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Fast detection of phosphorylation of human pepsinogen A, human pepsinogen C and swine pepsinogen using a combination of reversed-phase high-performance liquid chromatography and capillary zone electrophoresis for peptide mapping

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Abstract

A combination of reversed-phase high-performance liquid chromatography (RP-HPLC) and capillary zone electrophoresis (CZE) was used for characterization of α-chymotryptic digests of human pepsinogen A, human pepsinogen C (both isolated from stomach mucosa of patients suffering from gastric cancer), swine pepsinogen and their dephosphorylated forms. Combining RP-HPLC and CZE for peptide mapping allowed to detect phosphorylations in molecules of the above mentioned gastric zymogens. We have found one phosphate group in the molecule of human pepsinogen A and two phosphate groups in the molecule of human pepsinogen C. The investigated sample was obtained from stomach mucosa of a patient suffering from gastric cancer. An increased number of phosphate groups in molecules of human pepsinogen seems to be associated with gastric cancer. The developed method represent a suitable tool for studying relationships between specific phosphorylations of proteins and cancerogenesis or potentially could serve for early diagnosis of gastric cancer.

Keywords: Peptide mapping; Phosphorylation

COMPOUND INDEX:

Pepsinogens

1. Introduction

Pepsinogens are produced in stomach mucosa cells as prepepsinogens with an N-terminal signal sequence [1], which serves for transport across the endoplasmatic reticulum. Pepsinogen consists of a single polypeptide chain with an average relative molecular mass of 42 000. Its catalytic site contains

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a binding cleft extending through the whole length of the molecule. This cleft is formed in tertiary structure by folding of the polypeptide chain and it is covered by the N-terminal part of the molecule, which inhibits proteolytic activity [2]. The active site is formed by two aspartic acid residues, their side chains pointing out in the cleft [3]. Pepsinogens are converted to active pepsins by cleaving of the N-terminal part, when they are released from gastric mucosa to acid conditions in gastric juice.

Samloff [4] separated seven pepsinogen isozymogens from human gastric mucosa by agar gel electrophoresis and designated them PGA-1 through PGA-5 (pepsin I group, pepsinogens) and PGC-6,7 (pepsin II group, gastricsinogens). Groups PGA and PGC are significantly different in their amino acid composition and sequence [5]. On the other hand, Evers et al. [6] found only small differences between individual pepsinogens of group A. For example, PGA-3 differs from PGA-5 by substitution of a single amino acid residue in the activation segment (PGA-3 contains glutamic acid residue in position 43, PGA-5 contains lysin in the same position).

Both major human zymogens, pepsinogen A (PGA, pepsinogen) and pepsinogen C (PGC, gastricsinogen), may contain as a maximum three phosphate groups in the molecule [7]. This fact was found using immobilized metal chelate affinity chromatography for fractionation of pepsinogens according to their degree of phosphorylation. A higher number of phosphate groups was observed in pepsinogens isolated from stomach mucosa of patients suffering from gastric cancer [8]. The phosphorylation generally seems to be associated with cancerogenesis [9–11].

Cobb and Novotny [12] were able to distinguish phosphorylated and nonphosphorylated forms of β-casein using microcolumn HPLC (high-performance liquid chromatography) and CZE (capillary zone electrophoresis) for separations of peptide fragments after β-casein digestion with trypsin. 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 [13]. In our recent paper [14] we have used combination of RP-HPLC and CZE for separation of peptide fragments of α-chymotryptic digest of swine pepsin and its dephosphorylated form. 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 apply our previously developed approach for determination of differences between swine pepsin and its dephosphorylated form [14], to detect phosphorylations of human pepsinogens and swine pepsinogen. This is very important from a diagnostic point of view, since phosphorylation seems to be associated with early stages of carcinogenesis. RP-HPLC and CZE α-chymotryptic peptide maps of normal and dephosphorylated forms of human pepsinogen A and human pepsinogen C (gastricsinogen) from gastric cancer patients and swine pepsinogen were compared and used for determination of their phosphorylation degree.

2. Experimental

2.1. Chemicals

Swine pepsinogen, 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).

2.2. Isolation of human pepsinogens

Gastric mucosa was obtained from resected parts of stomach of one 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 tissue, at 4°C. Supernatant was separated by centrifugation at 5000 rpm for 60 min at 4°C. Proteolytic activity in supernatant was measured by Anson and Mirsky's method [15]. The supernatant was applied on anion-exchange column (100×4 mm

I.D.) filled in laboratory with DEAE cellulose (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 the peak was dialyzed against distilled water at 4°C for 16 h and lyofilized. Pepsinogen A was separated from pepsinogen C according to Foltmann [16].

2.3. Dephosphorylation of investigated pepsinogens

Phosphate groups of all described pepsinogens (human pepsinogen A, human pepsinogen C and swine pepsinogen) were removed using a modification of the method of Martin et al. [17]. 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.

2.4. α-Chymotryptic digest

α-Chymotryptic digests of pepsinogens and their dephosphorylated forms were prepared according to the method of Stone et al. [18]. A 100-μg amount of dry pepsinogen was dissolved in 100 μl of 0.4 mol/l ammonium hydrogencarbonate containing 8 mol/l urea, 10 μl of 45 mmol/l DTT were added and the mixture was incubated at 50°C for 15 min. After cooling to room temperature, 10 μl of 10 mmol/l IAA was added and the solution was incubated at 25°C for 10 min. Then 280 μl of water was added, followed by 10 μl of 0.3 mg/ml α-chymotrypsin. The mixture (final pH 8.3) was incubated at 37°C for 24 h. All reactions were stopped by freezing to −20°C.

2.5. Reversed-phase HPLC separation

Peptides were separated on Hewlett-Packard 1090 Series II liquid chromatograph on a reversed-phase column (250×4 mm I.D., OD-584, HP, LiChrospher 100 RP-18, 5 μm). The injection volume was 250 μl and the flow-rate 1.0 ml/min. 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 206 nm.

RP-HPLC was also used as preseparation method for CZE analyses. Peptides eluted from HPLC column were separated into three fractions with retention times: 24–40 min, 40–55 min and 55–78 min, respectively. These selected fractions were collected and freeze-dried prior to further CZE investigation.

2.6. Capillary zone electrophoresis

Analyses were performed in a home made apparatus for CZE equipped with an untreated fused-silica capillary (I.D. 0.050 mm, O.D. 0.150 mm, effective length 200 mm, total length 310 mm, polyimide outer coating) and a UV-photometric detector at 206 nm [19]. Background electrolyte (BGE) was 0.04 mol/l Tris, 0.04 mol/l Tricine, pH 8.1. Peptides were dissolved in BGE in the concentration range 0.1–0.4 mg/ml. The sample solution was manually introduced into the capillary using hydrostatic pressure (50 mm height difference between the capillary tips) for 20–30 s. The applied voltage was 12 kV (anode at the injection end of the capillary) and the current was 18–19 μA at ambient temperature 23–25°C. CZE analyses of the three collected chromatographic fractions of all investigated zymogens and their dephosphorylated forms were performed.

3. Results and discussion

3.1. Chromatographic and electrophoretic peptide maps of selected gastric zymogens

The RP-HPLC peptide maps of human pepsinogen A, human pepsinogen C (gastricsinogen) and swine

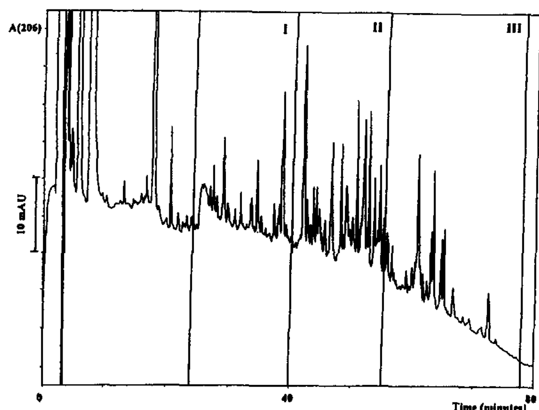

Fig. 1. RP-HPLC peptide map of human pepsinogen A digested by α-chymotrypsin. Three fractions I, II, III were collected and subjected to CZE analyses (see Fig. 4). A(206)=absorbance at 206 nm. For separation conditions see the text.

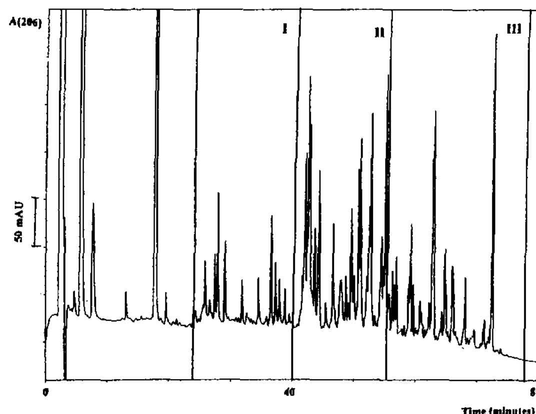

Fig. 3. RP-HPLC peptide map of swine pepsinogen digested by α-chymotrypsin. Three fractions I, II, III were collected and subjected to CZE analyses (see Fig. 6). A(206)=absorbance at 206 nm. For separation conditions see the text.

pepsinogen are shown in Fig. 1, Fig. 2 and Fig. 3, respectively. The peaks eluted up to 10 min are associated with buffer components, IAA and DTT. Peaks with retention times from 10 to 24 min correspond to α-chymotrypsin and its self-cleavage fragments. This was proved by a pepsinogen-free experiment. Peptide fragments of investigated zymogens are eluted at 24–78 min. This relevant area was split into three fractions (fraction I 24–40 min, fraction II 40–55 min, fraction III 55–78 min) which were collected for CZE analyses. This partial

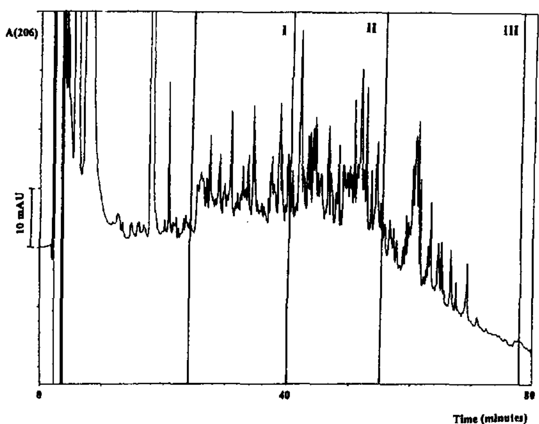

Fig. 2. RP-HPLC peptide map of human pepsinogen C digested by α-chymotrypsin. Three fractions I, II, III were collected and subjected to CZE analyses (see Fig. 5). A(206)=absorbance at 206 nm. For separation conditions see the text.

fractionation enables better electrophoretic separation of complex peptide mixtures resulting from pepsinogen digests. The electropherograms of chromatographically preseparated α-chymotryptic digests of mentioned gastric zymogens are illustrated in Fig. 4 (human pepsinogen A), Fig. 5 (human pepsinogen C) and in Fig. 6 (swine pepsinogen).

Comparisons of both RP-HPLC profiles (in Fig. 1 and Fig. 2) and CZE profiles (in Fig. 4 and Fig. 5) show very significant differences between peptide maps of pepsinogen A and pepsinogen C. This fact corresponds to their different amino acid composition and sequence [5,6].

3.2. Differences between peptide maps of original (phosphorylated) human pepsinogen A, human pepsinogen C, swine pepsinogen and their dephosphorylated forms

The relatively easy way to determine phosphorylations in protein molecules results from the comparison of the peptide maps of their original forms (potentially phosphorylated) and enzymatically dephosphorylated (e.g. by potato acid phosphatase [17]) forms. Phosphorylated peptide fragments in the digest of the original form are more hydrophilic and more negatively charged than corresponding peptide fragments of the dephosphorylated form, and for that

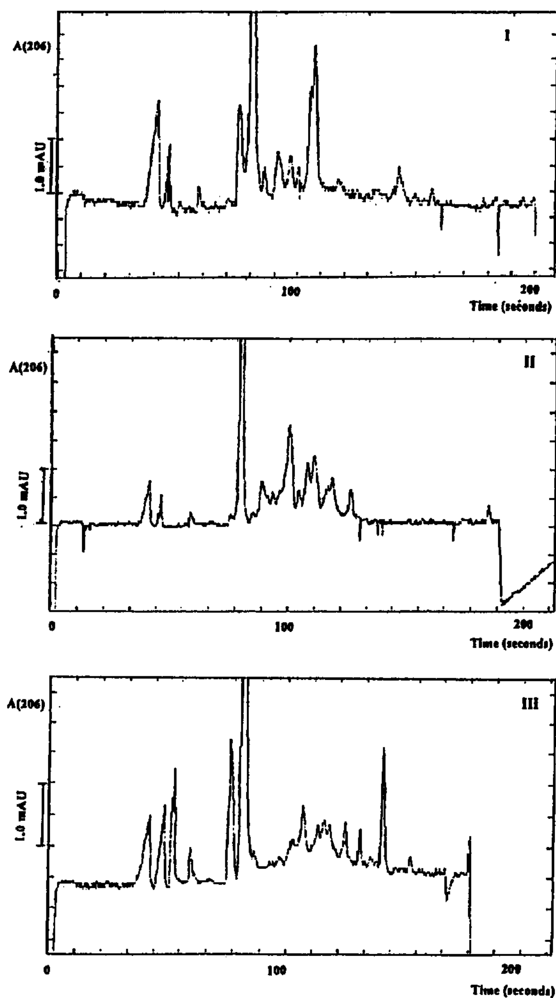

Fig. 4. CZE separation of RP-HPLC fractions I, II, III of human pepsinogen A digested by α-chymotrypsin. A(206) = absorbance at 206 nm. For separation conditions see the text.

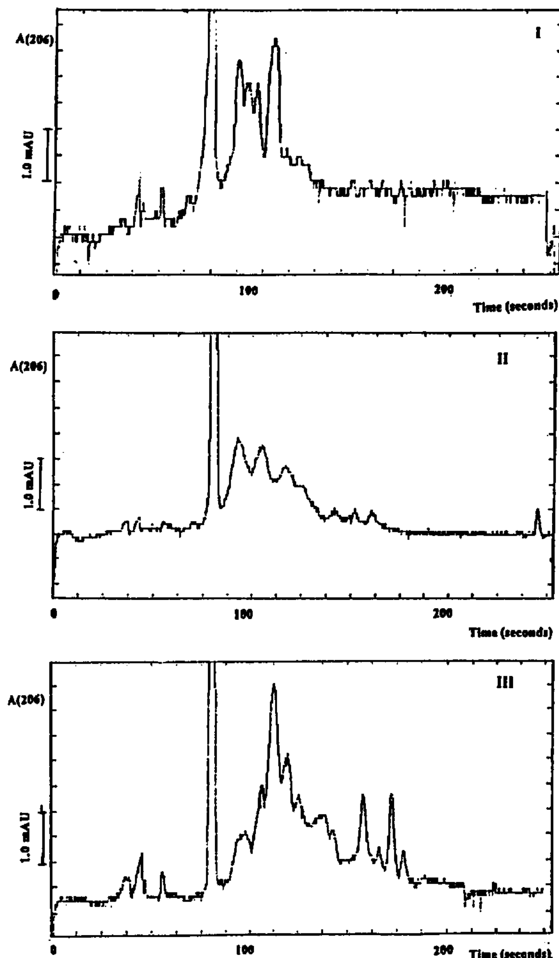

Fig. 5. CZE separation of RP-HPLC fractions I, II, III of human pepsinogen C digested by α-chymotrypsin. A(206) = absorbance at 206 nm. For separation conditions see the text.

reason they exhibit different chromatographic and electrophoretic behaviour.

RP-HPLC peptide maps of original forms of all investigated zymogens (Figs. 1–3) and maps of their dephosphorylated forms (not shown here) were found to be almost identical (only tiny shifts in the chromatograms). The high similarity of the chromatograms of phosphorylated and dephosphorylated forms can be explained by relatively small differences in the composition of very complex peptide mixtures of phosphorylated and nonphosphorylated pepsinogen digests (insufficient for significantly different chromatographic profiles). On the other hand, CZE analyses proved significant differences between both forms of all three zymogens. Electropherograms of selected RP-HPLC fractions in which differences were found are illustrated in Figs. 7–10. Differences between the original and dephosphorylated form were found in CZE maps of fractions I (24–40 min) in the case of human pepsinogen A digests (Fig. 7). The peaks which are shifted relative to each other are marked by arrows. The small differences between areas of peaks with migration times 80–120 s of the phosphorylated and dephosphorylated form (however their migration

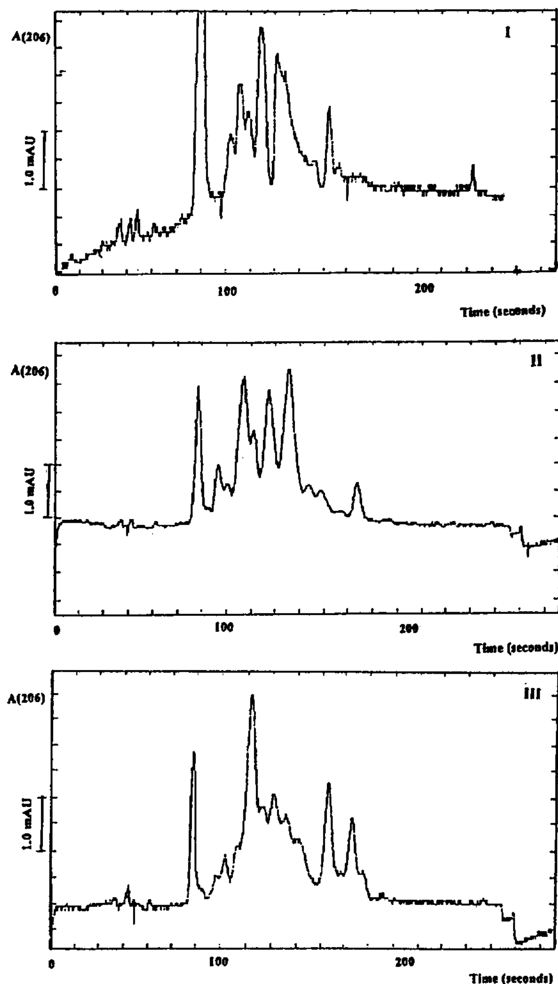

Fig. 6. CZE separation of RP-HPLC fractions I, II, III of swine pepsinogen digested by α-chymotrypsin. A(206)=absorbance at 206 nm. For separation conditions see the text.

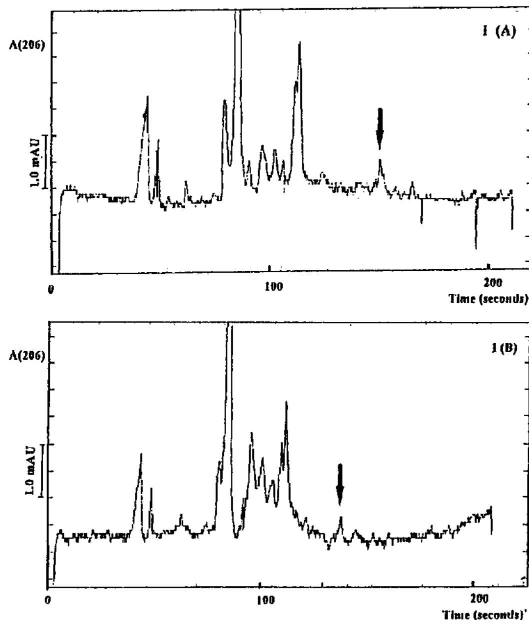

Fig. 7. Comparison of CZE separation of peptide fragments of chromatographic fraction I (24–40 min) of a chymotryptic digest of the phosphorylated (A) and the dephosphorylated (B) form of human pepsinogen A. Arrows point at the peaks which exhibit significantly different migration times in the peptide maps of the two pepsinogen forms. A(206)=absorbance at 206 nm. For separation conditions see the text.

times are identical) can be explained by slightly different ratios of α-chymotrypsin and human pepsinogen A (in very limited amounts in which human pepsinogens are available it is difficult to keep these ratios absolutely exact). Human pepsinogen C showed shifts in the CZE map of HPLC fractions II (40–55 min) (Fig. 8) and HPLC fractions III (55–78 min) (Fig. 9). Swine pepsinogen exhibited a shift in CZE of HPLC fractions I (24–40) (Fig. 10).

For the CZE separations of peptide fragments a relatively low-conductivity background electrolyte (0.04 *M* tris, 0.04 *M* tricine, pH 8.1) in the slightly alkaline pH region was selected similar to the background electrolyte for CZE separation of tryptic digest of β-casein [12]. On the basis of partial knowledge of amino acid sequences of analyzed pepsinogens, peptide fragments with phosphate group(s) were expected to be short peptides with high negative charge resulting in relatively high effective mobility (i.e. the region of relatively longer migration times in the counter-electroosmotic-flow regimen of CZE), whereas the other peptide fragments originating from pepsinogen digests should have lower mobilities and should migrate relatively close to the electroosmotic flow marker (noncharged component of sample mixture). Due to the great number of these peptide fragments and small differences in their mobilities, mixed zones (overlapping peaks) are present in the region just behind the electroneutral components.

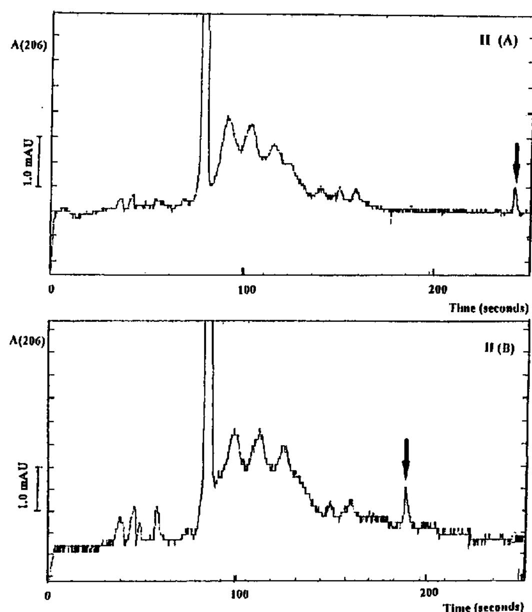

Fig. 8. Comparison of CZE separation of peptide fragments of chromatographic fraction II (40–55 min) of a chymotryptic digest of the phosphorylated (A) and the dephosphorylated (B) form of human pepsinogen C. Arrows point at the peaks which exhibit significantly different migration times in the peptide maps of the two pepsinogen forms. A(206)=absorbance at 206 nm. For separation conditions see the text.

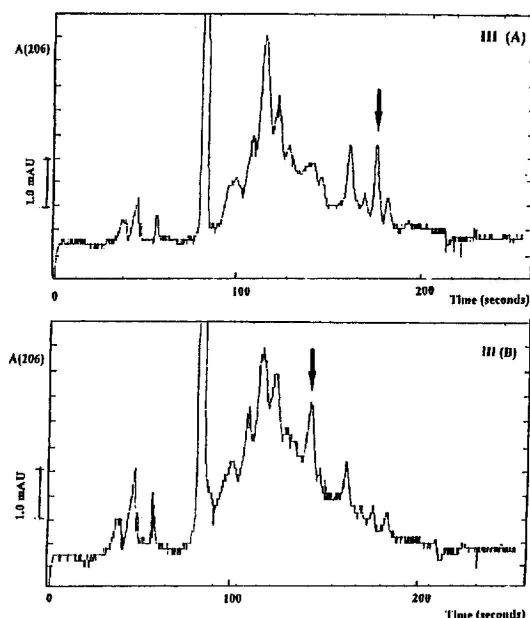

Fig. 9. Comparison of CZE separation of peptide fragments of chromatographic fraction III (55–78 min) of a chymotryptic digest of the phosphorylated (A) and the dephosphorylated (B) form of human pepsinogen C. Arrows point at the peaks which exhibit significantly different migration times in the peptide maps of the two pepsinogen forms. A(206)=absorbance at 206 nm. For separation conditions see the text.

The migration times of shifted peaks of dephosphorylated forms are shorter than the migration times of the original phosphorylated forms. This electrophoretic behaviour agrees with theory, because non-phosphorylated peptide fragments possess lower negative charge and thus exhibit lower electrophoretic mobility (i.e. a shorter migration time in the capillary with prevailing electroosmotic flow in the cathodic direction, which is opposite to the direction of the electrophoretic migration).

Both human pepsinogen A and human pepsinogen C can contain, as a maximum, three phosphate groups [7]. One shift was found in the peptide maps of phosphorylated and dephosphorylated form of human pepsinogen A (Fig. 7). Maps of both forms of human pepsinogen C (gastricsinogen) (Figs. 8 and 9), showed two shifts. This means that one phosphate group was present in the molecule of pepsinogen A and two phosphate groups in the molecule of pepsinogen C in the investigated sample. Swine pepsinogen maps (Fig. 10) showed one shift, which corresponds with the fact that serin in position 68 of swine pepsin is always phosphorylated [20].

4. Conclusions

Our recently developed method combining RP-HPLC and CZE peptide mapping [14] has been successfully applied to detect phosphorylations in molecules of human pepsinogen A, human pepsinogen C and swine pepsinogen.

The digestion of the above mentioned pepsinogens and their enzymatically dephosphorylated forms by α-chymotrypsin, followed by pre-separation of peptide fragments by RP-HPLC, and finally CZE analyses of individul collected chromatographic fractions provide comprehensive peptide maps. Comparison of CZE peptide maps of phosphorylated and dephosphorylated forms enables us to identify the phos-

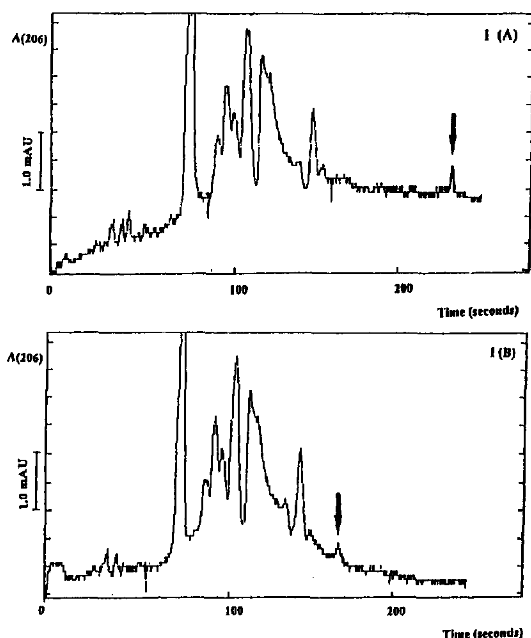

Fig. 10. Comparison of CZE separation of peptide fragments of chromatographic fraction I (24–40 min) of a chymotryptic digest of the phosphorylated (A) and the dephosphorylated (B) form of swine pepsinogen. Arrows point at the peaks which exhibit significantly different migration times in the peptide maps of the two pepsinogen forms. A(206)=absorbance at 206 nm. For separation conditions see the text.

phorylation in the investigated protein molecules because the phosphorylated and dephosphorylated peptide fragments differ from each other significantly in their electrophoretic mobilities.

This fast and handy method could serve for studying gastric cancerogenesis or could be potentially used for early diagnosis of gastric cancer.

Acknowledgments

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