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A SEPHAROSE DERIVATIVE COUPLED WITH A LEUPEPTIN-LIKE PEPTIDE ALDEHYDE, GLYCYLGLYCYL-L-ARGININAL, AND ITS USE AS AN AFFINITY ADSORBENT FOR TRYPSIN

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A Sepharose derivative containing a peptide aldehyde, glycylglycyl-L-argininal, the structure of which resembles that of leupeptin was prepared It was a strong affinity adsorbent for trypsin (EC 3 4 21 4) Bovine trypsin showed higher affinity for this adsorbent at the optimum pH of catalysis (8 2) than at lower pH (5 0) This observation was in good agreement with the pH dependence of the interaction of leupeptin and trypsin (Kuramochi, H , Nakata, H and Ishii, S (1979) J Biochem 86, 1403–1410) *Streptomyces griseus* trypsin was also adsorbed while trypsinogen, α-chymotrypsin and TLCK-trypsin were not adsorbed Though anhydrotrypsin, in which Ser-183 is converted to dehydroalanine, was not adsorbed, carbamoylmethylated (His-46) trypsin was adsorbed Ser-183 proved to be essential for the binding This adsorbent can also be used as a good tool to study the mechanism of action of leupeptin

Introduction

Leupeptin, a strong competitive inhibitor of trypsin (EC 3 4 21 4), was isolated from various strains of *Streptomyces* [1,2] Its structure is acetyl- or propionyl-L-leucyl-L-leucyl-L-argininal and the aldehyde group of L-argininal residue (Argal) proved to be essential for the strong inhibition [2,3] Various derivatives of amino acid aldehydes were also found to be specific inhibitors of hydrolyzing enzymes [4–11]

In our laboratory, affinity chromatography of trypsin has been extensively studied [12–20] Affinity adsorbents which contained various sorts of product-type oligopeptides terminated with L-arginine (e g , Gly-Gly-Arg Sepharose) proved to be good tools not only for the purification of trypsin but also for the basic study of enzyme-ligand interactions If a compound similar to leupeptin in structure is immobilized on an insoluble support, it would serve as a strong affinity adsorbent for trypsin and be valuable for the elucidation of the mechanism of leupeptin inhibition Thus, we prepared an affinity adsorbent coupled with a leupeptin analogue, Gly-Gly-Argal In the present paper, the properties of Gly-Gly-Argal Sepharose and some information on the mechanism of leupeptin action are described

Materials and Methods

The following materials were obtained from the indicated sources bovine trypsin (TRL 35J884, TRL 35P697) and bovine α-chymotrypsin (CRD 35A630) (Worthington Biochemical Corp), Pronase E (Kaken Chemical Co , Tokyo), benzoyl-DL-arginine *p*-nitroanilide (Boehringer, Mannheim), glutaryl-L-phenylalanine *p*-nitroanilide (Sigma

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Abbreviations Argal, L-argininal residue, TLCK, tosyl-L-lysine chloromethyl ketone, TLCK-trypsin, trypsin inactivated with TLCK

Chemical Co), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and L-leucine *p*-nitroanilide (Protein Research Foundation, Osaka), TLCK (Cyclo Chemical Corp), Sepharose 4B (Pharmacia Fine Chemicals Co) TLCK-trypsin was prepared by the incubation of trypsin with TLCK at pH 7 2 Carbamoylmethylated trypsin was prepared by the method of Inagami [21] using iodoacetamide in the presence of methylguanidine at pH 7 0 Remaining activity (less than 5% of the original value) was diminished by the treatment with TLCK to 0 25% Thus, prepared carbamoylmethylated trypsin was used without further purification Anhydrotrypsin was prepared by Dr Yokosawa from this laboratory This was a purified product by affinity chromatography [18] Leupeptin was a gift from Nippon Kayaku Co , Tokyo Benzyloxycarbonyl-L-nitroargininal semicarbazone was synthesized according to the method of Shimizu et al [22], m p 107–110°C (lit 107–109°C [22]) It was treated with liquid hydrogen fluoride and L-argininal semicarbazone thus obtained was used without further purification

Preparation of adsorbent

Gly-Gly Sepharose was prepared by coupling of CNBr-activated Sepharose 4B with glycylglycine according to Cuatrecasas [23] Amino acid analysis showed that 8 μmol glycylglycine were immobilized per ml gel L-Argininal semicarbazone was then coupled to Gly-Gly Sepharose by the method of Cuatrecasas and Parikh [24] Gly-Gly Sepharose (20 ml) was exhaustively washed with dry dioxane and suspended in the same medium (40 ml) To this suspension, *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide were added to give each final concentration of 0 1 M After being stirred for 90 min at room temperature, the gel was washed with dry dioxane over a period of 5 min Thus obtained *N*-hydroxysuccinimide ester of Gly-Gly Sepharose was added to an equal volume of previously cooled 0 1 M sodium bicarbonate/carbonate buffer, pH 8 5, containing 50 mg L-argininal semicarbazone hydrofluoride The coupling was allowed to proceed at 4°C for 16 h The gel was finally washed with 1 M NaCl and water Immobilized L-argininal semicarbazone was calculated to be 0 74 μmol/ml gel on the basis of recovered L-argininal semicarbazone in the washings, quantitated by the colorimetry with trinitrobenzenesulfonate [25] The color yield of L-argininal semicarabazone was assumed to be equal to that of L-arginine

Assay of enzymes

Trypsin activity was measured on benzoyl-DL-arginine *p*-nitroanilide Just before assay, a stock solution of the substrate (43 5 mg in 2 ml dimethylsulfoxide) was diluted 100-fold with 0 05 M Tris-HCl, pH 8 2/0 02 M $CaCl_2$ To 1 ml of this solution, 0 1 ml of the enzyme solution was added and the mixture was incubated at 37°C After an appropriate time, 0 4 ml of 30% acetic acid in dioxane was added and the increase in absorbance at 410 nm was measured Chymotrypsin and aminopeptidase activities were assayed on glutaryl-L-phenylalanine *p*-nitroanilide and L-leucine *p*-nitroanilide, respectively, essentially as described for trypsin assay 1 unit of enzymatic activity was defined as the quantity of enzyme which increases absorbance at 410 nm of 0 1 in 15 min by the above assay methods If trypsin was eluted from the adsorbent with leupeptin, samples were treated before enzyme assay with one-tenth volume of sodium borohydride aqueous solution (10 mg/ml) at room temperature for 10 min, in order to reduce the aldehyde group of leupeptin

Operation of chromatography

Before chromatography, Gly-Gly-Argal semicarbazone Sepharose was suspended in 0 1 N HCl/1% formalin, for 2 h at room temperature, in order to remove the semicarbazone group A column packed with Gly-Gly-Argal Sepharose (usually 2 ml) was equilibrated with an appropriate buffer and 0 1 ml enzyme solution (usually 1 mg or less) was applied Elution was started with the same buffer and effluent was collected by a fraction collector Desorption of adsorbed protein was effected in several ways After each operation of chromatography, the column was stored in 1 mM HCl at 4°C until next use This is strongly recommended because exposure of the adsorbent to alkaline solution (e g , pH 8 2) caused gradual leakage of the immobilized ligand Literature has already appeared concerning the leakage of immobilized ligands from affinity adsorbents [19,26–30] All chromatography was carried out in cold (4°C)

Results and Discussion

Chromatography of bovine trypsin

When commercial trypsin was applied on a column of Gly-Gly-Argal Sepharose at the optimum pH of catalysis (8 2), over 30% of protein passed through the column (Fig 1A) No activity was observed in the pass-through fraction This fraction corresponded to inactive protein usually contained in commercial trypsin preparations Most of the adsorbed trypsin was eluted with 50 mM HCl/1 M NaCl Nearly 90% of the applied protein was usually recovered Trypsin could also be eluted mildly and effectively with a low concentration of leupeptin (Fig 2C) This elution procedure is especially useful for acid-labile enzymes such as *S griseus* trypsin (see below)

Out of 8 μmol immobilized Gly-Gly/ml, only 0 74 μmol coupled with L-argininal semicarbazone So there remained more than 7 μmol of free Gly-Gly However, in a control experiment using Gly-Gly Sepharose, trypsin showed no interaction Therefore, the remaining Gly-Gly had little effect on trypsin Gly-Gly-Argal semicarbazone Sepharose showed only weak affinity for trypsin When this semicarbazone Sepharose was unmasked by treatment with HCl/

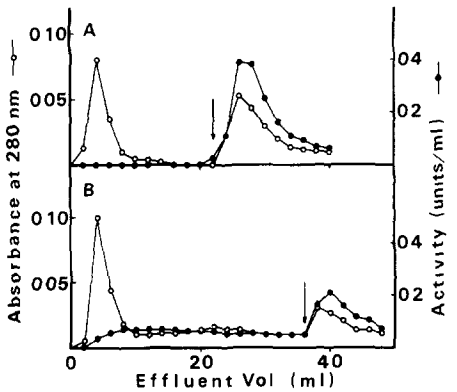

Fig 1 Chromatography of bovine trypsin on Gly-Gly-Argal Sepharose at different pH values (A) Trypsin (1 mg) dissolved in 0 1 ml of 1 mM HCl was applied on a column (0 6 × 7 2 cm) equilibrated with 0 05 M borate buffer, pH 8 2/0 02 M $CaCl_2$/0 04 M NaCl (I = 0 1) Fractions of 2-ml were collected The flow rate was 6 ml/h The eluent was changed to 50 mM HCl/1 M NaCl at the arrow Chromatography was done at 4°C (B) Trypsin (1 mg) was applied on the column equilibrated with 0 05 M acetate buffer, pH 5 0/0 02 M $CaCl_2$/0 01 M NaCl (I = 0 1) Other conditions are the same as in A

formalin, strong affinity for trypsin was generated (Fig 1A) However, this affinity was completely lost after reduction of the unmasked aldehyde group with sodium borohydride Furthermore, we have already reported that Gly-Gly-Arg Sepharose (L-arginine is located in the C-terminal) is a relatively weak affinity adsorbent for trypsin even at an optimum pH of chromatography (approx 5 0), where only retardation instead of adsorption is observed [16] Gly-Gly-Argal Sepharose showed stronger affinity though its ligand content was lower than that of Gly-Gly-Arg Sepharose (2 2–4 4 μmol/ml gel) Therefore, the free argininal moiety is essential for the strong affinity of Gly-Gly-Argal Sepharose for trypsin

When trypsin was applied on a column of Argal-Sepharose, which was prepared by direct immobilization of L-argininal semicarbazone by the CNBr method followed by HCl/formalin treatment, neither adsorption nor retardation of the enzyme activity was observed Gly-Gly seems to be important as a spacer

pH dependence

Fig 1A and B show chromatograms of trypsin on Gly-Gly-Argal Sepharose at pH 8 2 and 5 0, respectively Ionic strength was adjusted to 0 1 At pH 8 2, active trypsin was completely adsorbed, while at pH 5 0, large amounts of active trypsin leaked These observations are in good agreement with the pH dependence of leupeptin action The dissociation constants (K_d) of leupeptin-trypsin complex at pH 8 2 and 5 0 were determined to be 2.36 10^{-8} M and 4 88 10^{-7} M, respectively [31] On the other hand, when trypsin was chromatographed on Gly-Gly-Arg Sepharose, pH dependence was entirely the reverse, i e , trypsin showed higher affinity at pH 5 0 than at pH 8 2 [16]

Evidence that Gly-Gly-Argal Sepharose is an affinity adsorbent

Trypsin was hardly eluted even with 5 M NaCl, while it was eluted as a sharp peak with 8 M urea (Fig 2A) This suggests that the interaction between trypsin and Gly-Gly-Argal Sepharose is not a simple electrostatic one and that native conformation of the enzyme is essential Trypsin was not adsorbed in the presence of a low concentration of leupeptin (Fig 2B) Adsorbed trypsin was eluted with leupeptin

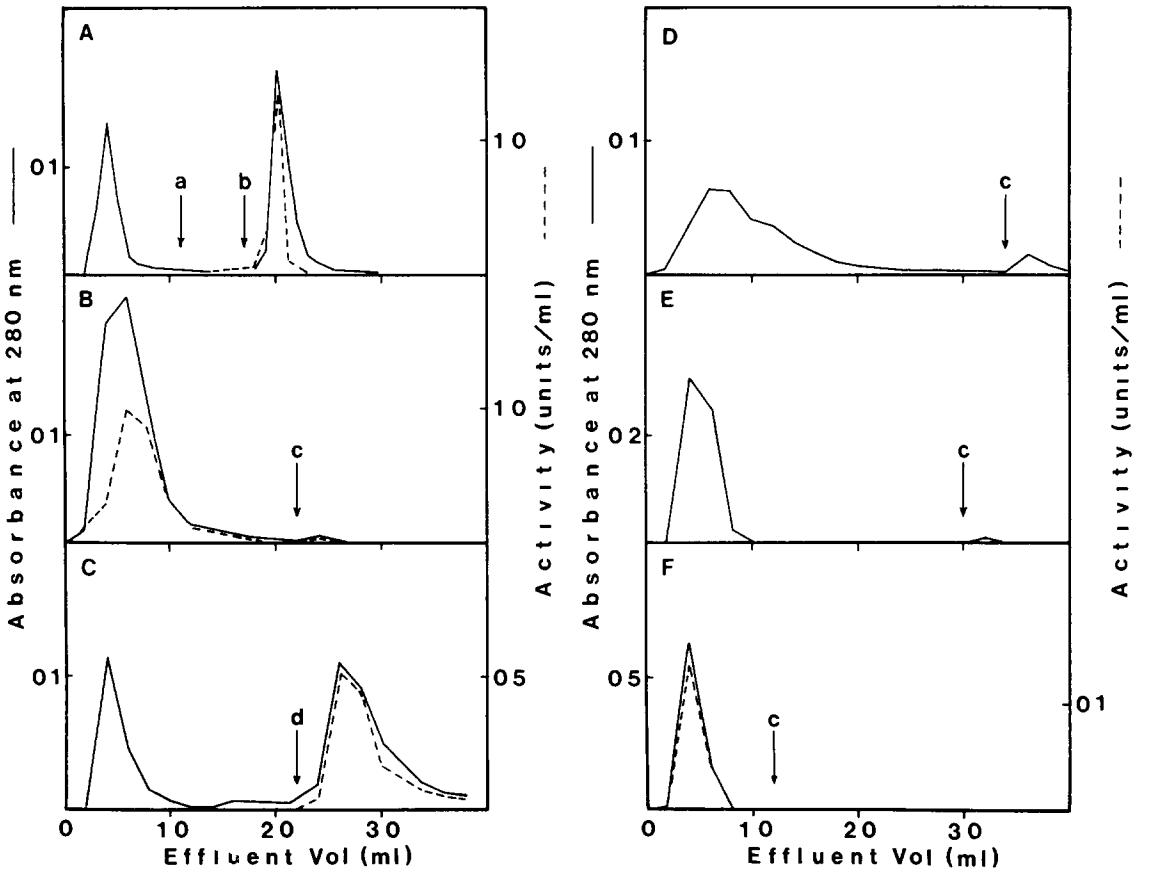

Fig 2 Chromatography of enzyme preparations on Gly-Gly-Argal Sepharose at pH 8 2 Samples (about 1 mg) were applied on a column (0 6 X 7 2 cm) equilibrated with 0 05 M borate buffer, pH 8 2/0 02 M $CaCl_2$/0 04 M NaCl (I = 0 1) Fractions of 1-ml (A) and 2-ml (B-F) were collected The flow rate was 6 ml/h Chromatography was done at 4°C The following samples were applied (A–C), bovine trypsin, (D), TLCK-trypsin, (E), bovine trypsinogen, (F), bovine α-chymotrypsin In B, chromatography was done in the presence of 0 1 mM leupeptin The eluent was changed to at the arrows a, 5 M NaCl, b, 8 M urea, c, 50 mM HCl/1 M NaCl, d, 0 1 mM leupeptin in the buffer

(Fig 2C) TLCK-trypsin was not absorbed (Fig 2D) Neither trypsinogen nor α-chymotrypsin were adsorbed (Fig 2E and F) It is now evident that Gly-Gly-Argal Sepharose is a biospecific affinity adsorbent for trypsin

Application to S griseus trypsin

Since Gly-Gly-Argal Sepharose proved to be an affinity adsorbent for bovine trypsin, an attempt was made to isolate *S griseus* trypsin [32] from Pronase E, a commercial preparation consisting of various proteases produced by the microbe As Fig 3 shows, trypsin activity was completely adsorbed The adsorbed enzyme could be eluted with leupeptin Nearly 80% of the trypsin activity was recovered and specific activity increased as much as 18-times Kasai and Ishii [15] and Yokosawa et al [17] had already succeeded in the purification of *S griseus* trypsin from Pronase by different affinity chromatographic techniques

Recently, we established a procedure to prepare leucyl-argininal from leupeptin by the action of thermolysin, and immobilized it to Sepharose with or without spacer Application of these adsorbents to plasmin, kallikrein, urokinase and clostripain as well as bovine and *S griseus* trypsins will be briefly reported elsewhere [33]

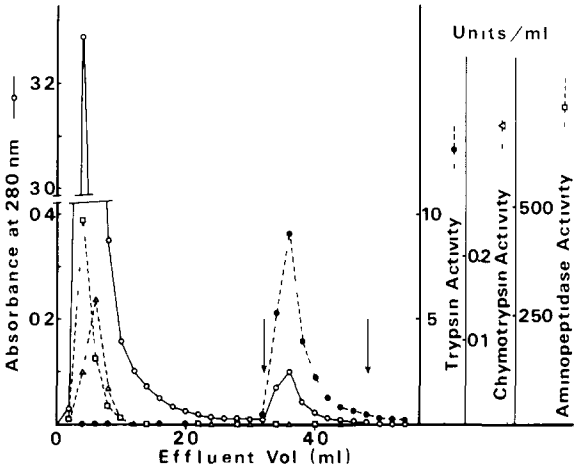

Fig 3 Chromatography of Pronase E on Gly-Gly-Argal Sepharose at pH 8 2 Pronase E (9 mg) in 0 3 ml 0 05 M borate buffer, pH 8 2/0 02 M $CaCl_2$/0 04 M NaCl (I = 0 1) was applied on a column (0 6 X 7 2 cm) equilibrated with the same buffer Fractions of 2-ml were collected The flow rate was 6 ml/h The eluent was changed to 0 1 mM leupeptin in the buffer at the first arrow and 1 mM leupeptin at the second arrow Chromatography was done at 4°C

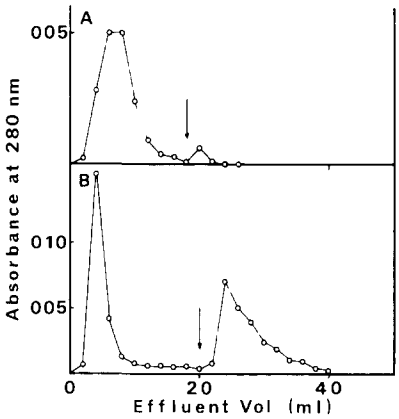

Fig 4 Chromatography of modified trypsins on Gly-Gly-Argal Sepharose at pH 8 2 (A) Anhydrotrypsin (0 4 mg) was applied on a column (0 6 X 7 2 cm) equilibrated with 0 05 M borate buffer, pH 8 2/0 02 M $CaCl_2$/0 04 M NaCl (I = 0 1) Fractions of 2-ml were collected The flow rate was 6 ml/h The eluent was changed to 50 mM HCl/1 M NaCl at the arrow Chromatography was done at 4°C (B) Carbamoylmethylated trypsin (1 mg) was applied on the column Other conditions are the same as in A In both cases, trypsin activity was below detectable limit

Estimation of essential amino acid residues in bovine trypsin for adsorption

Kasai and Ishii [13] found that anhydrotrypsin, in which Ser-183 was converted to dehydroalanine, and carbamoylmethylated-trypsin, in which His-46 was carbamoylmethylated, were adsorbed on an affinity adsorbent prepared by the immobilization of a mixture of several L-arginine-terminated oligopeptides (AP-Sepharose) This suggested that catalytically essential Ser-183 and His-46 are not important in the substrate binding The observed binding to AP-Sepharose was considered to be due to the interaction between the side chain of C-terminal L-arginine and the binding pocket of the enzyme

In order to clarify whether Ser-183 and His-46 are important in the present case, we carried out chromatography of anhydrotrypsin and carbamoylmethylated trypsin on Gly-Gly-Argal Sepharose When anhydrotrypsin was applied on a column of Gly-Gly-Argal Sepharose, almost all the protein passed through the column (Fig 4A) Evidently, Ser-183 is essential The same conclusion was reached in the case of leupeptin The dissociation constant of an anhydrotrypsin-leupeptin complex was higher by 5 orders of magnitude than that of a trypsin-leupeptin complex [31]

On the other hand, when a crude preparation of carbamoylmethylated trypsin was applied on the column, about half the amount of protein passed through the column, but the remaining portion was adsorbed (Fig 4B) The adsorbed protein was identified as carbamoylmethylated trypsin, because the amino acid analysis of its hydrolyzate indicated the appearance of 3-carboxymethyl histidine and disappearance of 1 mol histidine/mol trypsin The pass-through fraction was inactive protein which had lost both catalytic and binding abilities Thus, intact His-46 is not essential for the binding In our preliminary experiments, interaction of carbamoylmethylated trypsin and leupeptin was demonstrated Although carbamoylmethylated trypsin was inactive for almost all synthetic substrates, it could hydrolyze *p*-nitrophenyl *p'*-guanidinobenzoate It was not a catalytic reaction Carbamoylmethylated trypsin hydrolyzed only an equimolar amount of the substrate This reaction was inhibited by leupeptin Though the so-called charge relay system is absent, the enzyme derivative can bind with leupeptin so

long as Ser-183 is intact Similar results are reported for the methylchymotrypsin-benzoyl-L-phenylalaninal system [9]

It is suggested that leupeptin inhibits trypsin by forming a hemiacetal with the hydroxyl group of Ser-183 [31] The binding of trypsin to immobilized Gly-Gly-Argal will proceed in the same manner

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