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Application of reversed-phase high-performance liquid chromatography and capillary zone electrophoresis to the peptide mapping of pepsin isoenzymes

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Abstract

A combination of reversed-phase high-performance liquid chromatography (RP-HPLC) and capillary zone electrophoresis (CZE) was used for the characterization of peptide maps of swine pepsin after its digestion with α-chymotrypsin. Peptide maps obtained by both methods were compared and five selected chromatographic peaks were identified on an electrophoreogram. The different order of peaks found in RP-HPLC compared to CZE confirmed the complementarity of these two methods. More peptide fragments were resolved by RP-HPLC, which was also found to be less sensitive to salt content in peptide mixtures, than by CZE, but only CZE was able to separate and identify phosphorylated and dephosphorylated peptide fragments of swine pepsin digest. CZE provides faster separation than RP-HPLC, however, the salts have to be removed by ultrafiltration or by RP-HPLC pre-separation prior to CZE analysis. Combined use of RP-HPLC and CZE for peptide mapping makes it possible to distinguish between the phosphorylated and dephosphorylated forms of swine pepsin. This is important from a diagnostic point of view, because pepsin phosphorylation may be associated with gastric cancer.

Keywords: Peptide mapping; Peptides; Pepsin isoenzymes; α-Chymotrypsin; Enzymes

1. Introduction

Pepsins are aspartic proteases that are synthesized as zymogens by the cells of the gastric mucosa. Human gastric juice contains two major groups of aspartic proteases, now defined as pepsin A (pepsin I group, pepsin, EC 3.4.23.1) and pepsin C (pepsin II group, gastricsin, EC 3.4.23.3) [1] that differ in their amino acid composition and sequence and can be distinguished immunologically. Samloff [2] separated seven pepsinogen isozymogens from human gastric mucosa by agar gel electrophoresis and designated them as PGA-1–PGA-5 (pepsinogens) and PGC-6–7 (gastricsinogens) in order of their decreasing anodic mobility.

Low concentration levels of pepsinogen A in serum was found to be a marker of gastric cancer [3]. The ratios between individual human pepsins and their isozymogens are also very important from a diagnostic point of view. A low ratio of pepsinogen A to pepsinogen C was found to be a sensitive predictor of gastric cancer [4]. Huang et al. [5] used

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enzyme-linked immunosorbent assay (ELISA) for the determination of serum pepsinogen A and pepsinogen C. It was observed that the ratio of PGA to PGC was significantly higher in patients with duodenal ulcer but significantly lower in gastric cancer patients. The ratio of pepsinogen 3 to pepsinogen 5 in human gastric mucosa was found to be lower than one in patients with gastric cancer but higher than one in patients with ulcers [6]. Isozymogens of human pepsinogen may occur either in phosphorylated or in nonphosphorylated form. One molecule of pepsinogen can contain from zero to three phosphate groups [7]. The phosphorylation generally can be associated with carcinogenesis [8–12]. Higher phosphorylation of pepsinogens was found in patients suffering from gastric cancer [7]. For that reason, it is very important to have a fast and reliable method for the detailed investigation of pepsin isoenzymes. Peptide mapping is a very powerful method that is available for such studies. This method involves the enzymatic or chemical cleavage of a protein into a number of smaller peptide fragments, followed by their separation and detection. Reversed-phase high-performance liquid chromatography (RP-HPLC) is a widely used method for the separation of peptides following the cleavage of the protein under investigation. Capillary zone electrophoresis (CZE) is a relatively new method for the separation of peptide fragments, e.g. of human growth hormone [13] and β-casein [14]. These two methods are based on different separation principles. Peptides are separated according to their hydrophobicity by RP-HPLC and according to their electrophoretic mobility by CZE. Consequently, a combination of both methods may provide complementary results.

Human pepsins are available in very limited amounts and, for that reason, it is necessary to use an appropriate model protein for preliminary studies. For instance, swine pepsin can be used as a model protein, because it has a similar amino acid composition to that of human pepsins. The primary structure of swine pepsin was determined by Morávek and Kostka [15] (see Fig. 1) and Tang et al. [16]. Swine pepsin has a serine residue that is phosphorylated, at position 68 [17]. The phosphate group can be relatively easily removed by acid phosphatase [18]. Thus, swine pepsin is a suitable protein for studying the possibility of distinguishing pepsins that differ in the presence of a phosphate group.

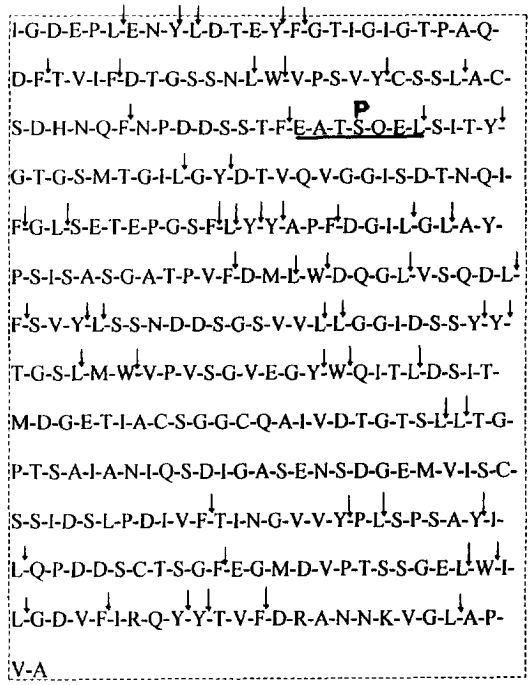

Fig. 1. Primary structure of swine pepsin. Arrows show the theoretical cleavage sites digested with α-chymotrypsin and "P" designates the phosphate group on the serine residue at position 68. The peptide fragment containing this phosphorylated serine is underlined.

Peptide mapping of five individual human pepsins, using RP-HPLC after protein digestion with either *Staphylococcus aureus* proteinase (V8) or α-chymotrypsin, was described previously [19]. However, a combination of RP-HPLC and CZE has not been used previously for the peptide mapping of pepsin.

The goal of this study was to compare RP-HPLC and CZE separations of peptide fragments of α-chymotryptic digests of swine pepsin and to develop a fast and reliable method that could be used to distinguish between the phosphorylated and unphosphorylated forms of pepsin. It is desirable to have such a method to investigate the relationship between phosphorylation of human pepsin isozymogens and carcinogenesis.

2. Experimental

2.1. Chemicals

Swine pepsin, α-chymotrypsin from bovine pancreas, acid phosphatase from potato and urea were obtained from Sigma (Prague, Czech Republic). Dithiothreitol (DTT), iodoacetamide (IAA) and trifluoroacetic acid (HPLC-grade) were obtained from Fluka (Buchs, Switzerland) and acetonitrile (HPLC-grade) was obtained from Merck (Darmstadt, Germany).

2.2. Dephosphorylation of swine pepsin

The phosphate group of swine pepsin was removed using a modification of the method of Martin et al. [18]. Freeze-dried swine pepsin was dissolved (to a concentration of 1 mg/ml) in 0.01 *M* sodium acetate buffer (pH 5.5) containing 0.02 *M* magnesium chloride. Then, 1 ml of potato acid phosphatase solution (2 mg/ml in 0.01 *M* sodium acetate buffer, pH 5.5) was added to 9 ml of pepsin solution (final concentration of phosphatase in the reaction mixture was 0.2 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 was then freeze-dried. Liquid chromatography on a Superose-12, HR 10/30 (Pharmacia LKB, Uppsala, Sweden) column was used for the isolation of the pure, dephosphorylated, form of swine pepsin.

2.3. α-Chymotryptic digestion

Chymotryptic digests of swine pepsin and its dephosphorylated form were performed according to the method of Stone et al. [20]. An aliquot containing 1 mg of dried pepsin was dissolved in 1 ml of 0.4 *M* ammonium hydrogencarbonate containing 8 *M* urea and then 100 μl of 45 m*M* DTT were added and the mixture was incubated at 50°C for 15 min. After cooling to room temperature, 100 μl of 100 m*M* IAA were added and the solution was incubated at 25°C for 10 min. Then 2.8 ml of water were added, followed by 33 μl of a 1 mg/ml solution of α-chymotrypsin. The mixture (final pH of 8.3) was incubated at 37°C for 24 h. All reactions were stopped by freezing to −20°C.

2.4. RP-HPLC separation

Peptides were separated on a Hewlett-Packard 1090 Series II Liquid Chromatograph using a LiChrospher 100 RP-18 (5 μm) reversed-phase column (250 × 4 mm I.D.) (OD-584, HP). The injection volume was 250 μl and the flow-rate was 1.0 ml/min. Solvent A was made up of trifluoroacetic acid–water (0.1:99.9, v/v) and solvent B consisted of solvent A–acetonitrile (40:60, v/v). The gradient consisted of 100% solvent A for 7 min followed by a 0–80% solvent B gradient, generated over 60 min. Peptides were detected at 206 nm.

The selected fractions were collected and freeze-dried prior to further CZE investigation.

2.5. CZE separation

The chymotryptic digest of pepsin was desalted by ultrafiltration (filter type PSAC, pore size NMWL: 10^3, Millipore) and was freeze-dried prior to CZE analysis.

Analyses were performed using 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, with a polyimide outer coating) and an UV-photometric detector that was set at 206 nm [21]. The background electrolyte (BGE) was 0.04 *M* Tris, 0.04 *M* Tricine, pH 8.1. Peptides were dissolved in BGE in the concentration range 0.2–0.8 mg/ml. The sample solution was introduced into the capillary manually, forming a hydrostatic pressure (with a 50 mm height difference of the capillary tips) for 20–30 s. The applied voltage was 14 kV (the anode was at the injection end of the capillary) and the current was 20 μA at an ambient temperature of 23–25°C.

CZE of five individual chromatographic peaks of a chymotryptic digest of swine pepsin, as well as of selected chromatographic fractions of a chymotryptic digest of normal and dephosphorylated forms of swine pepsin, was performed under the same conditions as those used for CZE of a mixture of the whole digest (see above).

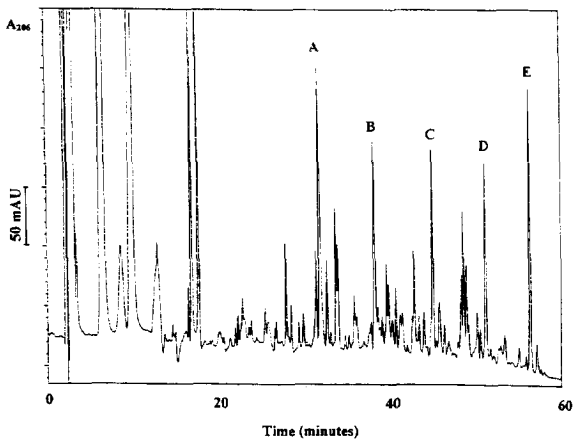

Fig. 2. Chymotryptic digest of swine pepsin separated by RP-HPLC. Peaks A, B, C, D and E relate to Fig. 3B. A_{206} = absorbance at 206 nm.

3. Results and discussion

The primary structure of swine pepsin is shown in Fig. 1. The arrows show the potential theoretical cleavage sites digested by α-chymotrypsin. The peptide fragment containing the phosphorylated serine is underlined.

3.1. RP-HPLC and CZE separation of chymotryptic digests of the normal (phosphorylated) form of swine pepsin

Fig. 2 shows the peptide map obtained by RP-HPLC. The early eluted peaks were associated with the buffer components, urea, IAA and DTT (0–7 min). A pepsin-free experiment showed peaks related to α-chymotrypsin and its self-cleavage fragments

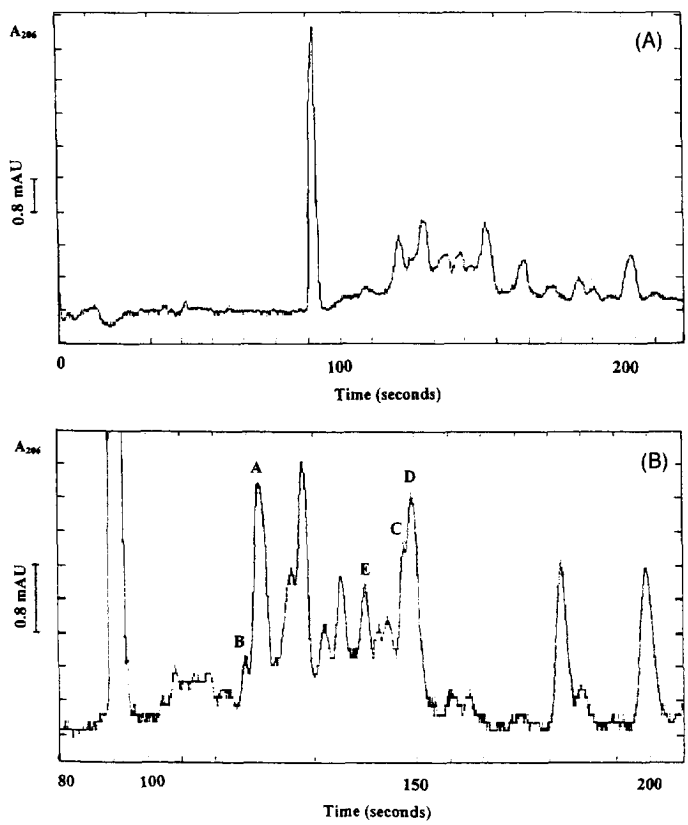

Fig. 3. Chymotryptic digest of swine pepsin separated by CZE. (A) represents a total record and (B) is a more detailed view. Peaks A, B, C, D and E in (B) relate to Fig. 2. A_{206} = absorbance at 206 nm.

Table 1
Retention times of five selected chromatographic peaks and their corresponding migration times on CZE

Peak	RP-HPLC retention time (min)	CZE migration time (s)
A	31.7	121
B	38.1	118
C	44.9	150
D	51.0	152
E	56.2	143

that eluted between 14 and 20 min. The time interval for the separated peptide fragments of pepsin is extended from 20 to 60 min. The major peaks A, B, C, D and E were collected and identified later in the corresponding electrophoreogram. The advantage of RP-HPLC is that the compounds used in the cleavage procedure do not have a negative influence on the separation of peptide fragments, and this allows for the direct application of the sample onto the chromatographic column immediately after digestion has occurred. The peptide map of the same digest obtained by CZE is shown in Fig. 3A and Fig. 3B. Peaks A, B, C, D and E correspond to the chromatographic peaks. It is obvious that their order in the electrophoreogram (Fig. 3B) is different than their order in the chromatogram (Fig. 2). Retention and migration times of the five peaks are summarized in Table 1. CZE of chromatographic peak D (Fig. 4) is shown as an example of the identification of collected chromatographic peaks in an electrophoreogram. The sample of α-chymotryptic digest of swine pepsin had to be desalted before CZE, since poor resolution was achieved when the digest was applied directly to the capillary. CZE is a very fast method for peptide separation, but on the other hand, the preparation of the protein digest prior to CZE separation is more complicated and time consuming than that required for RP-HPLC.

3.2. Comparison of peptide maps from normal (phosphorylated) swine pepsin and its dephosphorylated form

As previously mentioned, swine pepsin is a phosphoprotein, containing just one phosphorylated serine residue [15,17] (Fig. 1). The phosphate group can be removed e.g. by potato acid phosphatase [18]. The peptide maps of phosphorylated and dephosphorylated forms of swine pepsin should be distinguishable due to differences in the hydrophobicity and electrophoretic mobility of one peptide fragment containing either phosphoserine or serine. This fragment, containing seven amino acid residues, is underlined in Fig. 1. RP-HPLC peptide maps of α-chymotryptic digests of the two forms of swine pepsin are shown in Fig. 5. The peptide maps of both forms are almost identical. Only the peaks marked by arrows are slightly shifted from each other. This shift agrees with the theory, as the peak representing the dephosphorylated form is more hydrophobic and therefore is eluted later. However, the shift is so small that we cannot be sure if it is really caused by

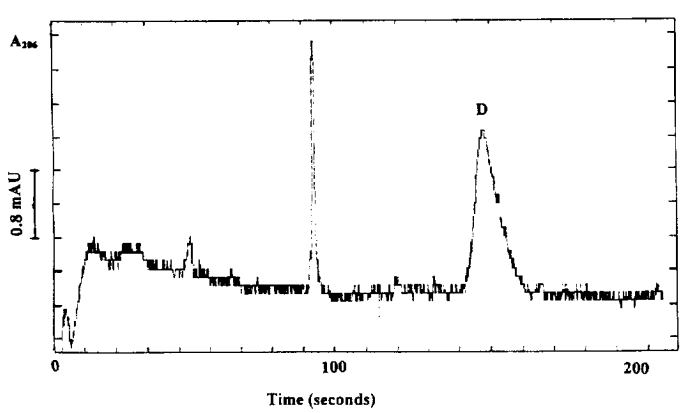

Fig. 4. Example of CZE identification of a chromatographic peak. Peak D was selected from RP-HPLC and its fraction was analyzed by CZE. A migration time of 152 s was determined. The other peaks were identified in the same way. A_{206} = absorbance at 206 nm.

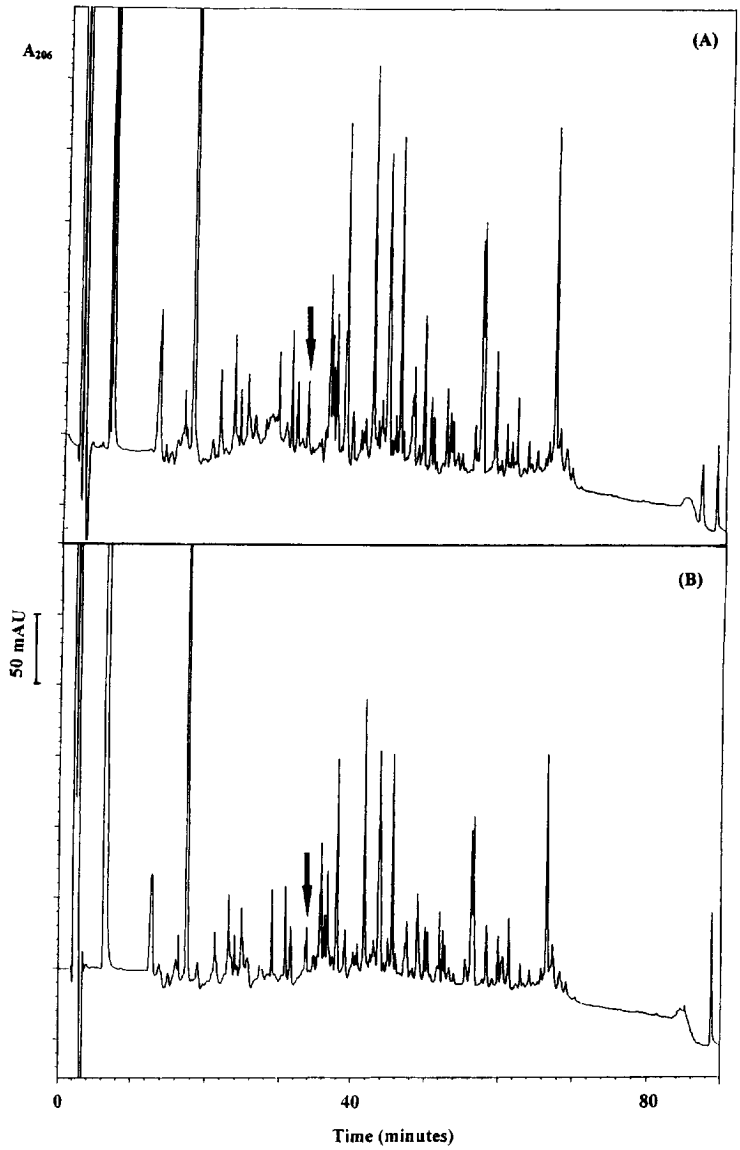

Fig. 5. Comparison of chymotryptic digests from phosphorylated (A) and dephosphorylated (B) forms of swine pepsin, separated by RP-HPLC. The arrows point at the peak which exhibits slightly different retention times in the peptide maps of the two pepsin forms. A_{206} = absorbance at 206 nm.

the absence of a phosphate group. Chromatographic fractions 20–65, 65–76 and 76–90 min of both digests were collected for CZE experiments. It was convenient to use RP-HPLC, when chromatographic and electrophoretic analyses of the same digest were performed, as it also removes salts from the sample. Collected fractions were freeze-dried and, after solubilization in the BGE, they were ready for CZE analysis.

Electrophoreograms of fractions 65–76 and 76–90

of both forms of swine pepsin were found to be identical, i.e. the suspected peptide was not present there. Differences between electrophoreograms of fractions 20–65 of the phosphorylated form (A) and the dephoshorylated form (B) of swine pepsin are illustrated in Fig. 6. Significant shifts between the peaks marked by arrows is obvious. The migration time of the peak for the phosphorylated form is higher than the migration time of the same peak for the dephosphorylated form. This behaviour agrees with the theory, as the phosphorylated peptide fragment has a higher negative charge and thus exhibits higher electrophoretic mobility (i.e., longer migration time in CZE with prevailing electroendoosmotic flow occurring in the opposite direction to that of electrophoretic migration). This means that we are able to distinguish phosphorylated and dephosphorylated forms of swine pepsin using CZE. RP-HPLC was repeated and fractions corresponding to 20–35, 35–50 and 50–65 min were collected for more distinct localization of the phosphorylated peptide fragment in the chromatogram. CZE of fractions 35–50 and 50–65 of both phosphorylated and dephosphorylated forms gave identical results. The significant shift between the peaks of fractions collected at 20–35 min is shown in Fig. 7. Thus, using CZE, we were able to localize the suspected peptide to the chromatographic fraction collected at 15 min. This fact corresponds with the slight shift of peaks seen in Fig. 3 because they are placed in this

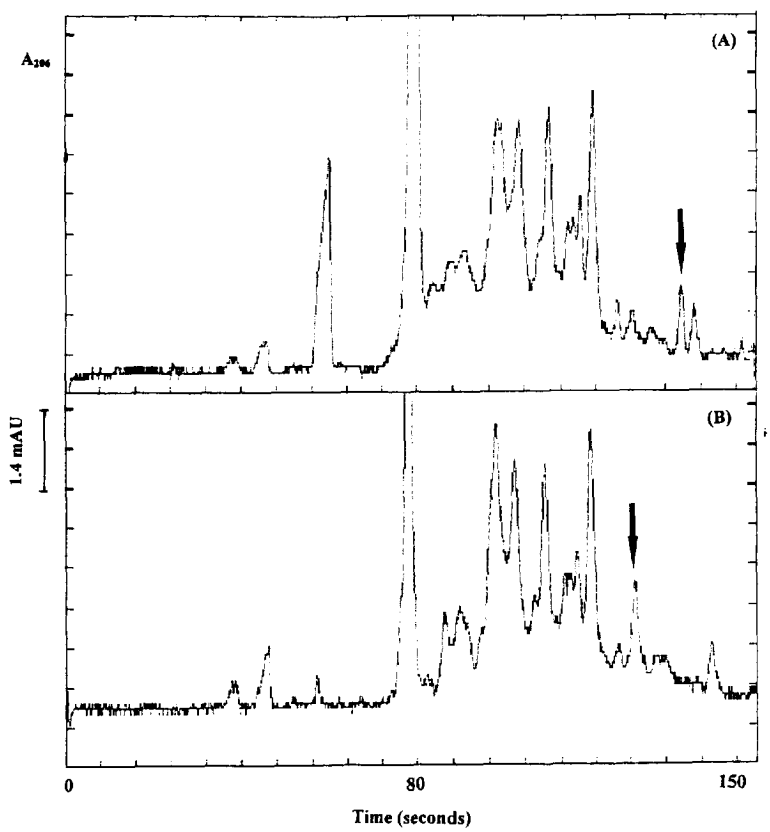

Fig. 6. Comparison of CZE separation of peptide fragments of corresponding to the 20–65 min chromatographic fraction of chymotryptic digest of the phosphorylated (A) and dephosphorylated (B) forms of swine pepsin. The arrows point at the peak that exhibits significantly different migration times in the peptide maps of the two pepsin forms. A_{206} = absorbance at 206 nm.

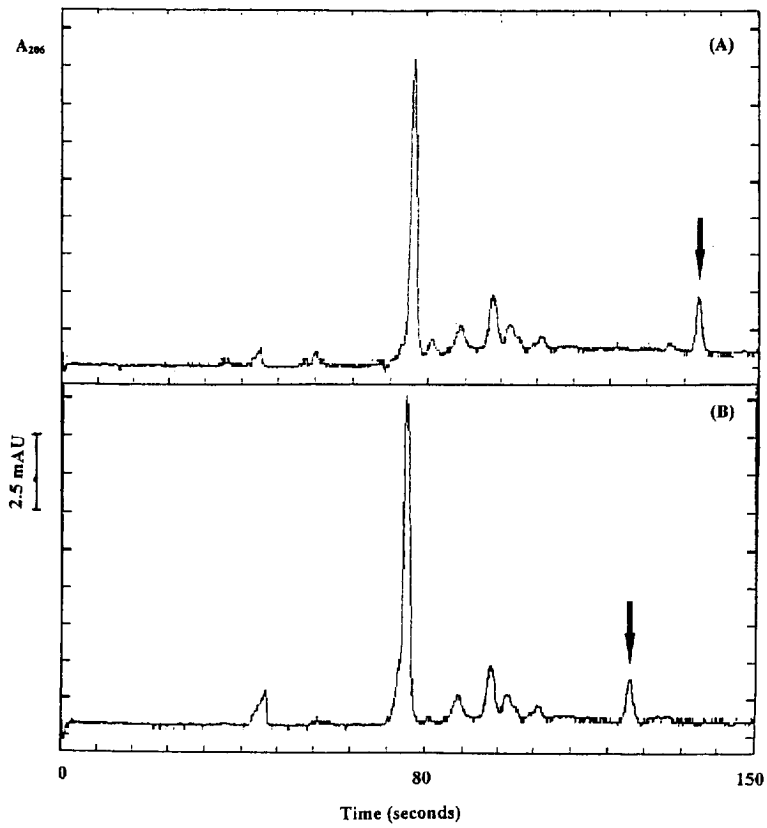

Fig. 7. Comparison of CZE separation of peptide fragments corresponding to the 20–35 min chromatographic fraction of chymotryptic digest of phosphorylated (A) and dephosphorylated (B) forms of swine pepsin. The arrows point at the peak that exhibits significantly different migration times in the peptide maps of the two pepsin forms. A_{206} = absorbance at 206 nm.

area (retention times of approximately 34 min). The peptide was eluted relatively early from the reversed-phase column. Such chromatographic behaviour can be explained by its relatively high polarity (see Fig. 1).

4. Conclusions

It is obvious that digestion of swine pepsin with α-chymotrypsin, followed by RP-HPLC pre-separation and CZE separation of peptide fragments, is a fast and reliable method that is capable of distinguishing between the phosphorylated and dephosphorylated forms of pepsin. RP-HPLC alone is not recommended for this purpose because differences in the retention times of both fragments are too small. Phosphorylated and dephosphorylated peptide fragments differ from each other more significantly in their negative charges, and therefore in their electrophoretic mobilities, than in their hydrophobicities. This fact can explain why better results are achieved using CZE than using RP-HPLC.

We have developed a procedure which allows one to distinguish between the phosphorylated and dephosphorylated forms of swine pepsin. It is very important to have such a fast method for studying the relationship between phosphorylation of human pepsins and gastric cancer. Potentially this method could be used for early diagnosis of gastric cancer.

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