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Reversed-phase high-performance liquid chromatography of peptides of porcine pepsin prepared by the use of various forms of immobilized α-chymotrypsin

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Abstract

Reversed-phase high-performance liquid chromatography (RP-HPLC) separation was used for the comparison of peptide maps of pepsin after its digestions by different forms of immobilized α-chymotrypsin. Porcine pepsin was hydrolysed with soluble α-chymotrypsin, with α-chymotrypsins glycosylated with lactose or galactose coupled to hydrazide derivative of cellulose, with α-chymotrypsin attached to poly(acrylamide-allyl glycoside) copolymer or to glycosylated hydroxyalkyl methacrylate copolymer Separon or to agarose gel Sepharose 4B. Efficiency of enzymatic protein cleavage with regard to peptide mapping of porcine pepsin has been examined by the use of α-chymotrypsins immobilized by different methods. Best results were achieved after hydrolysis with α-chymotrypsin immobilized on poly(acrylamide-allyl glycoside) copolymers. α-Chymotrypsin immobilized by this way has further three times higher relative specific activity in comparison with the soluble one. Modified α-chymotrypsin was not suitable for efficient pepsin cleavage.

Keywords: Peptides; Pepsin; α-Chymotrypsin

1. Introduction

Procedures for micro-scale analysis of proteins by peptide mapping have been developed which allow peptide maps to be obtained from picomole to femtomole quantities of proteins. Cobb and Novotny [1] determined peptide maps from tryptic digest of phosphorylated and dephosphorylated forms of β-casein by use of capillary zone electrophoresis or microcolumn HPLC. Peptide maps, obtained by enzymatic cleavage of proteins using immobilized proteinases, can be employed for a range of purposes: (a) detection of pathological changes of proteins occuring in physiological fluids in low concentrations (microgram amounts), (b) detection of posttranslation protein modification, (c) identification and localization of genetic variants, and (d) quality control and monitoring of genetically engineered protein products.

The use of immobilized proteinases overcome several disadvantages associated with enzymatic hydrolysis reactions performed in homogeneous free solutions consisting of a mixture of the proteolytic enzyme and the protein under investigation. First of

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all, the eluted digest is totally free from contamination because autolysis does not occur. The ability to easily separate immobilized enzymes from the reaction mixture offers the multiple use of the enzyme resulting in reduced cost. Immobilized enzymes can be more stable and can retain their catalytic activities for a much longer time than free enzymes .

Optimization of immobilized enzyme hydrolysis by use of peptidases, including chymotrypsin, thermolysin, trypsin, V_8 protease and carboxypeptidases A, B and Y, combined with HPLC/thermospray mass spectrometry was described for the determination of neuropeptides by Voyksner et al. [2]. Hydrolysis by chymotrypsin or thermolysin resulted in a complex, information-rich spectra due to the greater range of peptide bonds hydrolyzed by these two endopeptidases. The result of this publication is that the activity of the immobilized enzyme plays an important role in the quality of the spectra. Immobilized enzyme activity is dependent on pH, temperature, solvents, and buffers used for the hydrolysis.

Activity of the immobilized enzyme in a bioreactor is dependent not only on the operational conditions, but also on characteristics of the immobilization process, support, column size and sample concentration. An analytical procedure involving trypsin immobilized on the surface of a 50-μm I.D. fused-silica capillary has been developed for on-line digestion of protein by Amankwa and Kuhr [3,4]. They were able to perform on-line digestion and separation of picomole quantities of denatured β-casein by capillary zone electrophoresis in less than 3 h.

The combination of digestion by immobilized proteinases and liquid chromatography/mass spectral analyses was developed by Stachowiak et al. [5] for rapid protein sequencing. This method was demonstrated using basic pancreatic trypsin inhibitor as the substrate and trypsin, chymotrypsin, aminopeptidase M, and carboxypeptidase B and Y as the immobilized peptidases. The positions of all of the tryptic and chymotryptic fragments of the trypsin inhibitor could be deduced, and fully one-half of the amino acid residues could be assigned to their correct positions, none of which required over an hour of instrument time.

Based on the known primary structure of pepsin Hynek et al. [6] elaborated peptide mapping of porcine pepsin by use of digestion with soluble α-chymotrypsin and characterization by HPLC or capillary zone electophoresis. Vaňková et al. compared immobilized and soluble α-chymotrypsin for this purpose [7]. Glycosylation of trypsin described by Vaňková et al. [8] and coupling of enzymes through carbohydrate moiety [9] increased the enzymatic stability.

This paper reports the comparison of peptide maps of porcine pepsin after hydrolysis by α-chymotrypsin immobilized by different methods. In order to obtain high stability of proteinase used for hydrolysis of pepsin glycosylated α-chymotrypsin was coupled to hydrazide derivative of bead cellulose or α-chymotrypsin to solid supports containing saccharide residues.

2. Materials and methods

2.1. Materials

α-Chymotrypsin from bovine pancreas, porcine pepsin, *N*-succinyl-L-phenylalanine-*p*-nitro-anilide, dithiothreitol, iodoacetamide, acetonitrile and trifluoroacetic acid were products of Sigma (Prague, Czech Republic). Specific inhibitor benzyloxycarbonyl-glycyl-D-phenylalanine was obtained from the Institute of Organic Chemistry and Biochemistry of Acad. Sci. of CR (Prague, Czech Republic). Tessek (Prague, Czech Republic) provided the glycosylated Separon, the hydrazide derivate of cellulose was kindly donated by M.J. Beneš from the Institute of Macromolecular Chemistry of Acad. Sci. of CR (Prague, Czech Republic) and poly (acrylamide-allyl glycoside) copolymers by M. Tichá from the Department of Biochemistry, the Faculty of Nature Sciences Charles University (Prague, Czech Republic). Pharmacia (Uppsala, Sweden) was the supplier of Sepharose 4B. Other chemicals were of reagent grade.

2.2. Modification of α-chymotrypsin by reductive amination

Reductive amination was used to couple saccharide residues to α-chymotrypsin in the presence of its inhibitor. The modified method described by

Vaňková et. al. was applied [7]. Inhibitor benzyloxycarbonyl-glycyl-D-phenylalanine (21 mg), a saccharide (400 mg lactose or galactose) and natrium cyanoborohydrate (10 mg) were added to the solution of α-chymotrypsin (100 mg) in phosphate-buffered saline (PBS) pH 7.5 (2 ml). The solution was incubated at 37°C for 72 h, and then the reaction was stopped by acidification to pH 4.5 using 50% acetic acid. Glycosylated α-chymotrypsin was separated using gel permeation chromatography on Sephadex G-25.

2.3. Periodate-oxidation of carbohydrate moiety of glycosylated α-chymotrypsin

Glycosylated α-chymotrypsin (8 mg) was dissolved in acetate buffer (5 ml, pH 4.5), then 0.5 ml of 0.1 *M* $NaIO_4$ was added. The reaction mixture was stirred in the dark for 20 min, at 4°C. The reaction was stopped by the addition of ethylene glycol (2.5 ml).

2.4. Coupling of α-chymotrypsin containing oxidized carbohydrate to hydrazide derivative of cellulose

The solution of oxidized glycosylated α-chymotrypsin (8 mg of original α-chymotrypsin, 5 ml) was added to hydrazide derivative of cellulose (5 ml of wet matrix) equilibrated with 0.1 *M* acetate buffer pH 4.5. The suspension was kept for 20 h at 4°C in dark, then natrium borohydrate was added (8 mg in 2 ml of water). The solid material after standing 3 h at 4°C, was repeatedly washed with water and then kept in acetic solution pH 4.5.

2.5. Periodate-oxidation of carbohydrate moiety of supports

Supports containing saccharide residues — poly (acrylamide-allyl glycoside) copolymers, or glycosyl Separon, or Sepharose 4B — (approximately 5 ml of wet gel in all cases) were equilibrated with 0.1 *M* $NaHCO_3$ (pH 9), then 0.1 *M* $NaIO_4$ (10 ml) was added. The reaction mixtures were stirred in the dark for 30 min. The reaction was stopped by the addition of ethylene glycol (8 ml). The oxidized supports were washed with the 0.1 *M* $NaHCO_3$ (pH 9).

2.6. Coupling of α-chymotrypsin to supports containing oxidized carbohydrate moieties

The solution of α-chymotrypsin (100 mg in 10 ml 0.1 *M* $NaHCO_3$) was added to the oxidized supports (5 ml). The suspensions were kept for 20 h at 4°C in dark, then the solution of sodium borohydride was added (8 mg in 2 ml of water). After standing for 3 h at 4°C, the obtained gels were repeatedly washed with distilled water and then kept in diluted acetic acid solution (pH 3.5).

2.7. Determination of immobilization efficiency and α-chymotrypsin activity

The amount of immobilized α-chymotrypsin was determined after its complete hydrolysis (6 N HCl, 110°C, 20 h) and by amino acid analysis of the hydrolyzate by Durrum D-500 amino acid analyzer.

The efficiency of immobilization is defined as ratio of the amount of attached enzyme to the amount of enzyme applied for immobilization. The specific activity of α-chymotrypsin was determined using *N*-succinyl-phenylalanine-*p*-nitroanilide [10]. Relative specific activity, defined as the ratio of actvity of identical amounts of attached and soluble enzyme, was calculated. The corrected specific activity is the product of the relative specific activity and the ratio of amount of attached and used enzyme.

2.8. Hydrolysis of pepsin by immobilized α-chymotrypsin

α-Chymotryptic digests of porcine pepsin were prepared by modification of the method developed by Hynek et al. [6]. The solution of pepsin (2 mg in 2 ml of 0.4 *M* NH_4HCO_3 containing 8 *M* urea) was incubated with the dithiothreitol solution (200 μl of 0.045 *M* aqueous solution) at 50°C for 15 min. After cooling to a laboratory temperature, the solution of iodoacetamide (200 μl of 0.1 *M* aqueous solution) was added and the mixture was incubated at 25°C for 10 min. After dilution of the reaction mixture with distilled water (5.6 ml), immobilized enzyme (1 mg of bound α-chymotrypsin) was added. The mixture (final pH 8.3) was incubated at 37°C for 24 h. The reaction was stopped by acidification to pH 4.5 using

Table 1
Characteristics of modified α-chymotrypsin immobilized on a hydrazide derivative of cellulose

Type of support	Amount of immobilized α-chymotrypsin [μmol/g]	Efficiency of immobilization [%]	Relative specific activity [%]	Corrected specific activity [%]
gal-chymotrypsin	0.05	3.5	13.7	0.6
lac-chymotrypsin	0.12	2.2	77.6	2.7

50% acetic acid. The immobilized enzyme was removed from a solution of the peptide fragments using sintered glass filter. The control peptide map was prepared with soluble α-chymotrypsin (1 mg).

2.9. Reversed-phase HPLC separation

Peptides were separated on an Ecom Liquid Chromatograph (Ecom, Prague, Czech Republic) with a reversed-phase column a SGX C_{18} (5 μm, 250×4 mm I.D., Tessek, Prague, Czech Republic). The injection volume was 200 μl and the flow-rate was 1.0 ml/min. Solvent A was trifluoroacetic acid–water (0.1:99.9, v/v) and solvent B consisted of solvent A and acetonitrile (40:60, v/v). The linear gradient consisted of a 0–75% solvent B (60 min). Peptides were detected at 220 nm.

3. Results and discussion

3.1. Characteristics of immobilized preparations

The main characteristics of immobilized preparations are shown in Tables 1 and 2. The comparison the of modified and unmodified α-chymotrypsin demonstrates that the use of modified enzymes lead to supports with the worst characteristics (significantly lower amount of immobilized α-chymotrypsin and the lowest corrected specific activity). On the other hand it is evident (Table 2) that α-chymotrypsin immobilized on β-lactosyl polyacrylamide copolymer give the best results (significantly higher value of all parameters). Proteinases attached to membrane in living cell have also higher activity (several times) in comparison with soluble enzyme. Differences in hydrophobicity attached and soluble enzymes plays an important role. α-Chymotrypsin attached to supports with higher rigidity (polyacrylamide, Sepharose) has higher activity in comparison with attached to Separon. Supports containing lactosyl residues showed better results than those with galactosyl residues (Tables 1 and 2).

3.2. The peptide maps of porcine pepsin after hydrolysis with different types of α-chymotrypsin

The peptide maps were obtained by HPLC separation of peptides prepared by hydrolysis with soluble α-chymotrypsin or with different types of immobilized α-chymotrypsin. The chymotryptic maps for the porcine pepsin are shown in Figs. 1–5. The peaks associated with buffer components, urea, iodoacetamide and dithiothreitol were eluted first

Table 2
Characteristics of unmodified α-chymotrypsin immobilized on different supports containing saccharide residues

Type of support	Amount of immobilized α-chymotrypsin [μmol/g]	Efficiency of immobilization [%]	Relative specific activity [%]	Corrected specific activity [%]
α-D-gal-PAA	4.17	29.9	59.1	17.7
β-lac-PAA	2.40	14.27	306.0	79.3
gal-Separon	0.20	6.1	29.8	1.8
lac-Separon	0.20	4.6	68.8	3.3
Sepharose 4B	0.33	0.9	147.0	1.5

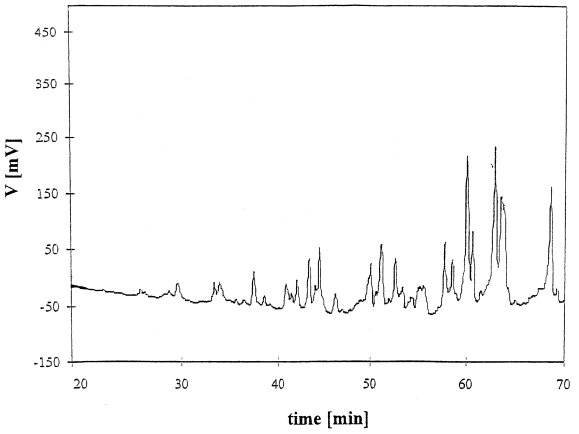

Fig. 1. RP-HPLC peptide map of porcine pepsin after hydrolysis by soluble α-chymotrypsin. For separation conditions see the text.

(0–15 min). The time interval of separated peptide fragments of pepsin is extended from 25 to 70 min.

The peptide map obtained after hydrolysis of pepsin with soluble α-chymotrypsin shows (Fig. 1) many peaks between 25 and 70 min. The peptide maps of pepsin hydrolyzed by the use of glycosylated α-chymotrypsin attached to hydrazide derivative of cellulose (Fig. 2) demonstrate only few small peaks. The peptide maps of pepsin after hydrolysis with α-chymotrypsin immobilized on poly-(acrylamide-allyl glycoside) copolymers (Fig. 3) display large amount of well resolved peaks. The peptide maps of pepsin prepared by the hydrolysis with α-chymotrypsin attached to glycosyl Separons are shown in Fig. 4. These profiles are very similar to map obtained with the use of soluble enzyme (Fig. 1). The number and height of the peaks of peptide fragments after hydrolysis of pepsin with α-chymotrypsin attached to Sepharose 4B (Fig. 5) are significantly reduced in comparison with soluble enzyme (Fig. 1).

3.3. Comparison of different types of immobilized α-chymotrypsin

From the comparison of different immobilized derivatives of α-chymotrypsin, it is evident that the use of modified α-chymotrypsin is not suitable for this purpose. The lower amount of bound enzyme leads to lower activity (Table 1) and only a few

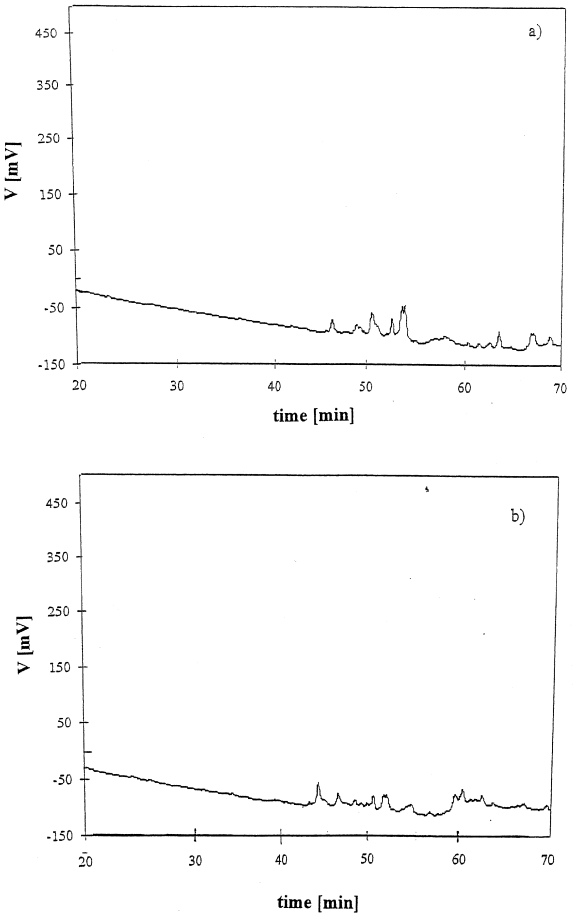

Fig. 2. RP-HPLC peptide maps of porcine pepsin after hydrolysis by α-chymotrypsin modified by galactose (a) or lactose (b) and attached to hydrazide derivate of cellulose. For separation conditions see the text.

small peaks on peptide maps (Fig. 2a, b). In addition, the use of galactose has a deleterious effect on catalytic activity.

α-Chymotrypsin immobilized on poly(acrylamide-allyl glycoside) copolymers containing glycosidically bound saccharide residues provide best results. The derivatives show high amount of bound enzyme, high corrected specific activity and efficience of immobilization (Table 2). The peptide maps of pepsin show good detectable peaks (Fig. 3a, b). Derivatives of β-lactosyl polyacrylamide copolymer have higher catalytic activity. With regard to the obtained results, it is evident that the use of this copolymer is most suitable for these purposes. It is

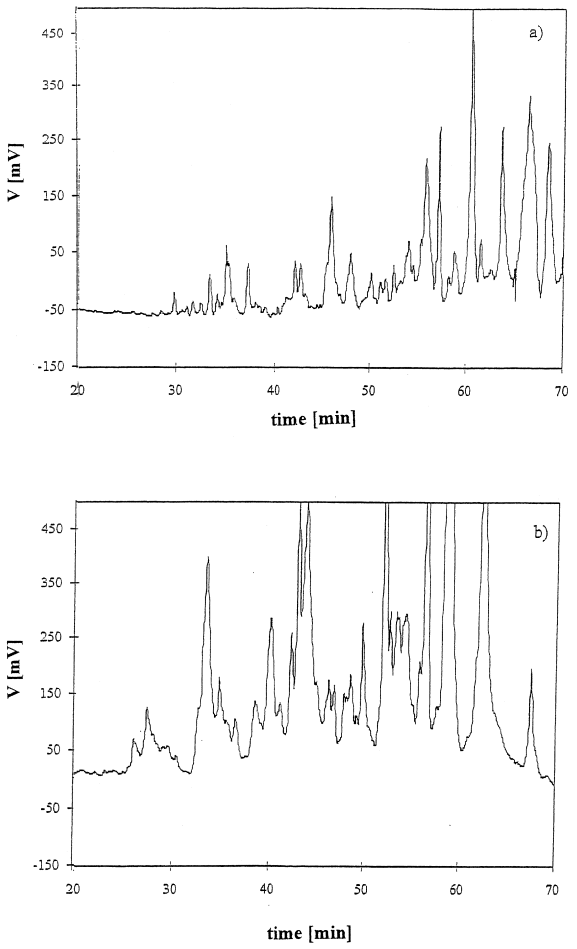

Fig. 3. RP-HPLC peptide maps of porcine pepsin after hydrolysis by α-chymotrypsin attached to either α-D-galactosyl (a) or β-lactosyl (b) polyacrylamide copolymer. For separation conditions see the text.

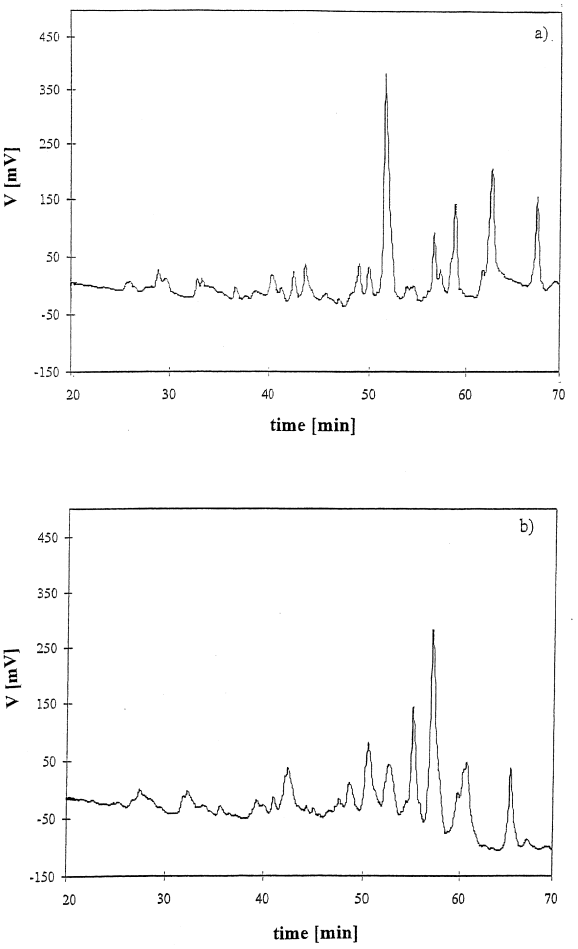

Fig. 4. RP-HPLC peptide maps of porcine pepsin after hydrolysis by α-chymotrypsin attached to Separon modified either with galactose (a) or with lactose (b). For separation conditions see the text.

probably caused by the higher stability of enzyme in the case of solid support containing disaccharide.

Both derivatives of modified Separon contain the same amount of α-chymotrypsin (Table 2). However, α-chymotrypsin attached to Separon modified with lactose provides double corrected specific activity in comparison with Separon modified with galactose.

The amount of immobilized α-chymotrypsin linked to Sepharose 4B is comparable with the amount immobilized to modified Separons, but it is significantly lower than in the case of poly (acrylamide-allyl glycoside) copolymers (Table 2). However, this derivative provided support with high catalytic activity gives significantly reduced map. (Fig. 5).

From the comparison of solid supports containing either lactosyl residues (disaccharide) or galactosyl residues (monosaccharide) (Tables 1 and 2), it is evident, that better results were provided by the supports containing lactosyl residues. It is probably caused by the size of the saccharide molecule. Galactose does not provide an adequately long spacer. Instead, it leads to steric hindrances causing

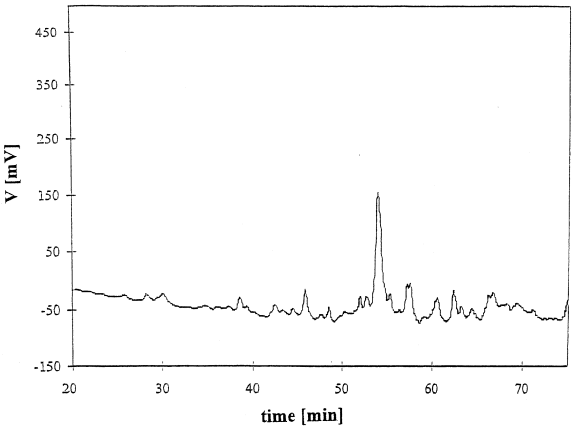

Fig. 5. RP-HPLC peptide maps of porcine pepsin after hydrolysis by α-chymotrypsin attached to Separose 4B. For separation conditions see the text.

low catalytic activity. This monosaccharide indicates lower thermostability, which is manifested during hydrolysis of pepsin (37°C, 24 h).

4. Conclusion

Efficiency of enzymatic protein cleavage with regard to peptide mapping of porcine pepsin has been examined by the use of α-chymotrypsin immobilized by different methods. Glycosylated α-chymotrypsin attached to hydrazide derivative of cellulose was not suitable for efficient pepsin cleavage. Peptide maps obtained by these α-chymotrypsin derivatives show a few small peaks only. α-Chymotrypsin attached to glycosyl Separon has very low corrected specific activity, similarly as α-chymotrypsin attached to Sepharose 4B. The best results were obtained using α-chymotrypsin immobilized on poly (acrylamide-allyl glycoside) copolymers. Supports containing lactosyl residues are more suitable than galactosyl ones. This modified support has the highest relative specific activity (three times higher in comparison with the soluble one).

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