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Immobilization of α-chymotrypsin to magnetic particles and their use for proteolytic cleavage of porcine pepsin A

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ABSTRACT

To detect phosphorylation state of pepsin, a simple and a rapid procedure for coupling of α-chymotrypsin to commercially available magnetic particles was elaborated. α-Chymotrypsin was immobilized to –CHO activated commercial magnetic particles via the protein free amino groups. The following properties of the immobilized proteinase were compared with those of the soluble enzyme: pH dependence of the activity, thermo-stability, self-cleavage activity, and possibility of repeated use.

The immobilized α-chymotrypsin was used to study the phosphorylation degree of porcine pepsin A, used as a model acidic protein and phosphoprotein. The use of enzyme immobilized to magnetic carriers has several advantages as compared with an application of soluble forms of enzymes: preferably an increased stability of enzymes, a possibility of direct use of enzyme reaction products for MALDI-TOF MS. The prepared proteolytic digest was separated using RP-HPLC and immobilized metal affinity chromatography (IMAC) with immobilized Fe(III) ions prior to MALDI-TOF analysis: the presence of phosphopeptide in porcine pepsin A was shown.

1. Introduction

Most cellular processes are regulated by the reversible phosphorylation of proteins on serine, threonine, and tyrosine residues [1]. About one-third of all proteins are supposed to contain covalently bound phosphate. Anomalous phosphorylation of proteins can be related to the development of cancer or to metabolic diseases [2,3]. Human pepsins belong to proteins, the extent of their phosphorylation is related to gastric diseases, and it can be used for early diagnosis of gastric cancer. Despite the importance and widespread occurrence of this modification, identification of sites of protein phosphorylation is still not fully solved, even when performed on highly purified protein [4–6]. An elaboration of methods for the separation and analysis of phosphoproteins is useful not only for better understanding the role of these modified proteins in living systems, but also for diagnostic purposes.

Immobilized metal affinity chromatography (IMAC) is the most widely used method for the separation or enrichment of phosphopeptides being formed after digestion of studied phosphoproteins with suitable proteinase [7–11]. In combination with subsequent mass spectrometry analysis, this approach represents the main tool for phosphoprotein studies.

Proteolysis is the first important step in the proteomics research integrated with MALDI-TOF MS. An efficiency of techniques of in-solution digestion of proteins is limited by lower enzyme stability, long incubation times and esp. by autolytic activity of the used proteinase. To solve many of these problems, proteinases immobilized to different solid supports have been widely used. A variety of methods have been described for proteinase immobilization on various supports, such as, e.g. polymer particles [12], glass [13], membrane [14], gel beads [15], sol–gel supports [16], porous silicon matrix [17], porous monolithic materials [18,19] or magnetic particles [20–24]. A certain drawback of some of immobilized proteinases consists in a decrease of enzyme activity or a change of enzyme specificity, esp. in the case of high molecular weight substrates [25,26]. Application of immobilized enzymes onto magnetic particles draws a great attention due to their easy manipulation and recovery.

Trypsin belongs to the most often used immobilized proteinase used for the preparation of peptide maps. However, much less information is available on α-chymotrypsin that is possible to use for the proteolytic digestion of proteins with low content of basic amino acids. Immobilization of this serine proteinase to different types of magnetic particles [26–30] or polyacrylamide copolymer [31] has been reported.

An evaluation of the phosphorylation state of phosphoproteins is not in all cases fully solved despite an increasing interest in these macromolecules. The objective of the present study is an elaboration of a simple procedure for the preparation of phosphoprotein proteolytic digest and for obtaining the phosphopeptide fraction

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from proteins with higher content of acidic amino acid residues, suitable for mass spectrometry analysis. For the phosphoprotein study, we have used in our experiments a simple way of coupling of α-chymotrypsin to commercial activated magnetic particles and porcine pepsin A as a model phosphoprotein.

2. Materials and methods

2.1. Materials and chemicals

α-Chymotrypsin from bovine pancreas (enzyme activity given by the producer: 54 units per mg of solid or 60 units per mg of protein) was obtained from (Sigma–Aldrich, St. Louis, MO, USA). Unless otherwise stated, all chemicals were of analytical grade and were also purchased from Sigma–Aldrich (St. Louis, MO, USA). Magnetic particles (Magnetic Glyoxal 4% Agarose Beads 20–75 μm) were obtained from BioScience Bead Division of CSS (West Warwick, RI, USA).

2.2. Preparation of α-chymotrypsin-modified magnetic particles

Previously described procedure [31] was used for the enzyme immobilization to magnetic particles. Magnetic Glyoxal 4% Agarose Beads 20–75 μm containing 20 μequiv. of aldehyde groups per 1 ml of magnetic beads was used for the enzyme coupling. Magnetic particle suspension (1 ml) washed with 0.1 M $NaHCO_3$ was mixed with α-chymotrypsin (Sigma–Aldrich, St. Louis, MO, USA) solution (23 mg in 2 ml 0.1 M $NaHCO_3$) and stirred for 24 h at laboratory temperature.

Magnetic particles with coupled α-chymotrypsin were washed three times with 0.1 M $NaHCO_3$ (~2 ml) and non-substituted –CHO groups were deactivated using glycine solution (20 mg in 2 ml 0.1 M $NaHCO_3$) for 60 min. After washing with distilled water and 0.1 M $NaHCO_3$, magnetic beads were reduced with sodium cyanoborohydride solution (15 mg in 2 ml 0.1 M $NaHCO_3$) for 30 min and washed with 0.1 M $NaHCO_3$, distilled water and finally with 0.2 M acetate buffer, pH 4.0.

The amount of coupled ligand was determined from a decrease of absorbance (at 280 nm) of the supernatant after the incubation with activated magnetic particles and from the absorbance of the first washing solution: 15–18 mg of the enzyme per 1 ml of bead suspension (calibration curve was constructed using soluble α-chymotrypsin). The enzyme activity of the immobilized α-chymotrypsin was 9.6 U/ml of magnetic particle suspension.

2.3. Determination of activity of the soluble and immobilized α-chymotrypsin

The α-chymotrypsin activity was determined using N-succinyl-L-phenylalanine-4-nitroanilide as a substrate [32].

The assay medium contained 0.1 M Tris–HCl buffer pH 7.8 containing 0.025 M calcium chloride (500 μl), the substrate solution (50 μl) in N,N′-dimethylformamide (1 mg in 0.1 ml), α-chymotrypsin (330 μg; enzyme activity 1 U) or immobilized enzyme (100 μl of suspension of magnetic particles, the enzyme activity: 0.96 U) and the reaction mixture was incubated at 37 °C for 10 min. The reaction was stopped by an addition of 30% acetic acid (250 μl) and magnetic particles were removed. Absorbance was read at 405 nm.

The enzyme activity was determined from the increase of absorbance of the released 4-nitroaniline (molar extinction coefficient 960 M^{-1}). One unit of enzyme activity (U) is defined as the amount of enzyme which hydrolyzes 1 μmol of substrate per min.

The effect of pH on the activity of the free and immobilized enzyme was investigated at 37 °C. Tris–HCl buffers (0.1 M) pH 7.2–8.7 were used. The relative activity was expressed in % of the enzyme activity determined at pH 8.1.

Thermal stability of free and immobilized enzyme was investigated by the determination of their residual activities after incubation for 30 min at 20–70 °C. The residual activity was assayed using standard assay conditions. The enzyme activity determined after incubation at 37 °C was taken as 100% activity. Determination of the enzyme activity under different conditions was carried out in four parallel measurements. Differences between obtained values were below 5%.

The retention of the immobilized α-chymotrypsin activity was examined after repeated use of the immobilized enzyme. The activity was measured using the standard procedure at 37 °C at intervals of 10 min. The effect of storage at 4 °C and repeated use was tested in the course of 2 months; the effect of repeated use during the long-term storage was examined 10 times. After each run, the immobilized enzyme was separated magnetically and washed 5 times with PBS (pH 7.2) and transferred to a fresh reaction medium and the activity determination was performed using the standard assay conditions.

2.4. Porcine pepsin A digestion with soluble and immobilized α-chymotrypsin

Porcine pepsin A (1 mg) was dissolved in 0.1 M acetate buffer pH 3.7 containing 1 M NaCl (1 ml) and pH of the solution was adjusted to pH 7.5–8.0 with 1.6 M NH_4HCO_3 containing 7 M urea. After an addition of 0.45 M dithiothreitol (20 μl), the mixture was heated at 50 °C under continuous stirring for 15 min. Then 0.1 M iodoacetamide solution (20 μl) was added to the mixture cooled to the laboratory temperature and resulting solution was incubated for 10 min at laboratory temperature. The proteolytic digestion was started by an addition either of the solution (100 μl) of α-chymotrypsin (1 mg per 1 ml) or of the suspension of magnetic particles containing immobilized enzyme (100 μl, 0.96 U). Molar ratio enzyme:protein was 1:7 in the case of soluble enzyme and 1:5 in the case of immobilized one. The mixture was then incubated at 37 °C for 24 h. The proteolytic digestion was terminated either by acidification to pH 4 with concentrated trifluoroacetic acid in the case of soluble enzyme or by a removal of magnetic particles from the incubation mixture.

2.5. RP-HPLC separation of α-chymotryptic digests of porcine pepsin A

Peptides obtained by α-chymotryptic digestion of porcine pepsin A were separated on liquid chromatograph using ZOBRAX Eclipse XDB-C-18 column (4.6 mm × 150 mm i.d.; 3.5 μm). The injection volume was 200 μl, the flow rate 0.5 ml/min and the column temperature 40 °C. Solvent A was made up of trichloroacetic acid–water (0.1:99.9, v/v) and solvent B is made up of trichloroacetic acid–water–acetonitrile (0.1:39.9:60, v/v). After the sample application, the column was eluted first with solvent A for 5 min, followed by linear gradient (0–75%) of solvent B in solvent A (60 min) and finally with solvent B (10 min). Peptides were detected at 220 nm.

2.6. Separation of phosphopeptide fraction using IMAC with immobilized Fe(III) ions

α-Chymotryptic digest of porcine pepsin A separated by RP-HPLC fractions and eluted in the retention time intervals of 1 min were applied to the IDA-Sepharose column (0.8 cm × 2 cm) with immobilized Fe(III) ions equilibrated with 0.5 M acetate buffer pH 4.0. Non-adsorbed peptides were eluted with the same buffer

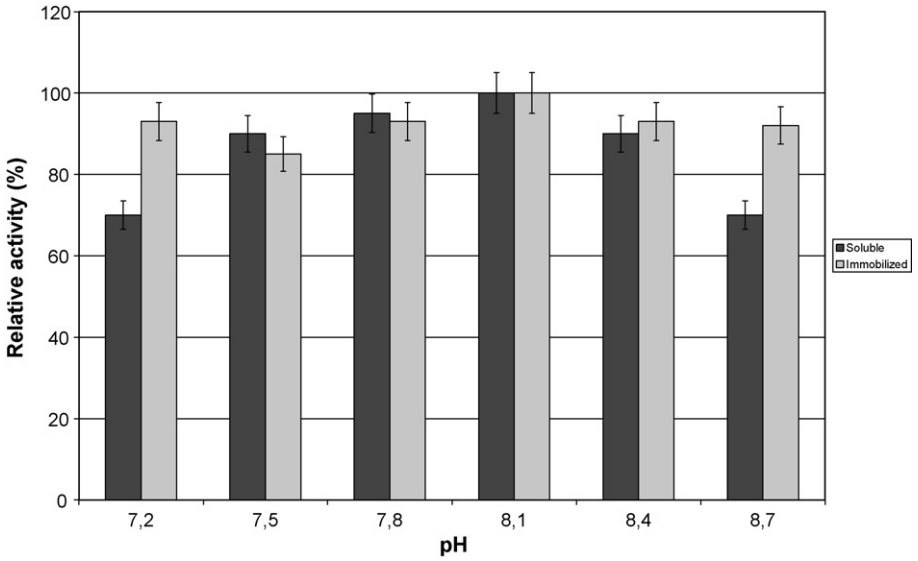

Fig. 1. Dependence of activity of soluble and immobilized α-chymotrypsin on pH. Relative activity expressed in % of the enzyme activity determined at pH 8.1; determination of the enzyme activity was carried out in four parallel measurements, differences among obtained values were below 5%; assay medium: 0.1 M Tris–HCl buffers pH 7.2–8.7 containing 0.025 M calcium chloride (500 μl), N-succinyl-L-phenylalanine-4-nitroanilide solution (50 μl) in N,N′-dimethylformamide (1 mg in 0.1 ml), α-chymotrypsin (330 μg) or immobilized enzyme (100 μl of suspension of magnetic particles); incubation at 37 °C for 10 min.

(20 ml); 0.5 ml fractions were collected. For the elution of adsorbed peptides, 0.05 M phosphate buffer pH 7.2 (2 ml) was used.

Fractions containing adsorbed peptides were analyzed by MS (MALDI-TOF). Prior to analysis peptides were purified using Perfect Pure C-18 Tip (Eppendorf, Hamburg, Germany): peptides were adsorbed and washed with 0.1% trifluoroacetic acid; adsorbed peptides were eluted with 0.1% trifluoroacetic acid in 50% acetonitrile.

2.7. MALDI-TOF MS analysis and database searching

MALDI-TOF and MALDI-TOF/TOF spectra were acquired on an Autoflex II TOF/TOF system (Bruker Daltonics, Bremen, Germany), using a nitrogen laser (337 nm) and the FlexControl software. The mass spectrometer was externally calibrated with the peptide calibration standard (Bruker Daltonics, Bremen, Germany) containing Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH (1–17), ACTH (18–39), Somatostatin 28.

Samples were deposited on an AnchorChip Steel 384-well target plate (Bruker Daltonics, Bremen, Germany). An amount of 0.5 μl of matrix solution (5 mg DHB (2,5-dihydroxybenzoic acid) in 1 ml of 30% ACN containing 1% phosphoric acid) was spotted onto the plate and allowed to air-dry at room temperature, then 0.5 μl of sample was added and again allowed to air-dry at room temperature. Afterwards, the plate was inserted in the mass spectrometer and subjected to MS analysis. The most intensive peptide ion from MS spectra was subjected to MS/MS. FlexAnalysis and BioTools (Bruker

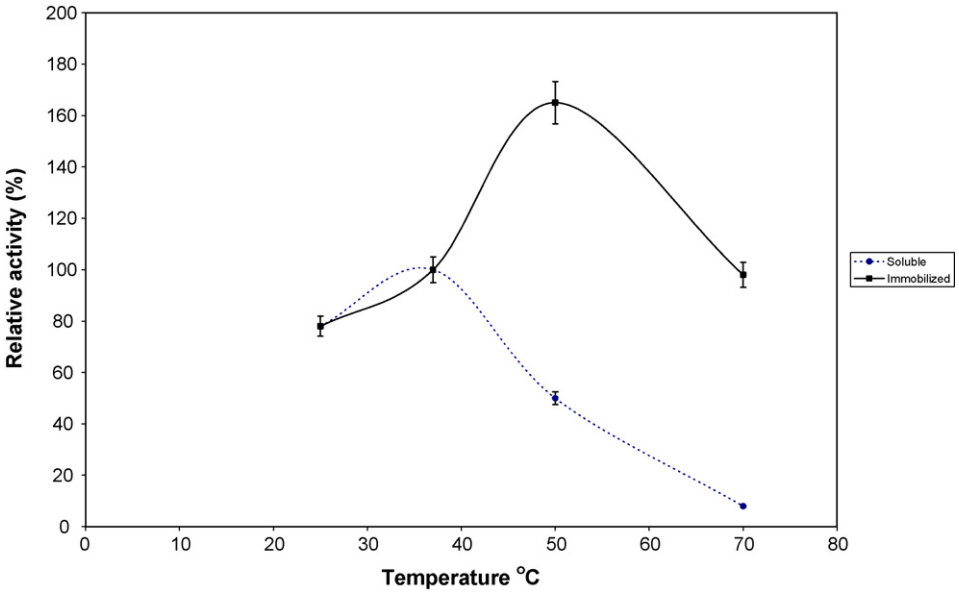

Fig. 2. Thermal stability of soluble and immobilized α-chymotrypsin. The residual activity after incubation at 20–70 °C for 30 min was determined; relative activity was expressed in % of the activity determined after incubation at 37 °C; determination of the enzyme activity was carried out in four parallel measurements, differences between obtained values were below 5%; assay medium: 0.1 M Tris–HCl buffer pH 7.8 containing 0.025 M calcium chloride (500 μl), N-succinyl-L-phenylalanine-4-nitroanilide solution (50 μl) in N,N′-dimethylformamide (1 mg in 0.1 ml), α-chymotrypsin (330 μg) or immobilized enzyme (100 μl of suspension of magnetic particles); incubation at 37 °C for 10 min.

Daltonics, Bremen, Germany) with Mascot (Matrix Sciences, London, UK) as a search engine and Swiss-Prot (Swiss Institute of Bioinformatics, Geneva, Switzerland) as a database were used to identify peptides from tandem mass spectra. The peptide mass tolerance was set to 150 ppm, the tandem mass tolerance was set to 0.7 Da, and missed cleavage was set to 5.

3. Results

3.1. Immobilization of α-chymotrypsin

α-Chymotrypsin was immobilized to magnetic particles (Magnetic Glyoxal 4% Agarose Beads 20–75 μm) by coupling free amino groups of the enzyme to active –CHO substituents of the magnetic carrier. The reduction with sodium cyanoborohydride results in the formation of stable linkage. The amount of the enzyme coupled to magnetic particles under the used experimental conditions was in the repeated experiments in the range from 15 to 18 mg/ml of the magnetic particle suspension. The specific activity of the immobilized α-chymotrypsin was 0.58 U/mg of protein as determined with N-succinyl-L-phenylalanine-4-nitroanilide as a substrate.

3.2. Properties of immobilized α-chymotrypsin

Comparison of the pH dependence of activity of soluble and immobilized forms of α-chymotrypsin presented in Fig. 1 did not show any effect of the immobilization procedure on the enzyme pH optimum. Contrary to these findings, coupling of the enzyme to magnetic particles significantly increased thermo-stability of the studied enzyme (Fig. 2). After incubation of the enzyme immobilized to magnetic particles at 50 °C for 30 min, its activity increased up to 150% as compared with that at 37 °C, while the activity of the soluble enzyme form reached only 50% of the original activity.

The possibility of repeated use of proteinases coupled to magnetic particles is an important factor in application of these immobilized enzymes for the protein digestion. The activity of α-chymotrypsin coupled to magnetic particles did not significantly change after 12 repeated uses (R.S.D. = 2.4%) (Fig. 3). Similarly, the immobilized enzyme fully retained its activity in suspension after 2 months of storage at 4 °C (not shown).

Besides that, the prolonged shaking of the suspension of magnetic particles containing immobilized α-chymotrypsin during the incubation had no effect on the activity of the immobilized enzyme (not shown).

3.3. RP-HPLC analysis of porcine pepsin A peptides obtained by digestion with immobilized α-chymotrypsin

RP-HPLC of peptides of porcine pepsin A obtained by α-chymotryptic cleavage with the enzyme immobilized to magnetic particles is shown in Fig. 4. Control experiments showed that peaks

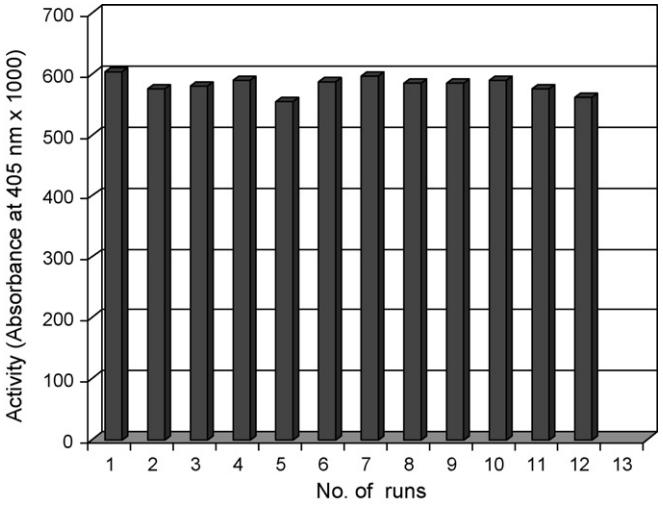

Fig. 3. Re-usability of α-chymotrypsin immobilized to magnetic particles. The enzyme activity expressed in absorbance measured at 405 nm corresponding to an amount of the product of the enzyme reaction; assay medium: 0.1 M Tris–HCl buffer pH 7.8 containing 0.025 M calcium chloride (500 μl), N-succinyl-L-phenylalanine-4-nitroanilide solution (50 μl) in N,N′-dimethylformamide (1 mg in 0.1 ml), α-chymotrypsin (330 μg) or immobilized enzyme (100 μl of suspension of magnetic particles); incubation at 37 °C for 10 min.

eluted in the retention time interval of 0–22 min were associated to buffer components (urea, iodoacetamide, dithiothreitol). Peaks with the retention times higher than 25 min corresponded to the porcine pepsin A peptides and the self-cleavage fragments of α-chymotrypsin.

Comparison of self-cleavage peptides obtained using only soluble or immobilized α-chymotrypsin is shown in Fig. 5. Immobilization of the enzyme to magnetic particles via its amino groups significantly decreased its self-cleavage activity.

3.4. Separation of phosphopeptides by IMAC with immobilized Fe(III) ions and MALDI-TOF mass spectrometric analysis

Affinity chromatography on immobilized Fe(III) ions (IMAC, IDA-Sepharose-Fe(III)) was used for adsorption of phosphopeptide containing fractions of porcine pepsin A obtained by RP-HPLC. Fractions obtained by RP-HPLC were pooled in 5 min intervals; each of them was separated by IMAC. Peptides adsorbed to immobilized Fe(III) ions were eluted with 0.05 M phosphate buffer pH 7.2 and analyzed by MALDI-TOF mass spectrometry. In MS spectra, the presence up to three potential phosphopeptide ions (*m*/*z* 857; 1321; 1720; 0-1 missed cleavage) derived form porcine pepsin A were assumed ([33], Expasy-PeptideMass, Swiss Institute of Bioinformatics, Switzerland). However, only phosphopeptide ion *m*/*z* 1321 was detected in RP-HPLC fractions and it was eluted in the retention time interval of 30–35 min (Fig. 6).

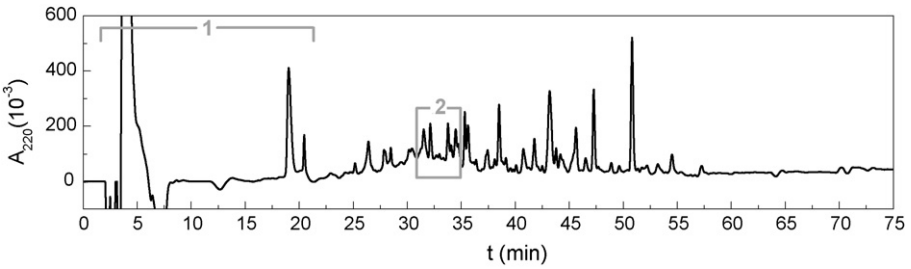

Fig. 4. RP-HPLC separation of porcine pepsin A peptides obtained by proteolytic digestion with α-chymotrypsin immobilized to magnetic particles. (1) Peaks corresponding to buffer components (urea, iodoacetamide, dithiothreitol, ammonium bicarbonate, sodium acetate); (2) the peptide fraction containing phosphopeptide; experimental conditions of the RP-HPLC separation in Section 2.5.

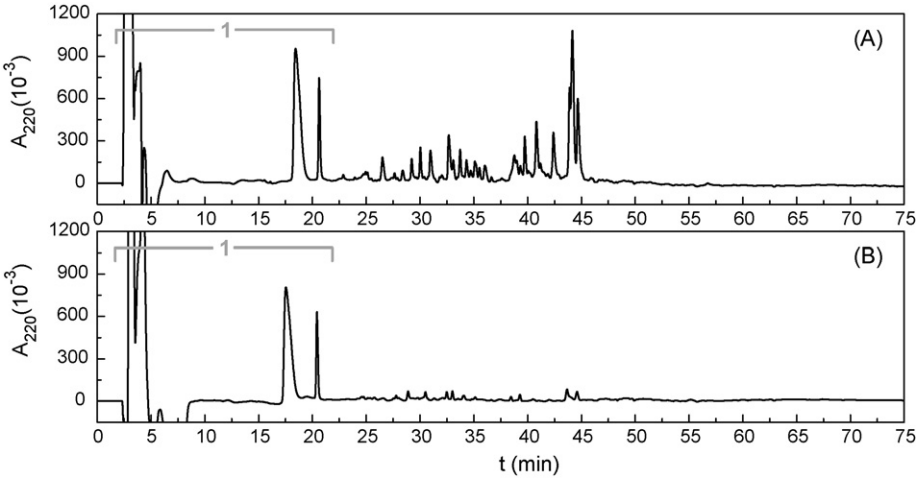

Fig. 5. Comparison of RP-HPLC of self-cleavage peptides of soluble (A) and immobilized (B) α-chymotrypsin. (1) Peaks corresponding to buffer components (urea, iodoacetamide, dithiothreitol, ammonium bicarbonate, sodium acetate); experimental conditions of the RP-HPLC separation in Section 2.5.

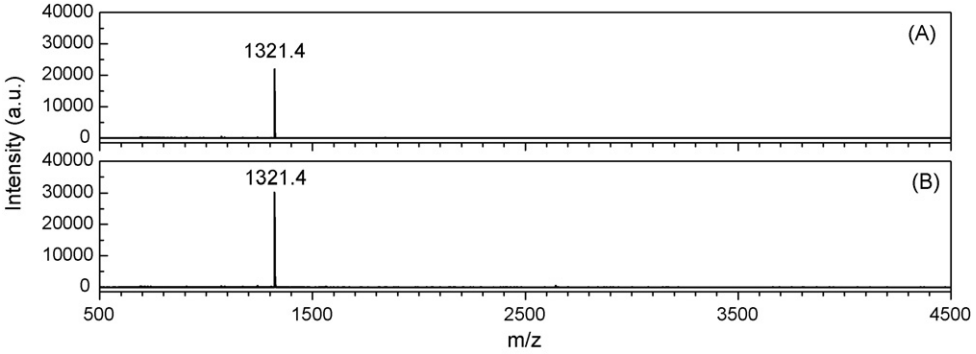

Fig. 6. MALDI-TOF MS analyses of peptides isolated by IMAC-Fe(III) after RP-HPLC separation of α-chymotryptic digest of porcine pepsin A. RP-HPLC fractions collected in the retention time interval of 31–35 min (A) or in the 34th min (B) were separated by IMAC-Fe(III); MS spectra were recorded in the mass range of *m*/*z* 500–4500 using positive ionization mode.

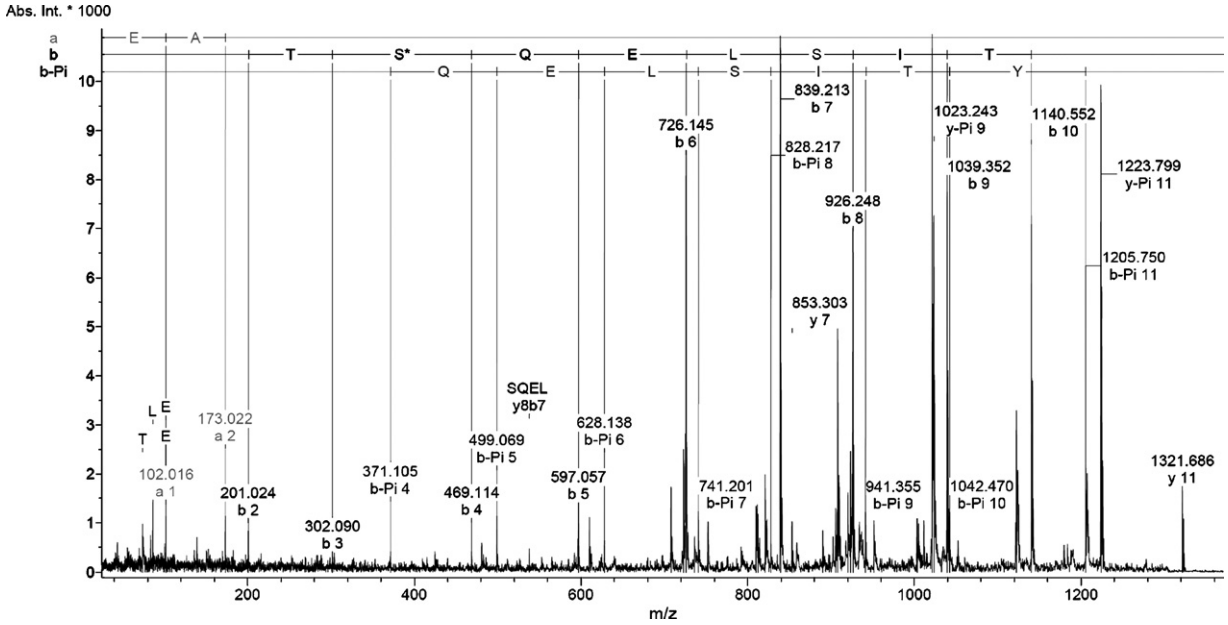

Fig. 7. MALDI-TOF/TOF MS spectra of the parent ion *m*/*z* 1321.7 separated from α-chymotryptic digest of porcine pepsin A. The amino acid sequence coverage is shown by 'a' and 'b' and (b-Pi) fragment ion series. A prominent loss of 98.0 Da (*m*/*z* 1223.8) indicates monophosphorylation. The mass difference 167.0 Da between fragment ions b4 (*m*/*z* 469.1) and b3 (*m*/*z* 302.1) corresponds to a phosphoserine residue, indicating the phosphorylation site at first serine.

3.5. MALDI-TOF/TOF MS analysis

MALDI-TOF/TOF MS analysis was used for the identification of the peptide ion of m/z 1321 (Fig. 7). The sequence of peptide ion was identified using Mascot and the Swiss-Prot database as EATS^{phos}QELSITY containing one phosphorylated serine residue. The determined sequence corresponds to residues 124–134 of porcine pepsinogen A with phosphorylated serine residue at the position 127. Mascot assigned a score of 79 for the matched sequence (EATS^{phos}QELSITY) that was above the statistical threshold of 37 for identity/extensive homology.

4. Discussion

Magnetic particles represent a valuable tool, not only because they facilitate rapid and easy separation by magnetic field but also they serve as very suitable carriers for enzymes used in proteomic studies, in modifications of proteins in vitro, etc. The use of enzymes immobilized to magnetic carriers has several advantages as compared with an application of soluble forms of enzymes: e.g. an increased stability of enzymes under different conditions, a decreased self-cleavage activity of the used proteinase, an easy removal of enzymes from a reaction mixture, a possibility of the enzyme repeated use, a possibility of direct use of enzyme reaction products for MALDI-TOF MS analysis or facilitation of the purification of a reaction product.

In the present paper, α-chymotrypsin immobilized to commercial magnetic particles has been used for the proteolytic digestion of porcine pepsin A before phosphopeptide analysis. In contrast to trypsin, much less attention has been paid to immobilization of α-chymotrypsin. This fact is related to lesser frequency of the use of α-chymotrypsin for the protein proteolytic digestion for the peptide analysis. As was already mentioned, trypsin (and also Lys C and Arg C proteinase) is not possible to use in the case of acidic proteins, due to low content of basic amino acid residues in these proteins. Therefore, we have used α-chymotrypsin in our studies of the phosphorylation state of aspartate proteinases.

α-Chymotrypsin was immobilized to various sorbents [26,27,29,30,34,35]. For the immobilization of this enzyme to magnetic particles of different types, either free amino or carboxyl groups of the protein were used that were coupled to carboxyl or amino groups, resp., being linked to magnetic particles. The carbodiimide reaction was used for coupling in both cases [26,30].

In our studies, we have employed –CHO activated commercial particles (Magnetic Glyoxal 4% Agarose Beads) and modified coupling reaction conditions that enabled very simple protein immobilization. α-Chymotrypsin was linked in this case to magnetic particles via its amino groups. Comparison of pH optimum of the immobilized enzyme with that of the soluble form did not show any difference, but the immobilized proteinase retained higher activity at pH far from pH optimum. Similar results were also described by Hong et al. [26,30].

The prepared immobilized enzyme was characterized by significantly lower self-cleavage activity that is very important for its use in proteolytic digestion of proteins for the preparation of peptide maps. Moreover, α-chymotrypsin coupled to magnetic particles exhibited higher thermo-stability and the immobilized enzyme could be repeatedly used without loss of the activity; similar results were described previously for immobilized α-chymotrypsin [26,30] or trypsin [23,36,37]. In the case of increased thermal stability of immobilized α-chymotrypsin, this observation was explained by an enzyme aggregation on the support and by an enhancement of hydrophobic interactions [38]. An increased stability of proteinases immobilized to solid supports via free amino groups of the protein were found to be similar to those of soluble enzymes with chemically modified lysyl residues by glycosylation [20,39,40], reductive methylation [41,42], or acetylation [36]. Both types of stabilized proteinases are used for the preparation of peptide maps.

5. Conclusions

α-Chymotrypsin was immobilized to commercial activated magnetic particles and its properties were compared with those of soluble enzyme. The immobilization resulted in an increased thermal stability of the enzyme, in a decrease of self-cleavage activity of the proteinase and the coupled enzyme retained its activity after repeated use.

Porcine pepsin A was used in our study as a model phosphoprotein for the elaboration of a simple procedure for the separation and analysis of phosphopeptide fraction of other proteins. This approach can be applied, e.g. to the study of the phosphorylation state of human pepsinogens and pepsins that is important esp. from the diagnostic point of view.

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