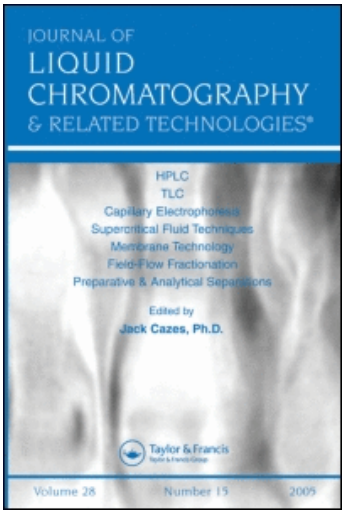

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Radovan Hynek[a]; Hana Vankova[b]; Marketa Tesarova[a]; Jan Kas[a]
[a] Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague 6, Czech Republic [b] Department of Pathological Physiology, 1st Faculty of Medicine, Charles University, Prague 2, Czech Republic

Online publication date: 30 November 2001

To cite this Article Hynek, Radovan , Vankova, Hana , Tesarova, Marketa and Kas, Jan(2001) 'DEGREE OF PHOSPHORYLATION OF PEPSINOGEN IN GASTRIC CANCER', Journal of Liquid Chromatography & Related Technologies, 24: 18, 2801 — 2814
To link to this Article: DOI: 10.1081/JLC-100106949
URL: http://dx.doi.org/10.1081/JLC-100106949

J. LIQ. CHROM. & REL. TECHNOL., 24(18), 2801–2814 (2001)

DEGREE OF PHOSPHORYLATION OF PEPSINOGEN IN GASTRIC CANCER

Radovan Hynek,[1,*] Hana Vankova,[2] Marketa Tesarova,[1] and Jan Kas[1]

[1] Department of Biochemistry and Microbiology, Institute of Chemical Technology, Technická 5, 166 28 Prague 6, Czech Republic
[2] Department of Pathological Physiology, 1st Faculty of Medicine, Charles University, U Nemocnice 5, 128 53 Prague 2, Czech Republic

ABSTRACT

Phosphorylation of amino acid residues in proteins and peptides is a very important modification playing a vital role in living organisms. That is a reason why it is so important to have an efficient tool for isolation and identification of phoshorylated peptides and proteins.

The efficient technique combining IMAC (immobilized metal ion affinity chromatography) and RP-HPLC (HPLC on reversed-phase) for determination of phosphorylated peptides in proteolytic digest was verified on model proteins with well defined phosphorylations. Peptide fragments created by digestion of β-casein from bovine milk with trypsin and swine pepsinogen A digested with α-chymotrypsin were separated in the first step by IMAC. Peptides containing phosphate group(s) were bound to ferric ions

*Corresponding author. E-mail: radovan.hynek@vscht.cz

(Fe3+) immobilized on iminodiacetate (IDA)–Sepharose 6B. Phosphorylated peptides were then released by 0.1 M sodium phosphate (pH 5.0). Reversed phase high performance chromatography (RP-HPLC) was applied as the subsequent step for the separation of individual phosphopeptides from the mixture obtained by immobilized metal ion affinity chromatography.

The developed method was then applied on pepsinogen A digests from stomach mucosa of patients suffering with gastric cancer, and pepsinogen A digests of patients suffering with peptic ulcer (without cancer). Significantly, higher amounts of phosphopeptides were found in samples obtained from patients with gastric cancer. Thus, it seems evident that the higher level of pepsinogen A phosphorylation seems to be associated with gastric cancer.

The method developed represents a reliable tool for the study of phosphorylation degree of proteins in general. In this particular case, it could serve, potentially, for an early diagnosis of gastric cancer.

INTRODUCTION

The significance of techniques for separation and characterization of phosphorylated biomolecules is now increasing, because phosphorylation plays a pivotal role in such processes as enzyme activity modulation, cell membrane permeability regulation, and molecular transport and secretion.

Pepsinogens are produced in cells of stomach mucosa as prepepsinogens with N-terminal signal sequence.(1) The active site of pepsinogen is formed by two aspartic acids.(2) Pepsinogens are converted to active pepsins by cleaving of the N-terminal part, when they are released from gastric mucosa to the acid environment of gastric juice.

SamLoff(3) separated seven pepsinogen isozymogens from human gastric mucosa by agar gel electrophoresis and designated them Pg1 through Pg5 (PGA, pepsin I group, pepsinogens) and Pg6, Pg7 (PGC, pepsin II group, gastricsinogen). PGA and PGC groups are significantly different in their amino acid composition and sequence.(4) On the other hand, Evers et al.(5) found only small differences between individual pepsinogens of A group. For example, Pg3 differs from Pg5 by substitution of a single amino acid residue in the activation segment (Pg3 contains glutamic acid residue in position 43, Pg5 contains lysine in the same position).

Both major human zymogens, pepsinogen A and pepsinogen C, may contain three phosphate groups in the molecule as a maximum.(6) This fact enabled

the use of immobilized metal ion affinity chromatography for fractionation of pepsinogens according to degree of their phosphorylation. A higher number of phosphate groups were observed in pepsinogens isolated from stomach mucosa of patients suffering from gastric cancer.(7) Aspartic proteinases, from patients with gastric cancer showed higher electrophoretic mobilities during PAGE (polyacrylamide gel electrophoresis), than those isolated from patients without gastric cancer.(6) It seems that the higher electrophoretic mobilities of proteinases in samples from gastric cancer patients were raised by a higher negative charge derived from a higher content of phosphoryl groups. Generally, the higher phosphorylation seems to be associated with cancerogenesis.(8-10)

Peptide mapping of five individual human pepsins using RP-HPLC (reversed-phase high performance liquid chromatography) after protein digestion with either Staphylococcus aureus proteinase V8 or α-chymotrypsin was described by Jones and Roberts.(11) Phosphorylated and dephosphorylated forms of swine pepsin, human pepsinogen A, and human pepsinogen C were distinguished using combination of RP-HPLC and CZE (capillary zone electrophoresis).(12,13) It was possible to distinguish these two forms due to different electrophoretic mobilities of phosphorylated and nonphosphorylated peptide fragments.

The goal of this study was to develop a very specific method, which would allow selective isolation and identification of phosphorylated peptides created by proteolytic digestion of human pepsinogen A. β-casein from bovine milk and swine pepsinogen A, were used as a model proteins, because of their well defined phosphorylation. The peptide fragments were prepared by proteolytic digestion with α-chymotrypsin in the case of pepsinogen and with TPCK treated trypsin (trypsin free of chymotryptic activity) in the case of β-casein. This method was used for the comparative study of the samples isolated from gastric mucosa of patients with gastric cancer and patients without cancer. This finding is very important from a diagnostic point of view, since phosphorylation seems to be associated with early stages of carcinogenesis, not only in the case of aspartic proteinases.

EXPERIMENTAL

Chemicals

β-casein from bovine milk, swine pepsinogen A (E.C.3.4.23.1), TPCK treated trypsin (EC 3.4.21.4) from bovine pancreas, α-chymotrypsin (EC 3.4.21.1) from bovine pancreas, potato acid phosphatase (EC 3.1.3.2.), urea, Tris and Tricine were obtained from Sigma (Prague, Czech Republic). Dithiothreitol (DTT), iodoacetamide (IAA), trifluoroacetic acid (HPLC grade)

were obtained from Fluka (Buchs, Switzerland) and acetonitrile (HPLC grade) from Merck (Darmstadt, Germany).

Isolation of Human Pepsinogens

Gastric mucosa was obtained from resected parts of stomach of a patient suffering from gastric cancer. The resected parts of stomach were processed immediately. After washing with 0.1 M phosphate buffer (pH 7.3), the sample was divided into several transversal strips. Dissected mucosa was homogenized in 0.1 M phosphate buffer (pH 7.3), 4 mL per 1 g of tissue, at 4°C. Supernatant was separated by centrifugation at 2000 g for 60 min at 4°C. Proteolytic activity in the supernatant was measured by the Anson and Mirsky method.(14) The supernatant was applied on a DEAE cellulose column (55 x 2.5 cm I.D.) filled in the laboratory with (Lachema, Prague, Czech Republic) (7 μm). Pepsinogens were eluted with 0.5 M NaCl containing 0.1 M sodium acetate (pH 5.6). The material from the apex of peak was dialyzed against distilled water at 4°C for 16 h and lyofilized. Pepsinogen A was separated from pepsinogen C according to Foltmann.(15)

Dephosphorylation of Swine Pepsinogen

Phosphate groups of all described pepsinogens (human pepsinogen A, human pepsinogen C, and swine pepsinogen) were removed using modification of the method of Martin *et al.(16)* Freeze-dried pepsinogen was dissolved (concentration 1 mg/mL) in 0.01 mol/L sodium acetate buffer (pH 5.5) containing 0.02 mol/L of magnesium chloride. Then 1 mL of potato acid phosphatase solution (1 mg/mL) in 0.01 mol/L sodium acetate buffer (pH 5.5) was added to 9 mL of pepsinogen solution (final concentration of phosphatase in reaction mixture 0.1 mg/mL) and the mixture was incubated at 37°C for 16 h. The reaction mixture was dialyzed against distilled water at 4°C for 24 h and freeze-dried. The liquid chromatography on Superose-12, HR 10/30 (Pharmacia LKB, Uppsala, Sweden) column was used for isolation of pure dephosphorylated forms of pepsinogens.

Proteolytic Digests

Digestion of β-casein with TPCK treated (free of chymotryptic activity) trypsin was performed in a solution of 0.1 M ammonium hydrogen carbonate

containing 2 M urea (pH 8.0). An amount of 1.5 mg of β-casein was dissolved in 1.5 mL of this solution. Then 14 µL of trypsin (2.3 mg/mL solution in the same buffer; activity 10,000 units/mg solid) was added. The reaction mixture was then incubated for 24 hours at 37 °C. The reaction was stopped by acidification to pH 4.5 using 50% acetic acid.

α-Chymotryptic digests of pepsinogens were prepared according to the method of Stone *et al.*(17) 2 mg of dry swine pepsin (human pepsinogen A) were dissolved in 2 mL of 0.4 mol/L ammonium hydrogencarbonate containing 8 mol/L urea, 200 µL of 45 mmol/L DTT were added, and the mixture was incubated at 50°C for 15 min. After cooling to room temperature, 200 µL of 10 mmol/L IAA was added, and the solution was incubated at 25°C for 10 min. Then 5.6 mL of water was added, followed by 1 mL of α-chymotrypsin (0.3 mg/mL). The mixture (final pH 8.3) was incubated at 37°C for 24 h. All reactions were stopped by acidification to pH 4.5 using 50% acetic acid.

Separation on IMA (Immobilized Metal Ion Affinity) Column

Peptides were separated on a Ecom Liquid Chromatograph using a chelating Sepharose column (25 x 20 mm i.d., HiTrap affinity column). 20 mM ferric chloride was applied on the column, which was then washed with distilled water and equilibrated with 0.1 M sodium acetate (pH 5.0) as the starting buffer. The injected volume of the sample was 200 µL and flow rate was 1.0 mL/min. Elution was performed with 100 mM sodium phosphate (pH 5.0). Peptides were detected at 220 nm.

Reversed-Phase HPLC Separation

Proteolytic digests (peptides) were separated on Hewlett-Packard 1090 Series II Liquid Chromatograph on reversed-phase column (250 x 4 mm I.D.) (OD-584, HP) LiChrospher 100 RP-18 (5 µm). Injected volume was 200 µL, flow-rate 1.0 mL/min.

Separation of digests of swine pepsinogen and casein: solvent A = trifluoroacetic acid - water (0.1: 99.9 v/v), solvent B = solvent A - acetonitrile (40:60 v/v). Linear gradient: 100% A for 5 min, 0 - 75% B generated over 75 min. Peptides were detected at 220 nm.

Separation of digests of human pepsinogen A: solvent A = trifluoroacetic acid - water (0.1:99.9 v/v), solvent B = solvent A - acetonitrile (20:80 v/v). Linear gradient: 100% A for 7 min, 0 - 80% B generated over 60 min. Peptides were detected at 220 nm.

RESULTS AND DISCUSSION

Separation of Proteolytic Digests of Model Proteins

Swine pepsin is a protein with well defined phosphorylation on serine in 68 positions.(18,19) This phosphate group can be removed by acid phosphatase. This means that swine pepsin can be available in phosphorylated (original) and in dephosphorylated form. For that reason, these two forms of swine pepsin were used for demonstrating the efficiency of IMAC (immobilized metal ion affinity chromatography). In the first step, α-chymotryptic digest of phosphorylated (dephosphorylated) form of swine pepsinogen diluted in acetate buffer (pH=5.7) was injected to IMA (immobilized metal ion affinity) column. Then the starting buffer, containing 20 mM of sodium phosphate, was used for elution of the bounded part of pepsinogen digest from the column (see Fig. 1). It is apparent, that the part of the peptides from the digest of phosphorylated form of pepsin, was bound via the phosphate group, to Fe3+ ions in the column and then eluted with 100 mM sodium phosphate (see high peak with retention time approx. 22 min in Fig. 1a), while no peptides from the dephosphorylated form were bound to Fe3+ ions. Thus, the unphosphorylated peptides passed through the column in the first step (no peak corresponding unphosphorylated peptides was recorded in the second step, see Fig. 1b).

A RP-HPLC chromatographic profile of whole α-chymotryptic digest of the original (phosphorylated) form of swine pepsinogen is shown in Fig 2a, while Fig. 2b shows RP-HPLC separation of the same digest, which was first pre separated on metal chelate column (peptides eluted with 50 mM sodium phosphate). A Chromatogram of pre separated digest of swine pepsinogen (Fig. 2b) contains only one large peak with retention time about 23 min. This finding agrees with the theory.

β-Casein from bovine milk is a protein, which is phosphorylated on serine residues. Two peptide fragments containing phosphorylated serines should be created by its specific cleavage with TPCK treated trypsin. Figure 3a shows RP-HPLC separation of whole proteolytic digest of β-casein from bovine milk. RP-HPLC separation of peptides from the same digest, which was previously retained on IMA column is shown on Fig. 3b. The chromatogram in Fig. 3b contains two significant peaks, as was expected.

Identification of Phosphorylated Peptides in the Peptide Map

Figure 4 shows comparison of the chromatographic profiles (reversed phase HPLC) of α-chymotryptic digests of human pepsinogen A, isolated from stomach mucosa of a gastric cancer patient, with a chromatographic profile

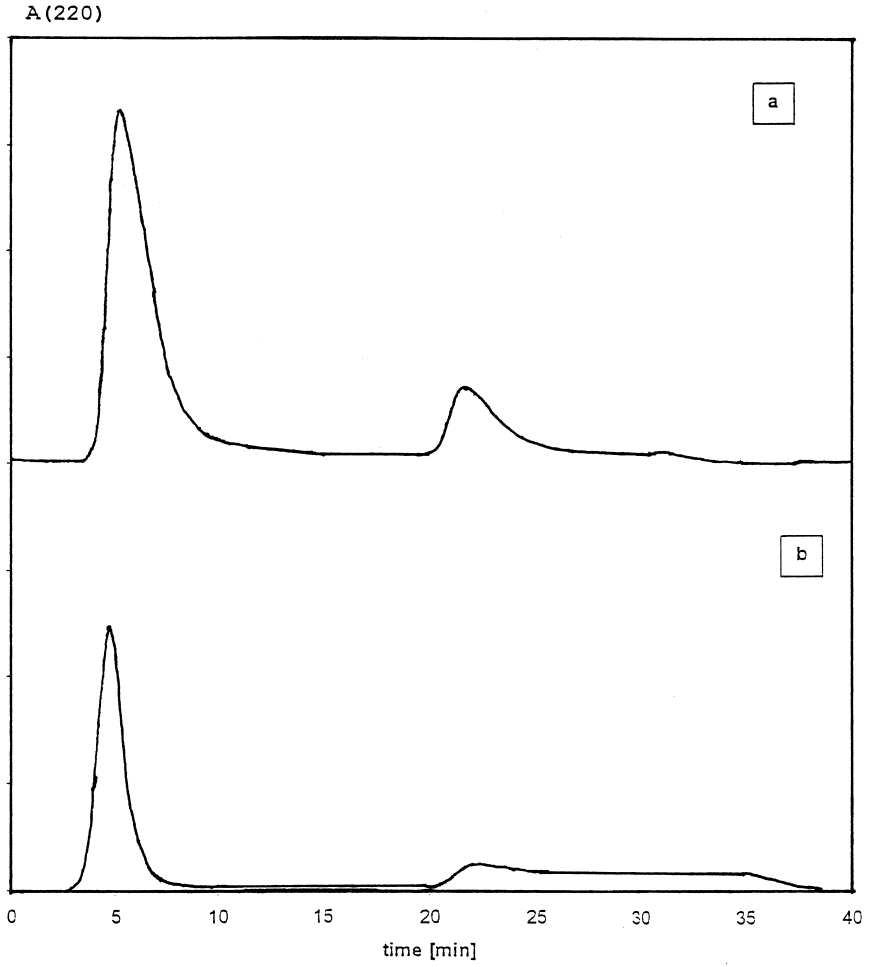

Figure 1. Metal ion affinity chromatography separation of α-chymotryptic digest of phosphorylated (a) and dephosphorylated (b) form of swine pepsinogen. Elution was performed by gradient of sodium phosphate. A_{220}= absorbance at 220 nm.

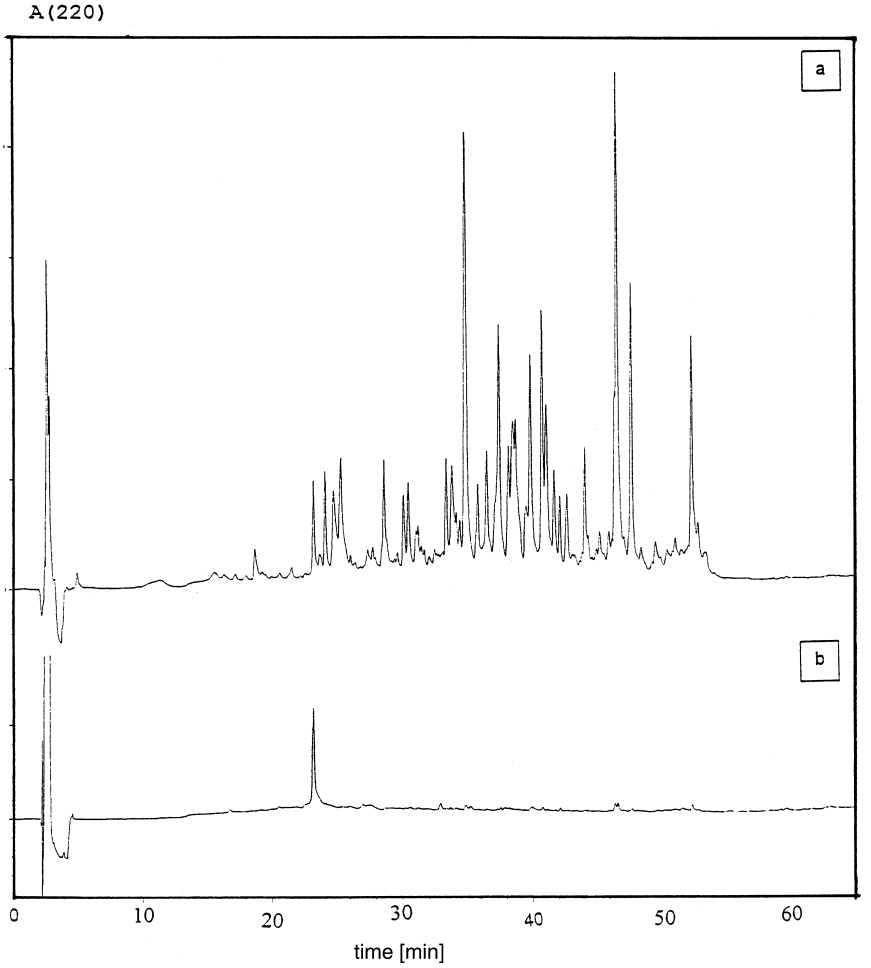

Figure 2. RP-HPLC separation of original (phosphorylated) form of swine pepsinogen after digestion with α–chymotrypsin (a) and separation of the same digest preseparated previously by metal ion affinity chromatography (b), A_{220}= absorbance at 220 nm.

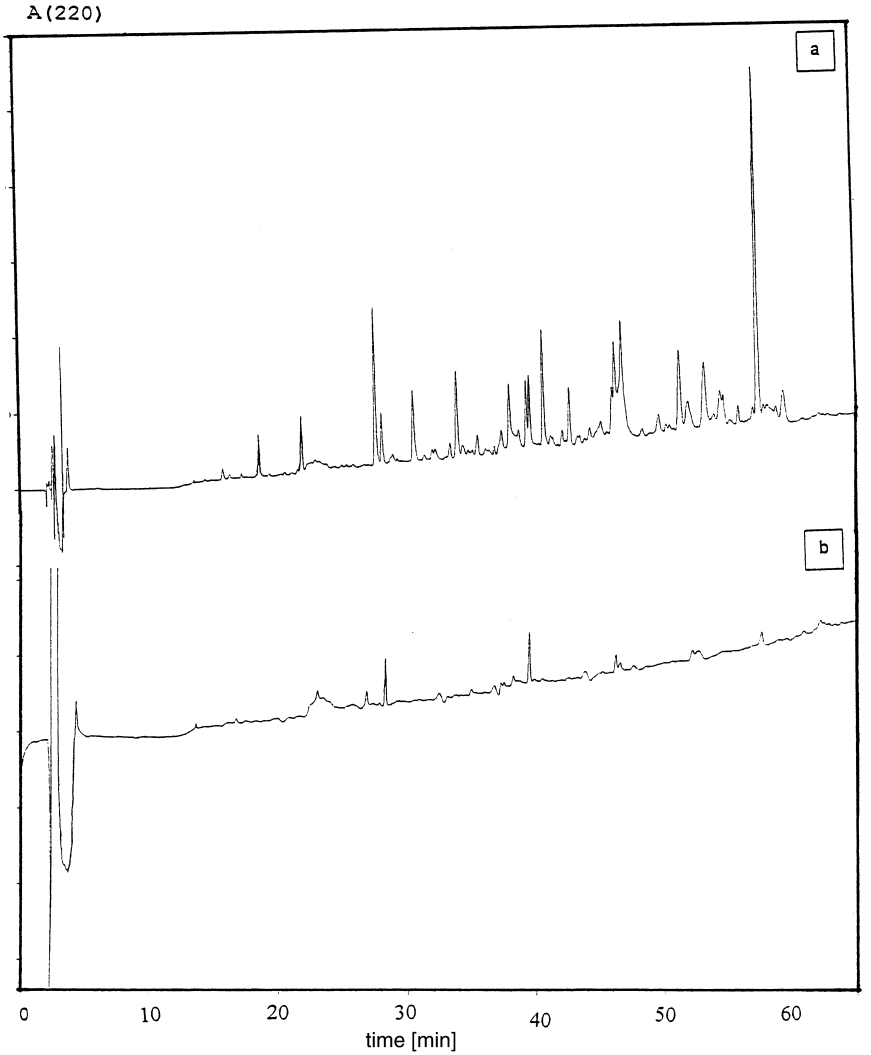

Figure 3. RP-HPLC separation of original (phosphorylated) form of bovine milk β-casein after digestion with TPCK treated trypsin (a) and separation of the same digest pre-separated previously by metal ion affinity chromatography (b), A_{220}= absorbance at 220 nm.

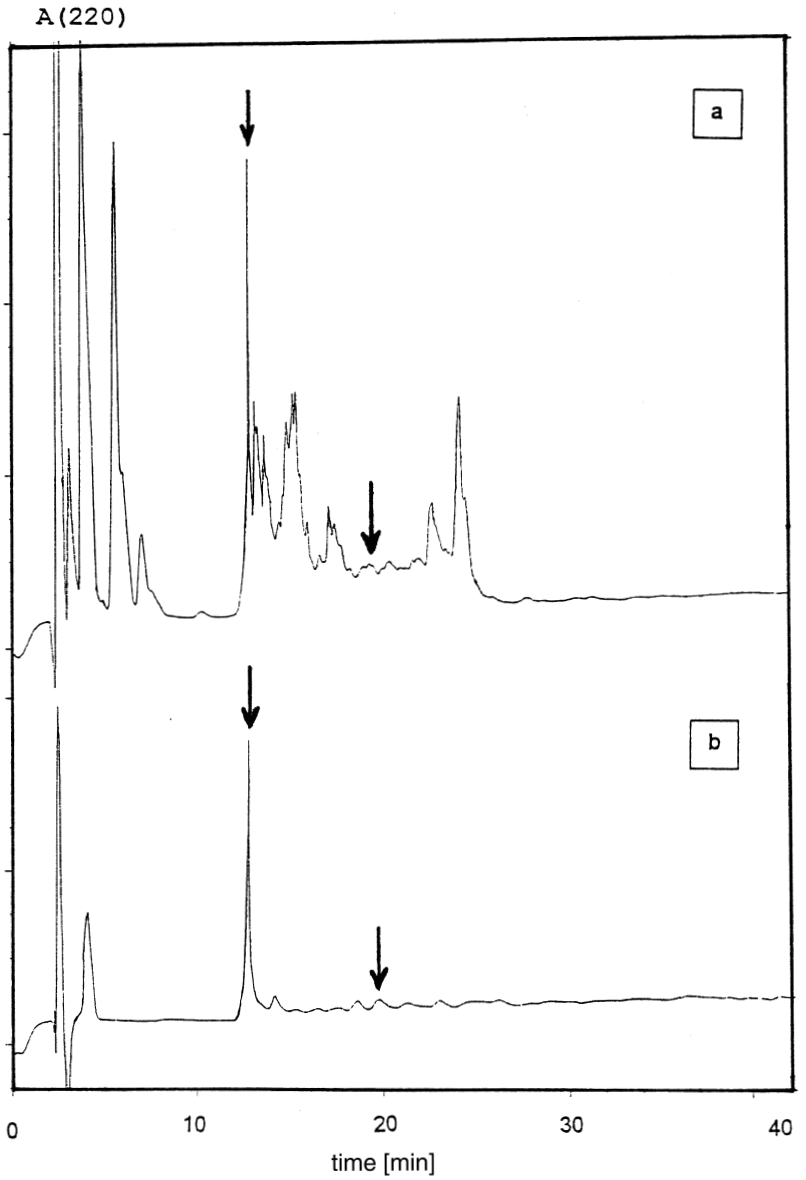

Figure 4. RP-HPLC separation of complete (a) α-chymotryptic digest of human pepsinogen A from stomach mucosa of patient with gastric cancer and (b) of the same digest pre separated first on metal ion affinity column. A_{220}= absorbance at 220 nm.

(reversed phase HPLC) of chymotryptic digest of the same sample preseparated on metal ion affinity column. The peaks eluted up to 8 min are associated with buffer components, IAA and DTT. The chromatogram on Fig. 4a shows an actual complete peptide map of human pepsinogen A, cleaved by α-chymotrypsin into a number of peptide fragments. On the other hand, Fig. 4b shows only phosphorylated peptides, which were created by cleavage of human pepsinogen A with α-chymotrypsin (peptide map of all phosphorylated peptide fragments). The chromatogram on Fig. 4b can be used for identification of phosphorylated peptides in a complete peptide map of human pepsinogen A (Fig. 4a). Peaks with corresponding retention times in both chromatograms are marked by arrows. These peaks correspond to phosphorylated fragments of a digested sample of human pepsinogen A.

Comparison of Chromatograms (RP-HPLC) of Pepsinogen A Samples from Gastric Cancer Patients with Chromatograms of Patients with Peptic Ulcers

Figure 5 shows a comparison of chromatographic (RP-HPLC) profiles (peptide maps of phosphorylated fragments) of pepsinogen A sample from a patient with a peptic ulcer, digested with α-chymotrypsin and preseparated on metal chelate column (Fig. 5a) with the sample from the patient with gastric cancer (Fig.5 b). The peaks, eluted up to 8 min, are associated with buffer components, IAA and DTT. The highest peak has a retention time of 12.7 min. in both chromatograms. The area of the peak with this retention time is obviously higher in the case of gastric cancer (see Fig. 5). The peak with retention time of 12.7 min was presented in chromatograms obtained from all samples. Seven samples from patients with peptic ulcers and seven samples from gastric cancer patients were evaluated. Areas of a characteristic peak with retention time 12.7 min from different samples are summarised in Table 1. It is apparent that areas of the mentioned peak are mostly higher in cases of gastric cancer in comparison with cases of peptic ulcer. In samples from gastric cancer patients, the average area of the peak with retention time 12.7 min was 3137 (mAU x sec), while in samples from peptic ulcer patients it was only 1194 (mAU x sec). This means that the main area of the characteristic peak is 2.6 x higher in the case of gastric cancer compared to the peptic ulcer. These findings indicate higher phosphorylation degree of pepsinogen A in gastric mucosa of patients suffering with gastric cancer. This observation is in agreement with the higher electrophoretic mobilities of pepsinogens from gastric cancer patients.(6) The above mentioned higher electrophoretic mobilities are related to higher phosphorylation degree, also leading to higher negative charge. Our findings indicate that higher phosphorylation degree of human pepsinogen A is related with gastric cancer.

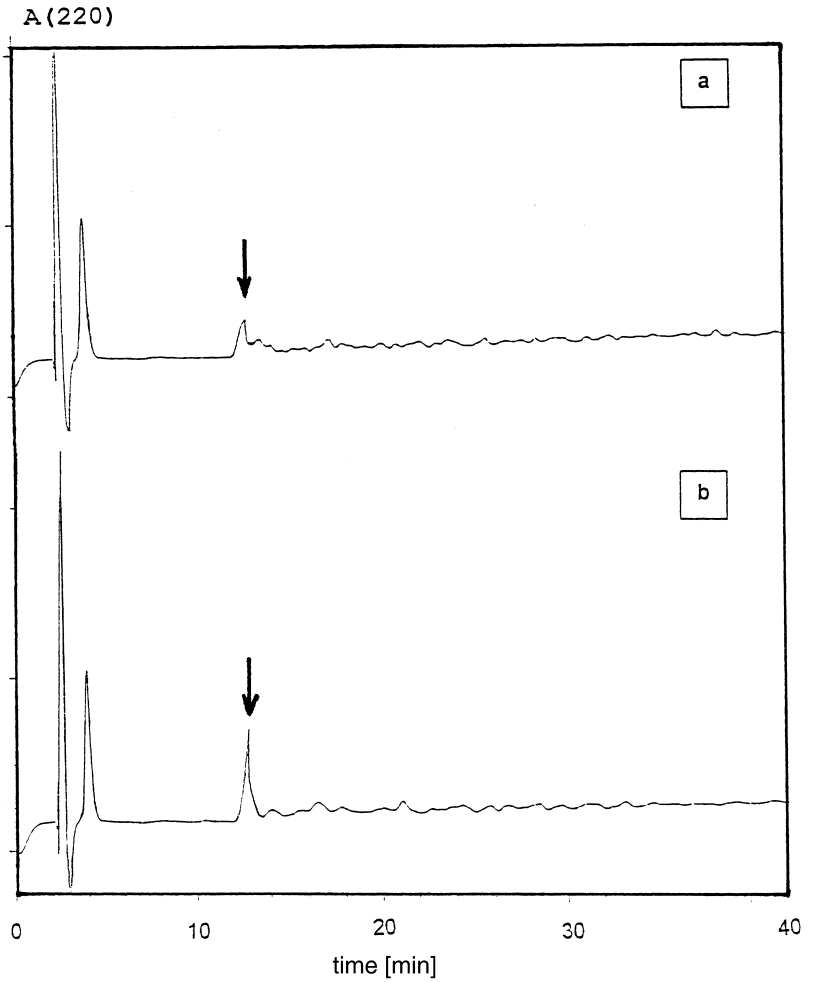

Figure 5. Comparison of RP-HPLC separations of phosphopeptides from α-chymotryptic digests of human pepsinogen A, isolated previously by metal ion affinity chromatography. Sample from peptic ulcer patient (a). Sample from gastric cancer patient (b). A_{220}= absorbance at 220 nm.

Table 1. Comparison of Characteristic Peak Areas (Retention Time 12.7) from Various Samples Obtained from Patient Suffering with Gastric Ulcer and Cancer

Sample	Diagnosis	Peak Area (mAU*sec)
1.	Peptic ulcer	1886
2.	Peptic ulcer	736
3.	Peptic ulcer	40
4.	Peptic ulcer	844
5.	Peptic ulcer	1329
6.	Peptic ulcer	2605
7.	Peptic ulcer	920
Average (7)	Peptic ulcers	Average 1194
8.	Gastric cancer	1975
9.	Gastric cancer	5472
10.	Gastric cancer	2330
11.	Gastric cancer	2577
12.	Gastric cancer	2374
13.	Gastric cancer	2575
14.	Gastric cancer	4656
Average (7)	Gastric cancers	Average 3137

40 μg of digested protein corresponds to each sample.

CONCLUSIONS

We have developed a fast and reliable method for isolation and characterization of individual phosphorylated peptides, as well as for localisation of phosphate groups in molecules of proteins.

The combination of immobilized metal ion affinity chromatography with Fe3+ ions on chelating Sepharose and RP-HPLC (reversed phase HPLC) represents a powerful tool for the separation and characterization of phosphorylated peptides or proteins. This technique has been successfully applied to detect phosphorylated peptides in α-chymotryptic digest of human pepsinogen A and it could be used in gastric cancer diagnosis.

ACKNOWLEDGMENT

The support of Grant Agency of the Czech Republic, grant no. 203/98/P201 and 303/98/1121, is gratefully acknowledged.

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Received December 20, 2000 Manuscript 5465
Accepted May 12, 2001