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ABSTRACT OF Ph.D. THESIS

STUDY OF HUMAN PEPSIN PHOSPHORYLATION

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CONTENT

SOUHRN V ČEŠTINĚ

Fosforylace je jednou z nejdůležitějších posttranslační modifikací proteinů. Proces fosforylace/defosforylace umožňuje regulaci téměř všech procesů probíhajících v buňce a je klíčovým mechanismem pro udržování homeostasy buněk. Abnormální fosforylace souvisí s mnoha závažnými lidskými onemocněními jako je např. rakovina.

Fosforylace hlavních žaludečních aspartátových proteas pepsinů a jejich prekurzorů pepsinogenů se liší v závislosti na živočišném druhu. Pepsiny a pepsinogeny některých živočichů obsahují fosfátové skupiny, jiné však tyto skupiny nemají. Fosforylace lidských pepsinů a pepsinogenů pravděpodobně souvisí s různými žaludečními onemocněními. Pacienti s rakovinou žaludku vykazují vyšší stupeň fosforylace pepsinogenů než zdraví lidé. Aby bylo možné využít tento poznatek pro diagnostické účely, je nejprve nezbytné vypracovat jednoduché a spolehlivé metody pro studium fosforylace pepsinů a pepsinogenů, které by byly použitelné i pro malá množství vzorku.

Předkládaná dizertační práce se zabývá vypracováním vhodného metodického přístupu pro studium fosforylace proteinů se zaměřením na lidské žaludeční aspartátové proteasy – pepsiny, pro které je charakteristický velmi nízký obsah bazických aminokyselinových zbytků a vyšší obsah kyselých aminokyselinových zbytků. Pozornost jsme zaměřili zejména na vypracování chromatografických metod pro selektivní (afinitní) separaci fosforylovaných pepsinů a pro izolaci z nich odvozených fosfopeptidů. V první části předkládané práce jsme se zabývali výběrem sorbentů vhodných pro separaci fosfoproteinů vysokotlakou afinitní chromatografií na imobilizovaných iontech. V další části jsme na vybraných sorbentech vypracovali metody pro separaci fosforylovaných a nefosforylovaných forem pepsinů. Tyto metody jsme následně použili pro separaci lidských pepsinů izolovaných ze žaludeční šťávy zdravého člověka a ze žaludeční sliznice pacienta s karcinomem žaludku. V poslední části práce jsme se zabývali vypracováním postupu pro selektivní izolaci fosfopeptidů z proteolyticky rozštěpených pepsinů pomocí afinitní chromatografie a jejich následnou detekci, identifikaci a určení místa fosforylace pomocí tandemové hmotnostní spektrometrie. Vypracovaný postup jsme použili k určení místa fosforylace lidského pepsinu A.

SUMMARY IN ENGLISH

Protein phosphorylation is one of the most important post-translational modifications in nature. The process of protein phosphorylation/dephosphorylation regulates almost all cellular processes and plays a key role in the maintaining of cell homeostasis. Abnormal phosphorylation is associated with many serious human diseases including cancers.

The phosphorylation of main gastric aspartic proteases pepsins and their precursors varies among animal species. Pepsins and pepsinogens of several species contain phosphate groups, while others have no phosphate groups. Phosphorylation of human pepsins and pepsinogens is probably associated with different gastric diseases. Gastric cancer patients show higher phosphorylation degree of pepsinogens compared to healthy subjects. To utilize this finding for diagnostic purposes, it is requisite to elaborate simple and reliable methods for the study of pepsin and pepsinogen phosphorylation, which could be applied to limited sample amounts.

This thesis focuses on the elaboration of appropriate methodical approach for the study of protein phosphorylation, particularly phosphorylation of human pepsins, which are characterized by a very low content of basic amino acid residues and a higher content of acidic amino acid residues. The attention was paid to the elaboration of affinity chromatography methods for the selective separation of phosphorylated pepsins and for the isolation of phosphopeptides derived from these proteins. In the first part of this thesis, we selected sorbents suitable for the separation of phosphoproteins by high-pressure immobilized metal affinity chromatography. In the next part, we elaborated methods for the separation of phosphorylated and non-phosphorylated forms of pepsins using selected high-pressure sorbents. The elaborated methods were applied to human pepsins isolated from gastric juice of a healthy individual and from gastric mucosa of a gastric carcinoma patient. In the last part of this thesis, we elaborated an approach for the identification of pepsin phosphorylation site. First, we optimized affinity chromatography methods for the selective enrichment of phosphopeptides from pepsin digest. The enriched peptides were then detected using tandem mass spectrometry and the phosphorylation site in pepsin was identified using database searching. Then we applied the elaborated procedure to human gastric juice proteins and successfully identified the phosphorylation site in human pepsin A.

1. INTRODUCTION

Protein phosphorylation is one of the most important and the most abundant post-translational modifications in nature. The process of protein phosphorylation/dephosphorylation regulates almost all aspects of cell life. It is involved in many cellular processes such as signal transduction, gene expression, proliferation, differentiation, cell cycle control, and metabolism and plays a key role in the maintaining of cell homeostasis [1-5]. It has been estimated that approximately one-third of all proteins in eukaryotic cells is phosphorylated at any given time [6] and that in eukaryotic proteome, there are several hundred thousand potential phosphorylation sites [7].

Eukaryotic proteins are usually phosphorylated on serine (S), threonine (T) and tyrosine (Y) residues. Serine and threonine residues undergo phosphorylation more often than tyrosine residues. The phosphoamino acid content ratio pS:pT:pY* is approximately 1800:200:1. Two different classes of enzymes control the phosphorylation status of proteins: protein kinases and protein phosphatases. The ratio of their activities determines the phosphorylation status of proteins [8].

Abnormal phosphorylation is associated with many serious human diseases such as cancer, diabetes or rheumatoid arthritis. Mutations in particular protein kinases and phosphatases, resulting in aberrant activities of these enzymes, give rise to a number of these disorders. In addition, many naturally occurring toxins and pathogens exert their effects by altering the phosphorylation states of intracellular proteins [6].

Phosphoproteomes of normal and cancer cells as well as phosphoproteomes of different tumors are distinct. It is possible to find phosphoproteins whose phosphorylation status is characteristics for a specific tumor. These phosphoproteins can serve as tumor-specific biomarkers. Cancer biomarkers such as protein kinases and their substrates are invaluable for their potential discriminatory power in molecular classification of cancers (diagnosis), in predicting clinical outcome (prognosis), and response to drugs (therapy) [9].

Human gastric juice contains two main biochemically and immunochemically distinct aspartic proteases – pepsin A and pepsin C. These enzymes are characterized by a low pH optimum of their proteolytic activities [10] and very low isoelectric points [11, 12] resulting from a very low content of basic amino acids and a high content of acidic amino acids [13-16]. Pepsins are synthesized in the form of inactive precursors – pepsinogens [10].

* pS - phosphoserine, pT - phosphothreonine, pY - phosphotyrosine

The phosphorylation of pepsins and their precursors varies among animal species. Pepsins and pepsinogens of several species contain phosphate groups, while others have no phosphate groups. For instance, porcine pepsin A contains one phosphate group bound to serine residue in position 68 [17]. Similarly, monkey pepsinogen A is phosphorylated [18]. In contrast, no phosphate groups were found in rat pepsinogen C [19] as well as in monkey pepsinogen C [20, 21], porcine pepsinogen C [22], and chicken pepsinogen A [23].

Phosphorylation of human pepsins and pepsinogens seems to be associated with different gastric diseases. No phosphate groups were detected in pepsinogen C of healthy people, while one phosphate group bound to serine residue was found in some isoforms of pepsinogen A. However, remaining isoforms of pepsinogen A were not phosphorylated [24]. In contrast, pepsinogens of gastric cancer patients showed higher phosphorylation degree, but there is no general agreement on the total number of phosphate groups. Hynek et al. found one phosphate group in pepsinogen A and two in pepsinogen C [25]. Another study showed the presence of one or two phosphate groups in pepsinogen A and one in pepsinogen C [26]. In addition, gastric cancer patients show higher phosphorylation degree of pepsinogens compared to gastric ulcer patients [27, 28]. However, phosphorylation sites are not known.

In the last decades, a number of chromatographic techniques were applied to separate or selectively enrich phosphoproteins and phosphopeptides. In general, three different effects can be distinguished and applied as selection principle. The first is the alteration of the isoelectric point or the net charge of the protein. This enables the separation of phosphorylated and non-phosphorylated forms of individual proteins by ion-exchange chromatography or chromatofocusing. Second, the phosphate group of proteins and peptides shows affinity for some metal ions. This enables the isolation, selective enrichment or separation by immobilized metal affinity chromatography or metal oxide affinity chromatography. Finally, a change of the protein immunogenic properties after phosphorylation allows the generation of antibodies, which in turn can be used for immunoprecipitations [29]. In addition, methods based on the modification of phosphate groups were developed for phosphoprotein isolation [30-32].

Beside that, a number of techniques were used to detect phosphoproteins, such as isotopic labeling of phosphoproteins, western blotting employing phosphospecific antibodies, detection based on the comparison of electrophoretic behavior of proteins before and after phosphatase treatment, or mass spectrometry [33]. Mass spectrometry also allows the identification of protein phosphorylation sites [8].

Despite a relatively high number of techniques used for the study of protein phosphorylation, only a few of them can be applied to study pepsin/pepsinogen phosphorylation due to the highly acidic character of these proteins (a high content of acidic amino acid, a low content of basic amino acids, very low isoelectric points). The separation of phosphorylated and non-phosphorylated forms of pepsins and pepsinogens was performed using low-pressure immobilized metal affinity chromatography (IMAC) [28, 34, 35]. For the detection of pepsinogen phosphate groups, three approaches were used. First approach was based on peptide mapping of phosphorylated and dephosphorylated forms of pepsinogens using the combination of RP-HPLC (reverse phase high performance liquid chromatography) and capillary zone electrophoresis. The phosphate group was detected by the comparison of both peptide maps [25]. Second approach was based on the separation of pepsinogens using isoelectric focusing and the following detection of phosphorylated pepsinogens using polyclonal antibodies against phosphoserine, phosphothreonine and phosphotyrosine [26]. The last approach was based on acidic hydrolysis of individual pepsinogens and the following determination of released phosphoamino acids using an amino acid analyzer [24]. However, no attempt was made to identify a phosphorylation site of human pepsins and pepsinogens.

Although above-mentioned methods succeeded in the separation of phosphorylated and non-phosphorylation forms of pepsinogens and in the detection of phosphate groups, they required relatively high amounts of sample (except for isoelectric focusing). Certain disadvantage also represents labor intensive and time-consuming work. Therefore, the elaboration of simple and reliable methods for the study of pepsin and pepsinogen phosphorylation, applicable to limited sample amounts, remains a great challenge.

2. AIM OF THE THESIS

The goal of this thesis was the elaboration of appropriate methodical approach for the study of protein phosphorylation, particularly phosphorylation of human gastric aspartic proteases – pepsins. These proteins are characterized by a higher content of acidic amino acids and a very low content of basic amino acids, which makes their phosphorylation analysis difficult. Therefore, the main part of this study was focused on the elaboration and optimization of affinity methods for the selective separation and isolation of such phosphoproteins and phosphopeptides. The rest of the study concerned the detection of phosphopeptides and identification of protein phosphorylation sites using tandem mass spectrometry.

To achieve the above-mentioned objectives, it was requisite to:

- compare the properties of several HP-IMAC* sorbents which differ in their matrices, chelating ligands, and metal ions and evaluate their abilities to separate native phosphoproteins
- elaborate a method for the separation of phosphorylated and non-phosphorylated forms of a model protein, porcine pepsin A, using selected HP-IMAC sorbents
- apply the elaborated method to human pepsins for the separation of their phosphorylated and non-phosphorylated forms
- elaborate a procedure for the selective enrichment of phosphopeptides from proteolytic digest of porcine pepsin A using IMAC sorbents and titanium dioxide and for phosphopeptide detection and identification of phosphorylation site using MALDI-TOF/TOF** mass spectrometry
- use the elaborated procedure to enrich phosphopeptides from proteolytic digest of human gastric juice proteins and identify protein phosphorylation sites, particularly in human pepsins

* HP-IMAC – high pressure immobilized metal affinity chromatography
** MALDI-TOF/TOF – matrix assisted laser desorption ionization - time of flight / time of flight

3. EXPERIMENTAL

Materials
- lyophilized human gastric juice
- extract of human gastric mucosa obtained from resected stomach of gastric carcinoma patient
- porcine pepsin A, chicken ovalbumin, and acid phosphatase from potato (Sigma-Aldrich, USA)

Immobilized metal affinity chromatography (IMAC) of proteins
- performed using Dionex Summit HPLC system (Dionex, USA)
- HP-IMAC columns: POROS MC20 (Applied Biosystems, USA) and MA-BP (Institute of Macromolecular Chemistry, AS, Czech Republic) loaded with Fe^{3+}, Ga^{3+} or UO_2^{2+} ions
- general protocol for protein analysis: (1) equilibration with the starting buffer; (2) protein loading (200 μL); (3) washing out non-adsorbed substances with the starting buffer; (4) elution of protein with the eluting buffer; flow rate: 0.5 mL/min; collected 0.5 mL fractions
- different buffers and solutions were tested to maximize protein recovery
- in collected fraction, protein content, eventually phosphorus content or proteolytic activity of pepsin were determined

Modification of pepsin carboxyl groups
- esterification with methanolic HCl was performed according to ref. [36]
- esterification with diazomethane was performed according to ref. [37, 38]
- amidation with dimethylamine, ethylamine and isopropylamine was performed according to ref. [39]

Dephosphorylation of porcine pepsin A
- performed according to modified method of Martin et al. [40]

Analytical methods
- protein concentration was determined using bicinchoninic acid protein assay [41]
- proteolytic activity was determined by Anson and Mirsky method [42]
- phosphorus content in pepsin was determined by phosphoprotein phosphate estimation assay kit (Pierce, USA)

Enrichment of phosphopeptides from pepsin digest
- on POROS MC20-Fe^{3+} column using following general protocol: (1) equilibration with the starting buffer; (2) loading of peptide sample (200 μL); (3) washing out non-adsorbed peptides with the starting buffer (10 mL); (4) elution of protein with the eluting buffer (7.5 – 10 mL); flow rate: 0.5 mL/min
- on TopTip IMAC (Fe^{3+}), TopTip IMAC (Ga^{3+}) tips (Glygen, USA) and Toptip TiO_2 tips (Eppendorf, Germany) using following general protocol: (1) equilibration with the starting buffer (100 μL); (2) loading of peptide sample (100 μL); (3) washing out non-adsorbed substances with the starting buffer (100 μL); (4) elution of protein with the eluting buffer (15 μL)

MALDI-TOF/TOF mass spectrometry
- performed using Autoflex II TOF/TOF mass spectrometer (Bruker Daltonics, Germany)
- identification of peptides was performed using Mascot searching algorithm (Matrix Sciences, UK) and the Swiss-Prot database (SIB, Switzerland); searching parametres: mass tolerance MS – 150 ppm, MS/MS tolerance – 0.7 Da, enzyme – chymotrypsin, missed cleavage – 5.

4. RESULTS AND DISCUSSION

This thesis is divided into three parts. The first part concerns with the choice of sorbents suitable for the separation of phosphoproteins by high-pressure immobilized metal affinity chromatography (HP-IMAC). In the second part, we focus on the elaboration of methods for the separation of phosphorylated and non-phosphorylated forms of pepsin and the application of these methods to the separation of human pepsins. In the last part of this thesis, we deal with the identification of pepsin phosphorylation sites using affinity chromatography for phosphopeptide enrichment and tandem mass spectrometry for detection (MALDI-TOF/TOF MS).

4.1 HP-IMAC of phosphoproteins

The aim of this part of the thesis was to evaluate and compare several IMAC sorbents in terms of their abilities to adsorb intact phosphoproteins under HPLC conditions. As a model phosphoproteins, porcine pepsin A and chicken ovalbumin were used. Porcine pepsin A contains one phosphate group bound to serine residue [17] and its isoelectric point is lower than one [11, 12]. Chicken ovalbumin contains two phosphate groups bound to serine residues [43] and its isoelectric point is 4.6 [44].

4.1.1 Sorbents suitable for HP-IMAC of phosphoproteins

The designation and composition of HP-IMAC sorbents used for the separation of intact model phosphoproteins is summarized in Tab. 1. The used sorbents were prepared by the immobilization of three different metal ions (ferric, gallic, and uranyl) to two different chelating ligands* (iminodiacetic acid (IDA) and (6-amino-1-hydroxyhexane-1,1-diyl) bisphosphonic

Tab. 1 IMAC sorbents

	Matrix	Ligand	Metal ion	Designation
BP-MA	poly(glycidyl methacrylate-*co*-ethylene dimethacrylate)	(6-amino-1-hydroxyhexane-1,1-diyl) bisphosphonic acid (BP)	Fe^{3+} Ga^{3+} UO_2^{2+}	BP-Fe^{3+} BP-Ga^{3+} BP-UO_2^{2+}
POROS MC20	poly(styrene-*co*-divinylbenzene)	iminodiacetic acid (IDA)	Fe^{3+} Ga^{3+} UO_2^{2+}	IDA-Fe^{3+} IDA-Ga^{3+} IDA-UO_2^{2+}

* Originally, three ligands (IDA, BP, and 8-hydroxyquinoline) were tested. However, pepsin was adsorbed directly on 8-hydroxyquinoline, therefore this ligand was not used for the immobilization of metal ions.

acid (BP)) covalently bound to rigid supports. Except for UO_2^{2+} and BP ligand, the metal ions formed with both ligands stable metal chelates; no leakage of metal ions from IMAC sorbents was observed. UO_2^{2+} did not form with BP ligand a stable metal chelate, therefore BP-UO_2^{2+} sorbent was not used anymore.

4.1.2 Adsorption and desorption of model phosphoproteins on HP-IMAC sorbents

The prepared HP-IMAC sorbents were compared in terms of their ability to adsorb model phosphoproteins – porcine pepsin A and chicken ovalbumin. We investigated the effect of pH (3.2 – 6.0) and ionic strength (0 and 1 M NaCl) on the adsorption of both phosphoproteins. We also searched for conditions for an effective desorption of both phosphoproteins from the used sorbents.

The chromatographic conditions were found for complete adsorption and desorption of both phosphoproteins from all prepared sorbents except for IDA-Ga^{3+}. On IDA-Ga^{3+} sorbent, the highest adsorption of pepsin was observed under acidic pH and low ionic strength; but conditions for complete adsorption of pepsin were not found. Tab. 2 summarizes the chromatographic conditions under which the highest adsorption and complete desorption of both phosphoproteins was achieved.

Tab. 2 HP-IMAC of porcine pepsin A and chicken ovalbumin: adsorption and desorption conditions

IMAC sorbent	Adsorption	Desorption
BP-Fe^{3+}	50 mM acetate buffer, pH 4.0, containing 0 – 1 M NaCl; 0.1% acetic acid, containing 0 – 1 M NaCl	50 mM EDTA-Na_2 containing 1 M NaCl
IDA-Fe^{3+}	50 mM acetate buffer, pH 4.0, containing 0 – 1 M NaCl; 0.1% acetic acid, containing 0 – 1 M NaCl	200 mM phosphate buffer, pH 7.2
BP-Ga^{3+}	50 mM acetate buffer, pH 4.0, containing 0 – 1 M NaCl; 0.1% acetic acid, containing 0 – 1 M NaCl	200 mM phosphate buffer, pH 7.2
IDA-Ga^{3+}	50 mM acetate buffer, pH 4.0; 0.1% acetic acid	200 mM phosphate buffer, pH 7.2
BP-UO_2^{2+}	NP	NP
IDA-UO_2^{2+}	50 mM acetate buffer, pH 4.0; 0.1% acetic acid	200 mM carbonate buffer, pH 10.7

NP: not possible to use sorbent due to the leakage of UO_2^{2+} ions

Pepsin and ovalbumin were more strongly adsorbed on sorbents containing ferric and gallic ions immobilized on BP than on sorbents containing ferric and gallic ions immobilized on IDA. Both phosphoproteins were desorbed from sorbents containing IDA under milder conditions. For instance, a small addition of sodium phosphate to the starting buffer was satisfactory for the desorption of both phosphoproteins from IDA-Fe^{3+} sorbent, while EDTA was required for the desorption of both phosphoproteins from BP-Fe^{3+} sorbent. Similarly, both phosphoproteins were desorbed from IDA-Ga^{3+} sorbent by a small addition of sodium phosphate to the starting buffer, while phosphate buffer with the simultaneous increase in pH was required for the desorption of both phosphoproteins from BP-Ga^{3+} sorbent.

The basic characteristics of the prepared HP-IMAC sorbents were determined under the chromatographic conditions given in Tab. 2 and are compared in Tab. 3. Under the conditions used, the sorbents were characterized by a high reproducibility as well as a high protein recovery. On BP-Ga^{3+} and IDA-Fe^{3+} sorbents, the dependence of the elution peak area on the concentration of loaded pepsin was also investigated. This dependence is linear in the concentration range 20-1000 μg/mL (R^2 = 0.9981) for BP-Ga^{3+} sorbent and 30-2000 μg/mL (R^2 = 0.9990) for IDA-Fe^{3+} sorbent.

Tab. 3 Chromatographic characteristics of IMAC sorbents containing immobilized Fe^{3+}, Ga^{3+} and UO_2^{2+} ions

IMAC sorbent	Capacity (mg/mL)	Recovery (%)		Reproducibility (RSD) (%)	
	pepsin	pepsin	ovalbumin	pepsin	ovalbumin
BP-Fe^{3+}	2.4	88	×	1.3	×
IDA-Fe^{3+}	2.4	95	100	0.8	1.3
BP-Ga^{3+}	2.2	100	100	1.2	2.0
IDA-Ga^{3+}	×*	100*	95	0.7	0.8
BP-UO_2^{2+}	NP	NP	NP	NP	NP
IDA-UO_2^{2+}	0.6	100	98	1.0	1.3

× was not determined; * pepsin was only partially adsorbed; NP not possible to determine due to the leakage of UO_2^{2+} ions

Capacity – expressed in mg of porcine pepsin A adsorbed to 1 mL of sorbent; recovery – amount of protein eluted from sorbent expressed in % of loaded protein; reproducibility – expressed as relative standard deviation (RSD) of elution peak area for ten repeated individual experiments. Chromatographic conditions: loaded 200 μL of protein solution (1 mg/mL of starting solution) per run; starting solution - 0.1% acetic acid or 50 mM acetate buffer, pH 4.0; elution buffer - 200 mM phosphate buffer, pH 7.2 and 200 mM carbonate buffer, pH 10.7 in the case of IDA-UO_2^{2+} sorbent.

The performed experiments showed that all tested sorbents except for BP-UO_2^{2+} are utilizable for HP-IMAC of phosphoproteins. Interactions of pepsin with a particular HP-IMAC sorbent did not significantly differ from interaction of ovalbumin with the same sorbent. However, the comparison of phosphoprotein behavior on the individual HP-IMAC sorbents revealed significant differences in the strength of interactions between individual sorbents and phosphoproteins. Pepsin and ovalbumin were very strongly adsorbed on BP-Fe^{3+} sorbent and for their desorption EDTA was required. In contrast, interactions of both phosphoproteins with IDA-Ga^{3+} sorbent were very weak; only a part of loaded pepsin was adsorbed. Both phosphoproteins were also only weakly adsorbed on IDA-UO_2^{2+} sorbent and in addition, this sorbent showed the lowest capacity for pepsin. Therefore, IDA-Fe^{3+} a BP-Ga^{3+} sorbents were evaluated as the most suitable sorbents for HP-IMAC of phosphoproteins.

4.2 Separation of phosphorylated and non-phosphorylated forms of pepsins

The next aim of this thesis was to elaborate a simple IMAC method for the separation of phosphorylated and non-phosphorylated forms of human pepsins. For this purpose, porcine pepsin A containing one phosphate group *per* molecule [17] was chosen as a model phosphoprotein due to its similarity to human pepsin A and the following high pressure IMAC sorbents were employed: *IDA-Fe^{3+} a **BP-Ga^{3+}. The elaborated method was then applied to the separation of human pepsins.

4.2.1 Separation of phosphorylated and non-phosphorylated forms of porcine pepsin A

Native porcine pepsin A was treated with potato acid phosphatase to obtain non-phosphorylated form of pepsin. Under the conditions used, pepsin was dephosphorylated only partially. The obtained dephosphorylated derivative of pepsin contained 2×10^{-5} moles of phosphorus *per* gram of a protein that means 0.7 moles of phosphate groups *per* a mole of pepsin. Just the partial dephosphorylation of pepsin could be caused by the digestion of phosphatase with pepsin or by the inhibition of phosphatase with released phosphate ions [45, 46].

Native pepsin and its partially dephosphorylated form were subjected to HP-IMAC using IDA-Fe^{3+} and BP-Ga^{3+} sorbents under the conditions given in Tab. 2. The chromatograms

* sorbent based on poly(styrene-*co*-divinylbenzene) containing iminodiacetic acid (IDA) and immobilized ferric ions

** sorbent based on poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) containing (6-amino-1-hydroxyhexan-1,1-diyl) bisphosphonic acid (BP) and immobilized gallic ions

obtained using IDA-Fe^{3+} sorbent are compared in Fig. 1. Similar results were obtained also using BP-Ga^{3+} sorbent (not shown). No differences were observed in the ability of both pepsin forms to adsorb on both sorbents. These results indicate that not only phosphate groups but also others contribute to the interaction of pepsin with immobilized Fe^{3+} and Ga^{3+} ions.

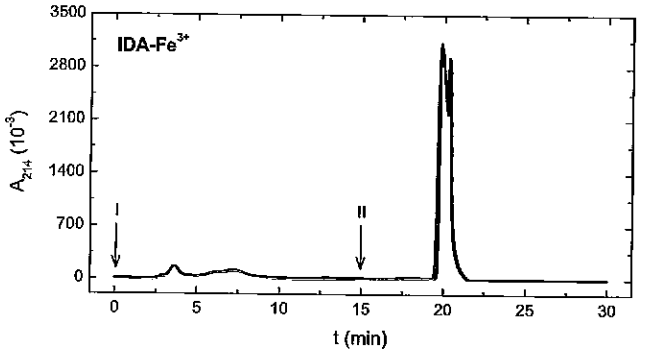

Fig. 1 HP-IMAC of native porcine pepsin A (red line) and its partly dephosphorylated form (black line) on IDA-Fe^{3+} sorbent

Chromatographic conditions: applied 200 µL of protein solution (1 mg/mL of 0.1% acetic acid); I – 0.1% acetic acid; II - 200 mM phosphate buffer, pufr pH 7.2; flow rate 0.5 mL/min.

Muszynska et al. [47] showed that proteins possessing clusters of carboxylic groups are bound to IMAC sorbents containing Fe^{3+} ions. Also non-phosphorylated peptides rich in aspartic and glutamic acid residues are adsorbed on these sorbents [36, 48-53] as well as on sorbents containing Ga^{3+} ions [49, 51, 54, 55]. If we take into consideration that 13% of all amino acid residues in pepsin contains a free carboxyl group and that these acidic amino acids are often localized next to each other [13], we can suppose that free carboxyl groups of pepsin will participate in interactions of pepsin with IMAC sorbents.

To suppress the participation of carboxyl groups in the interaction of pepsin with immobilized trivalent ions, the enzyme was modified by esterification with methanolic HCl or diazomethane or by amidation with dimethylamine, ethylamine or isopropylamine. The highest modification degree (98%) was achieved by esterification with diazomethane. The esterification of partially dephosphorylated pepsin with diazomethane resulted in almost the same modification degree (97%).

Native pepsin and its partially dephosphorylated form, both modified with diazomethane, were subjected to HP-IMAC using IDA-Fe^{3+} and BP-Ga^{3+} sorbents under the same conditions as their unmodified analogs mentioned above. The results of the chromatographic separations

on IDA-Fe^{3+} sorbent are shown in Fig. 2. Similar results were obtained also using BP-Ga^{3+} sorbent (not shown). Modification of native pepsin did not influence its ability to interact with immobilized Fe^{3+} and Ga^{3+} ions; pepsin was completely adsorbed (red chromatogram in Fig. 2). In the case of partially dephosphorylated pepsin, modification of its carboxyl groups resulted in the decrease in the amount of pepsin adsorbed on both immobilized metal ions (black chromatogram in Fig. 2). Non-adsorbed part of modified partially dephosphorylated pepsin represented 30-32% of applied protein. The amount of non-adsorbed protein corresponds to the decrease in phosphate content after the dephosphorylation of pepsin. These results suggest that the phosphate group of pepsin is after modification of carboxyl groups responsible for the interaction of pepsin with IMAC sorbents. Therefore, phosphorylated and non-phosphorylated forms of pepsins can be separated by affinity chromatography on immobilized ferric and gallic ions in case that free carboxyl groups are modified in advance.

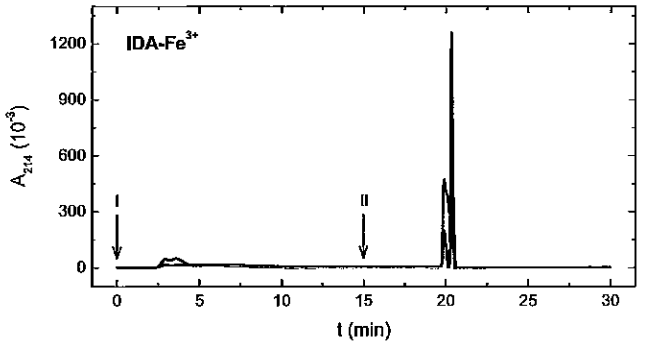

Fig. 2 HP-IMAC of esterified porcine pepsin A (red line) and its esterified partially dephosphorylated form (black line) on IDA-Fe^{3+} sorbent

Chromatographic conditions: applied 200 μL of protein solution (30 μg/mL of 0.1% acetic acid); I – 0.1% acetic acid; II - 200 mM phosphate buffer, pH 7.2; flow rate 0.5 mL/min.

4.2.2 Separation of phosphorylated and non-phosphorylated forms of human pepsins

The elaborated method for the separation of phosphorylated and non-phosphorylated forms of pepsin on HP-IMAC sorbents was applied to human pepsins. Human pepsins were isolated from gastric juice of a healthy individual and gastric mucosa of a gastric carcinoma patient. A part of isolated pepsins was subjected to HP-IMAC on IDA-Fe^{3+} sorbent, while the rest was at first esterified with diazomethane and then subjected to HP-IMAC.

The results obtained for modified and unmodified human pepsins of a healthy man are compared in Fig. 3. Both applied samples (modified and unmodified pepsins) contained

8.6×10^{-6} moles of phosphorus *per* gram of a protein which means 0.3 moles of phosphate groups *per* a mole of pepsin. If we take into consideration that pepsins in healthy individuals can possess one phosphate group *per* molecule as a maximum, we can assume that 30% of applied pepsin molecules was phosphorylated. Unmodified pepsins completely adsorbed on IDA-Fe^{3+} sorbent regardless their phosphate group content (pink chromatogram in Fig. 3). However, modification of pepsin carboxyl groups resulted in the decrease in pepsin adsorption; only 30% of applied proteins adsorbed (blue chromatogram in Fig. 3). Moreover, non-adsorbed pepsins did not contain any phosphate. Therefore, we can assume that only modified human pepsins containing phosphate groups were adsorbed on IDA-Fe^{3+} sorbent. Similarly, as in the case of porcine pepsin A, phosphorylated and non-phosphorylated human pepsins were successfully separated on IMAC sorbent after the modification of pepsin carboxyl groups.

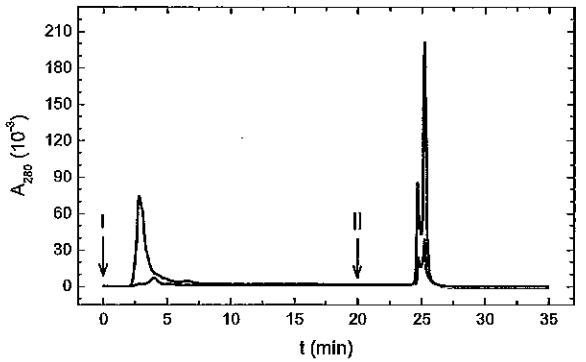

Fig. 3 Separation of human pepsins isolated from gastric juice (pink line) and their esterified derivatives (blue line) on IDA-Fe^{3+} sorbent

Chromatographic conditions: applied 200 μL of protein solution (0.3 mg/mL of 10 mM HEPES acidified to pH 4.0); I – 0.1% acetic acid; II - 200 mM phosphate buffer, pH 7.2; flow rate 0.5 mL/min.

Analogous results were also obtained when modified and unmodified human pepsins of a gastric carcinoma patient were analyzed (Fig. 4). In this case, the higher amount of modified pepsins adsorbed to IDA-Fe^{3+} sorbent. This observation fully corresponds with the higher phosphate group content determined in the applied sample (1.1 moles of phosphate groups *per* a mole of pepsin).

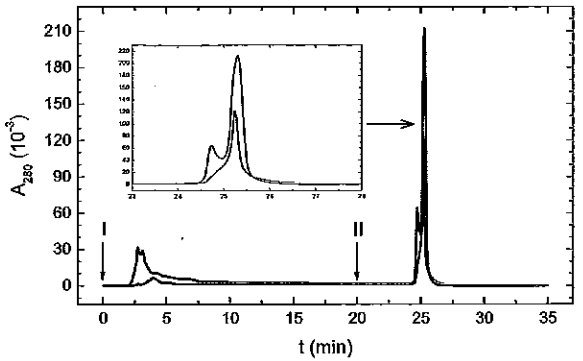

Fig. 4 Separation of human pepsins isolated from gastric mucosa (green line) and their esterified derivatives (red line) on IDA-Fe^{3+} sorbent

Inserted graph shows enlarged part of chromatogram. Chromatographic conditions: applied 200 μL of protein solution (0.3 mg/mL of 10 mM HEPES acidified to pH 4.0); I – 0.1% acetic acid; II - 200 mM phosphate buffer, pH 7.2; flow rate 0.5 mL/min.

Pepsins of the gastric carcinoma patient showed a higher phosphorylation degree in comparison with pepsins of the healthy individual. This finding is in agreement with the previous studies [25, 34, 35] which found out that gastric cancer patients exhibit higher phosphorylation of pepsinogen A and also pepsinogen C, whose phosphorylation was not observed in healthy individuals [24].

4.3 Enrichment of phosphopeptides and identification of phosphorylation sites in pepsins

For the characterization of protein phosphorylation at any given time, it is required not only to determine the stoichiometry of phosphorylation but also to identify protein phosphorylation sites [8, 56, 57]. As the stoichiometry of pepsin phosphorylation was investigated in the previous section, here we focused on the identification of pepsin phosphorylation sites. As mentioned above, no phosphate groups were detected in human pepsin C of healthy individuals, whereas one phosphate group bound to serine residue was found in some isoforms of human pepsin A [24]. However, the position of this phosphoamino acid residue remains unknown.

The aim of this part of the thesis was to elaborate an approach for the identification of human pepsin phosphorylation site(s). For this purpose, porcine pepsin A containing one phosphate group bound to serine residue (68. amino acid residue) [17] was chosen as a model phosphoprotein.

4.3.1 Identification of porcine pepsin A phosphorylation site

Porcine pepsin A was firstly digested with α-chymotrypsin. The enrichment of a phosphopeptide from pepsin digest was performed using immobilized metal affinity chromatography and metal oxide affinity chromatography. The following sorbents were employed:

- sorbent containing immobilized complex IDA-Fe^{3+} packed in a tip; commercial name: TopTip IMAC (Fe^{3+})
- analogical sorbent as in previous case but containing immobilized Ga^{3+} ions; commercial name: TopTip IMAC (Ga^{3+})
- HPLC sorbent containing immobilized complex IDA-Fe^{3+}; commercial name: POROS MC20-Fe^{3+}
- TiO_2 packed in a tip; commercial name: Toptip TiO_2

Phosphopeptide and other peptides desorbed from these sorbents were analyzed by MALDI-TOF mass spectrometry. The most intensive peptide ions from the obtained MS spectra were subjected to MALDI-TOF/TOF MS analysis. The obtained MS/MS data were searched against the Swiss-Prot database using Mascot searching algorithm to identify individual peptides.

The peptide ion *m/z* 1321 was identified as the searched phosphopeptide (Fig. 5). The sequence of this ion was identified as EATpSQELSITY containing one phosphorylated serine residue. The determined sequence corresponds to residues 124 – 134 of porcine pepsinogen A with phosphorylated serine residue in position 127. Mascot assigned a score of 79 for the matched sequence (EATpSQELSITY) that was above the statistical threshold of 37 for identity/extensive homology. The identified phosphorylation site of porcine pepsin/pepsinogen A was in agreement with previously published findings that phosphate group is bound to 68. amino acid residue of porcine pepsin A [17].

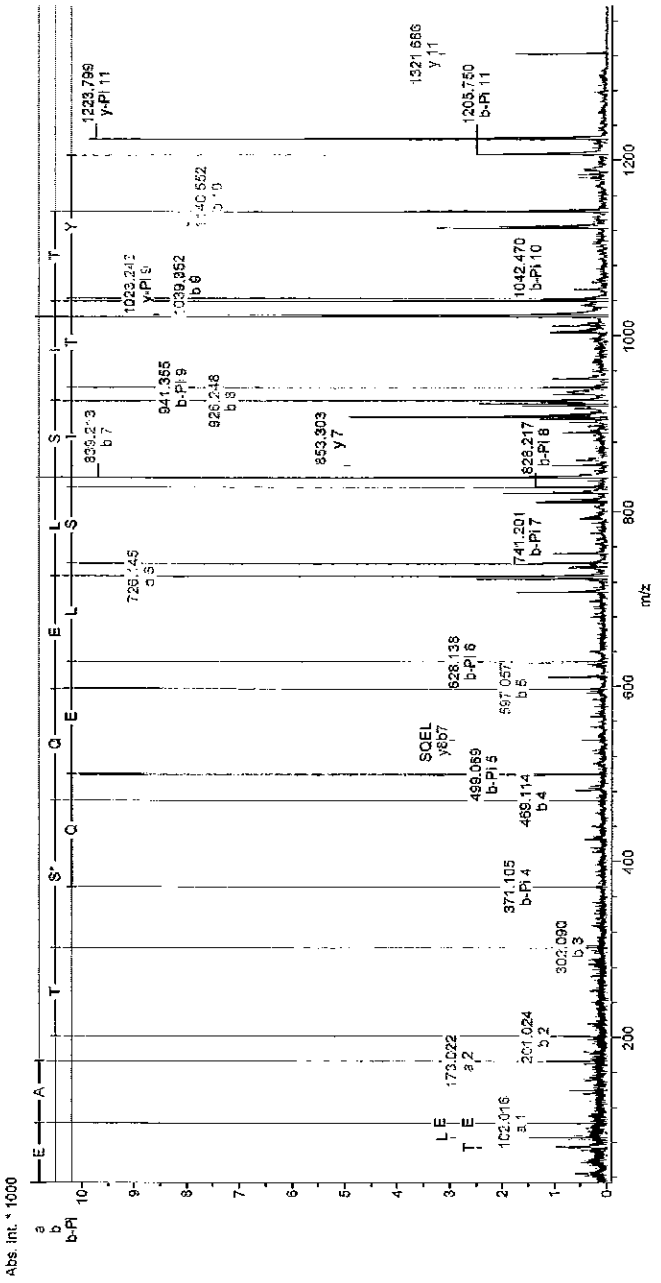

Fig. 5 MALDI-TOF/TOF MS spectrum of the parent ion *m/z* 1321.6
The amino acid sequence coverage is shown by 'a' and 'b' and (b-Pi) fragment ion series. A prominent loss of 97.9 Da (*m/z* 1223.7) indicates monophosphorylation. The mass difference 167.0 Da between fragment ions b4 (*m/z* 469.1) and b3 (*m/z* 302.0) corresponds to a phosphoserine residue, indicating the phosphorylation site at first serine.

Tab. 4 summarizes the adsorption and desorption conditions under which the highest enrichment of phosphopeptide (*m/z* 1321) from porcine pepsin A digest was achieved. The high enrichment of phosphopeptide (*m/z* 1321) and the low adsorption of non-phosphorylated peptides from porcine pepsin A digest was achieved on sorbents with immobilized Fe^{3+} and Ga^{3+} ions packed in tips (Fig. 6 A, B). The good separation of phosphopeptide (*m/z* 1321) was also observed on HPLC sorbent with immobilized Fe^{3+} ions (Fig. 6 C); however, the dilution of sample during the chromatography separation represented a certain disadvantage (from 200 µL of applied sample were gained about 2 mL of eluate). The worst results of phosphopeptide enrichment were obtained using TiO_2 (Fig. 6 D). A high number of non-phosphorylated peptides adsorbed on TiO_2 and co-eluted with phosphopeptide (*m/z* 1321) despite using of two-step elution.

Tab. 4 Optimized conditions for phosphopeptide enrichment from porcine pepsin A digest on sorbents with immobilized Fe^{3+} and Ga^{3+} ions and on TiO_2

Sorbent	Adsorption conditions	Elution conditions
HPLC sorbent with Fe^{3+} ions	0.1% acetic acid	5 mM **ammonium hydroxide**, pH 10.5 followed by 200 mM ammonium hydrogenphosphate, pH 7.9
Sorbent with Fe^{3+} ions in a tip	0.1% acetic acid	200 mM **ammonium hydrogenphosphate**, pH 7.9
Sorbent with Ga^{3+} ions in a tip	0.1% acetic acid	200 mM **ammonium hydrogenphosphate**, pH 7.9
TiO_2 in a tip	5% TFA containing 80% ACN	5 mM ammonium hydroxide, pH 10.5 followed by 200 mM **ammonium hydrogenphosphate**, pH 7.9

Solutions, that desorbed phosphopeptide, are marked in bold.

The obtained results showed that the detection of phosphopeptides by MALDI-TOF/TOF MS and the subsequent identification of a phosphorylation site is possible only after the enrichment of these peptides. In spectrum obtained by MALDI-TOF MS analysis of porcine pepsin A digest (Fig. 6 E), no phosphopeptide was found. This observation could by explained by the suppression of phosphopeptide signals by in the presence of other non-phosphorylated peptides [8, 56].

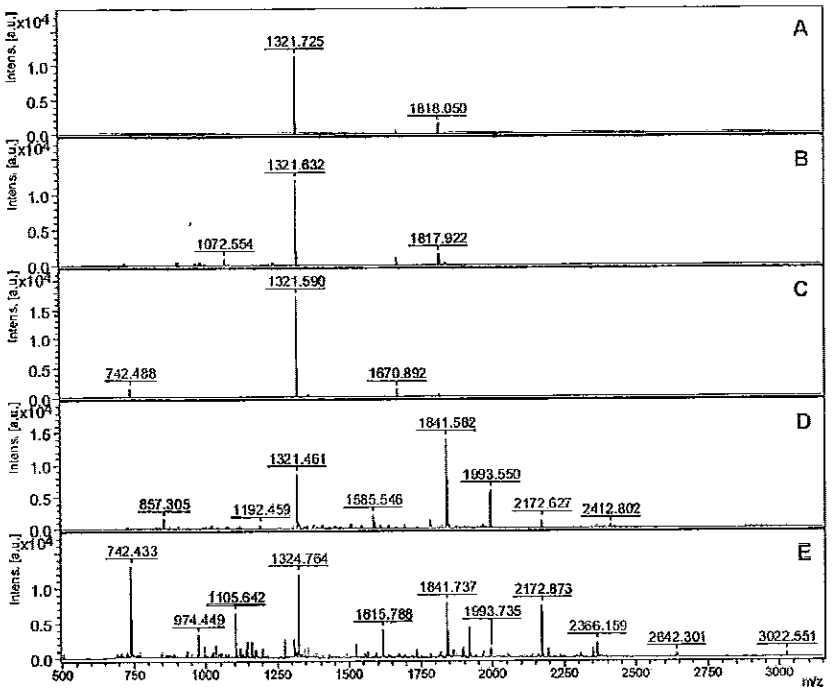

Fig. 6 MALDI-TOF MS analysis of peptides enriched from porcine pepsin A digest (E) on sorbent with Fe^{3+} ions packed in a tip (A), on sorbent with Ga^{3+} ions packed in a tip (B), on HPLC sorbent with Fe^{3+} ions (C) and on TiO_2 packed in a tip (D)

For chromatographic conditions see Tab. 4 (only MS spectra of peptides desorbed using solutions marked in bold are showed).

4.3.2 Identification of phosphorylation sites in human pepsin A and other proteins of human gastric juice

The identification of phosphorylation sites of proteins contained in human gastric juice was performed in the same way as the identification of a phosphorylation site in porcine pepsin A. Human gastric juice was firstly digested with α-chymotrypsin. Afterwards, phosphopeptides were enriched using the sorbents containing immobilized Fe^{3+} and Ga^{3+} ions, and TiO_2, analyzed by MALDI-TOF/TOF MS and identified using database searching. Six phosphopeptides were found (*m/z* 934, 1001, 1081, 1134, 1233, and 1295) and four of them were identified. Three phosphopeptides (*m/z* 1081, 1134, and 1233) were from human salivary acidic proline-rich phosphoprotein 1/2 and one (*m/z* 1295) from human pepsin/pepsinogen A.

Two different phosphorylation sites were identified in human salivary acidic proline-rich phosphoprotein 1/2. One phosphate group was bound to serine residue in position 24 as found out from MS/MS spectrum of peptide ion *m/z* 1081 (sequence DV**pS**QEDVPL). The second phosphate group was bound to serine residue in position 38 as resulted from MS/MS spectra of peptide ions *m/z* 1134 (sequence ISDGGD**pS**EQF) and *m/z* 1233 (sequence VISDGGD**pS**EQF). Identified phosphorylation sites are in agreement with previously published findings [58] and with data given in database Expasy (SIB, Switzerland).

The sequence of peptide ion *m/z* 1295 (MS/MS spectrum in Fig. 7) was identified as QST**p**SETVSITY containing one phosphorylated serine residue. The determined sequence corresponds to residues 127 – 137 of human pepsinogen A with phosphorylated serine residue in position 130. Mascot assigned a score of 59 for the matched sequence (QST**p**SETVSITY) that was above the statistical threshold of 39 for identity/extensive homology. The identified phosphorylation site of human pepsin/pepsinogen A is in agreement with previously published findings that some isoforms of human pepsinogen A of healthy individuals contain phosphoserine [24]. Furthermore, the identified phosphorylation site is in agreement with the position predicted by database Expasy (SIB, Switzerland) according to the homology with pepsinogens A of other animal species.

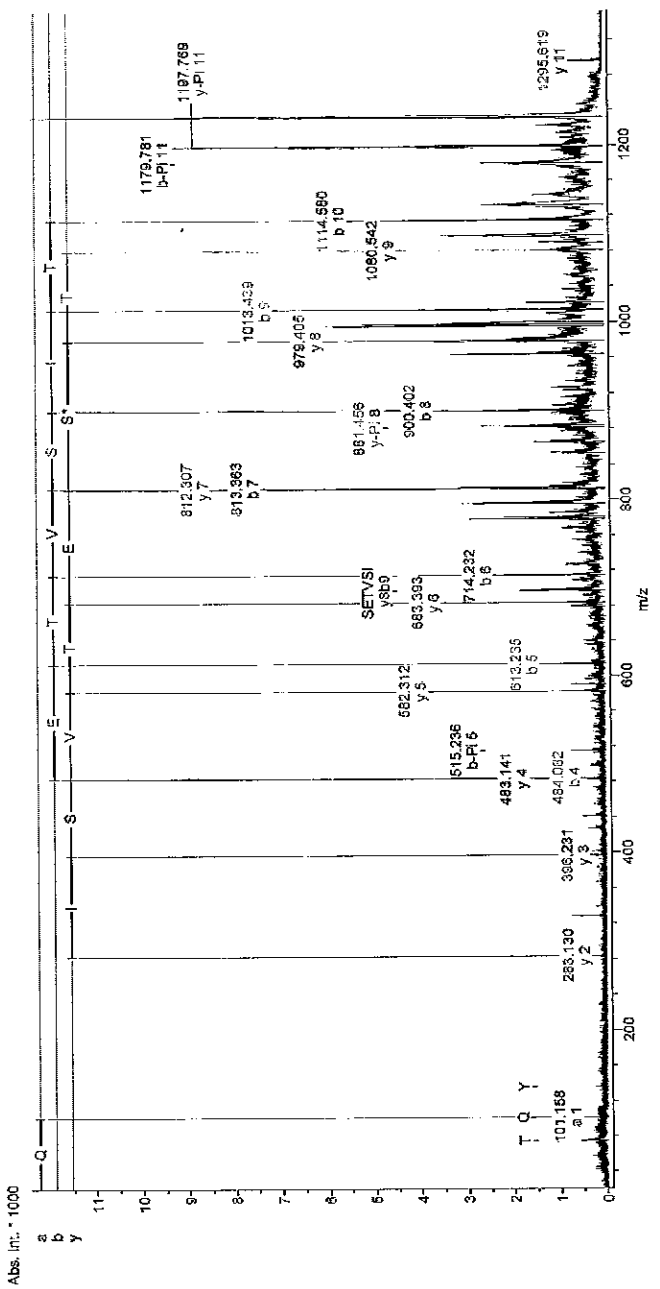

Fig. 7 MALDI-TOF/TOF MS spectrum of the parent ion *m/z* 1295.6
The amino acid sequence coverage is shown by 'a' and 'b' and 'y' fragment ion series. A prominent loss of 98 Da (*m/z* 1197.7) indicates monophosphorylation. The mass difference 167.1 Da between fragment ions y8 (*m/z* 979.4) and y7 (*m/z* 812.3) corresponds to a phosphoserine residue, indicating the phosphorylation site at second serine.

5. CONCLUSIONS

- We compared the ability of several high pressure immobilized metal affinity chromatography sorbents (HP-IMAC sorbents) to separate intact phosphoproteins – porcine pepsin A and chicken ovalbumin. We showed that except for BP-UO_2^{2+}, all tested sorbents can be utilized for HP-IMAC of phosphoproteins. However, the best results were achieved with IDA-Fe^{3+} and BP-Ga^{3+} sorbents.

- Using model phosphoprotein porcine pepsin A, we elaborated the method for the separation of phosphorylated and non-phosphorylated forms of pepsin on IDA-Fe^{3+} and BP-Ga^{3+} sorbents. We found that pepsin interactions with both sorbents are mediated not only through its phosphate group but also through its carboxyl groups. After esterification of pepsin carboxyl groups with diazomethane, we achieved the effective separation of phosphorylated and non-phosphorylated pepsin forms.

- We applied the elaborated separation method on human pepsins. Firstly, we isolated human pepsins from gastric juice of a healthy man and from gastric mucosa of a gastric carcinoma patient using ion-exchange chromatography. Afterwards, we esterified isolated pepsins with diazomethane. Finally, we separated modified pepsins using immobilized metal affinity chromatography according to the presence of phosphate groups in pepsin molecules. Furthermore, we found that pepsins of a gastric carcinoma patient were more phosphorylated than pepsins of a healthy subject.

- We elaborated an approach for the identification of pepsin phosphorylation site. This approach consisted of the following steps. Firstly, porcine pepsin A was digested with α-chymotrypsin. Afterwards, phosphopeptide was enriched from proteolytic digest using immobilized metal affinity chromatography or titanium dioxide affinity chromatography. Enriched phosphopeptide was than subjected to MALDI-TOF/TOF mass spectrometric analysis. Finally, phosphopeptide as well as the pepsin phosphorylation site was identified from the obtained tandem mass spectra using database searching.

- The elaborated procedure for the identification of phosphorylation sites was successfully applied to proteins of human gastric juice. We were able to enrich, detect and identify several phosphopeptides derived from different proteins of human gastric juice and we also succeed in the identification of the human pepsin A phosphorylation site.

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