins (isolated from the H+ fraction of boar seminal plasma separated by affinity chromatography on immobilized heparin; see Section 2) were gradually added to a fresh solution of H+ fraction and capillary runs were performed. Two iso-

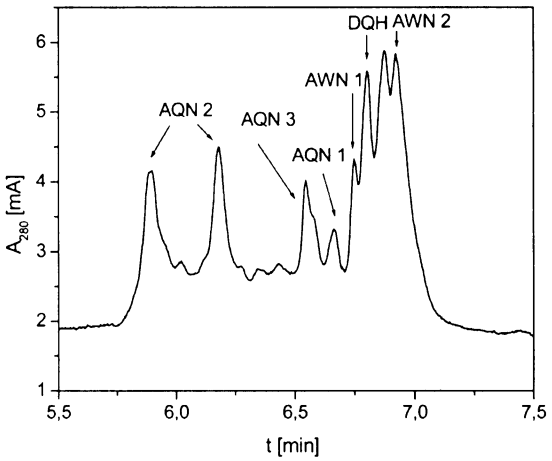

Figure 5. Identification of the components in the H+ fraction of boar seminal plasma in CE (unmodified fused-silica capillary – a micellar system). Peaks are marked directly in the electropherogram; mobile phase, 0.02 M borate buffer with 0.05 M SDS, pH 10.0.

forms of AQN 2 were observed (Fig. 4), which is in agreement with the literature [14]. According to our measurements, almost all the components in H+ boar seminal plasma could be identified (see Fig. 5; the peaks are labeled in the electropherogram), except for one peak, which may represent a differently glycosylated isoform of AWN spermadhesin.

Identification of the H– fraction of the boar seminal plasma proteins was impossible because PSP-I and PSP-II proteins consist of more isoforms which are difficult to differentiate.

4 Concluding remarks

Affinity chromatography with heparin immobilized to Toyopearl support proved to be useful for the analysis of binding properties of boar, bull, and human seminal plasma proteins. The elution of heparin-binding proteins was attained using a salt gradient while the seminal proteins interacting with phosphorylcholine were eluted with 0.05 M phsophorylcholine added to the mobile phase. Different interactions with heparin and phosphorylcholine in different species were confirmed.

A new micellar CE method has been developed for separation of the H+ and H– fractions of boar and bull seminal plasma and applied to the identification of the components in the H+ fraction of boar seminal plasma. The electropherograms demonstrated that even a single isolated protein is a mixture of different isoforms.

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5 References

[1] Evans, J. P., Kopf, G. S., *Andrologia* 1998, *30*, 297–307.

[2] Jansen, S., Ekhlasi-Hundrieser, M., Töpfer-Petersen, E., *Cells Tissues Organs* 2001, *168*, 82–92.

[3] Suarez, S. S., *Cells Tissues Organs* 2001, *168*, 105–112.

[4] Wassarman, P. M., Jovine, L., Litscher, E. S., *Nat. Cell Biol.* 2001, *3*, E59–E64.

[5] Jonáková, V., Tichá, M., *Collect. Czech. Chem. Commun.* 2004, *69*, 461–475.

[6] Maňásková, P., Liberda, J., Tichá, M., Jonáková, V., *Folia Biologica (Prague)* 2000, *46*, 143–151.

[7] Jelínková, P., Ryšlavá, H., Liberda, J., Jonáková, V., Tichá, M., *Collect. Czech. Chem. Commun.* 2004, *69*, 616–630.

[8] Gasset, M., Saiz, J. L., Laynez, J., Sanz, L., *et al.*, *Eur. J. Biochem.* 1997, *250*, 735–744.

[9] Manjunath, P., Thérien, I., *J. Reprod. Immunol.* 2002, *53*, 109–119.

[10] Malm, J., Hellman, J., Magmersson, H., Laurell, C.-B., Lilja, H., *Eur. J. Biochem.* 1996, *238*, 48–53.

[11] Lundwall, Å., Bjartell, A., Olsson, A. Y., Malm, J., *Mol. Hum. Reprod.* 2002, *8*, 805–810.

[12] Peter, A., Lilja, H., Lundwall, Å., Malm, J., *Eur. J. Biochem.* 1998, *252*, 216–221.

[13] Tichá, M., Železná, B., Jonáková, V., Filka, K., *J. Chromatogr. B* 1994, *656*, 423–426.

[14] Jonáková, V., Kraus, M., Veselský, L., Čechová, D., *et al.*, *J. Reprod. Fertil.* 1998, *114*, 25–34.

[15] Simpson, D. C., Smith, R. D., *Electrophoresis* 2005, *26*, 1291–1305.

[16] Heegaard, N. H. H., *Electrophoresis* 2003, *24*, 3879–3891.

[17] Liberda, J., Maňásková, P., Švesták, M., Jonáková, V., Tichá, M., *J. Chromatogr. B* 2002, *770*, 101–110.

[18] Calvete, J. J., Raida, M., Gentzel, M., Urbanke, C., *et al.*, *FEBS Lett.* 1997, *407*, 201–206.

[19] Calvete, J. J., Mann, K., Sanz, L., Raida, M., Töpfer-Petersen, E., *FEBS Lett.* 1996, *399*, 147–152.

[20] Reddy, J. M., Audhya, T. K., Goodpasture, J. C., Zaneveld, L. J. D., *Biol. Reprod.* 1982, *27*, 1076–1083.

[21] Li, S. F. Y., *Capillary Electrophoresis, Principles, Practise and Aplications, Journal of Chromatography Library*, Vol. 52, Elsevier, Amsterdam 1991.

[22] Pacáková, V., Hubená, S., Tichá, M., Maděra, M., Štulík, K., *Electrophoresis* 2001, *22*, 459–463.

Separation Media in Affinity Chromatography of Proteins – A Critical Review

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Abstract: The rapid development of proteomics has been made possible primarily by the progress in analytical and preparative separation methods. Biological systems are so complex that the separation procedures employed must be highly efficient as well as highly selective. The latter requirement is best met by affinity separations based on molecular recognition. The present review critically discusses the properties of the affinity sorbents employed in proteomics, namely, agarose gels, dextrans, modified methacrylate and acrylamide polymers, porous and nonporous silica, porous glass beads, monoliths, affinity membranes and magnetic beads. The physico-chemical properties of these materials, their preparation, application, approaches to their modification and their relative advantages and drawbacks are discussed.

Key Words: Affinity chromatography, proteins, supports, proteomic applications.

INTRODUCTION

Proteomics is one of the most dynamic and important scientific field at present. This would not be possible without rapid progress in the methods of analytical and preparative separations. The complexity of biological systems places great demands on the properties of the separation methods used: a high separation efficiency is required, but a very high selectivity, approaching the unattainable ideal of specificity, is even more important. The affinity methods, based on molecular recognition, definitely exhibit the highest selectivity attainable at present and thus represent the best choice for the purpose, even if their separation efficiency somewhat lags behind that of some other separation methods.

This review deals with one of the most important aspects of affinity separations, the choice and pretreatment of affinity sorbents; this field has recently been developing very rapidly. The most important sorbents are described and critically evaluated, involving agarose gels, dextrans, modified methacrylates and acrylamide polymers, porous and non porous silica and glass beads, monoliths, affinity membranes and magnetic beads. The physico-chemical properties of these materials are summarized, information is provided on their preparation, practical applicability as well as their relative advantages and drawbacks; special attention is paid to modification of these materials in order to meet specialized applications.

AGAROSE GELS

Agarose has widely been used as a stationary phase for various techniques involving affinity interactions. It is a linear polysaccharide consisting of alternating D-galactose and 3,6-anhydro-L-galactose units connected by β-(1,4)- O-glycosidic bond, Fig. (**1**). The reason why these gels have continuously received great attention lies in their physical and chemical properties, such as the very high chemical stability, hydrophilicity, high exclusion limit and low tendency to non-specific interactions. Agarose matrices are commercially available as Sepharose products; they differ in chemical and mechanical stability given by different level of cross-linking which extends their potential use in chromatography (their properties are listed in Table (**1**). The polysaccharide composition offers a high number of easily derivatizable hydroxyl groups which is one of the essential prerequisites for their use in affinity chromatography applications.

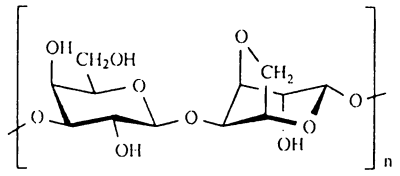

Fig. (1). The chemical structural unit of agarose.

However, agarose gels suffer from several drawbacks which are not critical, but must be taken into account when it is attempted to use agarose as an affinity matrix. Due to their natural composition (they are not synthetic polymers), agarose-based gels suffer from microbial attack, which can severely affect the structure of the gel. To avoid this, it is usually recommended to add 20% ethanol as a preservative and to store the gels at temperatures between 4 - 8 °C (Carlsson *et al.*, 1989). A limited mechanical stability is another very important drawback and even if partial cross-linking improves the robustness of agarose gels, this fact predetermines agarose only for affinity experiments not requiring columns or for low-pressure affinity chromatography applications.

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Table 1. Some properties of Sepharose Gels (Pharmacia-Biotechnology, 1995)

Sepharose	2B	4B	6B	CL-2B	CL-4B	CL-6B
% agarose	2	4	6	2	4	6
Optimal MW separation range (globular proteins)	70×10^3 - 40×10^6	70×10^3 - 20×10^6	10×10^3 - 4×10^6	70×10^3 - 40×10^6	70×10^3 - 20×10^6	10×10^3 - 4×10^6
Bead size range [μm]	60-200	45-165	45-165	60-200	45-165	45-165
Recommended linear flowrate [cm/h]	10	11.5	14	15	26	30
pH stability						
long term	4-9	4-9	4-9	3-13	3-13	3-13
short term	4-9	4-9	4-9	2-14	2-14	2-14
Chemical stability	Stable to all solutions commonly used in gel filtration including 8 M urea and 6 M guanidine hydrochloride.			Stable to all solutions commonly used in gel filtration including 8 M urea and 6 M guanidine hydrochloride. Also stable in organic solvents such as ethanol, DMF, chloroform, dichloromethane, dichloroethane, pyridine, triethyl phospkate and acetonitrile.		
Physical stability	Negligible volume variation due to changes in pH or ionic strength					
Sterilization	Chemical			Autoclavable, 20 min at 120 °C, pH 7		

The thermal stability up to 40°C is usually not considered as a very limiting factor, since most affinity interactions take place at ambient temperature or below it. An elevated temperature can sometimes speed up the elution of a strongly bound protein and obviously agarose cannot be used in such a situation. Gels also have a wide bead size distribution, roughly from 40 to 200 μm, and thus agarose cannot be used as a high-resolution stationary phase or in low-pressure affinity interaction experiments on micro scale. Another very important aspect is its tendency to non-specific binding. The use of agarose as a support for affinity interactions between immobilized ligands and samples in proteomics does not usually pose a risk of considerable non-specific interactions because most features involved in proteomic investigations are rather partially hydrophobic in character and thus they do not significantly interact with the agarose hydrophilic surface. However, even if non-specific binding based on hydrophobic interaction is not a major issue, other types of adsorption occur. Affinity interactions usually proceed under mild conditions where the pH is relatively close to the value in the physiological environment. Under such conditions a charge may be present on several proteins or peptides which can then be involved in non-specific ion-ion interactions. This is usually prevented from happening with agarose gels by adding 100 – 500 mM sodium chloride to the buffers used, thus introducing a substantial amount of ions into the system; these ions interact with hydrophilic surfaces rather than analytes.

As mentioned above, agarose and other polysaccharide matrices contain many hydroxyl groups that can be readily activated in order to immobilize the desired affinity ligand on the solid support. Activated agarose or agaroses with a wide variety of immobilized ligands are commercially available, but when a new ligand is about to be introduced as a tool for proteomic studies, activation or immobilization procedures must sometimes be carried out in the laboratory to attain an optimum yield. Probably the oldest method for activation of agarose was introduced by Axén and co-workers (1967), based on the reaction with cyanogen bromide at a high pH (around 12). The reaction mechanism has already been described, pointing out that the major reactive products that readily react with ligands containing primary amino groups are imidocarbonates and cyanate esters. The main advantages of this reaction are its simplicity, high yields attained and no necessity for using harsh conditions (except for a higher pH). On the other hand, substantial toxicity of cyanogen bromide with a high vapor tension requires working in a well-ventilated fume hood abiding by strict safety rules. For this reason, there was a need for developing a modified procedure that would have posed lower risk without negative impact on the reaction yields. Such a procedure was finally devised by Kohn and Wilchek (1983) who replaced strong inorganic bases maintaining a high pH by organic ones, such as triethylamine or diethylaminopyridine, whereby stable electrophilic complexes with cyanogen bromide were formed. The reactivity of these complexes remained almost unchanged, whereas their toxicity, hygroscopicity and volatility significantly decreased. Finally, 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate that is now commercially available as a stable, solid and almost non-volatile compound, was found to be one of the best cyanogen bromide replacements (Kohn and Wilchek, 1983).

Reactions with bisepoxiranes and epichlorhydrine introduce active epoxy groups onto an agarose-based support and allow immobilization of ligands containing amine, thiol and hydroxyl groups (Sundberg and Porath, 1974). However, it has been mentioned that this way of modification not only introduces partially hydrophobic spacer arm carrying active epoxy group, but may also cause, especially in case of using bisoxirane, noticeable level of cross-linking with a slight decrease in the gel porosity. Cleavage of oxirane ring, as the essential step for immobilization of a ligand, occurs rapidly at a pH around 4 or higher than 8 and most ligands may not be stable under such conditions. With respect to this, the reaction must be carried out at a neutral pH which obviously reduces the speed of the immobilization process; longer reaction times are thus necessary to achieve suitable yields.

Although there are many other ways of activating a polysaccharide matrix (Carlsson *et al.*, 1989), only activation with N,N'-disuccinimidyl carbonate (DSC) will be described which has been described by Wilchek and Miron (1987). This method introduces reactive N-hydroxysuccinimidyl groups into agarose beads and permits immobilization of ligands containing primary amino groups. The reaction is carried out in organic solvents in the presence of triethylamine as a basic catalyst and immobilization is usually carried out in aqueous solution at a neutral pH and offers satisfactory coupling yields.

Agarose-based gels have been used as affinity chromatography supports for more than three decades. Because of their convenient properties, these gels containing various immobilized ligands have played a key role when performing many protein purification processes, either mixing them with samples in tubes or displacing the bound compounds from the gel using elution buffers, or placing them in columns and thus permitting some degree of automation of the whole affinity purification step. The limited stability under high pressure and a large size of the beads prevent agarose from being used for automated high-throughput proteomic experiments and thus affinity purification is usually performed as an additional step to simplify various complex protein mixtures prior to analysis and identification. For example, agarose-based immobilized metal affinity chromatography (IMAC) with Cu(II) ion has been used successfully for the enrichment of histidine-containing peptides prior to their proteomic evaluation (Ren *et al.*, 2003) as well as examining the influence of various buffers and modifying the N-terminus to improve the efficiency and recovery of the affinity trapping. It has been found that the presence of 500 mM sodium chloride in 100 mM acetate buffer (pH 4.0) provided the best elution buffer for underivatized peptides. However, derivatization of N-terminus has slightly lowered the affinity towards Cu(II) ion but later this approach, together with acylation of tryptic peptides, has successfully been applied to quantitative proteomics (Ren *et al.*, 2004).

Agarose loaded with either Fe(III) or Ga(III) ions was used to isolate phosphopeptides from digests of standard phosphoproteins such as β-casein and α-casein (Zhou *et al.*, 2000). The eluted phosphopeptides were analyzed by MALDI-MS without any additional separation and it was found that while Ga(III) ion had better overall selectivity for phosphopeptides, Fe(III) ion exhibited stronger affinity towards monophosphorylated peptides. Even though the difference in affinity interaction of phosphopeptides towards these two ions is not sufficiently significant to separate monophosphorylated peptides from those with multiple phosphorylation sites, IMAC enrichment, followed by treatment of eluted phosphopeptides with phosphatase and carboxypeptidase Y, has led to successful assignment of even multiple phosphorylation sites and appeared promising for applications in biochemical analysis.

Agarose beads with immobilized Fe(III) ions have also been used for enrichment and subsequent identification of phosphopeptides (Zheng *et al.*, 2005); the authors mainly focused on a study of peptides containing phosphotyrosine from Jurkart cell cultures. After extraction of proteins followed by tryptic digestion and isotopic labeling, phosphopeptides were selectively enriched and identified. In this case, the use of IMAC on agarose significantly improved the number of identified phosphopeptides and revealed novel phosphorylation sites on some proteins involved in signal transduction.

In addition to phosphorylation, glycosylation is another post-translational modification of proteins which has been paid significant attention in the field of proteomics. To enrich glycoproteins from various biological samples for their further analysis and identification, agarose-based affinity matrices with immobilized lectins have widely been used, usually displacing the bound glycoproteins from the affinity media by washing with hapten sugar. For example, Con A agarose affinity chromatography was employed to fractionate glycoproteins present in various kinds of snake venoms (Nawarak *et al.*, 2004). Glycoproteins bound to Con A were eluted with 0.2 M mannose, subsequently deglycosylated, separated by 2D gel electrophoresis, and identified. It was found that all kinds of snake venom subjected to this study contained Con A-binding glycoproteins of moderate or higher molecular weights. This probably comes from the fact that Con A is mannose and glucose binding lectin and thus it also interacts with the chitobiose core of N-glycans. On the bases of database searches, the authors suggest that extensive glycosylation might protect the activity of some proteins rather than being responsible for their toxicity.

Glycopeptides derived from human plasma antithrombin has been enriched on Con A-agarose (Demelbauer *et al.*, 2005). Further analysis by tandem MS led to the assignment of glycosylation sites and identification of several glycoforms of antithrombin.

In another proteomic/glycomic study (Young *et al.*, 2002), SBA lectin-agarose played a key role in the purification of glycoproteins from glycine extracts of *Campylobacter jejuni* cell cultures. At least 22 glycoproteins, predominantly annotated as periplasmic proteins, were identified after 2D gel electrophoresis and tandem MS analysis. In addition, purified glycoproteins were subjected to pronase digestion and the glycopeptides generated were analyzed using nano-NMR techniques. This approach, involving β-elimination to find out if the glycan attached to the peptide backbone is N- or O-linked, allowed structural identification of the major glycan moiety. Further genetic mutation abolished the N-glycosylation of the protein and thus confirmed that the en-

zyme coded by this gene was responsible for formation of the N-linkage.

Cell-surface proteins shed by metalloproteases were successfully identified using WGA-agarose affinity chromatography (Guo *et al.*, 2002). Enriched glycoproteins were later deglycosylated with PNGAse F, separated by polyacrylamide gel electrophoresis, identified and relatively quantified *via* isotope dilution. The use of wide specific lectin binding glycans with GlcNAc and sialic acid provided quite a high degree of enrichment, as a majority of N-linked glycoproteins present in real samples contain N-acetyl-D-glucosamine or are sialylated. In this case, the affinity purification step improved the proteomic investigation and helped to identify several new proteins.

Con A-agarose was also used as an affinity medium for purification of glycoproteins from an extract of *Caenorhabditis elegans* (Kaji *et al.*, 2003). The uniqueness of this approach lies in the identification based on glycosylation-site specific tagging. After tryptic digestion of enriched glycoproteins, the glycopeptides were selectively labeled with ^{18}O isotope by treatment with N-glycanase in a buffer prepared in $H_2{}^{18}O$, thus implementing ^{18}O into the peptide backbone when N-linked asparagine was converted into aspartic acid during deglycosylation process. As many as 250 glycoproteins, including 83 putative transmembrane proteins, were then identified with simultaneous assignment of 400 unique N-glycosylation sites.

Wilkins and co-workers (Ghosh *et al.*, 2004) captured membrane glycoproteins using concanavalin A and wheat germ agglutinin lectin affinity chromatography. The glycoproteins were separated by RPLC and analyzed by MALDI-MS. The two lectins determined two different pools of membrane glycoproteins with a broad range of molecular mass and pI values. This technique not only demonstrated that approximately 30% of isolated proteins contained predictable transmembrane regions characteristic of integral membrane proteins but also revealed proteins resulting from genes of unknown function.

Urinary prostate-specific antigen (PSA) was subjected to a proteomic/glycomic study from the point of view of profiling the affinity towards lectins with different specificity (Jankovic and Kosanovic, 2005). It has been found that monitoring the changes in glycosylation microheterogeneity yields better results when lectins with narrow specificities are used instead of the ones with broad binding ranges. Whereas Con A-agarose affinity chromatography has provided rather inconsistent results, SNA-I, UEA-I, RCA, PHA-L and PHA-E lectins has been found to be more relevant for possible clinical evaluation of prostate.

Multidimensional agarose-immobilized lectin affinity chromatography was successfully applied to the determination of different levels of sialylation of serum glycoproteins (Qiu and Regnier, 2005). Glycopeptides from human blood serum tryptic digest were first enriched by Con A affinity chromatography, isotopically labelled and part of the sample was fractionated on an SNA column. The fractions were then mixed, deglycosylated, separated and analyzed by RPLC-ESI-MS. It was concluded that using serial affinity chromatography with lectins with broad and narrow selectivity allowed assessment of different degrees of sialylation at individual glycosylation sites. In addition, with samples from healthy donors, the degree of sialylation among glycoproteins did not probably exceed the variation in concentration between non-glycosylated proteins.

Multi-lectin affinity chromatography (M-LAC) was first introduced by Yang and Hancock (2004) where the affinity column was prepared by even mixing of four different lectins immobilized on agarose. Displacement of bound glycoproteins with different elution buffers showed the promising way for monitoring glycosylation changes of human blood serum glycoproteins and the importance of the distribution shift in affinity towards various lectins for diagnostic purposes (Yang and Hancock, 2005). Finally, M-LAC enrichment of human blood serum sample followed by elution with a buffer containing four different sugars led to identification of more than 150 glycoproteins with a high level of confidence (Yang *et al.*, 2005).

Despite the above-mentioned limitations of agarose media, applications can still be found trying to integrate these matrices into systems for high-throughput proteomic analyses. For example, on-line tryptic digestion was carried out on trypsin-immobilized agarose beads together with iminodiacetic agarose that were packed into sequential microchannels and facilitated on-line enrichment and identification of phosphopeptides (Yue *et al.*, 2005).

Bischoff and co-workers introduced a very efficient trypsin reactor based on acetylated trypsin immobilized on NHS-activated Sepharose (Freije *et al.*, 2005). Acetylation of trypsin after immobilization not only suppressed its tendency to autolysis but also significantly improved the reactor efficiency. Rapid on-line digestion of Cytochrome C on modified trypsin-agarose packed in 10 mm x 2 mm ID cartridges resulted in complete conversion of the protein to its component peptides within 4 seconds and turned out to have a great potential for high-throughput proteomic applications.

DEXTRANS

Dextran is a branched-chain glucose polymer. Soluble dextran, prepared by fractional precipitation with ethanol of partially hydrolyzed crude dextran, contains more than 90% of α-1,6-glucosidic linkages, and it is branched by 1,2-, 1,3- and 1,4-glucoside bonds. When crosslinked with 1-chloro-2,3-epoxypropane in alkaline solution, dextran affords a three dimensional gel (Fig. **2**) (Turková, 1993). Dextran gels are chemically very stable. On the other hand, a disadvantage of dextran and of other natural polymers is their sensitivity to bacterial attack. For this reason, it is recommended to store them with a bacteriostat. Furthermore, dextrans are not very mechanically stable.

The dextran gels are supplied by General Electric Healthcare (http://www1.amershambiosciences.com) under the trade names Sephadex and Superdex. Sephadex™ is hydroxypropylated, cross-linked dextran and is stable within a pH range of 2-10 (Sephadex G-10, G-15 and G-25 are stable up to pH 13). It is also stable in most aqueous and organic eluent systems. The matrix of Superdex™ is a spherical composite of cross-linked dextran and agarose. This matrix combines the excellent size exclusion properties of cross-

linked dextran (Sephadex™) with the physical and chemical stability of highly cross-linked agarose, to produce separation media with outstanding selectivity and high resolution. In addition, their low tendency to non-specific interactions permits high recoveries of biological material. It is stable for a long time within a pH interval from 3 to 12 and can withstand a brief exposure to media with a pH of 1 to 14. All common buffers including 8 M urea and 6 M guanidinium hydrochloride can be used.

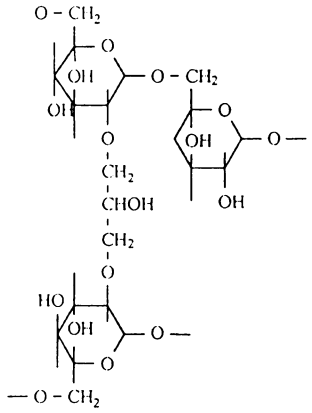

Fig. (2). The chemical structure unit of dextran gel.

Sephadex is not sufficiently porous for general use and it is mainly employed in gel-filtration or ion-exchange chromatography. However, there are also some applications to affinity chromatography. A two step purification of lysozyme in the urine of hemodialysis patients involving DEAE Sephadex chromatography followed by Sephacryl chromatography has been reported (Takai *et al.*, 1996). The concentrated urine was applied to a DEAE Sephadex A-50 column which was washed with a phosphate buffer. The fractions were collected to measure the lytic activity. Then the samples were dialyzed against deionized water and concentrated by lyophilization. The lyophilized sample was dissolved in a phosphate buffer saline (PBS) and applied to a Sephacryl column. The fraction indicating the lytic activity was concentrated as a finally purified lysozyme solution. The completeness of purification of lysozyme may be explained by its molecular affinity for Sephacryl.

Birkenmeier and Kopperschläger (1982) covalently bound Cibacron blue F3G-A to Sephadex G-100 for isolation of α-1-proteinase inhibitor and α-1-acid glycoprotein, the binding being affected by an ether kink between the triazine ring and the polysaccharide matrix. The dye-Sephadex conjugate (Blue Sephadex) was equilibrated with a phosphate buffer. This matrix is capable of adsorbing a great number of serum proteins, e.g. albumin, lipoproteins, blood coagulation factors, α-1-antichymotrypsin and complement factors. The modified Sephadex is sufficiently stable at high ligand concentrations; it permits sufficiently high flow-rates in a column and can also be used for large-scale chromatography.

A very interesting use of Sephadex matrix was published by Porath and Hansen (1991), namely a cascade-mode multiaffinity column chromatography (CASMAC) with human serum as a model mixture. More than 99% of of the serum proteins adsorbed in the same high salt-containing buffer on tandem columns consisting of (1) immobilized Zn^{2+} on tris-carboxymethyl diamine gel followed by (2) thiophilic (T) gel, (3) Zn^{2+} bound to the new tridentate chelating adsorbent dipicolylamine agarose (DPA), (4) hexyl-thioether C6-S agarose and (5) Ni^{2+}-DPA agarose. After the adsorption step, the immobilized metal ion affinity gels were attached to the top of tandem columns of other adsorbents – T gel, Sephadex G-25 and Mono-Q and the elution conditions were selected such that a further group separation was attained. A high resolution, high recovery, easy manipulation and a high capacity are characteristic features of the cascade process with these adsorbents.

Bizzozero and co-workers (1982) published a method for the purification of proteolipids from rat brain on a lipophilic dextran gel. Sephadex LH-60 and LH-120 were washed, swollen and packed into the glass columns. Crude proteolipid solutions were applied to the column and eluted with a chloroform-methanol mixture containing HCl.

METHACRYLATE POLYMERS AND MODIFIED ACRYLAMIDE

Hydrophilic hydroxyalkyl methacrylate gels were introduced by Wichterle and Lim (1960). In 1973, Coupek *et al.* (1973) described a spherical macroporous copolymer of 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate, Fig. (3). This copolymerization is carried out in a non-polar medium and leads to the formation of homogenous gels which swell in water and other polar solvents. The pore size and pore distribution of gels in a swollen state is determined exclusively by the content of the cross-linking monomer in the initial mixture.

The original patented technology of suspension polymerization of water-soluble monomers permits the preparation of spherical particles whose size and porosity is controlled over a wide range. The subsequently hydrophilized HEMA-BIO series of adsorbents exhibits pressure resistance up to 20 MPa, long-term stability in the pH range 2-12 and compatibility with most organic solvents; 1 M hydrochloric acid and 1 M sodium hydroxide solution can be used for short-term regeneration and purification. The highly hydrophilic surface results in a high protein recovery and a high activity of the isolated protein (Coupek and Vins, 1994). The supports are biologically inert and are not attacked by microorganisms. HEMA has a molecular weight exclusion limit ranging from 20.000 to 20,000,000 (Turková *et al.*, 1991).

Hydroxyalkyl methacrylates in the gels contain a large amount of nonionogenic hydroxyalkyl groups, the reactivity and solvation of which are analogous to those of primary aliphatic alcohols. These gels are sufficiently hydrophilic for the chromatography of biopolymers, but are more hydrophobic than cellulose, agarose or polydextran. The possibility of preparing these gels in the bead form and their mechanical strength and favorable parameters at high flow rates and pressures make them very suitable supports for HPLC stationary phases (Mikes *et al.*, 1978).

Owing to their high hydroxyl group content, HEMA sorbents are readily modified by immobilization of various ligands using common methods with cyanogen bromide,

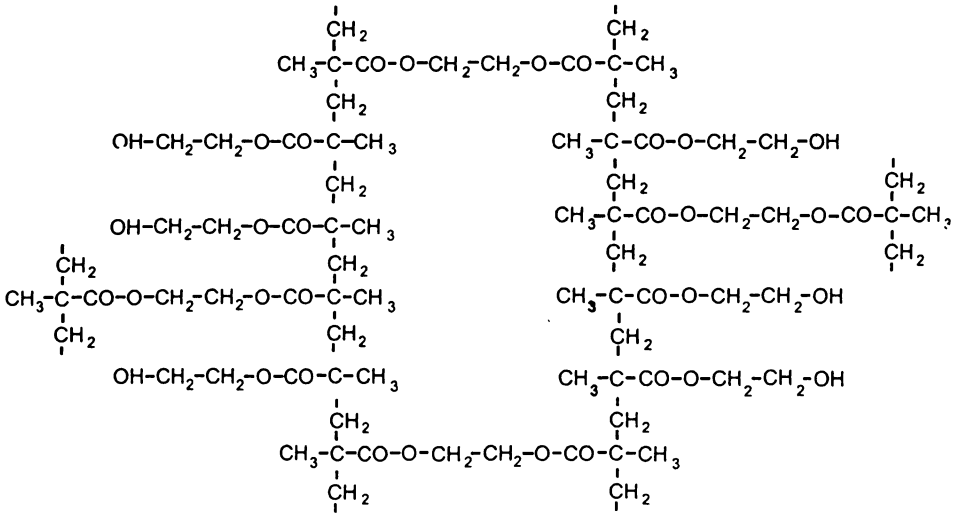

Fig. (3). The chemical structure of HEMA. Reprinted from (Coupek and Vins, 1994).

glutaraldehyde or benzoquinone. Amino acids, peptides and proteins can be bound to the activated gels through their amino groups (Coupek and Vins, 1994).

Hydroxyethyl methacrylate supports are commercially available under the trade name Separon HEMA from TESSEK, Ltd. (http://www.tessek.com/cat4.htm). They have a rigid, macroporous and hydrophilic polymer matrix, synthesized by suspension co-polymerization of 2-hydroxyethyl methacrylate (HEMA) and ethylene dimethacrylate (EDMA). As a result of the polymerization of hydrophilic HEMA units with alternate incorporation of crosslinking EDMA monomers, the growing backbones create a dense, non-penetrable three-dimensional network in the form of microspheres (nodules). Tessek offers preactivated affinity supports. Two different modifications are available - epoxy and vinysulphone with different levels of activation. Epoxy-activated supports (HEMA E) react under suitable conditions with a variety of functional groups, especially with amino-, thio-, carboxy- and hydroxy groups. Vinylsulphone-activated supports (HEMA VS) exhibit higher reactivity, particularly for hydroxyl groups (http://www.tessek.com/cat4.htm).

Lukás and co-workers (1977) investigated the basic physical and chromatographic properties of a number of glycidyl methacrylate-co-ethylendimethacrylate (GMA-co-EDMA) copolymers. These copolymers can be prepared with a broad range of specific surface areas and have sufficient thermal stability (up to more than 200 °C). With increasing proportion of the cross-linking agent EDMA, the specific surface area of the copolymer greatly increases. A change in the copolymer composition has no essential influence on its thermal stability.

Prádny *et al.* (2003) published different conditions for preparation of macroporous crosslinked copolymers of 2-hydroxylethyl methacrylate and methacrylic acid in the presence of fractionated particles of sodium chloride. The hydrogels were characterized by the number of pores in unit volume, the pore size, the water content in the pores and the diffusion parameters. The content of methacrylic acid higher than 25 mole % could not be measured because the hydrogel spontaneously decomposed during swelling. These hydrogels might be useful as implants in nervous tissues.

Alvarez *et al.* (2001) compared the behavior of two IMAC supports: poly(hydroxylated polybutadienic-hydroxyethyl methacrylate ([poly(PB-HEMA)] and poly(ethylene glycol dimethacrylate-hydroxyethyl methacrylate) [poly(EG DMA-HEMA)]. Both these matrices were epoxy-activated using 1,4-butanediol diglycidyl ether. Imidodiacetic acid was bound as a ligand to the activated matrices. The structural properties of poly(PB-HEMA) and poly(EGDMA-HEMA) are listed in Table (2). The poly(PB-HEMA) base support exhibited a higher functionality (epoxy and acid), a lower ligand occupation (L) and the presence of a larger proportion of macropores than poly(EGDMA-HEMA) which had a larger amount of mesopores and a higher total specific surface of pores.

The influence of the morphology of poly(EGDMA-co-HEMA) base support on various Fe^{3+}-contaning sorbents and on their properties in retention of O-phosphothreonine was investigated by Gomez and co-workers (2003). Three base poly(EGDMA-co-HEMA) supports were obtained using different quantities of the initiator in suspension polymerization reactions. These products were chemically treated with 1,4-dibutanediol diglycidyl ether (BDGE) for activation and several chelating agents (iminodiacetic acid, IDA; disodium ethylenediamine tetraacetate, EDTA; hexamethylenediamine tetrapropanoic acid, HMDTP) were used in coupling reactions to obtain Fe^{3+}-containing sorbents.

Serine proteinases from the human and bovine intervertebral discs were studied using affinity chromatography on the hydroxyalkyl methacrylate copolymer Separon HEMA 1000 E with immobilized basic pancreatic trypsin inhibitor (BPTI) (St'ovícková *et al.*, 1992).

Table 2. Structural Properties of Poly(PB-HEMA) and Poly(EGDMA-HEMA) (Gomez *et al.*, 2003)

Polymeric base support	Total S_s m^2/g	S_s of mesopores m^2/g	S_s of macropores m^2/g
poly(PB-HEMA)	21	7	14
poly(EGDMA-HEMA) I	136	134	2
poly(EGDMA-HEMA) II	92	83	9

Toyopearl is another type of support based on a methacrylate derivative. It is a hydrophilic vinyl polymer which can be used under high pressure. It is commercially available from Tosoh Bioscience under the trade-name TSKgel. TSK-gel affinity chromatography columns are based on the G5000PW (5PW) porous resin with 0.1 μm pores (a high exclusion limit) and with an estimated protein exclusion limit of $5x10^6$ Da. The small particle size (10 μm) makes it suitable for analytical applications (Tosoh Bioscience, 2003). The supports can be ordered either activated (e.g., epoxy, tresyl, formyl, amino, carboxy, and chelate) or with a ligand coupled (e.g., p-aminobenzadine ABA, heparin, blue HC) (Tosoh Biosciences, http://www.tosohbioscience.de).

Matsumoto *et al.* (1982) published the optimum conditions for activation of Toyopearl by the epoxy method, the subsequent derivatization and immobilization of various ligands. The epoxy activation was performed using epichlorhydrin in a NaOH solution at an increased temperature. Compared to agarose gel, it can withstand harsher conditions, a higher temperature and higher concentrations of NaOH and epichlorhydrin. The maximum amount of epoxy groups introduced into Toyopearl was found to correspond to 30% epichlorhydrin. The higher the reaction temperature, the shorter was the time for maximum incorporation and for degradation (at 60 ° C the maximum was reached in less than 1 h). The prepared gels were subsequently aminated (using concentrated ammonia solution at 40°C) or succinylated (using succinic anhydride). The ligands were introduced into the gel: lactose (binding by reductive amination with amino Toyopearl), D-galactose amine hydrochloride (GalNHCl; binding with succinyl Toyopearl) and soybean trypsin inhibitor (STI; binding with succinyl Toyopearl). The stationary phases prepared were tested with lectins.

Methacrylate polymers bearing bioactive compounds often show high levels of nonspecific interactions. Therefore, Tanaka and co-workers (Shiyama *et al.*, 2004) introduced new hydrophilic spacers to suppress non-specific binding of proteins. Spacer 1 was based on the poly(ethyleneglycol) (PEG). However, the nonspecific binding of proteins still remained even after introduction of a hexamer of the PEG-type spacer (Tamura *et al.*, 2003). Spacer 2 was also based on PEG but it contained a phenyl ring to join two PEG moieties. Spacers 3-5 were based on tartaric acid derivatives. In order to assess the effectiveness of these new spacers (2-5), the authors synthesized affinity matrices bearing a ligand FK506 (immunosuppressive drug) on Toyopearl. It was concluded that some tartaric acid derivatives were most effective. Introduction of derivatives 5 into Toyopearl phases reduced tubulin and actin nonspecific interactions by almost 65% and 90%, respectively.

The technique of dye ligand affinity chromatography has widely been used for protein purification but most of the examples are given for analytical applications (reviewed by Lowe and Pearson, 1984). Nakamura and co-workers (2003) described the basic properties of Toyopearl AF-Blue HC-650 M (supplied by Tosoh) and its application to the purification of proteins. The authors pointed out the high binding capacity of this stationary phase for human albumin and its high stability in acidic and alkaline solutions. It is a suitable adsorbent for preparative use because of its high mechanical stability, binding capacity, protein recovery and easy regeneration.

Another interesting study was concerned with immobilization of p-aminobenzamidine on a Toyopearl support, the basic properties of this stationary phase and some applications to protein purification (Nakamura *et al.*, 2003). ABA is a synthetic inhibitor for trypsin-like proteases and it was covalently immobilized on Toyopearl (three different Toyopearls with pore sizes within a range of 50-100 μm) by reductive amination. ABA-Toyopearl was used for high-performance affinity chromatography (HPAC) of trypsin-like proteases (trypsin, thrombin, tissue-type plasminogen activator, urokinase).

Zhuang *et al.* (1996) isolated a lectin (APL) from the toxic mushroom *Amanita pantherina*. The isolation procedure consisted of 4 steps: 1) A salt-extract was adjusted to 40% saturation with ammonium sulphate and 2) directly applied to Butyl-Toyopearl (hydrophobic chromatography). 3) The active fraction was subjected to affinity chromatography on bovine submaxillary mucin (BSM)-Toyopearl. And finally 4) the eluate from the column was further purified by gel filtration on Superose 12 HR10/30 using a FPLC system.

Varilová *et al.* (2005 a) published the preparation and testing of three different kinds of stationary phases with immobilized 3,5-diiodo-L-tyrosine (DIT) which were tested on a model sample of porcine pepsin A and applied to human pepsin. DIT was bound to the epoxy- and vinylsulphone-activated hydroxyethylmethacrylate supports and epoxy-activated Toyopearl. The results showed that the preparation of stationary phases with immobilized DIT was reproducible: two different columns of HEMA VS-DIT were prepared, the amounts of DIT were 48 and 51 mg per g of support, respectively. These phases exhibited sufficient selectivity for pepsin and were found to be applicable to practical samples. On the other hand, their lifetime was limited to only about two months.

Varilová and co-workers (2005b) also studied the interactions of boar, bull and human seminal plasma proteins with heparin and phosphorylcholine. The proteins were monitoried by affinity liquid chromatography using heparin immobilized to a Toyopearl support. The TOYOPEARL-heparin column was purchased from Watrex. The heparin-binding proteins were eluted using a salt gradient while the seminal proteins interacting with phosphorylcholine were eluted by adding 0.05 M phsophorylcholine to the mobile phase. Different interactions of various species with heparin and phosphorylcholine species were confirmed.

Acrylamide derivatives represent another type of synthetic polymers. They were synthesized by Jedlinski and Paprotny (1967) who investigated the polymerization rates of N-alkylacrylamides under homogenous conditions. Girot and Boschetti (1981) reported the physico-chemical properties of a carboxylic ion exchanger obtained by polymerization of N-[tris(hydroxymethyl)methyl]acrylamide. This macromolecule bears three hydroxymethyl groups, a carboxyl group and one alkylamide group per principal repeating unit. Due to these chemical functions, the polymer is very hydrophilic and suitable for separation of biological macromolecules, especially proteins. The molecular weight of N-[tris(hydroxymethyl)methyl]acrylamide is higher than that of acrylamide and thus the matrices obtained at identical molar concentrations contain more dry material and are mechanically more resistant.

The acrylamide derivatives are advantageous in their stability to most eluting agents including salts, urea, guanidinium hydrochloride and detergents. The support has many modifiable groups which can easily be derivatized. On the other hand, the disadvantage lies in a low degree of bead porosity. This material is commercially available from the Bio-Rad Laboratories under the tradename Bio-Gel. The gels are prepared by copolymerization of acrylamide and N,N'-methylene-bis-acrylamide. Bio-Gel P gels are supplied dry and are available in several particle size ranges with molecular weight exclusion limits ranging from 1,800 to 100,000. Owing to their synthetic character, they do not support microbial growth or leach carbohydrates in contrast to dextran and agarose gels. Bio-Gel P gels are extremely hydrophilic and essentially nonionic. They are autoclavable at pH 5.5–6.5 and operate over a pH range of 2–10 at room temperature. The flow rate and resolution increase with increasing temperature within a range of 4–80°C. Bio-Gel P gels are compatible with dilute organic acids, 8 M urea, chaotropic agents, detergents, and miscible organic solvents. Alcohol can be used up to 20% v/v to enhance the solubility of nucleotides, peptides, and tannins without altering the exclusion properties of the gels. Formamide may also be used at full strength, as Bio-Gel P gels are completely swollen in this solvent (Bio-Rad, www.bio-rad.com).

Liberda and co-workers (2002) prepared an affinity sorbent containing immobilized L-glyceryl phosphorylcholine attached to poly(acrylamide-allyl amine) copolymer for isolation of phosphorylcholine-binding proteins from seminal plasma (bull, boar and human). L-glyceryl phosphorylcholine was dissolved in dimethylformamide, mixed with maleic anhydride and shaken overnight at laboratory temperature. The solution was then mixed with 1-(3-dimethylaminopropyl)-3-ethylcarbodimide and shaken for 3 hours. Then a suspension of poly(acrylamide-allylamine) copolymer was added and shaken for 48 h. Finally, the gel was washed.

Ziegler and others (Ziegler *et al.*, 1994) synthesized 5-aminopentyl 4,6-O-[(R)-1-carboxyethylidene]-β-D-galactopyranoside and coupled this ligand to epoxypropyl-modified polyacrylamide beads. This stationary phase was used for the affinity chromatography of human serum amyloid P (SAP) protein.

Another interesting use of acrylamide derivative was published by Mattiasson (Arvidsson *et al.*, 2003). A continuous supermacroporous matrix was developed allowing direct capture of enzyme from non-clarified crude cell homogenate. The matrix was produced by radial co-polymerization of acrylamide, allyl glycidyl ether and N,N'-methylene-bis (acrylamide) which took place in aqueous solution of the monomers frozen inside a column (cryo-polymerization). After thawing, the column contained a continuous matrix having interconnected pores of 10-100 μm. The cryogel was mechanically very stable and it could be easily removed from the column, dried and kept in a dry state.

POROUS, NONPOROUS SILICA AND POROUS GLASS BEADS

Albeit silica exists in many different forms that are sometimes very difficult to classify, the formation of a silica gel is a well-described process comprising several subsequent steps. First, silica sols are prepared by adding sodium silicate into an acidic solution, followed by pH dependent formation of a gel when colloidal particles form three-dimensional aggregates called silica hydrogel. Afterwards, the hydrogel is dried out resulting in the formation of xerogel that is also commonly referred to as porous silica (Unger, 1979). The structure of silica is amorphous and usually expressed as $SiO_2.x\ H_2O$, with tetrahedral (SiO_4) basic units (Turková, 1993). Whereas the interior of silica particles contains very stable Si-O-Si bonds, the surface mainly comprises slightly acidic silanol groups (Si-OH) which maintain the tetrahedral configuration of the exposed silicon atom.

The high mechanical stability with capability of forming well-defined, regularly shaped particles belong to the main advantages of silica gels for their use in various chromatographic applications. Furthermore, in the case of porous silica, gel formation procedures can be modified in order to obtain particles with the required porosity which is very important, especially when silica is intended to be used as a solid support for affinity interactions. Countless bulk silica products are at present commercially available, differing not only in the particle size, which usually varies from 3.5 to more than 100 μm (1.5 – 10 μm for nonporous silica), but also in the pore size falling into a range of 60 – 4000 Å. Various properties of silica particles have also been thoroughly reviewed (Mikes, 1988; Ohlson *et al.*, 1989). On the other hand, despite excellent chemical stability of silica at lower pH values (up to 2), exposing the particles to pH values higher than 8 causes their partial dissolution that exponentially increases with the pH increasing toward more basic values. The solubility also increases linearly with the temperature, exponentially with decreasing particle size and it is

significantly influenced by the presence of bulk impurities (Unger, 1979).

Unfortunately, bulk silica does not contain reactive groups needed for the attachment of an affinity ligand. Surface silanol groups are capable of dissociation in aqueous solvents but that is obviously not enough to form a covalent bond with the ligand. In addition, the presence of a charge on the silica surface may cause significant tendency to nonspecific binding and thus it has to be minimized if not completely eliminated. For these reasons, it is always necessary to derivatize silica by introducing reactive functional groups, prior to the immobilization of an affinity ligand. This is usually done by treatment with appropriate silanes, such as γ-aminopropylsilane or γ-glycidoxypropylsilane in either organic (Larsson, 1984) or aqueous solvents (Voyksner *et al.*, 1990) with a trace of triethylamine as the catalyst. Three common ways to derivatize silica to make it suitable for immobilization of the ligand has been proposed (Larsson, 1984). Each procedure starts with the reaction of silica with γ-glycidoxypropylsilane to modify the surface with reactive epoxy groups. Then the ligand can be immobilized onto the resulting epoxy silica directly, or epoxy groups can further be modified prior to final use, as depicted in Fig. (**4**). Another way of derivatizing silica with simultaneous masking of the remaining silanol groups is the coating with a polymer containing functional groups capable of reaction with the ligand (Geng *et al.*, 2001). Grafting of silica with chitosan prior to ligand coupling was also successfully applied to minimizing the tendency to nonspecific binding of analytes to the silica surface (Xi *et al.*, 2005).

Excellent mechanical stability with the possibility of forming regular particles with well-defined porosity permit silica to be used as a stationary phase in high-performance affinity chromatography (HPAC). HPAC has been extensively applied to the purification of proteins and peptides. Compared to its low-pressure counterpart, HPAC offers several advantages, such as shorter analysis times, better resolution due to sharper peaks, better selectivity, more sensitive detection, and the possibility of interfacing to a variety of automated systems (Ohlson *et al.*, 1989). In spite of these advantages, the number of applications employing HPAC on silica-based materials in comprehensive proteomic measurements is surprisingly low. There may be several reasons responsible for this. First, in contrast to the wide availability of affinity media for low pressure affinity chromatography, there is a lack of commercially available silica-based affinity stationary phases with already immobilized ligands. This requires in-house attachment of a particular protein to the silica surface. Sometimes this can be time-consuming. Furthermore, the absolute majority of recently published methodologies utilizing HPAC in proteomics, and especially high-throughput proteomic approaches, prefer monoliths or affinity membranes as affinity media, which will be discussed later.

Nevertheless, there has been a successful attempt at characterizing the affinity binding of metal ions to the chelating agents immobilized on the controlled porous glass (CPG) surface (Howard and Holcombe, 2000). The authors conducted a study employing zonal and frontal chromatographic techniques resulting in generation of breakthrough curves based on the equilibrium constants and capacities of the immobilized species while taking into account the mass-transfer limit and axial dispersion imposed by the substrate. The theoretical results were in a good agreement with experimental data obtained on an affinity microcolumn loaded with 8-hydroxyquinoline. Thus this approach was considered

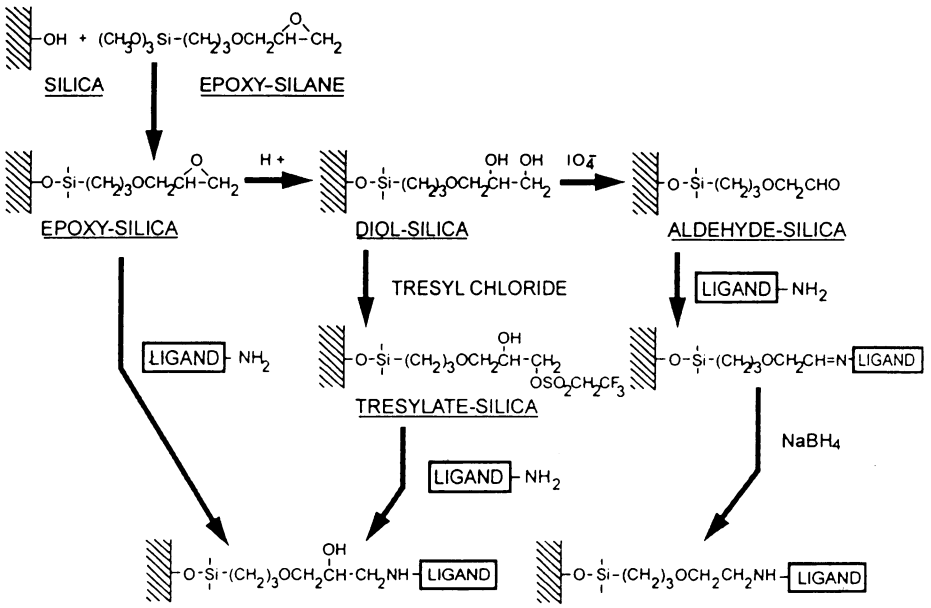

Fig. (4). Three routes for preparing ligand-substituted silica. Reprinted from (Larsson, 1984).

useful for characterization of other, even more complicated affinity systems.

Hage and co-workers (Sengupta and Hage, 1999) used HPAC on human serum albumin (HSA) covalently bonded to macroporous silica to identify and evaluate some drug binding regions previously predicted to exist and to be responsible for transport of low molecular weight compounds in the body. While HSA is known for its capability of carrying a wide variety of small proteins and drugs and thus controlling the transport and distribution of these compounds, comprehensive arrangement of minor and major drug binding sites was not proposed at that time. Researchers conducted zonal elution experiments employing a variety of agents known as potential markers for the HSA binding sites and successfully developed a model describing the relationship between the various binding regions on HSA. This methodology became very useful for characterizing HSA binding sites to other drugs or proteins.

The attachment of HSA to the porous silica has later been improved. (Kim *et al.*, 2004). Silica was treated with 3-aminopropyltrimethoxysilane in order to derivatize the surface with aminopropyl groups and then it was activated with disuccimidyl suberate (DSS) to introduce active N-hydroxysuccimidyl (NHS) esters capable of reacting with primary amino groups of affinity ligands. This method also offered very good coupling yields achievable within a short reaction time (2 hrs) and, compared to the Schiff-base coupling method devised by Larsson (Larsson, 1984). It provided a better activity and long-term stability of the immobilized ligand. Finally, HSA attached to NHS-silica has been successfully used as a chiral stationary phase for the separation of racemic mixtures of R/S-warfarin and D/L-tryptophan (see Fig. 5). This is made possible by a higher affinity of a certain isomer toward a particular drug binding site on HSA.

Affinity chromatography on silica-based supports has also been used for the predominantly small-scale metal ion affinity chromatography enrichment of phosphoproteins, phosphopeptides, or peptides containing certain amino acids in their sequences. For example, the use of a novel stationary phase consisting of a magnetic core and porous silica with nickel ions strongly incorporated into the matrix (SiMAC beads) as the stationary phase resulted in a very efficient enrichment of histidine-tagged recombinant proteins (Frenzel *et al.*, 2003). SiMAC-based affinity chromatography then offered twice as high yields compared with those obtained with the conventional materials. It completely removed in-house handling and immobilization of toxic Ni^{2+} salts.

A comprehensive study involving optimization of the affinity enrichment of standard phosphopeptides spiked with the tryptic digests derived from standard proteins on immobilized iminodiacetic acid-silica (IDA-silica) has been reported (Trojer *et al.*, 2005) . The bound phosphopeptides were later eluted

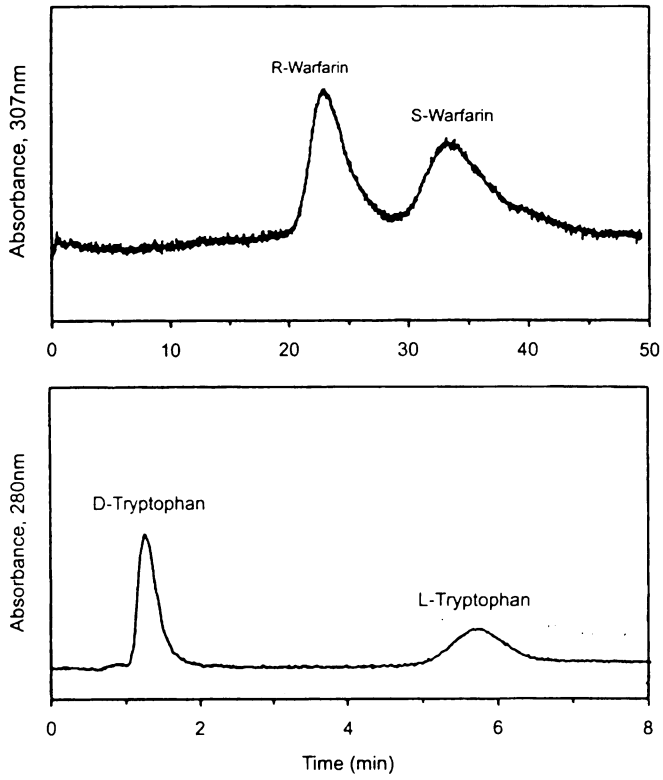

Fig. (5). Chiral separation of (a) R/S-warfarin and (b) D/L-tryptophan on an immobilized HSA column prepared using NHS-activated silica. Reprinted from (Kim *et al.*, 2004).

from the column with an appropriate elution buffer, and desalted on a C18 column prior to capillary-LC-MS/MS analysis. Since several parameters strongly influencing the recovery and selectivity of the process have been evaluated, the authors have found out that loading of a sample at pH 2.5 onto the IDA-silica with a pore size of 0.1 μm, followed by the elution with a phosphate buffer at pH 8.0, results in a 90% recovery and a minimum non-specific binding of non-phosphorylated peptides. However, in order to minimize the binding interference of the carboxylic acid present on then C-terminus of the peptide, the interfering groups were converted into the methylesters. Methylation successfully eliminated the interference but, on the other hand, made the analysis and identification of bound phosphopeptides more complicated due to the occurrence of partial derivatization.

A new IMAC matrix was prepared by coating non-porous silica with cross-linked chitosan permitting the association with Cu^{2+} ions (Xi and Wu, 2004). The problems connected with lower loading capacities of non-porous silica were overcome by the formation of a macroporous structure layer through imprinting polyethylene glycol (PEG) in a chitosan film. This approach turned out to substantially decrease the tendency to non-specific binding with significant improvement of Cu^{2+} loading capacities up to 82.4 $mmol.L^{-1}$ of wet beads. The prepared silica has finally been evaluated with bovine serum albumin (BSA) and trypsin which are well-known metal-binding proteins, and this novel stationary phase has been found to have a potential for being used in small-scale, rapid enrichment of Cu^{2+}-binding proteins and peptides in proteomic measurements.

Another interesting IMAC stationary phase was prepared by covalent attachment of tetraphenylporphyrine to aminopropyl-derivatized silica (Biesaga *et al.*, 1999). Due to the ability of porphyrin to form complexes with various metal ions, this stationary phase was utilized for an affinity separation of tyrosine-containing di- and tripeptides. The best separation was attained under isocratic conditions in a phosphate buffer of pH 7.0 with 1% acetonitrile as an additive. As a double mechanism of interaction has been observed, involving hydrophobic π-π interactions together with affinity complexation with the incorporated metal ions, this approach has been proposed as a valuable tool for the separation of large biomolecules.

Nanoscale Fe(III)-IMAC employing fabricated microcolumns loaded with Fe(III)-NTA-silica resin have been used for affinity enrichment of phosphopeptides (Stensballe *et al.*, 2001). Phosphopeptides, derived from in-gel tryptic digests of standard phosphoproteins, were displaced from IMAC microcolumns, desalted and unambiguously identified by the alkaline phosphatase treatment followed by MALDI-MS from 80 Da shifts. ESI-MS/MS was then used as a technique for phosphopeptide sequencing which permitted better identification of phosphorylation sites. This strategy has been used successfully for the identification of the tryptic phosphopeptides derived from human p47/phox phosphoprotein isolated by two-dimensional gel electrophoresis.

Affinity chromatography on silica-based resins has also found use in small-scale purification of glycoproteins and glycopeptides. Increased interest in purification of glycosylated compounds is strongly related to their strong association with various health disorders. Many glycoproteins are actually believed to be biomarkers and their purification and identification can often be very important for diagnostics of diseases in their early stages (Jankovic and Kosanovic, 2005). Furthermore, studying the changes in abundance of some glycoproteins in various body fluids or modulation of glycosylation patterns of particular glycoproteins (Sumi *et al.*, 1999; Dwek *et al.*, 2001) is also considered to be a valuable part of clinical glycoproteomics. Some other affinity purifications employing silica-based resins such as stationary phases for clinical purposes have been thoroughly reviewed (Hage, 1999).

High-performance lectin affinity chromatography on acrylate polymer-coated silica was used for the analysis of various types of glycoproteins expressed in either human blood serum or in a cancer cell line (Geng *et al.*, 2001). The experiment involved loading the peptides derived from tryptic digests on affinity columns with two different types of covalently linked lectins (Con A and BS-II), displacement of glycopeptides with appropriate elution buffers, off-line desalting and identification by MS. Several types of glycopeptides were analyzed by the above method: N-type glycoproteins with known primary structure, N-type glycoproteins with unknown primary structure, and O-type glycoproteins glycosylated with a single N-acetylglucosamine. Although this approach was found feasible and fast, it suffered from inability of discriminating among the glycoforms.

The same authors monitored the changes in fucosylation of some glycoproteins originating from dog blood serum in relation to the occurrence of cancer (Xiong *et al.*, 2003). The serum proteome was digested with trypsin, the fucosylated peptides were then enriched on *Lotus tetragonolobus agglutinin* (LTA), labelled with a global internal standard (GIST) and simultaneously analyzed by MALDI-MS and ESI-MS/MS. It was confirmed that overexpression of certain fucosylated glycoproteins like CD44, E-selectin, or Interleukin-4 strongly reflected presence of cancer. The authors also noticed that the number of diagnostic glycoproteins demonstrably decreased during chemotherapy treatment, which made this methodology indispensable in the diagnostics of disease remission or, on the other hand, its re-occurrence after the treatment failed. Some other applications employing comparative proteomics, together with off-line silica-based affinity chromatography for the study of various post-translational modifications, have also been reviewed (Regnier *et al.*, 2002).

Since the demands of contemporary proteomics have been leaning towards utilizing high-throughput automated systems, there have been several attempts to interface silica-based affinity chromatography on-line to high resolution techniques in order to monitor the protein expression, map distinct post-translational modifications in small quantities of samples, or evaluate binding constants characterizing the strength of interactions between the proteins and immobilized ligands (Lee and Lee, 2004). For example, sialic acid-specific mushroom *Polyporus squamosus* lectin immobilized on porous glass beads and connected on-line to mass spectrometry was used for determination of the binding constants quantifying the affinity toward various glycan structures (Zhang *et al.*, 2001).

Madera *et al.* (2005) interfaced silica-based lectin microcolumns on-line to capillary LC-ESI-MS/MS and demonstrated the efficient lectin binding even in 3% acetonitrile as the binding buffer which is compatible with mass spectrometry. They demonstrated that the proposed setup could be used for small-scale affinity enrichment of standard glycoproteins or glycopeptides derived from tryptic digests. On-line lectin affinity enrichment significantly facilitated the assignment of three N-glycosylaton sites on bovine fetuin and was assumed to be a useful tool for fast profiling of glycoproteins originating from real samples.

In addition to lectins, various enzymes, covalently attached to the porous silica surface, can be used for accelerated protease digestion allowing rapid identification of proteins in high-throughput measurements. First silica-based trypsin microreactor was employed for fast digestion of selected standard proteins (Davis *et al.*, 1995). The enzymatic reactor showed an excellent long-time stability and reusability with a low tendency to autolysis.

Trypsin immobilized to silica-based macroporous chitosan beads has been successfully applied to fast small-scale affinity purification of trypsin inhibitor (Xi *et al.*, 2005). The importance of trypsin inhibitors has recently increased, especially due to their importance for prevention or suppression of carcinogen-induced transformation. As discussed above in this chapter, a chitosan layer substantially reduced non-specific binding of interfering proteins which also offered a possibility of utilizing this new affinity media as fast and efficient trypsin reactors integrated into high-pressure valve-based automated systems for high-throughput proteomic measurements.

MONOLITHS

Monoliths, are commonly defined as "continuous polymer beds" (Hjertén *et al.*, 1989) or "continuous polymer rods" (Svec and Frechet, 1992). They represent a very popular type of stationary phase for a wide variety of chromatographic techniques. Perhaps the first attempts at using polymers as supports for chromatographic separations were made in the late 1960s, when polymerized cross-linked hydroxyethylmethacrylate, referred to as hydrogel, was successfully used for size-exclusion separations (Kubin *et al.*, 1967). Later on, the utility of monolithic stationary phases has been significantly improved, from compressed polymer beds (Hjertén *et al.*, 1989) through monolithic disks (Coupek *et al.*, 1973) to rigid monoliths in cylindrical shapes (Svec *et al.*, 1975) forming stationary phases packed in small columns (Svec and Frechet, 1992) or inserted into microfluidic devices (Peterson *et al.*, 2002a; Slentz *et al.*, 2003).

Monoliths provide a better performance than conventional particle-based chromatographic media, primarily because of enhanced mass transport (Iberer *et al.*, 1999). Also, rigid polymers offer further numerous advantages making them very popular in contemporary proteomics, especially when affinity purification is required. First, monolithic beds are easy to fabricate while the modification of the resultant stationary phase is in principle attained by suitable selection of the monomers containing functional groups essential for the desired interaction with the analytes. Despite the limitations in the particle size of some solid supports, monoliths can be used as stationary phases in unprecedentedly small-scale chromatographic experiments because they can be prepared by polymerization of a monomer solution previously filled into a column or a micro channel. Although extensive use of monolithic columns only started in the early 1990s (Svec and Frechet, 1992), their properties, ways of preparation, and applications to a wide variety of techniques have already been discussed in detail (Josic *et al.*, 2001; Svec *et al.*, 2003).

Monolithic affinity chromatography is a relatively new methodology. The initial experiments involving affinity interactions utilized polymer-based affinity membranes (Brandt *et al.*, 1988). Affinity chromatography with ligands immobilized onto rigid polymers was introduced several years later (Josic *et al.*, 1992). Even though the rapid expansion of high-performance monolithic affinity chromatography made some frequently used stationary phases which contained commercially available immobilized ligands (Josic *et al.*, 2001), in-house polymerization followed by covalent attachment of the ligand is still popular. This is primarily due to the simplicity and versatility of the process. The ligand can be introduced into the monolithic bed after the polymerization and flushing the residual monomers out of the column (Mallik *et al.*, 2004; Petro *et al.*, 1996). Alternatively, it can be included in the mixture of monomers prior to polymerization (Hodgson *et al.*, 2004; Palm and Novotny, 2004, 2005). An advantage of incorporation of a ligand after the completed polymerization lies in the controlled immobilization process. It permits the exposure of the ligand binding site to the analyte to be enhanced by introducing a suitable group capable of reaction with a functional group forming a polymer backbone. However, this is usually time-consuming process. On the other hand, the preparation of monoliths by polymerization of monomers that already contain the ligand is usually a fast, one-step procedure, however, the advantage of controlled immobilization of the ligand is lost in this case because the ligand is usually encapsulated in the polymer with no (Kato *et al.*, 2005) or only partial covalent attachment (Palm and Novotny, 2004). The large number of monomers with diverse functional groups permitting controlled immobilization of the ligand also provides various coupling procedures with different levels of feasibility and different coupling yields (Josic *et al.*, 2001).

The first high-performance monolithic affinity chromatography (HPMAC) experiments involved immobilization of low molecular weight ligands onto polymers based predominantly on polymethacrylate or acrylamide. The technique was at that time primarily directed toward affinity purifications prior to the identification of proteins present in either bound or unbound fractions, using protein A (Kumar *et al.*, 2003; Luo *et al.*, 2002; Pan *et al.*, 2002), protein G (Gupalova *et al.*, 2002), monoclonal antibodies (Hodgson *et al.*, 2005), organic dyes (Sun and Chai, 2002) and synthetic peptides (Hahn *et al.*, 2001; Pflegerl *et al.*, 2002; Vlakh *et al.*, 2003, 2004) as the affinity ligands. For example, Mattiasson and co-workers enhanced the affinity purification of cells bearing IgG antibodies on their surface using Protein A immobilized on an acrylamide-based monolithic cryogel (Kumar *et al.*, 2003). As the size of the pores is indirectly proportional to the temperature (Svec and Frechet, 1995), the authors demonstrated that the polymerization carried out

under low temperatures (-12 °C) resulted in a supermacroporous monolith with a pore size of 10 -100 μm, enabling purification of large biomolecules.

Immobilization of monoclonal antibodies permitted fast, small-scale immunoextractions resulting in enrichment of samples within milliseconds. Anti-FITC antibody was covalently attached to a monolith based on a copolymer of glycidyl methacrylate and ethylene dimethacrylate (Jiang *et al.*, 2005). The polymerization procedure was optimized and the yields obtained by several coupling methods were compared. The resultant stationary phase with immobilized antibody was fabricated as a micro disk allowing ultrafast immunoextractions with a 95% recovery within 100 milliseconds. A similar experiment was conducted by another group, where antibody was incorporated into the silica-based monolith through a sol-gel procedure and used as a support for microcolumn affinity chromatography with in-line laser-induced fluorescence detection (Hodgson *et al.*, 2005).

Although monoclonal antibodies are very selective, their production and implementation for affinity chromatography purposes is rather expensive. Moreover, their sensitivity to the reaction conditions limits the number of procedures that can be used for covalent attachment of the ligand to the monolith. Therefore, there was a need for finding other low molecular weight ligands that would be cheaper, easy to synthesize, and would retain their activities even after coupling procedures carried out at elevated temperatures and/or in organic solvents. For that purpose, small synthetic peptides were prepared, permitting immobilization and attainment of high ligand densities (Necina *et al.*, 1998). Affinity chromatography on monoliths with immobilized peptides from combinatorial library was then employed for purification of human blood coagulation factor VIII (Amatschek *et al.*, 2000). The protein under study was converted into its peptides which were electroblotted onto a nitrocellulose membrane and incubated with biotinylated ligands permitting exact identification of the binding site. The same protein was later immobilized onto CIM (Convective Interaction Media®) (Berruex *et al.*, 2000) monolithic micro disks based on a copolymer of glycidyl methacrylate and ethylenglycoldimethacrylate and utilized for fast screening of binding capabilities of a variety of the synthetic peptides (Pflegerl *et al.*, 2002).

With the growing interest in the purification of metal-binding proteins and studies of posttranslational modifications, especially phosphorylation, monoliths were found to be suitable supports for immobilized metal affinity chromatography. Iminodiacetic acid, a common chelating agent capable of complexing many metal ions, was coupled to poly(glycidyl methacrylate-co-ethylene dimethacrylate) molded monolith *via* the reaction with active epoxy groups (Luo *et al.*, 2001) The influence of the pH, the reaction time and temperature on the immobilization process was studied. The coupling efficiency reached a maximum at 70 – 80°C with the coupling period of at least 16 hours at pH 8.0. The stationary phase prepared under the optimum conditions with adsorbed Cu^{2+} was then used for the purification of crude human serum albumin. Different affinities of various proteins toward the immobilized metal ion were then demonstrated by the separation of standard proteins depicted in Fig. (6). The purity of the collected fractions was confirmed by MALDI-TOF mass spectrometry.

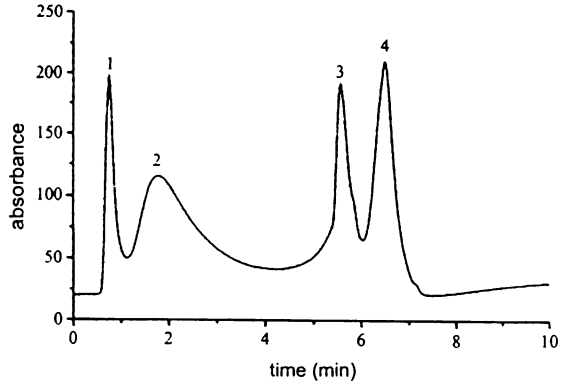

Fig. (6). Separation of proteins on Cu^{2+}-IDA monolithic column. Experimental conditions: Linear gradient in 6 min from 0 to 50% B; eluent A, 20 mmol/L phosphate buffer containing 1.0 mol/L sodium chloride (pH 7.0); eluent B, eluent A containing 100 mmol/L imidazole (pH 7.0). Peaks: 1 = trypsin; 2 = lysozyme; 3 = BSA; 4 = myoglobin. Reprinted from (Luo *et al.*, 2001).

The Cu^{2+}-IDA ligand was also successfully immobilized onto a supermacroporous monolithic matrix consisting of a cryogel of poly(acrylamide) crosslinked with methylenebis(acrylamide) (Dainiak *et al.*, 2005). The polymerization was carried out at a low temperature and thus the size of the resultant pores varied from 10 to 100 μm, allowing affinity profiling of the whole cells based on the amino acid composition of the surface proteins. This methodology was proposed as ideal for fast preparative separation of various types of cells, and also as very promising for fast screening of distinct post-translational modifications of surface proteins in relation to various health disorders.

The same affinity ligand was also immobilized onto macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) matrix (Zhang *et al.*, 2005) and the resulting capillary column was employed for on-line, nano-scale preconcentration of Cu^{2+} binding proteins and peptides and were subsequently analyzed by capillary electrophoresis. This approach has been found reproducible, with a relative standard deviation (RSD) of less than 5% for injection-to-injection and 12% for column-to-column, based on 3 preparations. A similar study dealing with on-line coupling of Cu^{2+}-IDA covalently attached to the cross-linked poly(glycidylmethacrylate) monolith to capillary zone elecrophoresis (Vizioli *et al.*, 2005) focused mainly on preconcentration of histidine-containing peptides and suggested that a monolithic microextractor could be an essential part of various high-throughput systems applied to biopharmacy, biotechnology and proteomics.

Recently, the Fe^{3+}-IDA ligand has been attached to a poly(glycidyl methacrylate/divinylbenzene) matrix in order to enrich phosphoproteins prior to their mass spectrometric analysis (Aprilita *et al.*, 2005). To simplify the extraction procedure, the monolithic stationary phase was combined

with a silica phase in a single cartridge, providing affinity enrichment and desalting at the same time without disturbing the process of binding of phosphopeptides to the affinity material. However, the carboxy groups on the C terminus had to be converted into the methylesters to avoid nonspecific binding of non-phosphorylated peptides and proteins. As evident from the affinity enrichments of synthetic peptide spikes in a myogloobin tryptic digest and from the selective pre-concentration of phosphopetides originating from a β-casein tryptic digest shown in Fig. (7), this methodology offered excellent recoveries (95%), with a minimum tendency to non-specific binding. For these reasons, monolithic Fe^{3+}-IDA affinity chromatography in conjunction with reversed-phase desalting appears to be an interesting alternative to other IMAC techniques. In addition, the proposed setup is ideal for incorporating into robotic high-throughput systems for routine analyses and satisfies the demands of the contemporary phosphoproteomics.

Encouraged by the successful use of monoliths as affinity stationary phases with covalently linked low molecular weight ligands, scientists proceeded to the replacement of the ligands used so far with different ones, such as saccharides (Bedair and Rassi, 2004), enzymes (Josic and Buchacher, 2001; Petro *et al.*, 1996), and various proteins (Mallik *et al.*, 2004). For example, a capillary packed with the dihydrofolate reductase enzyme, entrapped in a silica-based monolith prepared by sol-gel method and interfaced on-line to ESI mass spectrometer, was successfully employed for fast determination of the binding constants describing the interaction between the enzyme and related inhibitors (Hodgson *et al.*, 2004). A capillary column packed with the identical stationary phase was later coupled to MALDI mass spectrometer to overcome reproducibility problems associated with the use of low ionic strength buffers required in the previous case (Kovarik *et al.*, 2005).

A new type of monolith based on polymethacrylate with the inclusion of positively charged [2-(methacryloyloxy) ethyl]trimethyl ammonium chloride monomer was introduced by Bedair and Rassi (2005). After the covalent attachment of two lectins with different specificities (Con A

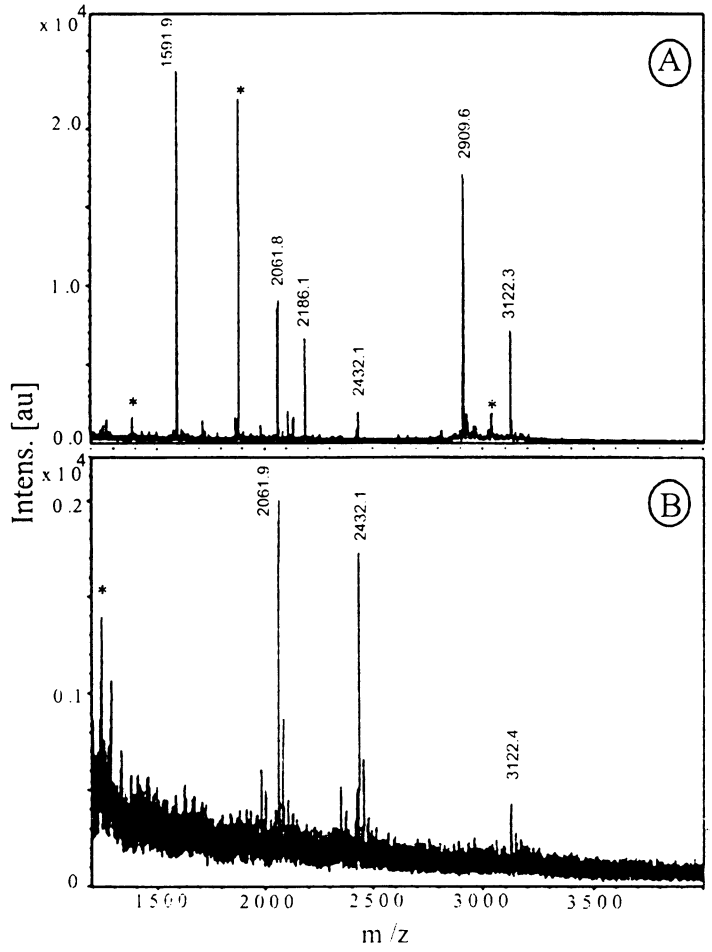

Fig. (7). MALDI-TOF mass spectra of β-casein digest. Asterisks indicate peptides that have not been identified. (A) 1 μg/μL β-casein digest before SPE (solid-phase extraction) (B) phosphopeptides after SPE originating from 0.2 μg/μL β-casein digest. Reprinted from (Aprilita *et al.*, 2005).

and WGA), the resultant affinity microcolumns were used for small-scale affinity purification of selected glycoproteins and glycans. Compared to the commonly used neutral monoliths, the presence of a positive charge offered sharp peak fractionation with a minimum tendency to non-specific binding. The Con A affinity microcolumn was thereafter connected to a C17 monolith, offering a novel 2D separation system for rapid analyses of glycoproteins and glycopeptides.

Nano-scale lectin affinity chromatography with *Pisum sativum agglutinin* (PSA) immobilized on glycidyl methacrylate / ethyleneglycol dimethacrylate monolith was performed on a microfluidic device schematically depicted in Fig. (**8**) (Mao *et al.*, 2004). A suitable combination of the elution buffers differing in the concentration of a hapten sugar resulted in electroosmotically driven separation of the glycoforms of fluorescently labelled turkey albumin, chicken albumin and ovomucoid.

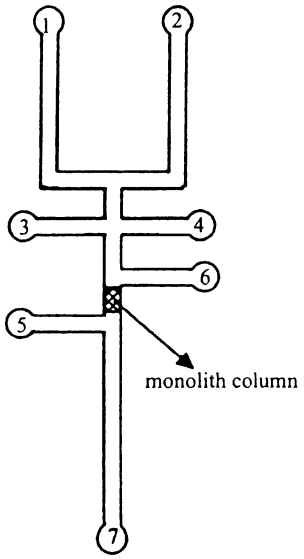

Fig. (8). Layout of the microfluidic chip: 1, running buffer reservoir 2, eluent buffer reservoir; 3, sample reservoir; 4, sample waste reservoir; 5,6, washing reservoir; 7, waste reservoir. Reprinted from (*Mao et al.*, 2004).

As pointed out above, modern proteomic methodologies rely on the development of automatic, high-throughput systems capable of processing small quantities of real samples within short periods of time. The proteins originating from such samples are usually identified by mass spectrometric analysis of the peptides obtained by enzymatic digestion which, if conducted in solution, definitely complicates the sample handling and makes the analysis longer. The need for shortening the digestion times and for on-line interfacing to various high-throughput systems has led to the development of protease reactors containing enzymes covalently attached to suitable solid supports, including monoliths. Probably the first monolithic enzyme reactor with trypsin immobilized on a poly(glycidyl methacrylate-co-ethylene dimethacrylate) matrix was introduced about a decade ago (Petro *et al.*, 1996) and the work on further optimization and miniaturization is still continuing (Bencina *et al.*, 2004; Kato *et al.*, 2005; Xie *et al.*, 1997). A trypsin reactor with a volume of 470 nL, immobilizing the enzyme onto a macroporous polymer monolith was prepared (Peterson *et al.*, 2002b). The efficiency of enzymatic conversion was tested with various standard proteins whose fragments were analyzed by off-line MALDI and on-line nano ESI mass spectrometer. Despite the residence time of only 1 min, the in-column tryptic digestion of all the individually injected proteins resulted in reliable identification with fairly high sequence coverages.

Palm and Novotny (2004) developed a very efficient acrylamide-based trypsin reactor capable of digestion of a 80 nM single protein solution, as deducted from the off-line MALDI-MS of derived peptides. Polymerization was conducted in an aqueous buffer so that the enzyme could be added to the mixture of monomers immediately after the reaction was initiated. The fast preparation procedure (1 h) and a good reproducibility (5% RSD), together with the stability for at least 20 weeks, made this microreactor an interesting alternative for small-scale proteomic applications requiring fast on-line tryptic digestions. The same stationary phase and a very similar coupling methodology were later used for the fabrication of a PNGase F reactor allowing the real-time enzymatic release and analysis of N-glycans originating from various glycoproteins (Palm and Novotny, 2005).

An enzymatic microreactor-on-a-chip based on trypsin immobilized on porous polymers consisting of 2-vinyl-4,4-dimethylazalactone, ethylene dimethacrylate, and acrylamide or 2-hydroxyethyl methacrylate was introduced (Peterson *et al.*, 2002a). The monoliths prepared by UV initiated photopolymerization showed very low backpressures and thus single mechanical pumping could be used for the enzyme attachment and digestion of the samples. The proteolytic activity of the enzyme was evaluated by the cleavage of fluorescently labeled casein, and the efficiency was determined from the characterization of the myoglobin digest by MALDI-MS. As a result, fast, on-line on-chip digestion of myoglobin led to the identification of 102 out of 153 peptides, yielding an excellent sequence coverage of 67%. This enzymatic reactor was later combined with a solid-phase extractor based on a hydrophobic poly(butyl methacrylate-co-ethylene dimethacrylate) monolith and interfaced on-line with an ESI mass spectrometer (Peterson *et al.*, 2003). This dual-function device offered operation in both flow directions; the protein concentration followed by digestion, or protein digestion followed by concentration. The system was tested with myoglobin as the standard and provided a very high sequence coverage of 80% for the highest loading.

AFFINITY MEMBRANES

An alternative to chromatographic affinity columns for bioseparation of large proteins are affinity membranes which can overcome certain disadvantages of affinity columns, i.e., their high cost and a high pressure drop along the column. Furthermore, compared to packed columns, membranes exhibit improved mass-transfer efficiencies, higher throughput values due to the presence of macropores that enable primarily convective flow, no intra-pore diffusion and compactness

of bed, a better accessibility of ligands for affinity interactions, a higher dynamic binding capacity (by more than one order of magnitude) (Teeters *et al.*, 2003) and easier scaling-up. The applications of affinity membranes in biotechnology are rapidly growing, as demonstrated in numerous review articles (Brandt *et al.*, 1988; Roper and Lightfoot, 1995; Thommes and Kula, 1995; Charcosset, 1998, Klein, 1990, 2000 Ghosh, 2002; Suen *et al.*, 2003; Hao *et al.*, 2004; Borcherding *et al.*, 2003; Zeng and Ruckstein, 1999; Piletsky *et al.*, 1999).

Affinity membranes are prepared analogous to affinity stationary phases, starting with the selection of a suitable membrane and following with its surface activation and coupling of affinity ligands.

The membrane matrices used for ligand immobilization should have the same properties as the supports in affinity chromatography, i.e., a high mechanical and chemical stability, hydrophilic character to avoid non-specific adsorption, a high adsorption capacity (a high surface area), large pore size and the presence of functional groups for ligand immobilization. The pore size is especially important. The pores should be sufficiently large to allow free flow of solutes to ligands and thus membranes for microfiltration (a pore size of 0.1 to 50 μm) are mostly used.

The choice of the membrane material is of a great importance. Various materials, both organic and inorganic, have been tested for affinity membrane preparation. Organic membranes based on natural or synthetic polymers are generally preferred over inorganic ones. Cellulose-based membranes are easy to prepare but they suffer from poor durability, which can be improved sometimes by chemical cross-linking (Guo and Ruckenstein, 2001, Ruckenstein and Guo, 2001). The cross-linked membranes have a high porosity (~ 50%) and large pores (0.4–1.0 μm). Membranes based on modified synthetic polymers, e.g., polyamide, polysulfone, polymethacrylate, polyvinylidene fluoride or polypropylene, are preferred for protein purification (Charcosset, 1998; Morais *et al.*, 1999). The physical and chemical properties of the membrane surface determine the membrane separation characteristics (Singh *et al.*, 2005). Commercially available membranes often have a broad distribution of the pore sizes and require surface modification prior to their use. Cellulose- and some copolymer-based membranes do not require surface pretreatment prior to ligand binding. Chemically inert materials, i.e., polyethylene or polypropylene, must first be treated to produce reactive groups for further surface modification. Several methods for such treatments have been developed. Graft modification has successfully been applied to tailoring of the membrane properties for certain applications (see the reviews, Charcosset, 1998; Klein, 2000; Nasef and Hegazy, 2004). Chemical or photochemical methods are mostly used, for example γ-ray irradiation to graft epoxy groups to glycidyl methacrylate GMA (Kim *et al.*, 1991, 1996; Iwata *et al.*, 1991; Kobayashi *et al.*, 1993), or ultraviolet grafting of polyacrylic acid on polyethylene (PE) membranes (Kiyohara *et al.*, 1997) to modify poly(ether sulfone) membranes (Taniguchi and Belfort, 2004; Pieracci *et al.*, 1999), poly(ethylene terephthalate) PET membranes (Yang and Yang, 2003), or polypropylene (PP) membranes (Borcherding *et al.*, 2003).

Atom transfer radical polymerization (ATRP) and reactive primary polymer monolayers have been employed to functionalize commercially available poly(vinylidenedifluoride) microporous membranes with weak ion-exchange groups (Singh *et al.*, 2005). The method produces more uniform surfaces than conventional radical polymerization and allows a manipulation of the chain molecular mass and, consequently, the membrane pore size.

Membranes produced from polycarbonate (PC), PET, polysulfone, poly(ethylene vinyl alcohol) (EVAL) and polyamides usually have sufficient numbers of end groups for surface modification (Klein, 2000). Membranes made of EVAL with a high specific surface area were applied to the preparation of affinity membranes for hormone removal (Urmenyi *et al.*, 2005). Klein and co-workers (1994) covalently bound hydroxyethyl cellulose (HEC) to the terminal phenol groups to reduce nonspecific sorption of proteins on microporous polystyrene hollow fiber membranes. Beeskowet and co-workers (1995) used HEC to modify microporous polyamide membranes. Immobilizing HEC also increases the number of binding sites for affinity ligands. Porous crosslinked chitosan (CS) membranes were prepared by extraction of polyethylene glycol (PEG) from CS/PEG membranes. These membranes are pH sensitive (Zeng and Fang, 2004).

Inorganic materials involve glass fibers for metal affinity membranes (Serafica *et al.*, 1994) or TiO_2 ion-exchange membranes (Li and Sencer, 2003). Commercial glass fiber filters were used to prepare large pore size affinity membranes. The filters were treated with a mixture of sulfuric acid and hydrogen peroxide to introduce additional silanol groups, followed by silanization and derivatization.

Immobilization of ligands to affinity membranes is governed by the same rules as in affinity chromatography and depends on both the matrix and the ligands: immobilization procedures should lead to stable bonds between the ligand and the membrane matrix and the specific affinity interaction must be preserved. The application of spacers makes the ligand more easily accessible to proteins. The spacer should have only two active sites, those for reactions with the ligand and with the membrane. The optimum spacer length has been discussed (Liu *et al.*, 2005). It has been found that the use of 1,4-diaminodecane and 1,8-diaminooctane spacers has led to a higher dye ligand density and lysozyme adsorption capacity on polyvinyldiene fluoride-based affinity membranes with immobilized copper ions (Suen *et al.*, 2000, Tsai *et al.*, 2002).

Molecularly imprinted affinity membranes occupy a special place. Their potential for separation of biomolecules is significant and has not yet been fully utilized (Piletsky *et al.*, 1999, Ulbricht *et al.*, 2002). They represent a synthetic recognition system specific for a given molecule. Their synthesis includes polymerization of the monomer in the presence of a cross-linking agent and a template and removal of the template. The properties of MIP membranes were studied. (Ulbricht *et al.*, 2002; Klein, 2000; Zou *et al.*, 2001; Wong, 1991; Huang *et al.*, 1996; Hennion and Pichon, 2003).

The prepared affinity membranes have various forms and are placed in modules of various shapes, e.g., flat sheets,

monolithic discs and hollow fibres. Flat sheets and hollow fibres are most common as they are inexpensive and readily available commercially.

Affinity membranes are not expected to provide new separations but primarily to speed up the separations of known species. An excellent review of application of immobilized metal affinity membranes to separations of biomolecules has been published by Suen *et al.*, 2003.

Guo and Ruckenstein (2003) used glass affinity membranes with immobilized papain to separate papain inhibitors; the inhibitor activity and the total protein amount were monitored (Fig. (**9**)). After adsorption of the inhibitor on the membrane, the elution was carried out with the mobile phase of 6 M urea in aqueous solution.

Liu and co-workers (2005) used immobilized metal affinity membranes (IMAM) for purification of penicillin G acylase. Cu(II) ions were immobilized on cellulose-based membranes *via* a 1,8-diaminooctane spacer. Fig. (**10**) depicts an application of IMAM under flow conditions, obtaining a 2.73-fold purification and a 70% recovery.

Guo and Ruckenstein (2003) applied glass membranes to separate fibronectin from human blood plasma using two successive cartridges. The yield of the method was 0.185 mg fibronectin per ml of plasma. Membranes prepared from

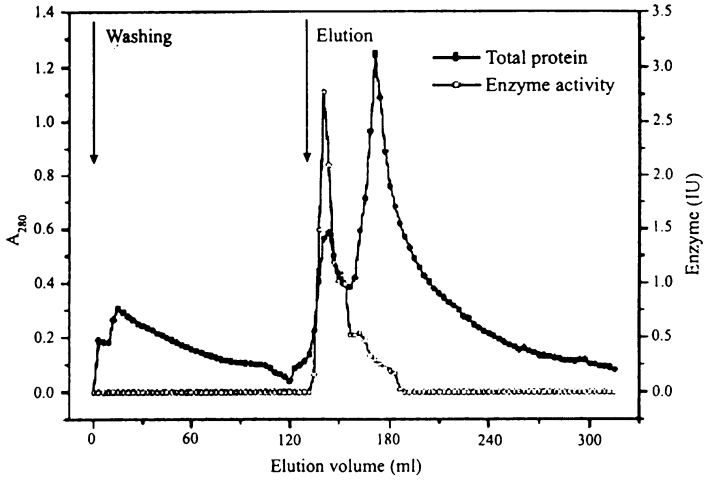

Fig. (9). Separation of papain inhibitors by membrane affinity chromatography. The amount of papain inhibitor was assayed by inhibition of the papain and the total protein content was determined by Coomassie Brilliant Blue method. The initial mobile phase was 0.05 M Tris.HCl buffer pH 7.40, containing 0.1 M NaCl and 10 mM Na_2SO_3, then the mobile phase was switched to 4 M urea after 50 min. Reprinted from (Guo and Ruckenstein, 2003).

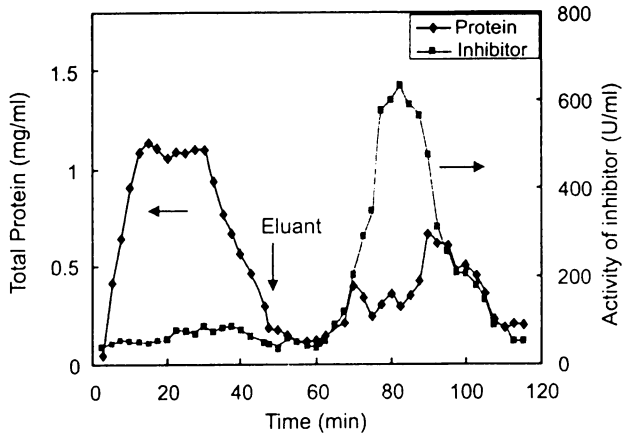

Fig. (10). Purification of penicillin G acylase using cartridge with 10 pieces of IMAMs employing 1,8-diaminooctane as spacer arm. Conditions: Loading protein: activity: 0.192 IU/mL; Loading buffer: 10mM phosphate, 0.5M NaCl, pH 8.5; wash buffer: 10mM phosphate, 0.1M NH_4Cl, 0.5M NaCl, pH 6.8; elution buffer: 10mM phosphate, 10mM imidazole, 0.5M NaCl, pH 6.8. While washing starts from fraction 1 elution starts from fraction 41. Reprinted from (Liu *et al.*, 2005).

polyamide activated with bisoxiran and with polylysine carrying chitosam as a ligand were applied to adsorb bilirubin (Shi *et al.*, 2005).

Borcherding and co-workers (2003) introduced a flat-sheet microfiltration affinity membrane based on polypropylene. They used UV irradiation grafting to make the surface more hydrophilic. Streptavidin was immobilized *via* epoxy groups, followed by immobilization of alkaline phosphatase. The enzyme is suitable for adsorption of biotinylated ligands, including oligonucleotides, biotin-tagged recombinant proteins etc.

A poly(hydroxyethylmethacrylate-co-methacrylylamide affinity membranes, containing L-histidine as the ligand, were used in the separation and purification of human immunoglobulin G (HIgG) from solutions and human serum (Arica *et al.*, 2004a) and the effects of the experimental parameters on the HIgG adsorption were studied. In the continuous mode, the purity of the HIgG was 93%, with a recovery of 58%. The affinity membranes were stable after treatment with sodium hydroxide between repeated adsorption-elution cycles.

Two dyes, Procion Brown MX-5BR (RB-10) and Procion Green H-4G (RG-5) were immobilized onto poly(2-hydroxyethylmethacrylate) (pHEMA) membranes and applied to purification of egg white lysozyme. The purity of the eluted lysozyme was 76% and 92%, with a recovery of 63% and 77% for RB-10 and RG-5 membranes, respectively (Arica *et al.*, 2004b).

MAGNETIC PARTICLES

Biocompatible magnetic particles used in chemistry, medicine and biotechnology are known since the 1970s (Mosbach and Schroder, 1979; Kronick *et al.*, 1978) and recently, the number of publications on their use has been increasing. The application of magnetic particles has already been reviewed, targeting the areas of isolation and purification of proteins and peptides (Safarik and Safarikova, 2004), drug discovery and biomedicine (Saiyed *et al.*, 2003), isolation of cells (Safarik and Safarikova, 1999) and bioassays (Haukanes and Kvam, 1993).

The principle of magnetic separation is simple. Magnetic carriers bearing an immobilized ligand are mixed with a sample containing target compounds (samples may be crude cell lysates, blood, plasma, milk, urine and many others). Following an incubation period when the target compounds bind to magnetic particles, the whole magnetic complex is easily and rapidly removed from the sample using an appropriate magnetic separator. After washing out the contaminants, the isolated target compounds can be eluted and used for further work (SafaRik and SafaRikova, 2004).

Materials used in this way can be defined as magnetizable particles, i.e., those that become magnetic under the influence of an external magnetic field and enable isolation of a target molecule from a mixture. The non-magnetic target species bind to the surface of the magnetizable solid-phase support through a specific affinity interaction or by another mechanism (e.g., ion exchange or hydrophobic interactions) (Bruce *et al.*, 2004).

Compared to the standard, mainly chromatographic separation procedures, magnetic separation techniques have several advantages as they are usually simple, efficient, cheap and do not involve toxic compounds. Moreover, magnetic separation is usually gentle toward the target proteins and enzymes. The reduced shearing forces and higher protein concentrations maintained throughout the isolation process are advantageous (Safarik and Safarikova, 2004).

Magnetic supports can be prepared by entrapping magnetic particles within a supporting polymer by copolymerization of magnetic particles during the synthesis of the supporting polymer, allowing the entrapment of the magnetic particles within polymers, onto which the biomolecules can be immobilized (Konecká *et al.*, 2002). Almost all systems employ micrometer or submicrometer magnetic particles coated with a suitable stabilizer (Mehta *et al.*, 1997). The typical diameter of particles ranges from about 50 nm to approximately 10 μm. However, larger magnetic affinity particles, with diameters up to a millimeter range, have also been used successfully. Magnetic particles with diameters larger than approximately 1 μm can be separated readily using single magnetic separators, whereas separation of smaller particles (magnetic colloids with the particle size ranging between tens and hundreds of nanometers) may require the use of high gradient magnetic separators (Safarik and Safarikova, 2004).

A ferrofluid is a stable colloidal suspension of subdomain magnetic particles in a liquid carrier. The particles are coated with a stabilizing dispersing agent (surfactant) which prevents particle agglomeration even when a strong magnetic field gradient is applied to the ferrofluid. In the absence of magnetic field, the magnetic moments of the particles are randomly distributed and the fluid has no net magnetization. When a magnetic field is applied to a ferrofluid, the magnetic moments of the particles orient along the field lines almost instantly. The magnetization of the ferrofluid responds immediately to the changes in the applied magnetic field and when the applied field is removed, the moments randomize quickly (Ferrotec Corporation, http://www.ferrotec.com).

The amount of magnetic particles in a given volume of a ferrofluid can be estimated thermogravimetrically or by measurements of magnetization curves. The particle size distribution can be determined by electron microscopy and magnetic measurements (Konecká *et al.*, 2002; Horák *et al.*, 2003).

Many supports are employed for the preparation of the magnetic separation phases, primarily polystyrene, agarose, cellulose, silica or porous glass. All of them are commercially available (Table 3). Safarik and Safarikova (2004) also provided an overview of immobilized ligands which are commercially available, such as streptavidin, antibodies, protein A and protein G, nitrilotriacetic acid (NTA), glutathione, trypsin, trypsin inhibitor, gelatin, etc. Ligands can, of course, be immobilized on both commercial and home-made magnetic particles. The functional groups present on the surface of magnetic particles e.g., –COOH, -OH or $-NH_2$, are used for immobilization of target species; magnetic particles are sometimes available in the already activated form (e.g., tosyl- or epoxy activated).

Table 3. Commercial Magnetic Particles

Support	Tradename	Supplier	Reference
Polystyrene	Dynabeads®	Dynal Biotech (Norway)	Dynal (http://www.dynalbiotech.com)
Polystyrene	Polybead®	Polysciences (USA)	Polysciences (http://www.polysciences.com)
Agarose	Ni-NTA Magnetic Agarose Beads®	Qiagen	Qiagen (http://www.qiagen.com)
Cellulose	MagaCell®	Cortex Biochem (USA)	Cortex (http://www.cortex.com)
Silica	MagneSil®	Promega (USA)	Promega (http://www.promega.com)
Porous glass	MPG®	Talron Biotech (Israel)	Talron (http://www.talron.co.il)

The recent publications in this field involve some important results: Horák and co-workers (2003) described the effect of the reaction parameters on the particle size during dispersion polymerization of 2-hydroxyethyl and glycidyl methacrylate in the presence of a ferrofluid; Bruce *et al.* (2004) published a study about synthesis and application of silica-magnetite nanocomposites; Chatterjee *et al.*, (2004) reported synthesis of a magnetic gel based on hydroxypropyl cellulose without a crosslinking agent; Odabasi *et al.*, (2004) reported preparation of magnetic poly(2-hydroxyethyl-methacrylate) (mPHEMA) modified by iminodiacetic acid (IDA), with subsequently bound Cu^{2+} ions for IMAC; Qiu *et al.* (2005) reported use of Fe_3O_4/poly(styrene-co-maleic anhydride) core-shell composite microspheres for binding enzymes. A novel preparation procedure was carried out for micrometer-size non-porous magnetic poly(methacrylate-divinylbenzene) (mPMA-DVB) with bound IDA and Cu^{2+} ions for IMAC (Ma *et al.*, 2005).

On the other hand, it has been shown that it is possible to bind protein molecules directly to unmodified magnetic particles: Bovine serum albumin (BSA) and selected enzymes have been coupled to freshly prepared magnetite particles in the presence of carbodiimide (Mehta *et al.*, 1997). Lauva *et al.* (1990) used colloidal magnetite with a polysaccharide heparin in cell fractionation.

Mehta *et al.* (1997) prepared magnetic particles by co-precipitation of di-valent ($FeSO_4.7H_2O$) and tri-valent ($FeCl_3.6H_2O$) ions in an alkaline solution (8 M NH_4OH). The particles obtained were black and exhibited a strong magnetic response. The average particle diameter, determined from an electron micrograph (Fig. 11), was 12 ± 2.0 nm. The binding of bovine serum albumin (BSA) to the magnetic particles was affected both by the concentration of carbodiimide and by that of the magnetic particles. The percentage binding did not depend on the reaction time within 20 hours. The optimum concentration ratio of BSA, carbodiimide and magnetic particles was 1:1:3 at pH 6.3. The particles synthesized by co-precipitation techniques were macro ions. The surface electric charge of these ions was due to specific adsorption of amphoteric hydroxyl group and was negative in alkaline media, with the point of zero charge at pH 7.5. Around this point the particles were not dispersed to form a colloid. Therefore, the presence of hydroxyl group on the surface of the particles was responsible for binding the protein. Then bovine alkaline phosphatase was immobilized onto the magnetic particles. The results showed that 75% of the enzyme activity was retained, compared to the original activity.

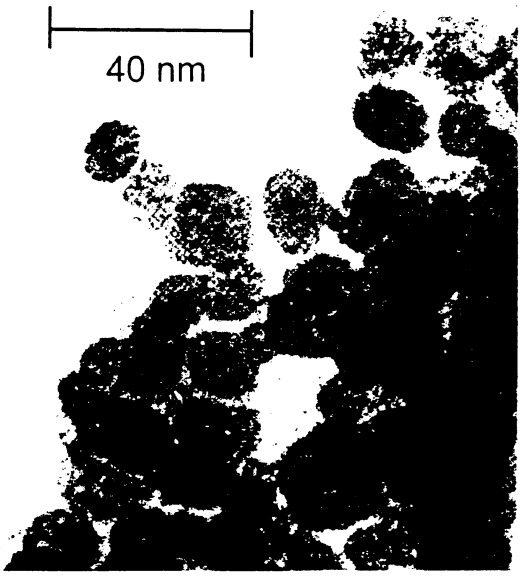

Fig. (11). Electron microphotograph of a protein bound to magnetic particles. Reprinted from (Mehta *et al.*, 1997).

Koneracká and co-workers (2002) also published a direct immobilization procedure for BSA and selected enzymes to magnetic particles (Fe_3O_4) using carbodiimide as the coupling agent. They published the optimum conditions for the binding of BSA to the magnetic particles at a ratio of BSA, carbodiimide and magnetic particles 1:2:2, at room temperature and pH 6.3. The following enzymes were also bound to magnetic particles: glucose oxidase (GOD), streptokinase, chymotrypsin and dispase. The optimum ratio of GOD, carbodiimide and magnetic particles was found to be 1:2:6. The higher magnetic particle-to-protein ratio used helped to minimize losses of the enzyme. Althought GOD could be bound to the magnetic particles at pH 6.3, no en-

zyme activity was observed. The enzyme showed the maximum activity at pH 4.5, but over 70% of the enzyme's activity was lost. This is a common observation for enzymes immobilized by covalent bonding. Table (4) summarizes the results obtained for immobilization of BSA and selected enzymes.

Table 4. The Optimized Concentration of Immobilizing Mixture Consisting of Proteins, Carbodiimide and Magnetic Particles. According to (Koneracká *et al.*, 2002)

Proteins/enzymes	Proteins:carbodiimide: magnetic particles (mass ratio)
BSA	1:2:2
GOD	1:2:6
streptokinase	1:2:2
chymotrypsin	1:2:2
dispase	1:2:2

Odabasi *et al.* (2004) prepared and used magnetic immobilized metal affinity separation media based on mPHEMA for adsorption of human serum albumin (HSA). The metal chelating ligand IDA was covalently attached to epichlorhydrin-activated mPHEMA beads *via* the reaction between the epoxide groups and the primary amine groups of the IDA and Cu^{2+} ions were subsequently bound to form metal chelate. This adsorbent was prepared in the laboratory. The equilibrium water content of mPHEMA beads was 34%, indicating that the magnetic beads had a hydrophilic skeleton. The highest adsorption of HSA was observed at pH 7.0 and more than 90% of the adsorbed HSA was easily removed from the magnetic beads using NaSCN for desorption. Under these conditions no Cu^{2+} was released, hence Cu^{2+} ions were bound as a strong chelate.

Saiyed and co-workers (2003) also reviewed some proteomic applications of magnetic particles. Proteins and enzymes can be bound covalently to naked magnetic particles in the presence of carbodiimide. The immobilized molecules can be used for a bioassay or as affinity ligands to capture or modify target molecules. On this basis, Ni-nitrilotriacetic acid tagged magnetic agarose beads have been used for versatile magnetocapture assays using 6xHis-tagged proteins. The procedure involves the use of metal chelating NTA groups covalently bound to the agarose surface, which contains strong magnetic particles. The beads are precharged by nickel, which is ready to capture 6xHis-tagged proteins for sensitive interaction assays or microscale purification of 6xHis-tagged proteins.

Another application of magnetic particles has been reported (Qui *et al.*, 2005). Fe_3O_4/poly(styrene-co-maleic anhydride) core-shell composite microspheres were prepared for immobilization of α-amylase (magnetic immobilized enzyme, MIE). The MIE had a higher optimum temperature and a higher pH optimum compared with those for free α-amylase, and exhibited excellent thermal, storage, pH and operational stability. The enzyme activity remained above 80% of the initial state after ten repeated experiments (the free enzyme can be used only once). Furthermore, MIE can easily be separated in a magnetic field and reused repeatedly.

CONCLUSION

It is clear from the survey above that the selection of an affinity system suitable for a particular purpose is always a complex problem in which a number of aspects must be considered. The most important is, of course, attainment of a sufficient selectivity and reproducibility of the separation. Furthermore, the separation must not adversely affect the biological activity of the substances separated; in this connection, great demands are often placed on the speed of the separation procedure. The separation system must be sufficiently resistant against microbial contamination and, depending on the technique used, must be adequately strong mechanically. There should be wide possibilities of functionalization of the sorbent to modify its selectivity as required. The cost is often an important parameter, as are the possibilities of automation of the procedures. Also, compatibility of the separation step with subsequent analytical measurements or scaling up to a preparative level must be considered.

On this basis, as well as according to the literature reviewed, it can be concluded that the importance of classical dextran gels (e.g., Sephadex) in affinity chromatography is decreasing because of their sensitivity to microbial attack. Also, they do not have very high mechanical strength. However, they still have important applications in size-exclusion chromatography. Synthetic hydrophilic polymers based on methacrylates and modified acrylamides are widely used as supports in affinity chromatography and it can be expected that their importance will not decrease. The main reasons are their high mechanical and chemical stability and easy modification of their surface with a great variety of affinity ligands.

The use of affinity membranes is growing because they permit rapid separations and are relatively cheap. Similarly, new monolithic materials will be used more, basically for the same reasons. A very promising area is imprinted polymers as synthetic receptors, as they are considerably more stable than natural ligands; their wider use at present is hindered by problems connected with their reproducible preparation. A very new approach involves magnetic particles that are attractive because they make separations simple, efficient, inexpensive and do not require the use of toxic chemicals. It can be expected that their use will grow rapidly.

ABBREVIATIONS

2D = Two dimensional

ABA = *p*-Aminobenzadine

ATRP = Atom transfer radical polymerization

BDGE = 1,4-dibutanediol diglycidyl ether

BPTI = Basic pancreatic trypsin inhibitor

BSA = Bovine serum albumin

BSM	=	Bovine submaxillary mucin
CASMAC	=	Cascade-mode multiaffinity column chromatography
CIM	=	Convective Interaction Media®
Con A	=	Concanavalin A
CPG	=	Controlled porous glass
CS	=	Chitosan
DEAE	=	Diethylaminoethyl
DIT	=	3,5-diiodo-L-tyrosine
DMF	=	Dimethylformamide
DPA	=	Dipicolylamine agarose
DSC	=	N,N'-disuccinimidyl carbonate
DSS	=	Disuccimidyl suberate
EDMA	=	Ethylene dimethacrylate
EDTA	=	Ethylenediamine tetraacetate
ESI	=	Electrospray ionization
EVAL	=	Poly(ethylene vinyl alcohol)
FPLC	=	Fast protein liquid chromatography
GalNHCl	=	D-galactose amine hydrochloride
GIST	=	Global internal standard
GlcNAc	=	N-acetylglucosamine
GMA-co-EDMA	=	Glycidyl methacrylate-co-ethylendimethacrylate copolymers
GOD	=	Glucose oxidase
HEC	=	Hydroxyethyl cellulose
HEMA	=	2-hydroxyethyl methacrylate
HEMA E	=	2-hydroxyethyl methacrylate epoxy-activated supports
HEMA VS	=	2-hydroxyethyl methacrylate vinylsulphone-activated supports
HIgG	=	Human immunoglobulin G
HMDTP	=	Hexamethylenediamine tetrapropanoic acid
HPAC	=	High-performance affinity chromatography
HPMAC	=	High-performance monolithic affinity chromatography
HSA	=	Human serum albumin
IDA	=	Iminodiacetic acid tetrapropanoic acid
IMAC	=	Immobilized metal affinity chromatography
IMAM	=	Immobilized metal affinity membranes
LTA	=	LOTUS tetragonolobus agglutinin
MALDI-MS	=	Matrix assisted laser desorption/ionization – mass spectrometry
MIE	=	Magnetic immobilized enzyme
MIP	=	Molecularly imprinted
M-LAC	=	Multi-lectin affinity chromatography
NMR	=	Nuclear magnetic rezonance
NTA	=	Nitrilotriacetic acid
mPHEMA	=	Magnetic poly(2-hydroxyethylmethacrylate)
mPMA-DVB	=	Magnetic poly(methacrylate-divinylbenzene)
MS	=	Mass spectrometry
MW	=	Molecular weight
NHS	=	N-hydroxysuccimidyl
NTA	=	Nitrilotriacetic acid
PBS	=	Phosphate buffer saline
PC	=	Polycarbonate
PE	=	Polyethylene
PEG	=	Poly(ethyleneglycol)
PET	=	Poly(ethylene terephthalate)
PHA-E	=	*Phaseolus vulgaris* erythroagglutinin
PHA-L	=	*Phaseolus vulgaris* leukoagglutinin
pHEMA	=	Poly(2-hydroxyethylmethacrylate)
poly(EGDMA-HEMA)	=	Poly(ethylene glycol dimethacrylate-hydroxyethyl methacrylate)
poly(PB-HEMA)	=	Poly(hydroxylated polybutadienic-hydroxyethyl methacrylate)
PP	=	Polypropylene
PSA	=	Prostate-specific antigen
RCA	=	*Ricinus communis* agglutinin
RPLC	=	Reversed phase liquid chromatography
RSD	=	Relative standard deviation
SAP	=	Serum amyloid P
SiMAC	=	Ion metal chromatography on silica based support
SNA	=	Lectin from *Sambucus nigra*
SPE	=	Solid-phase extraction
STI	=	Soybean trypsin inhibitor
TOF	=	Time of flight
UEA	=	Lectin from *Ulex europaeus*
WGA	=	Wheat germ agglutinin

REFERENCES

Alvarez, C.I., Gomez, C.G. and Strumia, M.C. (2001) Influence of the polymeric morphology of sorbents on their properties in affinity chromatography. *J. Biochem. Bioph. Meth.* **49**: 141-51.

Amatschek, K., Necina, R., Hahn, R., Schallaun, E., Schwinn, H., Josic, D. and Jungbauer, A. (2000) Affinity chromatography of human blood

coagulation factor viii on monoliths with peptides from a combinatorial library. *J. High Res. Chromatog.* **23**: 47-58.

Aprilita, N.H., Huck, C.W., Bakry, R., Feuerstein, I., Stecher, G., Morandell, S., Huang, H.-L., Stasyk, T., *et al.* (2005). Poly(glycidyl methacrylate/divinylbenzene)-IDA-FeIII in phosphoproteomics. *J. Proteome Res.* **4**: 2312-19.

Arvidsson, P., Plieva, F.M., Lozinsky, V.I., Galaev, I.Y. and Mattiasson, B. (2003). Direct chromatographic capture of enzyme from crude homogenate using immobilized metal affinity chromatography on a continuous supermacroporous adsorbent. *J. Chromatogr. A* **986**: 275-90.

Arica, M.Y., Yalçin, E. and Bayramoglu, G. (2004a). Preparation and characterisation of surfaces properties of poly(hydroxyethylmethacrylate-co-methacrylolyamido-histidine) membranes: application for purification of human immunoglobulin G. *J. Chromatogr. B*, **807**: 315-25.

Arica, M.Y., Yilmaz, M., Yalçin, E. and Bayramoglu, G.M. (2004b). Affinity membrane chromatography: relationship of dye-ligand type to surface polarity and their effect on lysozyme separation and purification. *J. Chromatogr. B* **805**: 315-23.

Axén, R., Porath, J. and Ernback, S. (1967). Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature* **214**: 1302-04.

Bedair, M. and Rassi, Z.E. (2004). Affinity chromatography with monolithic capillary columns I. Polymethacrylate monoliths with immobilized mannan for the separation of mannose-binding proteins by capillary electrochromatography and nano-scale liquid chromatography. *J. Chromatogr. A* **1044**: 177-86.

Bedair, M. and Rassi, Z.E. (2005). Affinity chromatography with monolithic capillary columns: II. Polymethacrylate monoliths with immobilized lectins for the separation of glycoconjugates by nano-liquid affinity chromatography. *J. Chromatogr. A* **1079**: 236-45.

Beeskow, T.C., Kusharyoto, W., Anspach, F.B., Kroner, K.H. and Deckwer, W.D. (1995). Surface modification of microporous polyamide membranes with hydroxyethyl cellulose and their application as affinity membranes. *J. Chromatogr. A* **715**: 49-65.

Bencina, K., Podgornik, A., Strancar, A. and Bencina, M. (2004). Enzyme immobilization on epoxy- and 1,1'-carbonyldiimidazole-activated methacrylate-based monoliths. *J. Separ. Sci.* **27**: 811-18.

Berruex, L.G., Freitag, R. and Tennikova, T.B. (2000). Comparison of antibody binding to immobilized group specific affinity ligands in high performance monolith affinity chromatography. *J. Pharmaceut. Biomed.* **24**: 95-104.

Biesaga, M., Orska, J., Fiertek, D., Izdebski, J. and Trojanowicz, M. (1999). Immobilized metal-ion affinity chromatography of peptides on metalloporphyrin stationary phases. *Fresen. J. Anal. Chem.* **364**: 160-4.

Birkenmeier G. and Kopperschläger G. (1982). Application of dye-ligant chromatography to the isolation of alpha-1-proteinase inhibitor and alpha-1-acid glycoprotein. *J. Chromatogr.* **235**:237-48.

Bizzozero, O., Besio-Moreno, M., Pasquini, J.M., Soto, E.F. and Gómez, G.J. (1982). Rapid purification of proteolipids from rat brain subcellular fractions by chromatography on a lipophilic dextran gel. *J. Chromatogr.* **227**: 33-44.

Borcherding, H., Hicke, H.G., Jorcke, D. and Ulbricht, M. (2003). Affinity membranes as a tool for life science applications. *Ann. N. Y. Acad. Sci.* **984**: 470-79.

Brandt, S., Goffe, R.A., Kessler, S.B., Oconnor, J.L. and Zale, S.E. (1988). Membrane-based affinity technology for commercial scale purifications. *Bio-Technol.* **6**: 779-82.

Bruce, I.J., Taylor, J., Todd, M., Davies, M.J., Borioni, E., Sangregorio, C. and Sen, T. (2004). Synthesis, characterization and application of silica-magnetite nanocomposites. *J. Magn. Magn. Mater.* **284**: 145-60.

Carlsson, J., Janson, J.-C. and Sparrman, M. (1989). Affinity chromatography. In protein purification, (eds. J.-C. Janson & L. Rydén), Uppsala, VCH Publishers, Inc., pp. 275-329.

Charcosset, C. (1998). Purification of proteins by membrane chromatography. *J.Chem. Technol Biotechnol.* **71**: 95-110.

Chatterjee, J., Haik, Y. and Chen, C.-J. (2004). pH-reversible magnetic gel with a biodegradable polymer. *J. Appl. Polym. Sci.* **91**: 3337-41.

Coupek, J., Kriváková, M. and Pokorný, S. (1973). New hydrophilic materials for chromatography: glycol methacrylates. *J. Polym. Sci. Pol. Sym.* **42**: 185-90.

Coupek, J. and Vins, I. (1994). Hydroxyethyl methacrylate-based sorbents for high-performance liquid chromatography of proteins. *J. Chromatogr. A* **658**: 391-98.

Daniak, M.B., Plieva, F.M., Galaev, I.Y., Hatti-Kaul, R. and Mattiasson, B. (2005). Cell chromatography: Separation of different microbial cells using IMAC supermacroporous monolithic columns. *Biotechnol Progr.* **21**: 644-49.

Davis, M.T., Lee, T.D., Ronk, M. and Hefta, S.A. (1995). Microscale immobilized protease reactor columns for peptide-mapping by liquid-chromatography mass-spectral analyses. *Anal. Biochem.* **224**: 235-44.

Demelbauer, U.M., Plematl, A., Josic, D., Allmeier, G. and Rizzi, A. (2005). On the variation of glycosylation in human plasma derived antithrombin. *J. Chromatogr. A* **1080**: 15-21.

Dwek, M.V., Ross, H.A. and Leathem, A.J.C. (2001). Proteome and glycosylation mapping identifies post-translational modifications associated with aggressive breast cancer. *Proteomics* **1**: 756-62.

Freije, R.J., Mulder, P.P.M.F.A., Werkman, W., Rieux, L., Niederlander, H.A.G., Verpoorte, E. and Bischoff, R. (2005). Chemically modified, immobilized trypsin reactor with improved digestion efficiency. *J. Proteome Res.* **4**: 1805-13.

Frenzel, A., Bergemann, C., Köhl, G. and Reinard, T. (2003). Novel purification system for 6xHis-tagged proteins by magnetic affinity separation. *J. Chromatogr. B* **793**: 325-9.

Geng, M., Zhang, X., Bina, M. and Regnier, F. (2001). Proteomics of glycoproteins based on affinity selection of glycopeptides from tryptic digests. *J. Chromatogr. B* **752**: 293-306.

Ghosh, R. (2002). Protein separation using membrane chromatography: opportunities and challenges. *J. Chromatogr. A* **952**: 13-27.

Ghosh, D., Krokhin, O., Antonovici, M., Ens, W., Standing, K.G., Beavis, R.C. and Wilkins, J.A. (2004). Lectin affinity as an approach to the proteomic analysis of membrane glycoproteins. *J. Proteome Res.* **3**: 841-50.

Girot, P. and Boschetti, E. (1981). Physico-chemical and chromatographic properties of new ion exchangers. *J. Chromatogr.* **213**: 389-96.

Gomez, C.G., Alvarez, C.I. and Strumia, M.C. (2003). Influence of the morphology of poly(EGDMA-co-HEMA) on the chemical modifications and retention of O-phosphothreonine. *J. Biochem. Biophys. Meth.* **55**: 23-36.

Guo, W. and Ruckenstein, E. (2001). A new matrix for membrane affinity chromatography and its application to the purification of concanavalin A. *J. Membr. Sci.* **182**: 227-34.

Guo, W. and Ruckenstein, E. (2003). Modified glass fiber membrane and its application to membrane affinity chromatography. *J. Membr. Sci.* **215**: 141-55.

Guo, W. and Ruckenstein, E. (2003). Crosslinked glass fiber affinity membrane chromatography and its application to fibronectin separation. *J. Chromatogr. B*, **795**: 61-72.

Guo, W., Eisenman, J.R., Mahimkar, R.M., Peschon, J.J., Paxton, R.J., Black, R.A. and Johnson, R.S. (2002). *Mollecular & Cellular Proteomics* **1**: 30-36.

Gupalova, T.V., Lojkina, O.V., Pâlâgnuk, V.G., Totolian, A.A. and Tennikova, T.B. (2002). Quantitative investigation of the affinity properties of different recombinant forms of protein G by means of high-performance monolithic chromatography. *J. Chromatogr. A* **949**: 185-93.

Hage, D.S. (1999). Affinity chromatography: A review of clinical applications. *Clin. Chem.* **45**: 593-615.

Hahn, R., Podgornik, A., Merhar, M., Schallaun, E. and Jungbauer, A. (2001). Affinity monoliths generated by in situ polymerization of the ligand. *Anal. Chem.* **73**: 5126-32.

Hao, W.Q., Chen, Z.A., Wang, J.D. and Liu X.L. (2004). Modeling of protein adsorption in membrane affinity chromatogrpahy. *Anal. Letters* **37**: 1319-38.

Haukanes, B.I. and Kwam, C. (1993). Application of magnetic beads in bioassays. *Biotechnology* **11**: 60-63.

Hennion, M.-C. and Pichon, V. (2003). Immuno-based sample preparation for trace analysis. *J. Chromatogr. A* **1000**: 29-52.

Hjerten, S., Liao, J.L. and Zhang, R. (1989). High-performance liquid-chromatography on continuous polymer beds. *J. Chromatogr.* **473**: 273-5.

Hodgson, R.J., Chen, Y., Zhang, Z., Tleugabulova, D., Long, H., Zhao, X.M., Organ, M., Brook, M.A., *et al.* (2004). Protein-doped monolithic silica columns for capillary liquid chromatography prepared by the sol-gel method: Applications to frontal affinity chromatography. *Anal. Chem.* **76**: 2780-90.

Hodgson, R.J., Brook, M.A. and Brennan, J.D. (2005). Capillary-scale monolithic immunoaffinity columns for immunoextraction with in-line laser-induced fluorescence detection. *Anal. Chem.* **77**: 4404-12.

Hogan, J.M., Pitteri, S.J., Chrisman, P.A. and McLuckey, S.A. (2005). Complementary structural information from a tryptic N-linked glyco-

peptide *via* electron transfer ion/ion reactions and collision-induced dissociation. *J. Proteome Res.* **4**: 628-32.

Horák, D., Semenyuk, N. and Lednicky, F. (2003). Effect of the reaction parameters on the particle size in the dispersion polymerization of 2-hydroxyethyl and glycidyl methacrylate in the presence of a ferrofluid. *J. Polym. Sci. Pol. Chem.* **41**: 1848-63.

Howard, M.E. and Holcombe, J.A. (2000). Model for nonequilibrium binding and affinity chromatography: Characterization of 8-Hydroxychinoline immobilized on controlled pore glass using a flow injection system with a packed microcolumn. *Anal. Chem.* **72**: 3927-33.

Huang, S.C., Caldwell, K.D., Lin, J.-N., Wang, H.-K. and Herron, J.N. (1996). Site-specific immobilization of monoclonal antibodies using spacer mediated antibody attachment. *Langmuir* **12**: 4292-98.

Iberer, G., Hahn, R. and Jungbauer, A. (1999). Monoliths as stationary phases for separating biopolymers - Fourth-generation chromatography sorbents. *LC GC N. Am.* **17**: 998.

Iwata, K., Saito, K., Furusaki, S., Sugo, T. and Okamoto, J. (1991). Adsorption characteristics of an immobilized metal affinity membrane. *Biotechnol. Prog.* **7**: 412-18.

Jankovic, M.M. and Kosanovic, M.M. (2005). Glycosylation of urinary prostate-specific antigen in benign hyperplasia and cancer: assessment by lectin-binding patterns. *Clin. Biochem.* **38**: 58-65.

Jedlinski, Z. and Paprotny, J. (1967). Synthesis and polymerization studies of some N-alkylacrylamides. III. Polymerization of 2-methacrylamido-2-methylpropandiol-1,3 and 2-methacrylamido-2-methylpropanol-1. *J. Polym. Sci. A1* **5**: 2957-60.

Jiang, T., Mallik, R. and Hage, D.S. (2005). Affinity monoliths for ultrafast immunoextraction. *Anal. Chem.* **77**: 2362-72.

Josic, D. and Buchacher, A. (2001). Application of monoliths as supports for affinity chromatography and fast enzymatic conversion. *J. Biochem. Bioph. Meth.* **49**: 153-74.

Josic, D., Reusch, J., Loster, K., Baum, O. and Reutter, W. (1992). High-performance membrane chromatography of serum and plasma-membrane proteins. *J. Chromatogr.* **590**: 59-76.

Josic, D., Buchacher, A. and Jungbauer, A. (2001). Monoliths as stationary phases for separation of proteins and polynucleotides and enzymatic conversion. *J. Chromatogr. B* **752**: 191-205.

Kaji, H., Saito, H., Yamauchi, Y., Shinkawa, T., Taoka, M., Hirabayashi, J., Kasai, K., Takahashi, N., *et al.* (2003). Lectin affinity capture, isotope-coded tagging and mass spectrometry to identify N-linked glycoproteins. *Nat. Biotechnol.* **21**: 667-72.

Kato, M., Inuzuka, K., Sakai-Kato, K. and Toyo'ka, T. (2005). Monolithic bioreactor immobilizing trypsin for high-throughput analysis. *Anal. Chem.* **77**: 1813-18.

Kim, M., Saito, K., Furusaki, S., Sugo, T. and Ishigaki, I. (1991). Protein adsorption capacity of porous phenylalanine-containing membrane based on a polyethylene matrix. *J. Chromatogr.* **586**: 27-33.

Kim, M., Kiyohara, S., Konishi, S., Tsuneda, S., Saito, K. and Sugo, T. (1996). Ring-opening reaction of poly-GMA chain grafted onto a porous membrane. *J. Membr. Sci.* **117**: 33-38.

Kim, H.S., Kye, Y.S. and Hage, D.S. (2004). Development and evaluation of N-hydroxysuccinimide-activated silica for immobilizing human serum albumin in liquid chromatography columns. *J. Chromatogr. A* **1049**: 51-61.

Kiyohara, S., Kim, M., Toida, Y., Saito, K., Sugita, K. and Sugo, T. (1997). Selection of a precursor monomer for the introduction of affinity ligands onto a porous membrane by radiation-induced graft polymerization. *J. Chromatogr. A* **758**: 209-15.

Klein, E. (1990). Affinity membranes: their chemistry and performance in adsorptive separation processes. J. Wiley, New York.

Klein, E., Eichholz, E. and Yeager, H. (1994). Affinity membranes prepared from hydrophilic coatings on microporous polysulfone hollow fibers. *J. Membr. Sci.* **90**: 69-80.

Klein, E. (2000). Affinity membranes: a 10-year review. *J. Membr. Sci.* **179**: 1-27.

Kobayashi, K., Tsuneda, S., Saito, K., Yamagishi, H., Furusaki, S. and Sugo, T. (1993). Preparation of microfiltration membranes containing anionexchange groups. *J. Membr. Sci.* **76**: 209-18.

Kohn, J. and Wilchek, M. (1983). Activation of polysaccharide resins by cyanogen bromide. *Chem. Anal.* **66**: 599-630.

Koneracká, M., Kopcansky, P., Timko, M., Ramchand, C.N., de Sequeira, A. and Trevan, M. (2002). Direct binding procedure of proteins and enzymes to fine magnetic particles. *J. Mol. Catal. B-Enzym.* **18**: 13-8.

Kovarik, P., Hodgson, R.J., Covey, T., Brook, M.A. and Brennan, J.D. (2005). Capillary-scale frontal affinity chromatography/MALDI tandem mass spectrometry using protein-doped monolithic silica columns. *Anal. Chem.* **77**: 3340-50.

Kronick, P.L., Campbell, G.LeM and Joseph, K. (1978). Magnetic microspheres prepared by redox polymerization used in a cell separation based on gangliosides. *Science* **200**: 1074-6.

Kubin, M., Spacek, P. and Chromece, R. (1967). Gel permeation chromatography on porous poly(ethylene glycol methacrylate). *Collect. Czech Chem. C.* **32**: 3881-7.

Kumar, A., Plieva, F.M., Galeav, I.Y. and Mattiasson, B. (2003). Affinity fractionation of lymphocytes using a monolithic cryogel. *J. Immunol. Methods* **283**: 185-94.

Larsson, P.-O. (1984). High-Performance Liguid Affinity Chromatography. *Methods Enzymol.* **104**: 212-23.

Lauva, M., Auzans, E., Levickis, V. and Plavins, J. (1990). Selective HGMS of colloidal magnetite-binding cells from whole blood. *J. Magn. Magn. Mater.* **85**: 295-8.

Lee, W.-C. and Lee, K.H. (2004). Applications of affinity chromatography in proteomics. *Anal. Biochem.* **324**: 1-10.

Li, Y.H. and Sencer, H. G. (1992). Selective protein separations using formed-in-place anion-exchange membranes. *J.Biotechnol.* **26**: 203-11.

Liberda, J., Manásková, P., Svestàk, M., Jonáková, V. and Tichá, M. (2002) Immobilization of L-glyceryl phosphorylcholine: Isolation of phosphorylcholine-binding proteins from seminal plasma. *J. Chromatogr. B* **770**: 101-10.

Liu, Y.-C., Suen, S.-Y., Huang, C.-W. and ChangChien C.-C. (2005). Effects of spacer arm on penicillin G acylase purification using immobilized metal affinity membranes. *J. Membr. Sci.* **251**: 201-7.

Lowe, C.R. and Pearson, J.C. (1984). Affinity chromatography on immobilized dyes. *Method. Enzymol.* **104**: 97-113.

Lukás, J., Svec, F. and Kálal, J. (1977). Polar polymeric sorbents based on glycidyl methacrylate copolymers. *J. Chromatogr. A* **153**: 15-22.

Luo, Q.Z., Zou, H.F., Xiao, X., Guo, Z., Kong, L. and Mao, X.Q. (2001). Chromatographic separation of proteins on metal immobilized iminodiacetic acid-bound molded monolithic rods of macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate). *J. Chromatogr. A* **926**: 255-64.

Luo, Q.Z., Zou, H.F., Zhang, Q., Xiao, X. and Ni, J. (2002). High-performance affinity chromatography with immobilization of protein A and L-histidine on molded monolith. *Biotechnol. Bioeng.* **80**: 481-9.

Ma, Z.-Y., Guan, Y.-P., Liu, X.-Q. and Liu, H.-Z. (2005). Preparation and characterization of micron-sized non-porous magnetic polymer microspheres with immobilized metal affinity ligands by modified suspension polymerization. *J. Appl. Polym. Sci.* **96**: 2174-80.

Madera, M., Mechref, Y. and Novotny, M.V. (2005). Combining lectin microcolumns with high-resolution separation techniques for enrichment of glycoproteins and glycopeptides. *Anal. Chem.* **77**: 4081-90.

Mallik, R., Tao, J. and Hage, D.S. (2004). High-performance affinity monolith chromatography: Development and evaluation of human serum albumin columns. *Anal. Chem.***76**: 7013-22.

Mao, X.Z., Luo, Y., Dai, Z.P., Wang, K., Du, Y. and Lin, B. (2004). Integrated lectin affinity microfluidic chip for glycoform separation. *Anal. Chem.* **76**: 6941-47.

Matsumoto, I., Ito, Y. and Seno, N. (1982). Preparation of affinity adsorbents with Toyopearl gels. *J. Chromatogr. A* **239**: 747-54.

Mehta, R.V., Upadhyay, R.V., Charles, S.W. and Ramchand, C.N. (1997) Direct binding of protein to magnetic particles. *Biotechnol Tech.* **11**: 493-96.

Mikes, O. (1988). High-performance liquid chromatography of biopolymers and biooligomers (2nd ed.). Amsterdam: Elsevier Science Publishers B.V.

Mikes, O., Strop, P. and Coupek J. (1978). Ion-exchange derivatives of Spheron. *J. Chromatogr.* **153**: 23-6.

Miron, T. and Wilchek, M. (1987). Immobilization of proteins and ligands using chlorocarbonates. *Method. Enzymol.* **135**: 84-90.

Morais, S., Maquieira A. and Puchades R. (1999). Selection and characterisation of membranes by means of an immunofiltration assay. Application to the rapid and sensitive determination of the insecticide carbaryl. *J. Immunol. Meth.* **224**: 101-09.

Mosbach, K. and Schroder, U. (1979). Preparation and application of magnetic polymers for targeting of drugs. *FEBS Lett.* **102**: 112-16.

Nakamura, K., Suzuki, T., Kamichika, T., Hasegawa, M., Kato, Y., Sasaki, H. and Inouye, K. (2002). Evaluation and application of a new dye affinity adsorbent. *J. Chromatogr. A* **972**: 21-25.

Nakamura, K., Suzuki, T., Hasegawa, M., Kato, Y., Sasaki, H. and Inouye, K. (2003). Characterization of p-aminobenzamidine-based sorbent and

its use for high-performance affinity chromatography of trypsin-like proteases. *J. Chromatogr. A* **1009**: 133-39.

Nasef, M. M. and Hegazy, E.-S. A. (2004). Preparation and applications of ion exchange membranes by radiation-induced graft co-polymerization of polar monomers onto non-polar films. *Prog. Polym. Sci.* **29**: 499-561.

Nawarak, J., Phutrakul, S. and Chen, S.-T. (2004). Analysis of lectin-bound glycoproteins in snake venom from the Elapidae and Viperidae families. *J. Proteome Res.* **3**: 383-92.

Necina, R., Amatschek, K., Schallaun, E., Schwinn, H., Josic, D. and Jungbauer, A. (1998). Peptide affinity chromatography of human clotting factor VIII Screening of the vWF-binding domain. *J. Chromatogr. B* **715**: 191-201.

Odabasi, M., Uzun, L. and Denizli, A. (2004). Porous magnetic chelator support for albumin adsorption by immobilized metal affinity separation. *J. Appl. Polym. Sci.* **93**: 2501-10.

Ohlson, S., Hansson, L., Glad, M., Mosbach, K. and Larsson, P.-O. (1989). High performance liquid affinity chromatography: a new tool in biotechnology. *Trends Biotechnol.* **7**: 179-86.

Palm, A.K. and Novotny, M.V. (2004). Analytical characterization of a facile porous polymer monolithic trypsin microreactor enabling peptide mass mapping using mass spectrometry. *Rapid Commun. Mass Sp.* **18**: 1374-82.

Palm, A.K. and Novotny, M.V. (2005). A monolithic PNGase F enzyme microreactor enabling glycan mass mapping of glycoproteins by mass spectrometry. *Rapid Commun. Mass Sp.* **19**: 1730-8.

Pan, Z., Zou, H., Mo, W., Huang, X. and Wu, R. (2002). Protein A immobilized monolithic capillary column for affinity chromatography. *Anal. Chim. Acta* **466**: 141-50.

Peterson, D.S., Rohr, T., Svec, F. and Frechet, J.M.J. (2002a). Enzymatic microreactor-on-a chip: Protein mapping using trypsin immobilized on porous polymer monoliths molded in channels of microfluidic devices. *Anal. Chem.* **74**: 4081-8.

Peterson, D.S., Rohr, T., Svec, F. and Frechet, J.M.J. (2002b). High-throughput peptide mass mapping using a microdevice containing trypsin immobilized on a porous polymer monolith coupled to MALDI TOF and ESI TOF mass spectrometers. *J. Proteome Res.* **1**: 563-8.

Peterson, D.S., Rohr, T., Svec, F. and Frechet, J.M.J. (2003). Dual-function microanalytical device by in situ photolithographic grafting of porous polymer monolith: Integrating solid phase extraction and enzymatic digestion for peptide mass mapping. *Anal. Chem.* **75**: 5328-35.

Petro, M., Svec, F. and Frechet, J.M.J. (1996). Immobilization of trypsin onto "molded" macroporous poly(glycidyl methacrylate-co-ethylene dimethacrylate) rods and use of the conjugates as bioreactors and for affinity chromatography. *Biotechnol. Bioeng.* **49**: 355-63.

Pflegerl, K., Podgornik, A., Berger, E. and Jungbauer, A. (2002). Screening for peptide affinity ligands on CIM monoliths. *Biotechnol. Bioeng.* **79**: 733-40.

Pharmacia-Biotechnology. (1995). Gel Filtration Principles and Methods (5th ed.): Pharmacia Biotechnology.

Pieracci, J., Crivello, J. V. and Belfort, G. (1999). Photochemical modification of 10-kDa polyethersulfone ultrafiltration membranes for reduction of biofouling. *J. Membr. Sci.* **156**: 223-40.

Piletsky, S.A., Panasyuk, T.L., Piletskaya, E. V., Nicholls I. A. and Ulbricht, M. (1999). Receptor and transport properties of imprinted polymer membranes - a review. *J. Membr. Sci.* **157**: 263 - 78.

Porath, J. and Hansen, P. (1991). Cascade-mode multiaffinity chromatography. Fractionation of human serum proteins. *J. Chromatogr.* **550**: 751-64.

Prádny, M., Lesny, P., Fiala, J., Vacík, J., Slouf, M., Michálek, J. and Syková, E. (2003). Macroporous hydrogels based on 2-hydroxyethyl methacrylate. Part I. Copolymers of 2-hydroxyethyl methacrylate with methacrylic acid. *Collect. Czech. Chem. Commun.* **68**: 812-22.

Qiu, G.-M., Zhu, B.-K. and Xu, Y.-Y. (2005). A-amylase immobilized by Fe_3O_4/poly(styrene-co-maleic anhydride) magnetic composite microspheres: preparation and characterization. *J. Appl. Polym. Sci.* **95**: 328-35.

Qiu, R. and Regnier, F.E. (2005). Use of multidimensional lectin affinity chromatography in differential glycoproteomics. *Anal. Chem.* **77**: 2802-9.

Regnier, F.E., Riggs, L., Zhang, R.J., Xiong, L., Liu, P.R., Chakraborty, A., Seeley, E., Sioma, C., *et al.* (2002). Comparative proteomics based on stable isotope labeling and affinity selection. *J. Mass Spectrom.* **37**: 133-45.

Ren, D., Penner, N.A., Slentz, B.E., Mirzaei, H. and Regnier, F. (2003). Evaluating immobilized metal affinity chromatography for the selection of Histidine-containing peptides in comparative proteomics. *J. Proteome Res.* **2**: 321-9.

Ren, D., Penner, N.A., Slentz, B.E. and Regnier, F.E. (2004). Histidine-rich peptide selection and quantification in targeted proteomics. *J. Proteome Res.* **3**: 37-45.

Roper, D.K. and Lightfoot, E.N. (1995). Separation of biomolecules using adsorptive membranes. *J. Chromatogr. A* **702**: 3 - 26.

Ruckenstein, E. and Guo, W. (2001). Crosslinked mercerized cellulose membranes and their application to membrane affinity chromatography. *J. Membr. Sci.* **187**: 277-86.

Safarík, I. and Safaríková, M. (1999). Use of magnetic techniques for the isolation of cells. *J. Chromatogr. B* **722**: 33-53.

Safarik, I. and Safarikova, M. (2004). Magnetic techniques for the isolation and purification of proteins and peptides. *Biomagn. Res. Technol.* **2**:7.

Saiyed, Z.M., Telang, S.D. and Ramchand, C.N. (2003). Application of magnetic techniques in the field of drug discovery and biomedicine. *Biomagn. Res. Technol.* **1**: 2.

Sengupta, A. and Hage, D.S. (1999). Characterization of minor site probes for human serum albumin by high-performance affinity chromatography. *Anal. Chem.* **71**: 3821-7.

Serafica, G.C., Pimbley, J. and Belfort, G. (1994). Protein fractionation using fast-flow immobilized metal chelate affinity membranes. *Biotechnol. Bioeng.* **43**: 21-36.

Shi, W., Zhang, F. and Zhang, G. (2005). Adsorption of bilirubin with polylysine carrying chitosan-coated nylon affinity membranes. *J. Chromatogr. B* **819**: 301-6.

Shiyama, T., Furuya, M., Yamazaki, A., Terada, T. and Tanaka, A. (2004). Design and synthesis of novel hydrophilic spacers for the reduction of nonspecific binding proteins on affinity resins. *Bioorgan. Med. Chem.* **12**: 2831-41.

Singh, N., Husson, S. M., Zdyrko, B. and Luzinov, I. (2005). Surface modification of microporous PVDF membranes by ATRP. *J. Membr. Sci.* **262**: 81-90.

Slentz, B.E., Penner, N.A. and Regnier, F.E. (2003). Protein proteolysis and the multi dimensional electrochromatographic separation of histidine-containing peptide fragments on a chip. *J. Chromatogr. A* **984**: 97-107.

Stensballe, A., Andersen, S. and Jensen, O.N. (2001). Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. *Proteomics* **1**: 207-22.

St'ovícková, J., Podrazky, V. and Trnavsky, K. (1992). Affinity chromatography of serine proteinases from the bovine intervertebral disc on BPTI-Separon HEMA 1000. *J. Chromatogr. B* **575**: 229-35.

Suen, S.-Y., Lin, S.-Y. and Chiu, H.-C. (2000). Effects of spacer arms on cibacron blue 3GA immobilization and lysozyme adsorption using regenerated cellulose membrane discs. *Ind. Eng. Chem. Res.* **39**: 478-87.

Suen, S.-Y., Liu, Y.-C., Chang, C.-S. (2003). Review Exploiting immobilized metal affinity membranes for the isolation or purification of therapeutically relevant species. *J. Chromatogr. B* **797**: 305-19.

Sumi, S., Arai, K., Kitahara, S. and Yoshida, K.-I. (1999). Serial lectin affinity chromatography demonstrates altered asparagine-linked sugar-chain structures of prostate specific antigen in human prostate carcinoma. *J. Chromatogr. B* **727**: 9-14.

Sun, X.F. and Chai, Z.K. (2002). Urea-formaldehyde resin monolith as a new packing material for affinity chromatography. *J. Chromatogr. A* **943**: 209-18.

Sundberg, L. and Porath, J. (1974). Preparation of adsorbents for biospecific affinity chromatography. *J. Chromatogr. A* **90**: 87-98.

Svec, F. and Frechet, J.M.J. (1992). Continuous rods of macroporous polymer as high performance liquid chromatography separation media. *Anal. Chem.* **64**: 820-2.

Svec, F. and Frechet, J.M.J. (1995). Kinetic control of pore formation in macroporous polymers - Formation of molded porous materials with high-flow characteristics for separations or catalysis. *Chem. Mater.* **7**: 707-15.

Svec, F., Hradil, J., Coupek, J. and Kalal, J. (1975). Reactive polymers. I. Macroporous methacrylate copolymers containing epoxy groups. *Angew. Makromol. Chem.* **48**: 135-43.

Svec, F., Tennikova, T.B. and Deyl, Z. (2003). Monolithic materials: Preparation, properties and applications (1st ed. Vol. 67). Amsterdam: Elsevier Science Publishers B.V.

Takai, I., Oda, O., Shinzato, T., Ohbayashi, K., Zamanaka, N. and Maeda, K. (1996). Effective method for purification of lysozyme from human urine. *J. Chromatogr. B* **685**: 21-5.

Tamura, T., Terada, T. and Tanaka, A. (2003). A quantitative analysis and chemical approach for the reduction of nonspecific binding proteins on affinity resins. *Bioconjugate Chem.* **14**: 1222-30.

Taniguchi, M. and Belfort, G. (2004). Low protein fouling synthetic membranes by UV-assisted surface grafting modification: varying monomer type. *J. Membr. Sci.* **231**: 147-57.

Teeters, M.A., Conrardy, S.E., Thomas, B.L. Root, T.W. and Lightfoot, E.N. (2003). Adsorptive membrane chromatography for purification of plasmid DNA. *J. Chromatogr. A* **989**: 165-73.

Thommes, J. and Kula, M.-R. (1995). Membrane chromatography - an integrative concept in the downstream processing of proteins. *Biotech. Prog.* **11**: 357 - 67.

Tosoh Bioscience. (2003). Laboratory products catalogue, 2003, Europe Tosoh Biosciences.

Trojer, L., Stecher, G., Feuerstein, I., Lubbad, S. and Bonn, G.K. (2005). Characterisation and evaluation of metal-loaded iminodiacetic acid-silica of different porosity for the selective enrichment of phosphopeptides. *J. Chromatogr. A* **1079**: 197-207.

Tsai, Y.-H,. Wang, M.-Y. and Suen, S.-Y. (2002). Purification of hepatocyte growth factor using polyvinyldiene fluoride-based immobilized metal affinity membranes: equilibrium adsorption study. *J. Chromatogr. B* **766**: 133-43.

Turková, J. (1993). Bioaffinity Chromatography (2nd ed. Vol. 55). Amsterdam: Elsevier Science Publishers B.V.

Ulbricht, M., Belter, M., Langenhangen, U., Schneider, F. and Weigel, W. (2002). Novel molecularly imprinted polymer (MIP) composite membranes *via* controlled surface and pore functionalizations. *Desalination* **149**: 293-5.

Unger, K.K. (1979). Porous silica, its properties and use as support in column liquid chromatography. Amsterdam: Elsevier Scientific Pub. Co.

Urmenyi, A.M., Poot, A.A., Wessling M. and Mulder, M.H.V. (2005). Affinity membranes for hormone removal from aqueous solutions. *J. Membr. Sci.* **259**: 91-102.

Varilová, T., Vránková, A., Pacáková, V., Tichá, M. and Stulík, K. (2005a). Preparation and testing of stationary phases and modified capillaries for affinity chromatography and affinity capillary electrophoresis of pepsin. *J. Chromatogr. A* **1084**: 207-13.

Varilová, T., Seménková, H., Horák, P., Madera, M., Pacáková, V., Tichá, M. and Stulík, K. (2005b). Affinity liquid chromatography and capillary electrophoresis of seminal plasma proteins. *J. Sep. Sci.*, **in press**.

Vizioli, N.M., Rusell, M.L., Carbajal, M.L., Carducci, C.N. and Grasselli, M. (2005). On-line affinity selection of histidine-containing peptides using a polymeric monolithic support for capillary electrophoresis. *Electrophoresis* **26**: 2942-8.

Vlakh, E., Ostryanina, N., Jungbauer, A. and Tennikova, T. (2004). Use of monolithic sorbents modified by directly synthesized peptides for affinity separation of recombinant tissue plasminogen activator (t-PA). *J. Biotechnol.* **107**: 275-84.

Vlakh, E.G., Platonova, G.A., Vlasov, G.P., Kasper, C., Tappe, A., Kretzmer, G. and Tennikova, T.B. (2003). *In vitro* comparison of complementary interactions between synthetic linear/branched oligo/poly-L-lysines and tissue plasminogen activator by means of high performance monolithic-disk affinity chromatography. *J. Chromatogr. A* **992**: 109-19.

Voyksner, R.D., Chen, D.C. and Swaisgood, H.E. (1990). Optimization of immobilized enzyme hydrolysis combined with high-performance liquid-chromatography thermospray mass-spectrometry for the determination of neuropeptides. *Anal. Biochem.* **188**: 72-81.

Wilchek M. and Miron T. (1987). Use of N-hydroxysuccinimide esters in affinity-chromatography. *Biol. Chem. H.-S.* **368**: 783-3.

Wichterle, O. and Lím, D. (1960). Hydrophilic gels for biological use. *Nature* **185**: 117-8.

Wong, S.S. (1991). Chemistry of protein conjugation and cross-linking, CRS Press Inc.

Xi, F.N. and Wu, J.M. (2004). Macroporous chitosan layer coated on nonporous silica gel as a support for metal chelate affinity chromatographic adsorbent. *J. Chromatogr. A* **1057**: 41-7.

Xi, F.N., Wu, J.M. and Luan, M.M. (2005). Silica-supported macroporous chitosan bead for affinity purification of trypsin inhibitor. *Chin. Chem. Lett.* **16**: 1089-92.

Xie, S., Svec, F. and Frechet, J.M.J. (1997). Monolithic poly(2-vinyl-4,4-dimethylazlactone co-acrylamide-co-ethylene dimethacrylate) support for design of high throughput bioreactors. Polymer Reprints **38**: 211-2.

Xiong, L., Andrews, D. and Regnier, F. (2003). Comparative proteomics of glycoproteins based on lectin selection and isotope coding. *J. Proteome Res.* **2**: 618-25.

Yang, B. and Yang, W. (2003). Photografting modification of PET nucleopore membranes. *J. Macromol. Sci. Pure* **40**: 309-20.

Yang, Z. and Hancock, W.S. (2004). Approach to the comprehensive analysis of glycoproteins isolated from human serum using a multi-lectin affinity column. *J. Chromatogr. A* **1053**: 79-88.

Yang, Z. and Hancock, W.S. (2005). Monitoring glycosylation pattern changes of glycoproteins using multi-lectin affinity chromatography. *J. Chromatogr. A* **1070**: 57-64.

Yang, Z., Hancock, W.S., Chew, T.R. and Bonilla, L. (2005). A study of glycoproteins in human serum and plasma reference standards (HUPO) using multilectin affinity chromatography coupled with RPLC-MS/MS. *Proteomics* **5**: 3353-66.

Young, M.N., Brisson, J.-R., Kelly, J., Watson, D.C., Tessier, L., Lanthier, P.H., Jarrel, H.C., Cadotte, N., *et al.* (2002). Structure of the N-Linked glycan present on multiple glycoproteins in the gram-negative bacterium, Campylobacter jejuni. *J. Biol. Chem.* **277**: 42530-9.

Yue, G., Balchunas, C.J., Jeffery, E., Coon, J., Landers, J.P. and Ferrance, J.P. (2005). Phosphopeptide enrichment on a microchip integrated with mass spectrometry. *Special Publication - Royal. Soc. Chem.* **2**: 291-3.

Zeng, X. F. and Ruckstein, E. (1999). Membrane chromatography: preparation and applications to protein separation. *Biotechnol Prog.* **15**: 1003-19.

Zeng, M. and Fang, Z. (2004). Preparation of sub-micrometer porous membrane from chitosan/polyethylene glycol semi-IPN. *J. Membr. Sci.* **245**: 95-102.

Zhang, B., Palcic, M.M., Schriemer, D.C., Alvarez-Manilla, G., Pierce, M. and Hindsgaul, O. (2001). Frontal affinity chromatography coupled to mass spectrometry for screening mixtures of enzyme inhibitors. *Anal Biochem.* **299**: 173-82.

Zhang, L.Y., Zhang, L.H., Zhang, W.B. and Zhang Y.K. (2005). On-line concentration of peptides and proteins with the hyphenation of polymer monolithic immobilized metal affinity chromatography and capillary electrophoresis. *Electrophoresis* **26**: 2172-8.

Zheng, H., Hu, P., Quinn, D.F. and Wang, K.Y. (2005). Phosphotyrosine proteomic study of interferon α signaling pathway using a combination of immunoprecipitation and immobilized metal affinity chromatography. *Mol. Cell. Proteom.* **4**: 721-30.

Zhou, W., Merrick, A.B., Khaledi, M.G. and Tomer, K.B. (2000). Detection and sequencing of phosphopeptides affinity bound to immobilized metal ion beads by matrix-assisted laser desorption/ionization mass spectrometry. *J. Am. Soc. Mass Spectr.* **11**: 273-82.

Zhuang, C., Murata, T., Usui, T., Kawahishi, H. and Kobayashi, K. (1996). Purification and characterization of a lection from the toxic mushroom *Amanita pantherina*. *Biochim Biophys. Acta* **1291**: 40-4.

Ziegler, T., Eckhardt, E., Strayle, J. and Herzog, H. (1994). Synthesis of 5-aminopentyl 4,6-O-[(R)-1-carboxyethylidene]-β-D-galactopyranoside and its use as a ligand for the affinity chromatography of human serum amyloid P protein. *Carbohyd. Res.* **253**: 167-83.

Zou, H., Luo, Q. and Zhou, D. (2001). Affinity membrane chromatography for the analysis and purification of proteins. *J. Biochem. Biophys. Meth.* **49**: 199-240.

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