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HIGH-PERFORMANCE AFFINITY CHROMATOGRAPHY OF ANHYDRO-CHYMOTRYPSIN ON A HYDROPHILIC VINYL-POLYMER GEL COUPLED WITH TRYPTOPHAN

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SUMMARY

Anhydrochymotrypsin was purified by high-performance affinity chromatography based on its increased affinity for peptides containing an aromatic amino acid at the carboxyl terminal, *i.e.*, product-type ligands of chymotrypsin. Tryptophan was attached via its α-amino group to a hydrophilic vinyl-polymer gel, Toyopearl HW65S, through a spacer of 6-aminohexanoic acid and this affinity adsorbent was used for the chromatography. A crude anhydrochymotrypsin preparation was applied at pH 5 and the adsorbed proteins were eluted with an ascending pH gradient. This procedure resulted in complete separation of anhydrochymotrypsin not only from denatured protein and the remaining active enzyme, but also from a protein species which is catalytically inactive but has some binding affinity for the ligand.

INTRODUCTION

Chemical transformation of the active-site serine of α-chymotrypsin and trypsin to dehydroalanine generates anhydrochymotrypsin[1] and anhydrotrypsin[2], respectively. These products are catalytically inactive but retain binding abilities for substrates and inhibitors. Since no bulky chemical group is introduced into the enzymes, these anhydro derivatives are of great value in studies of the catalytic mechanisms of the enzymes.

Ishii and co-workers[3,4] reported that the transformation of trypsin and α-chymotrypsin to anhydro derivatives enhances their affinities toward their own product-type ligands, *e.g.*, peptides containing Arg and Trp, respectively, with a free carboxyl group. Since this enhancement of affinity is common to the anhydro derivatives of both α-chymotrypsin and trypsin, it may be helpful in elucidating the mechanism of enzymatic action of serine proteases. On the other hand, it can be applied to the separation of peptides by utilizing immobilized anhydro derivatives[3,4]. Thus, im-

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mobilized anhydrotrypsin can be used as an affinity adsorbent for peptides having arginine or lysine at the carboxyl terminal, and immobilized anhydrochymotrypsin can be used similarly for peptides having an aromatic amino acid at the carboxyl terminal.

For such studies and applications an effective purification procedure for these anhydro derivatives is desirable. Ako *et al.*[2,5] reported the purification of the anhydro derivatives by affinity chromatography on immobilized protein proteinase inhibitors. This procedure, however, cannot separate the anhydro derivatives from residual or regenerated active enzymes. Yokosawa and Ishii[6] found that affinity chromatography on a column bearing product-type peptides of trypsin is very effective for the purification of anhydrotrypsin. The latter is completely separated from denatured proteins and active enzymes owing to its enhanced affinity for these peptides.

The utility of the hydrophilic vinyl-polymer gel Toyopearl® as an insoluble support in high-performance affinity chromatography was recently reported by our laboratory. In the present study, the use of this technique on a column of Toyopearl coupled with a product-type ligand of α-chymotrypsin allowed the complete separation of anhydrochymotrypsin from not only denatured protein and active enzyme contaminants, but also some protein species that are catalytically inactive but differ from the major anhydrochymotrypsin species in their binding affinity for the ligand.

EXPERIMENTAL

α-Chymotrypsin from bovine pancreas (crystallized three times, Type II, Lot 49C-8015), phenylmethanesulphonyl fluoride (PMSF) and 7-(N-glutaryl-L-phenylalanineamido)-4-methylcoumarin (Glt-Phe-AMC) were purchased from Sigma (St. Louis, MO, U.S.A.). Toyopearl® HW65S (exclusion limit molecular weight $5 \cdot 10^6$, particle size 20–40 μm) was obtained from Toyo Soda (Tokyo, Japan). Toyopearl is also supplied by E. Merck (Darmstadt, F.R.G.) under the trade-mark Fractogel TSK.

N-Chloroacetyl-6-aminohexanoic acid (CAAHA) was synthesized as previously described[7]. L-Tryptophan methyl ester hydrochloride was synthesized according to Boissonnas *et al.*[8]. Dimethyl sulphoxide (DMSO) was dehydrated by the use of molecular sieves 4A. Methanesulphinyl carbanion was prepared basically according to Greenwald *et al.*[9].

Enzyme activity towards N-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) was determined spectrophotometrically[10]. Protein concentrations for all forms of α-chymotrypsin were determined from the absorbance at 280 nm based on an extinction coefficient of $5.0 \cdot 10^4\ M^{-1}\ cm^{-1}$ (ref. 11).

Preparation of affinity adsorbents

The affinity adsorbent for anhydrochymotrypsin was prepared in three steps. First, an ω-carboxyl spacer was introduced onto Toyopearl by O-alkylation with CAAHA. Secondly, L-Trp methyl ester was coupled to the terminal carboxyl group of the spacer through its α-amino group by the formation of an amide bond. Thirdly, the ester moiety of the ligand was hydrolyzed with dilute alkali to form a product-type ligand of α-chymotrypsin.

The procedure for the introduction of the ω-carboxyl spacer was the same as

that previously described[7] except that CAAHA was used as the alkylating reagent in place of N-chloroacetylglycylglycine. With 36 mmol of methanesulphinyl carbanion as a catalyst, 7.2 mmol of CAAHA were used to alkylate 18 g (corresponding to 100 g wet weight) of dried Toyopearl HW65S suspended in DMSO. The content of the spacer in the product, Toyopearl HW65S-AAHA, was 55 μmol/g wet gel (from amino acid analysis).

Toyopearl HW65S-AAHA (100 g wet weight) was suspended in 100 ml of water and the pH was adjusted to 4.75 with 1 *M* hydrochloric acid. L-Tryptophan methyl ester hydrochloride, 2.56 g (10 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, 3.84 g (20 mmol), were added consecutively to the suspension with stirring. The pH of the mixture was maintained at 4.7–4.8 with 1 *M* sodium hydroxide for 30 min and the mixture was gently shaken overnight at room temperature. The gel was successively washed with 1 l each of water, 1% acetic acid containing 0.5 *M* sodium chloride, 0.1 *M* sodium phosphate buffer (pH 7.5) containing 0.5 *M* sodium chloride and water. The amount of L-Trp methyl ester recovered in the combined washings was determined by absorption measurement at 278 nm after adjustment of the pH to 7.0 with 1 *M* sodium hydroxide (ε_{278} = $5.6 \cdot 10^3\ M^{-1}\ cm^{-1}$, pH 7.0). The amount of immobilized ligand was estimated by subtraction to be 49 μmol per g wet gel.

The washed gel was suspended in 100 ml of 4 *M* 2-aminoethanol–hydrochloric acid buffer (pH 4.75). After addition of the carbodiimide, the gel was allowed to react in order to block the free carboxyl groups of any residual spacer and washed as above. In order to detach the ester function of the immobilized ligand, the gel was suspended in 100 ml of 0.1 *M* sodium hydroxide and left for 30 min at 20°C with occasional shaking. The resulting gel (Toyopearl HW65S-AAHA-Trp) was washed with 1 l each of 1% acetic acid containing 0.5 *M* sodium chloride, 0.1 *M* sodium hydroxide containing 0.5 *M* sodium chloride and water until the washing became neutral.

Anhydrochymotrypsin

Phenylmethanesulphonyl-α-chymotrypsin[12] was prepared according to Ako *et al.*[5]. PMSF was used in a ratio of 1.4 mol to 1 mol of α-chymotrypsin. The residual activity toward glutaryl-L-phenylalanine *p*-nitroanilide was less than 1% of the original activity. Phenylmethanesulphonyl-α-chymotrypsin solution (730 mg in 100 ml) was dialyzed against 1 m*M* hydrochloric acid, then 6 *M* potassium hydroxide was added to the ice-cold solution to give a final concentration of 0.1 *M*. The solution was left in an ice-bath for 1 h. The pH was readjusted to 3 with 6 *M* hydrochloric acid and the solution was dialyzed against 1 m*M* hydrochloric acid. Anhydrochymotrypsin was directly purified from this crude anhydrochymotrypsin solution by high-performance affinity chromatography on a column of Toyopearl HW65S-AAHA-Trp.

Anhydrochymotrypsin was also purified according to Ako *et al.*[13] by affinity chromatography on a column of LBI-Sepharose after treatment of the crude product with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) to inactivate the active enzyme species. The resulting product is referred to as LBI-anhydrochymotrypsin.

Alkali-treated α-chymotrypsin was prepared under the same alkaline condi-

tions as used in the conversion of phenylmethanesulphonylchymotrypsin into anhydrochymotrypsin.

High-performance affinity chromatography

Pyrex glass columns (250 × 20 mm, GCH-20; 100 × 3 mm, GCH-03) and a packer for each column (Umetani Precision Co., Osaka, Japan), an eight-way valve connector (Omnifit, U.K.), a peristaltic pump (Minipuls II, HP4; Gilson, France); an UV spectrometer (Uvidec 100-III; Japan Spectroscopic, Hachiohji, Tokyo, Japan) and a fluorescence spectromonitor equipped with a xenon lamp (RF-530; Simadzu, Kyoto, Japan) were employed.

The procedure for preparative chromatography is described here; that specific for analytical chromatography is described in the legend to Fig. 2.

Toyopearl HW65S-AAHA-Trp was suspended in an equal volume of buffer L (0.1 *M* sodium dihydrogenphosphate, 0.1 *M* boric acid, 0.1 *M* acetic acid and 0.2 *M* sodium chloride, titrated to pH 5.0 with 6 *M* sodium hydroxide). The suspension was poured into a Pyrex glass column (250 × 20 mm I.D.) equipped with a packer and the column was packed by pumping buffer L at a rate of 10 ml/min for 2 h. In the chromatography the column was operated in the opposite diretion to that in the packing. The tubing before the pump was of 1.5 mm I.D. (polyethylene) and that after the pump was of 0.5 mm I.D. (PTFE). Eluents were connected to the pump through an eight-way valve connector and pumped to the column at a rate of 4 ml/min (pressure, 1.5–2.5 kg/cm^2). The absorbance of the effluent was continuously measured at 280 nm with the UV spectrometer. All operations were carried out at room temperature (20–25°C) except for the collection of the effluents (4°C).

The container of sample solution was connected to the pump also through the eight-way valve connector. Prior to the application of a sample, the column was washed with buffer H (0.1 *M* trisodium phosphate, 0.025 *M* sodium tetraborate and 0.3 *M* sodium chloride, titrated to pH 12.5 with 6 *M* sodium hydroxide). Crude anhydrochymotrypsin solution was applied to the column equilibrated with buffer L. A solution containing 100–150 mg of protein in 40–50 ml was prepared by adding a one-tenth volume of buffer L to the solution of dialyzed crude anhydrochymotrypsin (see above). The column was washed with 350 ml of buffer L and the adsorbed proteins were eluted with an ascending pH gradient formed by the addition of buffer H to a constant-volume mixing chamber (670 ml) filled with buffer L. The effluent corresponding to the main peak eluted at pH 9–9.5 (containing anhydrochymotrypsin) was pooled and the pH of the solution was reduced to 3 by adding 6 *M* hydrochloric acid. The solution was dialyzed against 1 m*M* hydrochloric acid (3 × 5 l) at 4°C and freeze-dried. The column was ready for the next operation after irrigation with buffer L (160 ml).

On-line assay of chymotrypsin activity

Chymotrypsin activity in the effluent from affinity chromatography of anhydrochymotrypsin was measured by using the on-line assay system described previously[7]. A portion of the effluent, 0.05 ml/min, was mixed with the substrate solution, 0.15 ml/min, consisting of 20 μM Glt-Phe-AMC in 0.5 *M* Tris–HCl buffer (pH 8.0) and allowed to react by passing it through a coiled tube (4 m × 0.25 mm I.D.) at 37°C. The concentration of 7-amino-4-methylcoumarin (AMC) produced was

measured with the fluorescence spectromonitor (RF-530); excitation at 380 nm, emission at 460 nm.

RESULTS

The separation of anhydrochymotrypsin on a column of Toyopearl HW65S-AAHA-Trp is shown in Fig. 1. The dialyzed crude anhydrochymotrypsin was mixed with a buffer of pH 5 (buffer L) and applied to the column equilibrated with buffer L. A part of the protein emerged from the column without retardation. No enzyme activity was detected in this fraction by the on-line assay system with an amide substrate, Glt-Phe-AMC. However, esterase activity towards Ac-Tyr-OEt was found in this fraction. On application of the ascending pH gradient, active chymotrypsin was first detached from the column. Then anhydrochymotrypsin was eluted as a large peak after a minor one. The absence of enzyme activity towards Glt-Phe-AMC in this peak of anhydrochymotrypsin is clear from the flat baseline of the on-line assay system. The peak fraction was dialyzed and freeze-dried.

The content of dehydroalanine residue in the freeze-dried preparation was determined spectrophotometrically after the conversion of this residue into pyruvate according to the method of Weiner *et al.*[14]. The preparation of anhydrochymotrypsin produced 1.09 mol of pyruvate per mol of protein, while chymotrypsin and alkali-treated chymotrypsin produced 0.20 and 0.26 mol, respectively. When the conversion yield (about 85%) of acetyldehydroalanine to pyruvate under these conditions[14] is taken into account, it can be concluded that the preparation contains 1.0 mol of dehydroalanine per mol of protein.

The esterase activity of the purified anhydrochymotrypsin towards Ac-Tyr-OEt was as low as 0.02% of the activity of native chymotrypsin. Our LBI-anhydro-

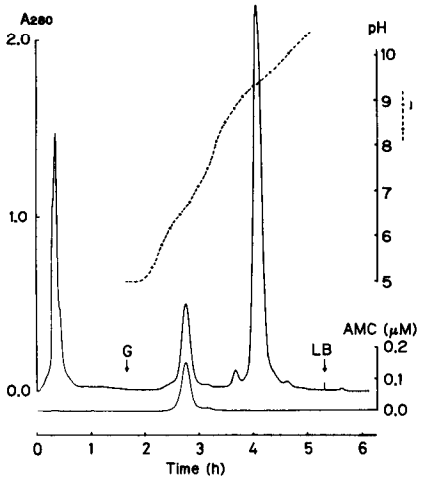

Fig. 1. Preparative high-performance affinity chromatography of crude anhydrochymotrypsin on a column of Toyopearl HW65S-AAHA-Trp. A dialyzed solution of crude anhydrochymotrypsin (45 ml, 124 mg of protein) was mixed with 4.5 ml of buffer L and applied to the column (250 × 25 mm I.D.) at time 0. Elution with a pH gradient was started at the arrow, G, and the eluent was changed back to the equilibrating buffer (buffer L) at the arrow, LB. Upper curve: A_{280}. Lower curve: chymotrypsin activity. – · – · –, effluent pH.

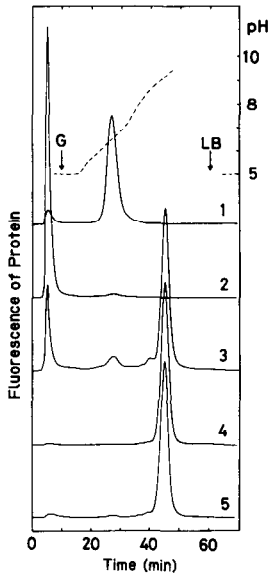

Fig. 2. Analytical high-performance affinity chromatography of anhydrochymotrypsin and related proteins. Toyopearl HW65S-AAHA-Trp was packed in a chromatographic column (100 × 3 mm I.D.) made of Pyrex glass. The column was equilibrated with buffer L at a flow-rate of 0.27 ml/min by using a peristaltic pump. Proteins (60 μg) in about 50 μl of 1 m*M* hydrochloric acid were applied to the column at time 0 from a sample injector. The elution with a pH gradient was started at 10 min (the arrow, G) as described in the Experimental section except that a mixing chamber with a volume of 6.5 ml was used. At the arrow, LB, the eluent was changed back to the equilibrating buffer. Proteins were detected by measurement of tryptophan fluorescence with the fluorescence spectromonitor (RF-530): excitation at 285 nm, emission at 340 nm. ---, effluent pH. Curves: 1, chymotrypsin; 2, phenylmethanesulphonylchymotrypsin; 3, crude anhydrochymotrypsin (100 μg); 4, anhydrochymotrypsin purified by the high-performance affinity chromatography; 5, LBI-anhydrochymotrypsin.

chymotrypsin preparation which had been subjected to treatment with TPCK prior to the affinity chromatography on the inhibitor column showed as much as 2.4% of the activity of the native enzyme.

The purified anhydrochymotrypsin and related proteins were subjected to high-performance affinity chromatography on an analytical column of Toyopearl HW65S-AAHA-Trp. The chromatographic conditions were essentially identical to those of the preparative chromatography. The chromatography was completed within 1 h with samples of less than 100 μg. Chymotrypsin was adsorbed on the column and eluted at pH 6–7 (Fig. 2, curve 1). Thus the active enzyme eluted at 2.8 h in the preparative chromatography is considered to be the native chymotrypsin which survived or was regenerated during the chemical modification process (Fig. 1). Phenylmethanesulphonylchymotrypsin was not adsorbed on the column (Fig. 2, curve 2). This shows the importance of the specific affinity of the substrate binding site of chymotrypsin for binding to the column. Fig. 2, curve 3 shows the result obtained with crude anhydrochymotrypsin. The chromatogram is almost identical to that obtained on the preparative column.

Anhydrochymotrypsin purified by preparative high-performance affinity chromatography was rechromatographed on the analytical column. It was eluted from

the column as a single peak (Fig. 2, curve 4). The majority of LBI-anhydrochymotrypsin was also eluted from the column at the same position as the anhydrochymotrypsin purified by high-performance affinity chromatography, but the presence of minor components which precede the major component is apparent (Fig. 2, curve 5). The amount of the minor component that was eluted just before the main peak varied from lot to lot of LBI-anhydrochymotrypsin and was sometimes as much as 10% of the total protein. The reason for the variability is unknown.

LBI-anhydrochymotrypsin was reported to contain a high-molecular-weight species ($M_r \approx 50\,000$) which is resolved by gel filtration on a column of Sephadex G-75[15]. The anhydrochymotrypsin purified by high-performance liquid chromatography did not show any high-molecular-weight contaminant on gel filtration, while our preparation of LBI-anhydrochymotrypsin contained about 3% of such species (data not shown).

An higher, but variable, value of ε_{240} compared to the native enzyme has been reported for freeze-dried and redissolved LBI-anhydrochymotrypsin[16]. This finding was attributed to the variable content of anhydrochymotrypsin species that has a different conformation from native chymotrypsin and a higher value of ε_{240} than chymotrypsin. Whether the separation of this subspecies can be achieved by the present method is of great concern. Anhydrochymotrypsin purified by high-performance affinity chromatography, however, had the same value of ε_{240}, $4.3 \cdot 10^4$ M^{-1} cm^{-1}, as LBI-anhydrochymotrypsin, while that of native chymotrypsin was $3.6 \cdot 10^4$ M^{-1} cm^{-1}. Therefore the present method cannot separate conformational variants of anhydrochymotrypsin which might be present.

DISCUSSION

The present high-performance affinity chromatography method, which utilizes a product-type ligand of chymotrypsin, is very effective for the purification of anhydrochymotrypsin. The separation from the active enzyme species was nearly complete. A fraction of LBI-anhydrochymotrypsin which is inactive and has lower affinity for the product-type ligand was also separated.

The binding of proteinase by a protein proteinase inhibitor such as LBI is extremely tight, with K_d values in the range of 10^{-9}–10^{-10} M. The high affinity is due primarily to the sum of many weak forces over a relatively wide region of the complementary contact surface of the two molecules[13,17]. Consequently, immobilized protein inhibitors cannot necessarily discriminate effectively between the anhydro derivatives and active enzymes. On the other hand, in affinity chromatography utilizing a product-type ligand, the interactions between the affinity ligand and protease derivatives are not so strong, but are restricted to the active sites. Thus, subtle changes in the conformation of the active site can result in sufficient differences in the affinities for the ligand to enable the separation of these protease derivatives. In addition to this advantage, the stability of these affinity adsorbents bearing low-molecular-weight ligands means that many chromatographic analyses can be carried out without any apparent change in the separation characteristics.

In anhydrochymotrypsin the configuration of the α-carbon atom of the 195th residue is changed from tetrahedral to planar by the conversion from serine into dehydroalanine. The effect of this alteration on the three-dimensional structure of

the active site would be difficult to distinguish from that brought about by elimination of the hydroxyl group of the active serine residue, and this might limit the usefulness of anhyrochymotrypsin in investigations of the role of the hydroxyl group in the catalytic function of the enzyme. From this point of view, deoxychymotrypsin, in which serine-195 is converted into alanine, is of great interest. The high-performance affinity chromatography described here could also be an effective method for the purification of deoxychymotrypsin.

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