PEPTIDE MAPS OF PORCINE PEPSIN PREPARED USING SOLUBLE AND IMMOBILIZED α-CHYMOTRYPSIN

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Reverse-phase high performance liquid chromatography (RP-HPLC) was used for characterization of peptide maps of porcine pepsin using soluble or immobilized α-chymotrypsin. The immobilized enzyme was prepared by its coupling to periodate-oxidized poly(acrylamide-*co*-allyl α-D-galactoside or allyl β-lactoside) copolymer (*O*-glycosylated acrylamide copolymer). After sodium cyanoborohydride reduction, a stable linkage was formed. Specific activity of α-chymotrypsin, linked to the copolymer containing bound α-D-galactosyl or β-lactosyl residues was 59.1 or 314.4% activity of soluble enzyme.

Key words: Immobilization; α-Chymotrypsin; Peptide maps; Reverse-phase HPLC; Porcine pepsin; Proteins; Enzymes.

Peptide mapping is a very powerful method available for detailed studies of proteins. The method involves the enzymic or chemical cleavage of a protein into a number of smaller peptide fragments followed by their separation and detection. The reverse-phase high performance liquid chromatography (RP-HPLC) is a method widely used for the separation of peptides after the cleavage of the investigated protein. Using peptide mapping it is possbile to distinguish proteins which differ in substitution of a single amino acid or even in the presence of one phosphate group[1–3]. This method might be useful to determine a composition of isoenzymogens of human pepsin. The ratio between individual human pepsins and their isozymogens is very important from a diagnostic point of view; *e.g.* low concentration of pepsinogen A in serum was found as a marker of gastric cancer[4]. The porcine pepsin studied in the present communication is used as a model protein with amino acid composition similar to human pepsin.

For the digestion of studied proteins, soluble or immobilized forms of protease can be used. The immobilized forms of enzymes offer a number of

advantages over their soluble forms; *e.g.* better reproducibility of peptide mapping, potentially higher stability of an enzyme, and absence or a decreased amount of autocatalytic products.

Polyacrylamide-based affinity carriers are useful tools both in affinity chromatography of various proteins and in the protein–ligand binding studies. Their easy preparation makes them suitable matrices for the binding of a chosen ligand with the desired degree of substitution. Polyacrylamide derivatives of simple saccharides were widely used for isolation of lectins[5,6]. Polyacrylamide-based carriers can be also used for the immobilization of proteins. Glycosylated trypsin, after its periodate oxidation, was immobilized to polyacrylamide carrier containing covalently bound amino groups[7]. The present communication describes a more general method for the immobilization of protein to the polyacrylamide carrier containing covalently bound saccharide residues. α-Chymotrypsin coupled to such polyacrylamide carrier was used for digestion of porcine pepsin.

EXPERIMENTAL

Chemicals

α-Chymotrypsin from bovine pancreas (EC 3.4.21.1), porcine pepsin (EC 3.4.23.1), α-chymotrypsin attached to macroporous acrylic beads (1 400 units/g of support), *N*-succinyl-L-phenylalanine-4-nitroanilide, dithiothreitol, iodoacetamide, acetonitrile and trifluoroacetic acid were obtained from Sigma (Prague, Czech Republic).

Preparation of *O*-Glycosylated Acrylamide Copolymers

O-Glycosylated acrylamide copolymers were prepared by copolymerization of acrylamide and an allyl glycoside in the presence of cross-linking reagent *N,N*-methylenebisacrylamide as described by Hořejší and Kocourek[6]. Allyl α-D-galactopyranoside or allyl β-lactoside were used for the copolymerization; the content of coupled saccharides was 7.5 and 8.1%, respectively.

Immobilization of α-Chymotrypsin

Periodate oxidation of O-glycosylated acrylamide copolymer. Poly(acrylamide-*co*-allyl glycoside) copolymer (approximately 5 ml of wet gel) equilibrated with 0.1 M $NaHCO_3$ (pH 9) was mixed with 0.1 M $NaIO_4$ (10 ml) and the suspension was stirred in the dark for 30 min. The reaction was stopped by addition of ethylene glycol (8 ml). The oxidized copolymer was washed with 0.1 M $NaHCO_3$ (pH 9).

α-*Chymotrypsin coupling.* The solution of α-chymotrypsin (100 mg in 10 ml 0.1 M $NaHCO_3$) was added to the oxidized acrylamide copolymer. The suspension was kept for 20 h at 4 °C in the dark, then a solution of sodium cyanoborohydride was added (8 mg in 2 ml of water) to stabilize the enzyme linkage to the support and to deactivate the residual

aldehyde groups. After standing for 3 h at 4 °C, the obtained gel was repeatedly washed with distilled water and then kept in dilute acetic acid solution (pH 3.5).

Hydrolysis of Pepsinogen with Immobilized α-Chymotrypsin

α-Chymotrypsin hydrolysis of porcine pepsin was performed according to Hynek *et al.*[2]. A solution of lyophilized pepsin (2 mg in 2 ml of 0.4 M NH_4HCO_3 containing 8 M urea) was incubated with a dithiothreitol solution (200 μl of 0.045 M aqueous solution) at 50 °C for 15 min. After cooling to laboratory temperature, 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), either soluble α-chymotrypsin (1 mg) or the preparation of immobilized enzyme (an amount of gel corresponding to 1 mg of enzyme) 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 50% acetic acid.

Reverse-Phase HPLC Separation

Peptides were separated on Ecom liquid chromatograph using an SGX C18 (5 μm) reverse-phase column (250 × 4 mm i.d.). The injection volume was 200 μl and the flow rate 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% A followed by a 0–75% solvent B gradient (60 min). Peptides were detected at 220 nm.

Analytical Methods

The α-chymotrypsin specific activity was determined using *N*-succinylphenylalanine 4-nitroanilide as a substrate[8]. Relative specific activity was expressed in percentage of the activity of the soluble α-chymotrypsin; its activity taken as 100%. The amount of immobilized α-chymotrypsin in polyacrylamide derivatives was determined after hydrolysis of freeze-dried samples (6 M HCl, 20 h, 110 °C) with a Durrum D-500 amino analyzer.

RESULTS

Coupling of α-Chymotrypsin to Periodate Oxidized O-Glycosylated Acrylamide Copolymers

Poly(acrylamide-*co*-allyl glycoside) copolymers containing glycosidically bound saccharide residues have been shown to be suitable matrices for coupling proteins *via* their free amino groups. Reactive aldehyde groups formed after periodate oxidation of saccharide residues reacted with free amino groups of α-chymotrypsin. The reduction with sodium cyanoborohydride results in the formation of a stable linkage between the protein and polyacrylamide matrix and in the deactivation of unsubstituted aldehyde groups.

Two types of immobilized α-chymotrypsin were prepared: the enzyme linked either to oxidized α-D-galactosyl or β-lactosyl residues. The amount of immobilized α-chymotrypsin on polyacrylamide matrices and its relative specific activity are shown in Table I.

The specific activity of immobilized α-chymotrypsin linked to the oxidized α-D-galactosyl residues was lower than that of the soluble enzyme. On the other hand, immobilization of α-chymotrypsin to the β-lactosyl residues resulted in an increase in the enzyme specific activity.

Application of Immobilized α-Chymotrypsin

The immobilized α-chymotrypsin was used for preparation of peptide maps of porcine pepsin. The RP-HPLC peptide maps are shown in Figs 1 and 2. The peaks eluted for up to 10 min are associated with buffer components (iodoacetamide and dithiothreitol). Peaks with retention times from 10 to 25 min corresponded to α-chymotrypsin and its self-cleavage fragments. This was proved by a pepsin-free experiment. Peptide fragments corresponding to investigated pepsin are eluted in the interval of retention times from 25 to 65 min.

Comparison of the maps showed some differences between the peptide maps of soluble and immobilized α-chymotrypsin (Figs 1 and 2). In the case of soluble α-chymotrypsin, the peptide maps contained the peaks probably corresponding to its self-cleavage fragments, which are not present in peptide maps obtained with the immobilized enzyme. The peptide maps obtained using two different preparations of immobilized α-chymotrypsin did not differ qualitatively. However, the amount of peptides formed upon hydrolysis with the enzyme immobilized to oxidized β-lactosyl residues was

TABLE I
Content and specific activity of immobilized α-chymotrypsin on the acrylamide copolymer carrier

Copolymer	Coupled protein[a], mg/g of dry gel	Specific activity[b], %
α-D-Gal-PAA	96.1	59.1
β-Lact-PAA	31.1	314.4

[a] From amino acid analysis. [b] Activity of soluble α-chymotrypsin is 100%.

much higher than that obtained with the enzyme coupled to α-D-galactosyl residues or with commercial α-chymotrypsin immobilized to macroporous acrylic beads (not shown); this fact is in agreement with values of specific activity of immobilized α-chymotrypsin (Table I).

DISCUSSION

Polyacrylamide-based carriers were shown to be useful for the immobilization of proteins. For such immobilization, two approaches can be used which are based on the same principle: saccharide residues after periodate oxidation yield active aldehyde groups which are allowed to react with amino groups; after reduction, a stable secondary amino group is formed.

In the first case of protein immobilization on polyacrylamide, glycosylated protein after periodate oxidation is bound to polyacrylamide con-

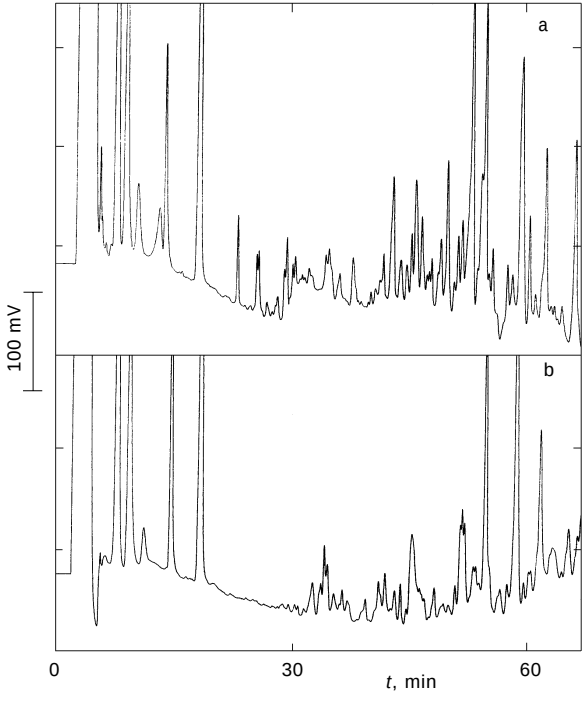

FIG. 1
Comparison of RP-HPLC peptide maps of porcine pepsin obtained with soluble α-chymotrypsin (a) or α-chymotrypsin coupled to oxidized α-D-galactosyl polyacrylamide copolymer (b)

taining covalently bound amino groups. This procedure has been used previously for the immobilization of trypsin modified by glycosylation; in this case reductive amination with reducing disaccharides was used for glycosylation[7]. A disadvantage of this approach is the necessity of glycosylation of a protein being immobilized, unless a native glycoprotein is used.

In the present communication, the second type of the procedure was used. As a general carrier, *O*-glycosylated acrylamide copolymer (α-D-galactosyl or β-lactosyl) is oxidized with periodate; the formed active aldehyde groups react with free amino groups of a protein. The resulting products of both types of the immobilization procedure differ slightly in the carbohydrate moiety.

The degree of substitution with ligands depends on the amount of oxidized saccharide residues on the polyacrylamide matrix. This amount can

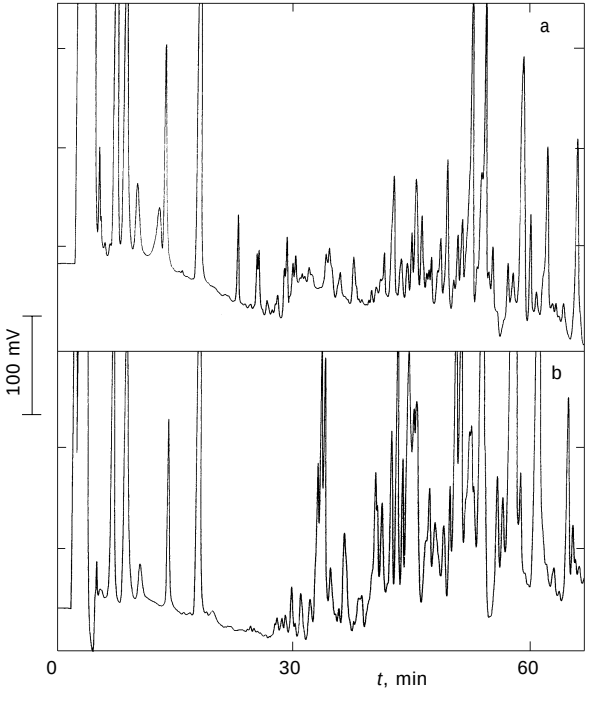

FIG. 2
Comparison of RP-HPLC peptide maps of porcine pepsin obtained with soluble α-chymotrypsin (a) or α-chymotrypsin coupled to oxidized β-lactosyl polyacrylamide copolymer (b)

be changed in two ways: (i) by changing the saccharide content in the polyacrylamide carrier (increasing or decreasing the allyl glycoside concentration in the copolymerization mixture); (ii) or by changing conditions of periodate oxidation (*e.g.* concentration of periodate, duration of oxidation).

An increased specific activity of α-chymotrypsin immobilized to oxidized β-lactosyl residues might be explained by a better steric accessibility of the enzyme binding site and also possibly by a stabilizing effect of the saccharide.

The solid matrix (poly(acrylamide-*co*-allyl glycoside) copolymer gel), originally used for the affinity chromatography of lectins[5], is a versatile carrier for coupling different types of proteins. The presence of saccharide residues can have a favorable effect on the protein stability.

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REFERENCES

1. Cobb K., Novotny M. : *Anal. Chem.* **1989**, *61*, 226.
2. Hynek R., Kašička V., Kučerová Z., Káš J.: *J. Chromatogr., Biomed. Appl.* **1996**, *681*, 37.
3. Hynek R., Kašička V., Kučerová Z., Káš J.: *J. Chromatogr., Biomed. Appl.* **1996**, *688*, 213.
4. Hollisey M. T., Dun J. A., Fielding J. W. L.: *Scand. J. Gastroenterol.* **1984**, *29*, 1129.
5. Kocourek J.: *Acta Histochem.* **1982**, *71*, 57.
6. Hořejší V., Kocourek J.: *Biochim. Biophys. Acta* **1978**, *538*, 299.
7. Vaňková H., Pospíšilová M., Tichá M., Turková T.: *Biotechnol. Tech.* **1994**, *8*, 375.
8. Nagel W., Willing F., Peshke W., Schmidt F. H.: *Hoppe-Seyler's Z. Physiol. Chem.* **1965**, *340*, 1.