**CHARLES UNIVERSITY IN PRAGUE**
**FIRST FALCULTY OF MEDICINE**

Abstract of Ph.D. Thesis

# The Separation of Gastric Aspartic Proteases Using Affinity Chromatography

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## Ph.D. Study Programs in Biomedicine

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## Souhrn

Lidská žaludeční šťáva obsahuje zejména aspartátové proteasy - pepsin A a pepsin C. Oba pepsiny jsou produkovány v žaludeční sliznici jako inaktivní pepsinogeny (pepsinogen A a pepsinogen C), které se liší svými fyzikálně-chemickými a imunologickými vlastnostmi. Oba pepsinogeny obsahují několik izozymogenů. K aktivaci pepsinogenů na příslušné pepsiny dochází v kyselém prostředí žaludku.

Hladiny pepsinogenů v krevním séru odrážejí morfologický a funkční stav žaludeční sliznice. Již mnoho let je pozorována souvislost mezi hladinami lidských pepsinogenů a některými žaludečními onemocněními (např. rakovinou žaludku, gastritidou). Z diagnostického hlediska je významný především vzájemný poměr pepsinogenu A a pepsinogenu C nejen v žaludeční sliznici, ale také v krevním séru. Pacienti s rakovinou žaludku nebo s atrofickou gastritidou mají významně snížený poměr pepsinogenu A a pepsinogenu C v krevním séru oproti zdravým jedincům. Během žaludečních onemocnění dochází také ke změnám zastoupení jednotlivých izozymogenů. Určení těchto změn by proto mohlo být významné pro včasnou diagnostiku zmíněných onemocnění.

Téma dizertační práce je součástí projektu zabývajícího se zejména vypracováním metody pro separaci žaludečních aspartátových proteas, která by byla vhodná pro sledování jejich změn během zmíněných onemocnění. Dizertační práce se konkrétně zabývala přípravou vhodných afinitních sorbentů pro separaci pepsinů a pepsinogenů. Při výběru vhodného afinitního ligandu pro tyto proteasy jsme vycházeli ze známého substrátu N-acetyl-L-fenylalanyl-3,5-dijod-L-tyrosinu, pomocí kterého lze stanovit aktivitu pepsinu A ve směsi s pepsinem C. Byly připraveny tři afinitní sorbenty: jodovaná L-tyrosin-Sepharosa, 3,5-dijod-L-tyrosin-Sepharosa a N-acetyl-L-fenylalanin-Sepharosa. Připravené sorbenty byly charakterizovány pomocí modelového enzymu (prasečího pepsinu A). Při studiu interakce prasečího pepsinu A a jeho komplexu s pepstatinem A s připravenými sorbenty bylo prokázáno, že se aktivní místo enzymu nepodílí na jeho interakci s těmito sorbenty. Dále byla prokázána účast fosfátové skupiny v molekule pepsinu na jeho interakci s N-acetyl-L-fenylalanin-Sepharosou. Všechny připravené sorbenty byly použity k separaci pepsinu A a pepsinu C z okyseleného homogenátu lidské žaludeční sliznice pacientů s různými žaludečními chorobami, případně žaludeční šťávy zdravého jedince. Výsledky ukázaly, že nejvhodnějším sorbentem pro separaci pepsinů je N-acetyl-L-fenylalanin-Sepharosa. Pomocí tohoto sorbentu se také podařilo částečně separovat jeden z izozymogenů pepsinogenu A z lidské žaludeční sliznice.

## Summary

Human gastric juice contains mainly aspartic proteases - pepsin A and pepsin C. Both pepsins are produced by gastric mucosa as inactive pepsinogens (pepsinogen A and pepsinogen C) that differ in their physico-chemical and immunological properties. Both pepsinogens consist of molecular variants, isozymogens. Pepsinogens are activated to the corresponding pepsins in the acidic environment of the gastric lumen.

The levels of pepsinogens in serum reflect the morphological and functional status of gastric mucosa. The levels of pepsinogens are associated with gastric diseases (e.g. gastric cancer, gastritis). For a diagnostic purpose, the ratio of pepsinogen A to pepsinogen C not only in the gastric mucosa but also in serum is relevant. The gastric cancer patients or the atrophic gastritis patients have significantly lower ratio of serum pepsinogen A to pepsinogen C in comparison with the healthy subjects. In addition, proportion of individual isozymogens reflects the type of the gastric disease. Determination of changes in pepsinogen levels could be useful for early diagnostics of gastric diseases mentioned above.

A subject of this Ph.D. thesis is a part of a long-term investigation that focuses on the elaboration of methods for the separation of gastric aspartic proteases that would be suitable for monitoring of their changes in mentioned diseases. This thesis was mainly focused on preparation of affinity sorbents suitable for separation of pepsins and pepsinogens. The choice of ligands was based on the substrate N-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine that is used to differentiate pepsin A and pepsin C. The following three affinity sorbents were prepared: iodinated L-tyrosine-Sepharose, 3,5-diiodo-L-tyrosine-Sepharose, and N-acetyl-L-phenylalanine-Sepharose. The basic characteristics of the prepared affinity sorbents were determined using the model enzyme (porcine pepsin A). The comparison of the chromatographic behavior of porcine pepsin A and its complex with pepstatine A showed that the enzyme active site was not involved in the enzyme interaction with ligands. In addition, participation of a phosphate group of the pepsin molecule was proved in the interaction of porcine pepsin A with N-acetyl-L-phenylalanine-Sepharose. All prepared sorbents were used for the separation of human pepsin A and pepsin C from acidified extract of gastric mucosa of patients with various types of diseases, eventually from gastric juice of healthy subjects. Out of the prepared sorbents, N-acetyl-L-phenylalanine-Sepharose was found to be the most suitable for this purpose. In addition, one of the isozymogens of pepsinogen A from gastric human mucosa was partially separated using this sorbent.

# 1. Introduction

The human gastric juice contains two major groups of aspartic proteases: pepsin A (EC 3.4.23.1) and pepsin C (3.4.23.3). Pepsins are endopeptidases which attack native proteins at acidic pH (ref. [1]); maximally active around pH 2.0 - 2.3, as determined using hemoglobin as the substrate [2]. The denaturation of protein substrates at low pH has been shown to contribute to their efficient hydrolysis [3]. Pepsin A specifically cleaves peptide bonds involving especially hydrophobic and aromatic amino acids [1, 3, 4]. Pepsin C preferentially cleaves Tyr-Xaa bonds (Xaa - various amino acids) [3]. Different synthetic peptides have been tested as substrates for pepsin A and pepsin C [5 - 8]. N-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine is the substrate for differentiation of pepsin A and pepsin C [3, 9].

Members of the family of aspartic proteases are inhibited by synthetic inhibitors diazoacetyl norleucine methylester and 1,2-epoxy-3-(*p*-nitrophenoxy) propane, and by a native inhibitor - pepstatin A [1]. The potency of these inhibitors varies for different aspartic proteases [2, 10]. The inhibition of pepsins by these two synthetic inhibitors is irreversible [1]. Pepstatin A was isolated from culture filtrates of various *Streptomyces* [11]. Pepstatin A is a heptapeptide [12] that non-covalently interacts with the active site of pepsin and thus it blocks the accessibility of a substrate to the active site. [1, 13, 14]

Pepsin A and pepsin C are secreted as inactive zymogens, pepsinogen A (PGA) and pepsinogen C (PGC), in the gastric mucosa. PGA is produced by cells of the gastric body and fundus, while PGC production was found throughout the stomach (the cardia, the fundus, the body and the antrum). In addition, PGC was found also in the proximal duodenum and in prostate [1, 15]. PGC is completely reabsorbed and metabolized by the kidneys. On the other hand, the kidneys metabolize only two-thirds of PGA. Both PGA and PGC can be detected in the blood, but only PGA is present under the physiological conditions in the urine [15].

Both PGA and PGC consist of isozymogens that differ in net charge, molecular weight or both [16, 17]. Seven isozymogens of pepsinogens were found in human gastric mucosa using the non-denaturing agar gel electrophoresis with stanining for proteolytic activity. They were designated Pg1 to Pg7 according to their decreasing anodal mobility. PGA consists of five isozymogens, Pg1 – Pg5, whereas PGC consists of two isozymogens, Pg6 – Pg7 (ref. [17]).

The levels of both pepsinogens in serum reflect the morphological and functional status of gastric mucosa; changes in pepsinogen levels are associated with gastric diseases [18 - 20]. Therefore, pepsinogens and their active forms are of a great medical and pharmaceutical interest. In healthy subject, the serum levels of pepsinogens are relatively constant but they considerably vary between different individuals. These variations are due to, i.e. age [20 - 22], sex [21, 22], diet, and nationality [15]. In fact, the PGA and PGC ratio could be considered more important than absolute values of PGA and PGC levels for diagnostic purposes.

Serum PGA levels and the ratio PGA/PGC significantly decreased in both the atrophic gastritis patients and gastric cancer patients as compared with the healthy controls, the gastric ulcer patients and the duodenal ulcer patients. Serum PGA levels and the ratio PGA/PGC in the atrophic gastritis patients were linked with the site and the grade of atrophic gastritis; it decreased with the progression of atrophic process [18]. Serum PGA levels were significantly higher in duodenal ulcer [20, 23] and gastric ulcer [20] patients, and significantly lower in the gastric cancer patients [18, 20] compared to the healthy controls. Serum PGC levels were higher in the duodenal ulcer or gastric ulcer patients [20, 21], whereas either no significant difference [20] or significantly higher [21, 24] PGC levels were found in the gastric cancer patients in comparison with the healthy controls. The ratio of PGA/PGC was significantly higher in the duodenal ulcer patients [20] but significantly lower in the gastric cancer patients [18, 20, 21, 24] compared to the healthy controls. In addition, PGA values were significantly lower in patients with advanced gastric cancer than in patients with early stage of gastric cancer [18, 20]. Levels of pepsinogens reflect also the cancer tissue type; both serum PGA and PGC levels were found to be significantly lower in intestinal-type cancer than in diffuse-type cancer. However, regardless the histological type, the ratio PGA/PGC was significantly lower in the gastric cancer patients in comparison with the healthy controls [24].

The presence and levels of isozymogens Pg3 to Pg5 also reflect the type of various gastric diseases. The patients with ulcer disease have the Pg3 to Pg5 ratio higher than 1, while gastric cancer patients lower than 1. In addition, the gastric cancer patients have significantly higher concentration of Pg5 (ref. [19]).

In clinical practice, the serum concentrations of PGA and PGC are determined by immunochemical methods (e.g. ELISA*), which utilize different immunochemical properties of these pepsinogens. Currently, many immunochemical kits for the determination of both pepsinogens in serum are available [25, 26].

No simple one-step chromatographic technique has been found yet for the isolation and separation of human pepsins and their zymogens. To obtain individual pepsins or their zymogens, or even individual isozymogens, by chromatographic methods, a multi-step chromatography has been required [27-32].

Affinity chromatography represents a valuable tool for the purification of proteins, for the separation of various proteins differing only slightly in their properties, and for the studies on structure of proteins or enzyme active site. This technique is based on the unique ability of biologically active substances to specifically and reversibly bind to a complementary substance (ligand), immobilized by a covalent bond to inert matrix. The choice of a suitable ligand is evidently the most important factor in the successful application of this technique. However, other components also influence the affinity separation, e.g. nature of matrix, matrix activation, spacer arm properties, mechanism of

* „enzyme linked immunosorbent assay“

ligand coupling, etc.[33] Only a limited number of ligands were described for the separation of pepsins, e.g. antibodies [30, 31], inhibitors [32, 34 - 36] and derivatives of substrate. Immobilized ligands belonging to the last group are derived from the substrate – N-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine [3, 9] used for distinguishing pepsin A and pepsin C; the following ligands were derived: L-phenylalanine, L-tyrosine, their iodinated analogs, and N-acetyl-L-phenylalanine. The affinity of pepsins and pepsinogens to these ligands was influenced by iodine content and the manner of ligand coupling [37 - 42].

Besides chromatography, electromigration methods have been used for separation of human pepsinogens and pepsins, e.g. non-denaturing polyacrylamide [16] or agar [17] gel electrophoresis, and isoelectric focusing [43, 44].

## 2. Aim of the Thesis

The object of this thesis forms a part of a long-term investigation that focuses on a study of a relationship between gastric diseases and changes in abundance of individual aspartic proteases in human gastric juice and their zymogens in gastric mucosa. The estimation of pepsinogen A and pepsinogen C ratio belongs to an important step in diagnosis of pre-malignant state of gastric cancer.

Aim of this thesis was to prepare the suitable affinity sorbent for the separation of human gastric aspartic proteases (pepsins) and their zymogens (pepsinogens). The solution of this task was separated in the following steps:

- Preparation of affinity sorbents for the separation of human pepsins and pepsinogens and finding of the optimum conditions for their adsorption and elution.

- Characterization of basic parameters of the prepared affinity sorbents using model protease – porcine pepsin A.

- Study of porcine pepsin A separation and the participation of the enzyme active site in its interaction with affinity sorbents.

- Application of the prepared affinity sorbents for the separation of human pepsins and pepsinogens from human gastric juice and human gastric mucosa, respectively.

# 3. Materials and Methods

**Materials**

- Biologic samples: lyophilized human gastric juice obtained from healthy subject, extracts of human gastric mucosa obtained from resected stomach of patients with gastric diseases, extracts of rat gastric mucosa
- Model proteins: porcine pepsin A (EC 3.4.23.1), porcine pepsinogen A, trypsin (EC 3.4.21.4) from bovine pancreas, α-chymotrypsin (EC 3.4.21.1) from bovine pancreas, chicken ovalbumin, bovine serum albumin (all from Sigma-Aldrich, Czech Republic)
- Sepharose 4B, N-acetyl-L-phenylalanine, Boc-3,5-diiodo-L-tyrosine, L-tyrosine, pepstatin A, acid phosphatase (EC 3.1.3.2) from potato (all from Sigma-Aldrich, Czech Republic); monoclonal antibody againts pepsinogen A and monoclonal antibody against pepsinogen C (Medix Biochemica, Finland)

**Preparation of affinity sorbents**

- L-tyrosin was directly coupled to the divinyl sulfone-activated Sepharose 4B. Immobilized L-tyrosine was iodinated after coupling [40].
- For the immobilization of N-acetyl-L-phenylalanine and 3,5-diiodo-L-tyrosine with protected amino group (Boc)*, ethylene diamine was first coupled to divinyl sulfone activated Sepharose 4B. Afterwards, the ligands were linked using a carbodiimide reaction. Then the protective group was removed from Boc-3,5-diiodo-L-tyrosine-Sepharose 4B (ref. [41]).

**Affinity chromatography of proteins**

- performed using: FPLC System (Ecom, Czech Republic) or BioLogic DuoFlow System (Bio-Rad, USA).
- the Bio-Scale MT2 High-Resolution Column (52 mm × 7 mm i.d.; Bio-Rad, USA) was packed with the prepared affinity sorbents
- general protocol for protein analysis: (1) equilibration with the starting buffer; (2) protein loading (1 ml); (3) washing out non-adsorbed substances with the starting buffer; (4) elution of protein with the eluting buffer (linear gradient or isocratic); flow rate: 1 ml/min; collected 1 ml fractions
- different buffers and solutions were tested to maximize protein recovery and achieve the best separation of proteins
- in collected fraction protein content and proteolytic activity of pepsin were determined

* Boc – *tert* – butyloxycarbonyl

- identification of separated proteins was performed using MALDI-TOF/TOF MS* or non-denaturing polycrylamide gel electrophoresis with staining for proteolytic activity [29]

**Analytical methods**

- protein concentration was determined using bicinchoninic acid protein assay [45]
- proteolytic activity was determined by modified Anson and Mirsky method [46]
- phosphorus content in pepsin was determined by „Phosphoprotein Phosphate Estimation Assay Kit" (Pierce, USA)

* MALDI-TOF/TOF MS – matrix assisted laser desorption ionization - time of flight/time of flight mass spectrometry

# 4. Results and Discussion

## 4.1. Preparation of Affinity Sorbents

In this study, the choice of ligands was based on a substrate (N-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine) used for the differentiation of pepsin A and pepsin C [3, 9]. N-acetyl-L-phenylalanine, 3,5-diiodo-L-tyrosine or L-tyrosine were immobilized to divinyl sulfone activated Sepharose 4B (ref. [40, 41]). L-tyrosine was linked to the activated matrix *via* its free amino group. For the immobilization of the other two ligands (that is N-acetyl-L-phenylalanine, 3,5-diiodo-L-tyrosine with protected amino group) *via* their free carboxyl groups, ethylene diamine was first coupled to divinyl sulfone activated Sepharose 4B. Afterwards, the ligands were linked using a carbodiimide reaction. After the immobilization of the aromatic amino acid derivatives, L-tyrosine-Sepharose 4B was further modified by iodination performed *via* the standard chloramine method, and Boc-3,5-diiodo-L-tyrosine-Sepharose* 4B was hydrolyzed to remove the Boc protective group.

Previous studies showed that the pepsin interactions with ligands derived from aromatic amino acids depended on the manner of coupling [40, 41]. The other factor that affects the affinity of aspartic proteases to aromatic amino acids is the presence of iodine atoms in the ligand molecule. The substitution by iodine into the phenyl ring of immobilized aromatic amino acids increased their affinity for pepsins compared to un-substituted compounds [40].

Divinyl sulfone** activated Sepharose was found to be a suitable matrix for coupling of different ligands [39 - 41]. Results of this study showed an advantageous application of the divinyl sulfone activated Sepharose for a linkage of amino acid derivatives both *via* their amino and the carboxyl groups. In the second case, aromatic amino acid derivatives were coupled to the activated matrix *via* an ethylene diamine spacer arm. In both cases, the amino group was coupled with the vinyl group of the divinyl sulfone activated agarose matrix. The structure of the resulting affinity sorbents corresponds to the general formula of thiophilic sorbents: agarose matrix–O–$CH_2$–$CH_2$–$SO_2$–$CH_2$–$CH_2$–X–Y (ref. [49]). Where X, besides S, can also be O or N and Y is an aromatic or heteroaromatic compound. Studies on the use of these thiophilic sorbents were mainly directed to the separation of different antibodies from various sources [48 - 51].

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* Boc – *tert* – butyloxycarbonyl

** Divinyl sulfone was originally used for cross-linking of agarose gel [47, 48].

## 4.2. Optimization of Chromatographic Conditions for Porcine Pepsin A on Affinity Sorbents

We focused on investigation of adsorption/desorption conditions for porcine pepsin A to/from prepared affinity sorbents. The choice of chromatographic conditions was limited by porcine pepsin A inactivation at pH higher than 6.0 (ref. [52]). We investigated effect of pH, ionic strength (NaCl, concentration of mobile phase) and composition of mobile phases (phosphate buffer, acetate buffer, MES[*]) on adsorption/desorption of porcine pepsin A. Affinity chromatography of porcine pepsin A under the optimum conditions is shown in figure 1. Porcine pepsin A adsorbed to all the prepared affinity sorbents, but under different conditions. No protease activity was detected in the flow-through fractions.

The increase in buffer pH was required for the desorption of porcine pepsin A from iodinated L-tyrosine–Sepharose (coupled *via* amino group) while the increase in buffer ionic strength released the pepsin from N-acetyl-L-phenylalanine-Sepharose and 3,5-diiodo-L-tyrosine-Sepharose (both coupled *via* carboxyl groups). The basic characteristics of the prepared affinity sorbents for porcine pepsin A were determined (tab. 1).

Tab. 1 Comparison of some characteristics of the prepared affinity sorbents

| Characteristics | N-acetyl-L-phenylalanine-Sepharose | 3,5-diiodo-L-tyrosine-Sepharose | Iodinated L-tyrosine-Sepharose |
|---|---|---|---|
| Reproducibility RSD (%) | 2 | 4 | 5 |
| Capacity (mg/ml) | 76.5 | 49.7 | 41.5 |
| $R^2$ | 0.993 | 0.998 | 0.999 |
| Recovery (%) | 96 | 88 | 96 |

Reproducibility – expressed as RSD (relative standard deviation; in percent) of elution peak area for ten repeated individual experiments performed under the optimum chromatographic conditions for porcine pepsin A; capacity – expressed in mg of porcine pepsin A adsorbed to 1 ml of sorbent; $R^2$ - regression coefficient of the linear dependence of the elution peak area on the applied amount of porcine pepsin A; recovery – the proteolytic activity of porcine pepsin A desorbed from affinity sorbent expressed in percentage of applied porcine pepsin A.

[*] 2-(N-Morpholino)ethanesulfonic acid

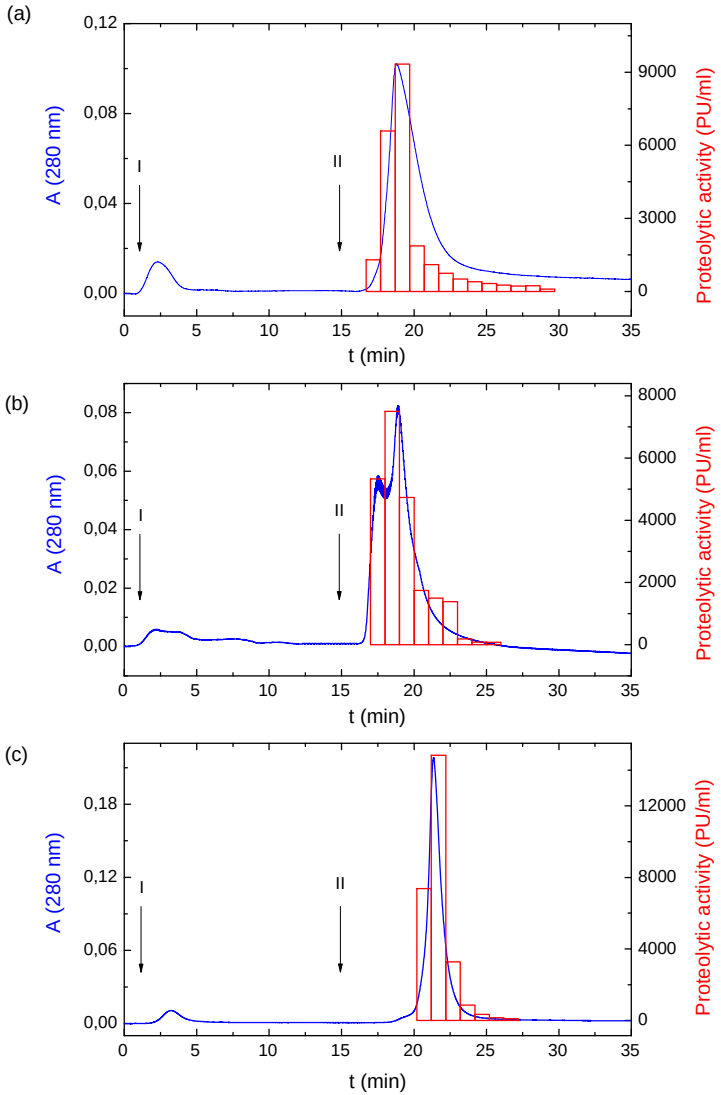


Fig. 1 Affinity chromatography of porcine pepsin A on affinity sorbents under the optimum conditions

**(a)** N-acetyl-L-phenylalanine-Sepharose: I – 100 mM acetate buffer, pH 3.7; II – 100 mM acetate buffer, pH 3.7, containing 0.5 M NaCl; **(b)** 3,5-diiodo-L-tyrosine-Sepharose: I – 50 mM acetate buffer, pH 6.0; II – 50 mM acetate buffer, pH 6.0, containing 1.0 M NaCl; **(c)** iodinated L-tyrosine-Sepharose: I – 25 mM acetate buffer, pH 4.0; II – 50 mM phosphate buffer, pH 6.0, containing 0.3 M NaCl; sample – 1 ml of porcine pepsin A solution (1mg/ml of starting mobile phase); flow rate – 1 ml/min; column graph – proteolytic activity, the enzyme activity determined by the modified Anson and Mirsky method; solid line – absorbance measured at 280 nm.

## 4.3. Non-specific Adsorption of Proteins to Affinity Sorbents

Affinity chromatography is a selective method that has been also frequently used for simplification of complex mixtures in proteomics. It is based on a reversible specific interaction of proteins present in a sample with an affinity ligand immobilized onto a matrix. Any other interactions of proteins from a sample, e.g. with matrix or spacer arm, are undesirable.

The non-specific interactions of the prepared affinity sorbents with various types of proteins were compared under the optimum conditions for the separation of porcine pepsin A. The behavior of the following proteins besides porcine pepsin A was examined: bovine serum albumin (BSA), chicken ovalbumin, trypsin and α-chymotrypsin from bovine pancreas. Under the conditions used, complete amount of BSA and ovalbumin were retained on 3,5-diiodo-L-tyrosine-Sepharose and iodinated L-tyrosine-Sepharose. In contrast, N-acetyl-L-phenylalanine-Sepharose adsorbed only a low amount of these proteins (up to 19%) and therefore it was the most ideal from this point of view.

As was already mentioned, the structure of prepared affinity sorbents resembles to N-containing aromatic thiophilic sorbents. Porath and Oscarsson [53] suggested that such sorbents are intermediates between thiophilic and hydrophobic sorbents. The similarity of our sorbents to these structures might explain the observed strong adsorption of bovine serum albumin and chicken ovalbumin to some of the prepared sorbents.

## 4.4. Study of Porcine Pepsin A Interactions with Affinity Sorbents

### 4.4.1. Participation of Pepsin Active Site in Its Interactions with Sorbents

The chromatographic behavior of porcine pepsin A in the presence and absence of pepstatin A on the prepared affinity sorbents was compared to get more information on interactions of the studied enzyme with all immobilized ligands. Pepstatin A is a peptide inhibitor of aspartic proteases, which binds tightly to the active site of the enzyme [13, 14]. In our experiments, the behavior of porcine pepsin A and its complex with pepstatin A on all affinity sorbents was the same: the enzyme and the inactive complex were completely adsorbed to the affinity sorbents under the conditions used. The results obtained with N-acetyl-L-phenylalanine-Sepharose are shown in (fig. 2). This observation might indicate that the enzyme active site is not involved in the adsorption of

the enzyme to the immobilized ligands. The behavior of pepsin and its complex with pepstatin A on immobilized ligands that are based on derivatives of aromatic amino acids differs from that of the same enzyme and its complex on Phenyl-Sepharose [54] (tab. 2). The complex of porcine pepsin A with pepstatin A exhibited reduced binding to Phenyl-Sepharose in comparison with the enzyme in the absence of the inhibitor [54]. Phenyl-Sepharose is very often used as a sorbent for hydrophobic chromatography.

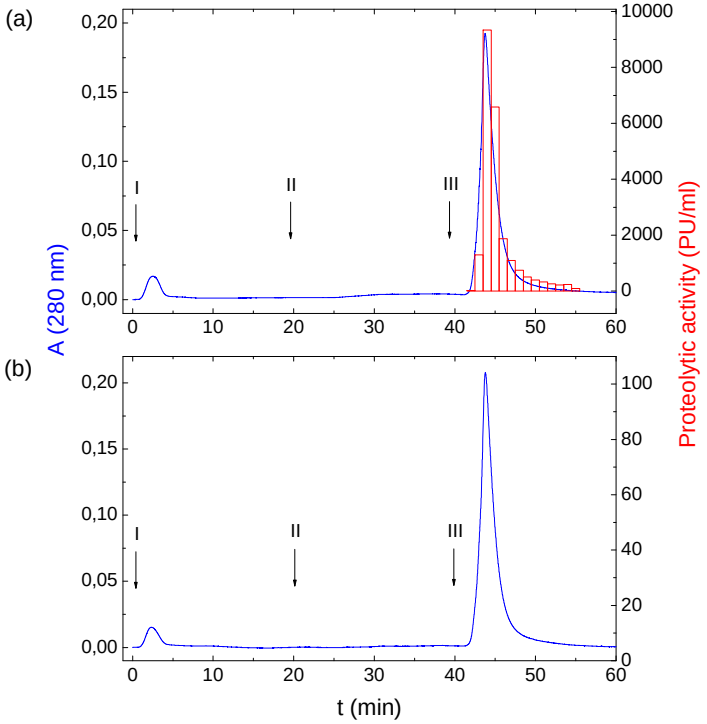


Fig. 2 Affinity chromatography of porcine pepsin A on N-acetyl-L-phenylalanine-Sepharose in the absence **(a)** and presence **(b)** of pepstatin A (inhibitor)
Chromatographic conditions: I – 100 mM acetate buffer, pH 3.7; II – 100 mM acetate buffer, pH 3.7, containing N-acetyl-L-phenylalanine ((1 mg + 2 µl N,N-dimethylformamide)/1 ml); III – 100 mM acetate buffer, pH 3.7, containing 0.5 M NaCl; flow rate – 1 ml/min; **(a)** sample – 1 ml of porcine pepsin A solution (1 mg/1 ml of 100 mM acetate buffer, pH 3.7; **(b)** sample – 1 ml of mixture solution ((1 mg of porcine pepsin A and 1 mg of pepstatin A)/ml of 100 mM acetate buffer, pH 3.7); column graph – proteolytic activity, the enzyme activity determined by the modified Anson and Mirsky method; solid line – absorbance measured at 280 nm.

Porcine pepsinogen A was completely adsorbed to N-acetyl-L-phenylalanine-Sepharose and 3,5-diiodo-L-tyrosine-Sepharose, while it was not adsorbed to Phenyl-Sepharose [54]. Porcine pepsinogen A consists of the prosegment (activation segment) and the pepsin moiety [52, 55]. The prosegment covers the pepsin active site and prevents entry of the substrate to the active site. Therefore, we could suppose that the active site of pepsin does not participate in the interaction with the prepared affinity sorbents, whereas it is involved in the interaction with Phenyl-Sepharose [54] (tab. 2). Contrary to immobilized ligands linked to Sepharose *via* carboxyl group, only a negligible amount of porcine pepsinogen A was adsorbed to iodinated L-tyrosine-Sepharose under the conditions used (pH 6.0 and pH 6.5).

The binding of porcine pepsin A to immobilized derivatives of aromatic acids was also not influenced by the presence of the corresponding free ligand in buffer. Porcine pepsin A was not released with N-acetyl-L-phenylalanine solution from N-acetyl-L-phenylalanine-Sepharose (fig. 2). Similarly, 3,5-diiodo-L-tyrosine solution had no effect on porcine pepsin A adsorbed to 3,5-diido-L-tyrosine-Sepharose and iodinated L-tyrosine-Sepharose (results not shown).

Tab. 2 Comparison of chromatographic behavior of porcine pepsin A and pepsinogen A on prepared affinity sorbents and Phenyl-Sepharose [54]

| Sorbent | Adsorbed protein (%) | | | Ref. |
|---|---|---|---|---|
| | Pepsin A | Pepsin A + pepstatin A | Pepsinogen A | |
| Phenyl-Sepharose | 95 | 61 | 0 | 54 |
| N-acetyl-L-phenylalanine-Sepharose | 100 | 100 | 100 | * |
| 3,5-diiodo-L-tyrosine-Sepharose | 100 | 100 | 100 | * |
| Iodinated L-tyrosine-Sepharose | 100 | 100 | 0 | * |

* This work

### 4.4.2. Effect of Porcine Pepsin A Phosphate Group in Interactions with N-acetyl-L-phenylalanine-Sepharose

Porcine pepsin A contains one phosphate group bound on serine residue in position 68 (ref. [56]). Porcine pepsin A was dephosphorylated by potato acid phosphatase (EC 3.1.3.2) immobilized on magnetic beads. The behavior of native porcine pepsin A ($2.8 \times 10^{-5}$ mol of phosphorus *per* 1 g of protein), its partially dephosphorylated derivate ($0.2 \times 10^{-5}$ mol of phosphorus *per* 1 g of protein) and mixture of both (1:1) on N-acetyl-L-phenylalanine-Sepharose is shown in figure 3. As evident from figure 3 the phosphate group of porcine pepsin A participated in interaction of enzyme with N-acetyl-L-phenylalanine-Sepharose which resulted from the retention time of both pepsin forms; dephosphorylated porcine pepsin A derivate had a shorter retention time than native form.

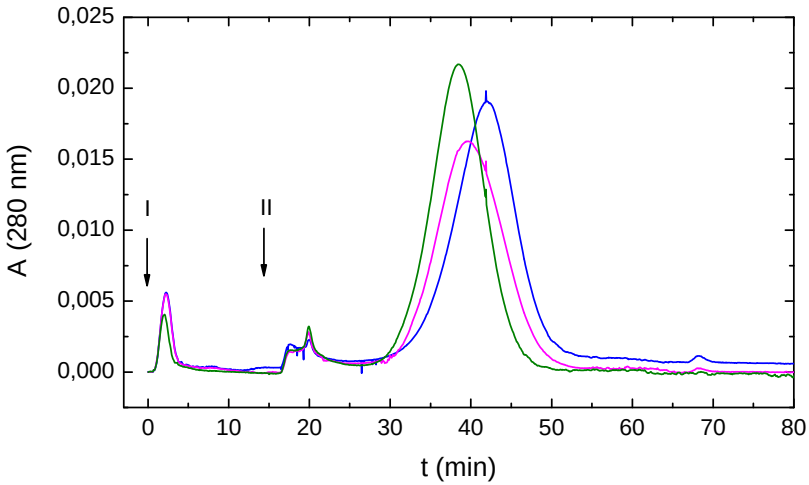


Fig. 3 Effect of dephosphorylation on porcine pepsinogen A behavior on N-acetyl-L-phenylalanine-Sepharose

Chromatographic conditions: I – 100 mM acetate buffer, pH 3.7; II – 100 mM acetate buffer, pH 3.7, containing 0.5 M NaCl; flow rate – 1 ml/min; sample – 1 ml of protein solution (1mg/ml 100 mM acetate buffer, pH 3.7): native porcine pepsin A (blue chromatogram); partially dephosphorylated derivative of porcine pepsin A (green chromatogram); mixture of both forms in ratio 1:1 (pink chromatogram).

## 4.5. Affinity Chromatography of Human Pepsins and Pepsinogens

Not much information is available on the use of affinity chromatography for the separation of pepsin A and pepsin C. Affinity chromatography using synthetic inhibitor (Val-D-Leu-Pro-Phe-Phe-Val-D-Leu) containing D-amino acid residues represents the only example. This inhibitor immobilized to Sepharose was used for the purification of human, porcine and chicken pepsin A, human pepsin C and bovine cathepsin D [35] and the separation of human pepsin A and pepsin C [32].

Preliminary studies in our laboratory showed differences in the interaction of human pepsinogens with aromatic amino acids and their iodinated derivatives [40]. In this study first, we used proteases from acidified extract of human gastric mucosa (pepsin A and pepsin C) and N-acetyl-L-phenylalanine-Sepharose, 3,5-diiodo-L-tyrosine-Sepharose, iodinated-L-tyrosine-Sepharose (results not shown). Our results showed that these enzymes differ in their interaction with the immobilized aromatic acid and could be separated using different elution conditions.

We mainly focused on separation of pepsins on N-acetyl-L-phenylalanine-Sepharose, the best sorbent for pepsin A. First, model proteases (porcine pepsin A and rat pepsin C) were separate (fig. 4). Then we optimized the separation of human pepsins from human gastric juice and acidic extract of human stomach mucosa on N-acetyl-L-phenylalanine-Sepharose. Individual pepsins were identified by mass spectrometry MALDI-TOF/TOF. Fig. 5 shows the separation of human pepsins from gastric juice with the designation of individual pepsins. The pepsins were eluted in the following order: pepsin C (between minutes 19 and 21), mixture of pepsin A and pepsin C (between minutes 22 and 24) and pepsin A (between minutes 25 and 36). The recovery was 96%.

Pepsinogen A and pepsinogen C from the extract of human gastric mucosa were separated on N-acetyl-L-phenylalanine-Sepharose (results not shown). Identification of individual pepsinogens was performed by non-denaturing polyacrylamide gel electrophoresis with staining for proteolytic activity. Pepsinogen A and pepsinogen C were identificated using monoclonal antibody against individual pepsinogens (data not shown). Partial separation of one pepsinogen A isozymogen was achieved. The separation of other isozymogens was not successful. After adjusting the pH of the collected fractions to 3.0

(pepsinogens converted to pepsins) the proteolytic activity was assayed. The recovery of pepsinogens was 91%.

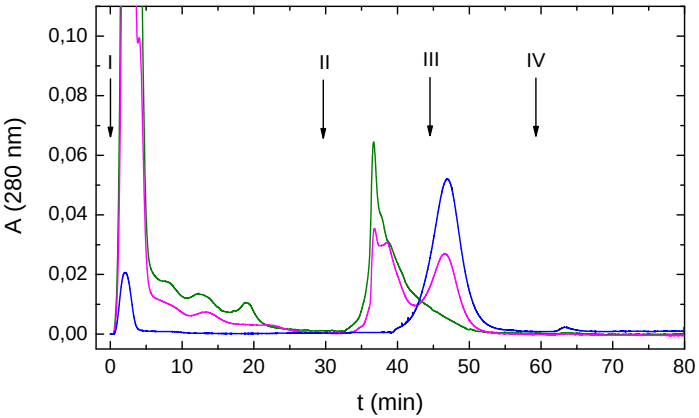


Fig. 4 Separation of porcine pepsin A and rat pepsin C on N-acetyl-L-phenylalanine-Sepharose
Chromatographic conditions: I – 100 mM acetate buffer, pH 3.7; II – a linear gradient of 0 – 0.5 M NaCl in 100 mM acetate buffer, pH 3.7 (15 min); III – 100 mM acetate buffer, pH 3.7, containing 0.5 M NaCl; IV – 100 mM acetate buffer, pH 3.7, containing 1 M NaCl; flow rate – 1 ml/min; sample – 1 ml of sample solution: acidified extract of rat gastric mucosa (1.3 mg of protein/ml; green chromatogram); mixture of porcine pepsin A and acidified extract of rat pepsin C in a ratio 1:1 (0.99 mg/ml; pink chromatogram); porcine pepsin A (0.89 mg/ml; blue chromatogram). The identification of individual pepsins was performed by the comparison of obtained chromatograms.

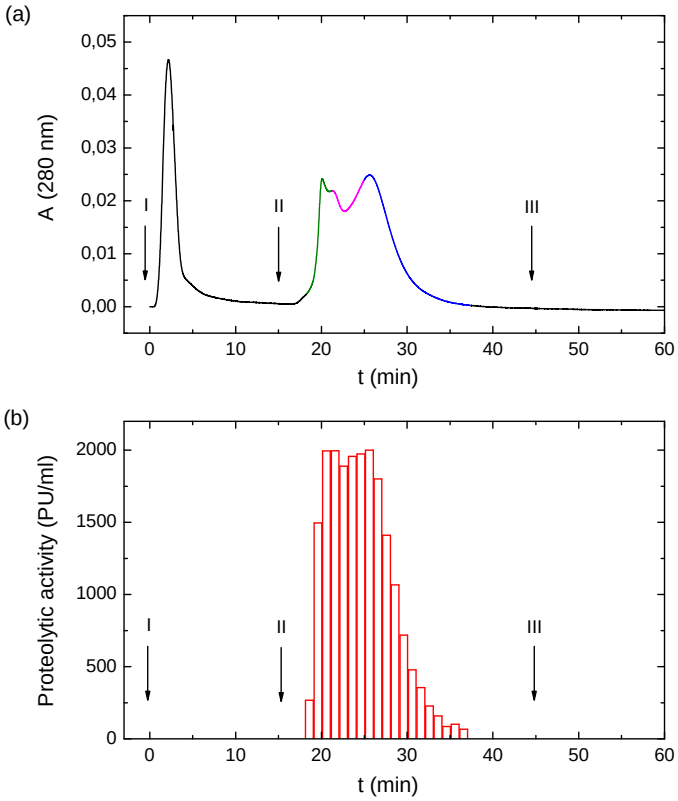


Fig. 5 Separation of pepsins from human gastric juice on N-acetyl-L-phenylalanine-Sepharose Chromatographic conditions: I – 100 mM acetate buffer, pH 3.7; II – a linear gradient 0.1 – 0.5 M NaCl in 100 mM acetate buffer, pH 3.7 (30 min); III – 100 mM acetate buffer, pH 3.7, containing 0.5 M NaCl; flow rate – 1 ml/min; sample – 1 ml of solution of lyophilized human gastric juice (0.679 mg protein/ml 100 mM acetate buffer, pH 3.7); **(a)** chromatogram with designation of human pepsins: blue – pepsin A; pink – mixture of both pepsin A and pepsin C; green – pepsin C; **(b)** proteolytic activity – the enzyme activity determined by the modified Anson and Mirsky method.

# 5. Conclusions

- The following sorbents for the separation of pepsins and pepsinogens were prepared: iodinated L-tyrosine-Sepharose, 3,5-diiodo-L-tyrosine–Sepharose and N-acetyl-L-phenylalanine-Sepharose. The ligands were immobilized to divinyl sulfone-activated Sepharose 4B either *via* free amino group or *via* free carboxyl group. In the case of immobilization of N-acetyl-L-phenylalanine or 3,5-diiodo-L-tyrosine *via* their free carboxyl groups, first ethylene diamine was linked to divinyl sulfone-activated Sepharose 4B and then the carbodiimide reaction was used for the coupling of ligand. L-tyrosine was directly immobilized to activated matrix *via* its free amino group and the resulting L-tyrosine-Sepharose was then iodinated using chloramine method.

- Interactions of porcine pepsin A with immobilized derivatives of aromatic amino acids were studied. The optimum conditions for the separation of porcine pepsin A using the prepared affinity sorbents were found and the following parameters were established: enzyme recovery, reproducibility of analyses, capacity and dependence of the elution peak area on the concentration of the applied enzyme.

- The ability of the prepared affinity sorbents to retain various types of proteins (bovine serum albumin, chicken ovalbumin, trypsin and α-chymotrypsin from bovine pancreas) was compared under the optimum conditions for porcine pepsin A separation. Out of the prepared affinity sorbents, the lowest non-specific interactions were observed in the case of N-acetyl-L-phenylalanine-Sepharose.

- The interaction of porcine pepsin A active site with immobilized ligands was investigated. It was shown that the pepsin active site did not participate in interaction with immobilized ligands. The behavior of porcine pepsin A was the same as its complex with pepstatin A on the prepared affinity sorbents.

- The participation of porcine pepsin A phosphate group in the interaction with N-acetyl-L-phenylalanine-Sepharose was investigated. It was shown

that pepsin phosphate group affected the interaction with N-acetyl-L-phenylalanine-Sepharose.

- All prepared affinity sorbents were used for the separation of pepsin A and pepsin C. Out of them, N-acetyl-L-phenylalanine-Sepharose was found to be the most suitable for this purpose. Using this sorbent, we were able to partially separate the model mixture of porcine pepsin A and rat pepsin C, as well as pepsins from human gastric juice and from acidified extract of human gastric mucosa.

- The ability of immobilized ligands to interact with porcine pepsinogen A was investigated. Interactions of this zymogen were proved only in the case of N-acetyl-L-phenylalanine-Sepharose and 3,5-diiodo-L-tyrosine-Sepharose.

- N-acetyl-L-phenylalanine-Sepharose was used for the isolation of human pepsinogen A and pepsinogen C from human gastric mucosa extract. In addition, one of the pepsinogen A isozymogen was partially separated.

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# 7. List of Publications

## Thesis-related publications

### *a) Publications with IF*

- **Frýdlová J.**, Tichá M., Kučerová Z.: Chromatographia 67 (2008) S41-S45. ($IF_{2007}$ 1,145)
- **Frýdlová J.**, Kučerová Z., Tichá M: J. Chromatogr. B 863 (2008) 135-140. ($IF_{2007}$ 2,935)
- **Frýdlová J**., Kučerová Z., Tichá M.: J. Chromatogr. B 800 (2004) 109-114. ($IF_{2004}$ 2,176)

### *b) Publications without IF*

### *Poster presentations*

- Kubosek L., **Frydlova J.**, Ticha M., Kucerova Z.: *Immobilization of Acid Phosphatase to Magnetic Particles and Their Use for Dephosphorylation of Proteins.* 34th FEBS Congress, Praha, Česká republika 4. – 9. 7. 2009.
- **Frýdlová J.**, Tichá M., Kučerová Z.: *N-acetyl-L-fenylalanin-Sepharosa-afinitní sorbent pro analýzu lidských pepsinů.* Analytické metódy a zdravie človeka, Nový Smokovec, Slovensko 20. – 23. 10. 2008.
- **Frýdlová J.**, Tichá M., Kučerová Z..: *Separation of Human Pepsins Using Affinity Chromatography on N-Acetyl-L-Phenylalanine – Sepharose.* 14th International Symposium On Separation Sciences, New Achievements in Chromatography, Primošten, Chorvatsko 30.9. – 3. 10. 2008.
- **Frýdlová J.**, Tichá M., Kučerová Z.: *Affinity Sorbent for Human Pepsins.* 17. konference českých a slovenských patofyziologů, Praha 11. – 12. 9. 2008.
- **Frýdlová J.**, Tichá M., Kučerová Z.: *Characterization of Pepsin Binding to Immobilized Aromatic Amino Acids.* 1st Central and Eastern European Proteomic Conference and 3rd Czech Proteomic Conference, Praha 29. – 31. 10. 2007.
- **Frýdlová J.**, Kučerová Z., Tichá M.: *Interaction of Pepsin with Aromatic Amino Acids and their Derivatives Immobilized to Sepharose.* 13th International Symposium on Separation Sciences, Štrbské pleso, Slovensko 27. – 29. 6. 2007.
- **Frýdlová J.**, Kučerová Z., Tichá M.: *Optimalization of Affinity Chromatography of Pepsins on Iodinated L-Tyrosine-Sepharose.* Advances in Chromatography and Electrophoresis 2007 & Chiranal 2007, Olomouc 24. – 27. 6. 2007.
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