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CHROMATOGRAPHY OF DIPEPTIDYL AMINOPEPTIDASE I ON INHIBITOR-SEPHAROSE COLUMNS

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Summary

A number of affinity materials for the purification of dipeptidyl aminopeptidase (dipeptidylpeptide hydrolase, EC 3.4.14.1) have been prepared and tested. These materials include peptide and amino acid inhibitors bound to agarose and reversible sulfhydryl adsorbents. Several of these materials are effective affinity adsorbents. The most useful material to be employed in combination with earlier purification methods is acetoxy-anilinomercuri-Sepharose. This removes proteins which are contaminants of some preparations and yields consistently high specific activities. Results with affinity and hydrophobic columns indicate that the primary interactions of the enzyme with amino acid and peptide derivative inhibitors are ionic in nature. This result is in agreement with the conclusions reached in studies of the interactions of these inhibitors with dipeptidyl aminopeptidase in solution.

Introduction

Dipeptidyl aminopeptidase (dipeptidylpeptide hydrolase, EC 3.4.14.1) has been purified to a high degree of homogeneity [1]. Some batches of enzyme have been found to contain small amounts of contaminants which are difficult to remove. The problem has been increased in our laboratory because, in an effort to obtain cathepsins B1 and B2 as well as dipeptidyl aminopeptidase from the same batches of crude enzyme, we have eliminated the heat treatment which inactivates the cathepsins B. This procecure yields dipeptidyl aminopeptidase I preparations which tend to be slightly lower in specific activity and more often contain small amounts of the contaminating protein.

While it is possible to eliminate the contaminant by careful fractional precipitation with $(NH_4)_2SO_4$ [2], the procedure is time consuming and recoveries are low. These problems, coupled with the importance of this enzyme in intra-

cellular protein degradation [3] and as a tool in protein and peptide sequencing [4], led us to explore methods for its further purification by affinity chromatography.

A number of peptide and amino acid derivatives have been shown to be effective inhibitors of dipeptidyl aminopeptidase I [5,6]. These derivatives seemed to be potential starting materials on which to base affinity adsorbents for the purification of this enzyme. Furthermore, dipeptidyl aminopeptidase is a sulfhydryl-requiring enzyme [5] and probably has a cysteine at its active site. It is known to have eight free sulfhydryls per molecule [2]. Adsorbents have been prepared [7–9] which form reversible covalent bonds to the sulfhydryl groups of proteins and are useful for the separation of proteins which contain sulfhydryl groups from those which do not. Consequently, materials of both types, peptides and amino acid derivatives and sulfhydryl specific adsorbents, were prepared and tested.

Materials and Methods

Materials. Benzoyl-L-arginine and glycyl-D-phenylalanine were obtained from Fox Chemical Co. (Los Angeles); 4-phenylbutylamine was from ICN-K and K Laboratories (Plainview, New York); 1,6-diaminohexane and [3-(dimethylamino)propyl]ethyl carbodiimide was from Eastman Kodak Co. (Rochester, New York); glycylglycine ethyl ester hydrochloride, glycyl-L-phenylalanine amide hydrochloride, bovine serum albumin and ovalbumin were from Sigma Chemical Co. (St. Louis, Mo.); trypsin was a twice-crystallized Worthington preparation. Ficin was prepared according to Englund et al. [10].

Assays. The activity was assayed by the transamidation method of Metrione et al. [1], with glycylglycine ethyl ester or glycyl-L-phenylalanine amide as the substrate. All specific activities were determined with glycyl-L-phenylalanine amide as the substrate. The protein concentration was determined by the method of Lowry et al. [11].

Preparation of affinity adsorbents. The affinity adsorbents were prepared first by coupling 1,6-diaminohexane to Sepharose 4B by the method of Cuatrecasas [7]. The product was either employed directly as an affinity column or amino acid or peptide derivatives were coupled to the amino group through a free carboxyl using [3-(dimethylamino)propyl]ethyl carboiimide [7].

The *p*-chloromercuribenzoate derivative was prepared according to Cuatrecasas [7] and the *p*-(acetoxymercuri)-anilino derivative was prepared according to Sluyterman [8]. 4-Phenylbutylamine-substituted Sepharose was prepared according to Hofstee [12].

Purification procedure. The enzyme was prepared according to Metrione et al. [1] through the G-200 fraction or CM-cellulose fraction, with omission of the heat treatment. This material was then concentrated on an Amicon ultrafiltration apparatus using a UM-10 filter and equilibrated with 0.02 M sodium phosphate buffer at pH 6.8, (containing 0.05 M mercaptoethanol in the case of columns using amino acid or peptide derivative adsorbents), by either dialysis or passage through a Sephadex G-25 column equilibrated with the buffer. This material was then applied to the affinity columns.

Results and Discussion

Columns containing L-phenylalanine, glycyl-D-phenylalanine and benzoyl-L-arginine coupled to Sepharose through an arm of diaminohexane were prepared and tested for the purification of dipeptidyl aminopeptidase (G-100 fractions). Typical elution patterns are shown in Fig. 1A, B and C. All have very similar elution characteristics. The chemical characteristics which these columns have in common is the presence of a positively charged (amino or guanidino) group. Since aminohexane has also been shown to be an inhibitor of this enzyme [6], a column of diaminohexyl-Sepharose was also run (Fig. 1D). Again the elution profile was very similar suggesting that similar interactions, i.e. ionic interactions, between column (or inhibitor) and the enzyme are involved for amino hexane and for the columns (and equivalent inhibitors) shown in Fig. 1A, B and C.

The specific activity of dipeptidyl aminopeptidase obtain by purification of the G-100 fraction (qith a specific activity of 5.4 units/mg) on L-phenylalanine-Sepharose, glycyl-D-phenylalanine-Sepharose, benzoyl-L-arginine Sepharose or diaminohexyl-Sepharose are 15—21 units/mg with recoveries of activity of 45—67%.

To test whether hydrophobic interactions are important a hydrophobic affinity column [12] was prepared and tested for the purification of dipeptidyl aminopeptidase I. The elution profile from a column of 4-phenylbutylamine-substituted Sepharose is shown in Fig. 2. The binding of dipeptidyl aminopeptidase to the hydrophobic column (4-phenylbutyl-Sepharose) was very strong. The enzyme was not eluted with 0.5 M NaCl, 0.25 M pyridine or by the inhibitor, L-phenylalanine amide [6]. It has previously been demonstrated [12] that serum albumin, ovalbumin, γ-globulin and α-chymotrypsin have similar elution characteristics. Hydrophobic affinity chromatography on this material

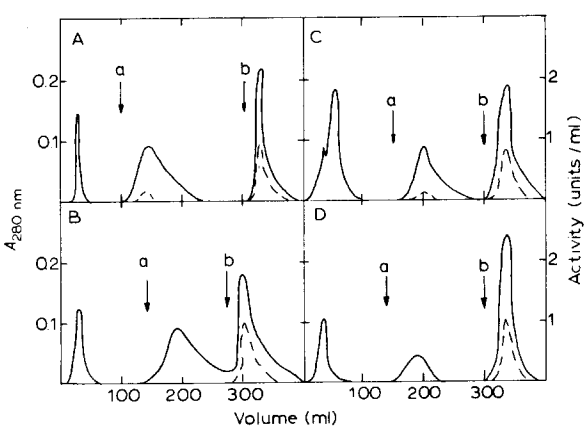

Fig. 1. Chromatography of dipeptdyl aminopeptidase on affinity columns (1.0 × 23 cm) prepared by coupling amino acid and peptide derivatives to agarose. Approx. 33 units of activity were applied to each column. A, Glycyl-D-phenylalanine-Sepharose; B, benzoyl-L-arginine-Sepharose; C, L-phenylalanine-Sepharose; D, diaminohexylSepharose. All columns were run in 0.02 M sodium phosphate buffer (pH 6.8). At (a) 0.14 M NaCl in the same buffer was begun, and at (b) 0.50 M NaCl in the same buffer was begun. The solid line is A_{280nm} and was measured on an LKB Uvicord II monitor, and the dashed line is dipeptidyl aminopeptidase activity.

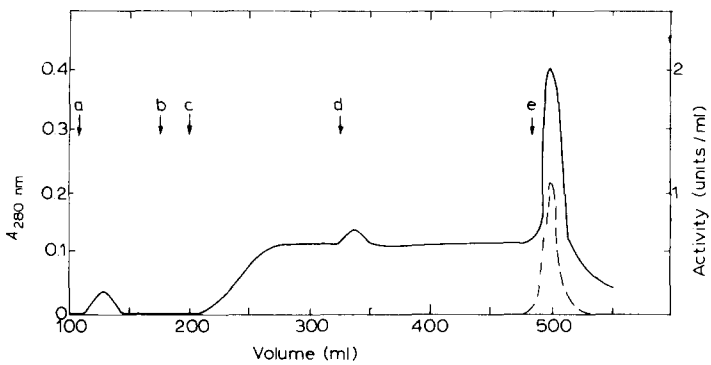

Fig. 2. Chromatography of dipeptidyl aminopeptidase on a 4-phenyl butyl-Sepharose column (1.0 × 23 cm). Approx. 33 units of activity were applied. The buffer was 0.02 M sodium phosphate (pH 6.8) and contained 0.14 M NaCl at 50 ml, (a) 0.5 M NaCl, (b) 1 mM L-phenylalanine amide (c) 0.25 M pyridine, (d) 0.25 M pyridine and 1 M NaCl and (e) 33% ethylene glycol and 1 M NaCl. The solid line is A_{280nm} and was measured on an LKB Uvicord monitor and the dashed line is dipeptidyl aminopeptidase activity.

does not appear to be a useful preparative procedure, however, since inert protein is not eluted under any conditions other than the conditions which elute dipeptidyl aminopeptidase obtained by purification of the G-100 fraction on 4-phenylbutyl-Sepharose was 10.9 units/mg with a 50% recovery.

The difference in elution characteristics between the hydrophobic affinity and the columns containing amino acid and peptide inhibitors suggests that different types of interactions are involved. Thus, hydrophobic interactions do not appear to be of major significance between amino acid- and peptide-containing columns and dipeptidyl aminopeptidase I. It therefore seems unlikely that such interactions are the major interactions in the inhibition of these enzyme derivatives of amino acids and peptides. This conclusion, that the major interaction between inhibitor and enzyme is ionic and that hydrophobic interactions are secondary, is in agreement with the conclusions reached by Metrione and MacGeorge [6] in their studies on the inhibition of dipeptidyl aminopeptidase by amino acid and peptide derivatives in solution.

The elution characteristics of a variety of proteins was tested on a column containing glycyl-D-phenylalanyl-Sepharose. Trypsin and ficin were not adsorbed to the column and eluted with the starting buffer. Ovalbumin, bovine serum albumin and beef spleen cathepsin B1 eluted with 0.14 M NaCl. It therefore seems unlikely that the affinity of these columns for dipeptidyl aminopeptidase I can be ascribed solely to non-specific ion exchange.

An attempt was also made to use 2,2'-dipyridine disulfide-glutathione-Sepharose [9] to purify dipeptidyl aminopeptidase. Although the column bound 27.8 μmol bis-dinitrodithiobenzoic acid, the enzyme did not bind to the column but eluted with the inactive protein in the initial buffer.

A series of adsorbents which are known to bind sulfhydryl-containing proteins was prepared and tested for the ability to purify dipeptidyl aminopeptidase. Both *p*-chloromercuribenzoate-Sepharose and acetoxymercurianilino-Sepharose (Fig. 3) were effective in binding the enzyme which then eluted with buffer containing 20 mM β-mercaptoethanol or 2 mM $HgCl_2$. When enzyme was applied as the CM-cellulose fraction [1], as in Fig. 3, only a single peak was

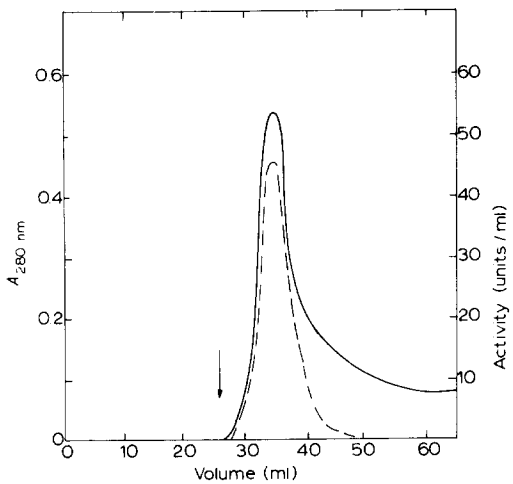

Fig. 3. The chromatography of dipeptidyl aminopeptidase on an acetoxy anilino-Sepharose column (0.5 X 17 cm). 89.6 units of activity were applied. The buffer was 0.05 M sodium phosphate (pH 7.0) and contained 20 mM mercaptoethanol where indicated by the arrow. The solid line is A_{280nm} and was measured on an LKB Uvicord monitor, and the dashed line is enzyme activity. *p*-Chloromercuribenzoate-Sepharose gave an identical profile.

eluted with mercaptan, no material being eluted with the starting buffer. Yields for such columns are 80—100% of the activity the specific activity of the material eluted from a typical acetoxymercurianilino-Sepharose column was 27.3 units/mg protein. Some material remained bound to the adsorbent, as can be seen by brown band at the top of the column. When Sephadex fraction [1] was applied to the column some material did elute with the starting buffer. Yields for these columns were 40—60% of the activity.

The procedure described for the use of acetoxmercurianilino-Sepharose is a convenient method for the elimination of small amounts of impurities which occasionally contaminate the preparations of dipeptidyl aminopeptidase purified by gel filtration and ion-exchange chromatography or to be used in place of the ion exchange columns [1]. It would not be expected that other sulfhydryl-containing proteins such as cathepsin B1 and B2 [13] and catheptic carboxypeptidase [14], would be eliminated by this procedure and these proteins must therefore be removed by other methods.

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