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Affinity Chromatography of Alkinonase A on *N*-Carbobenzoxy–Glycyl–Leucyl–Aminohexyl-Sepharose

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N-Carbobenzoxy–glycyl–leucyl–aminohexyl-Sepharose was found to be an effective affinity adsorbent for alkinonase A, an alkaline metalloendopeptidase of *Streptomyces violaceorectus*. The enzyme was adsorbed on this affinity adsorbent at pH 9.0 and eluted at pH 7.0. The purified enzyme was shown to be homogeneous by polyacrylamide gel electrophoresis, and the molecular weight of the enzyme was estimated to be 35000.

The kinetics of the enzyme was studied using *N*-carbobenzoxy–glycyl–leucine amide as a substrate. The K_m value decreased with increase of pH in the range of 6.0—9.0. The optimum pH for casein hydrolysis was 9.0—9.5, but the specificity rate constant (k_{cat}/K_m) in Tris-HCl (pH 7.0) was 9 times higher than that in Tris-HCl (pH 9.0) due to the much higher ratio of k_{cat} values.

No remarkable change in the relative rate constant was observed, when the leucine residue was replaced by phenylalanine in *N*-carbobenzoxy–glycyl–leucine amide. The replacement of the glycine moiety of the peptide with tryptophan or proline, however, markedly decreased the relative rate constant.

Keywords——alkinonase A; alkaline metalloendopeptidase; *Streptomyces violaceorectus*; affinity chromatography; *N*-carbobenzoxy–glycyl–leucyl–aminohexyl-Sepharose

Generally, alkaline proteinases of microbial origin, including those from *Streptomyces fradiae*[1] and *Streptomyces griseus*,[2] are considered to be serine enzymes.[3] Nevertheless, alkinonases A and AF[4] produced by *Streptomyces violaceorectus* are unique because of their alkaline metalloendopeptidase nature, and they exhibit substrate specificities which resemble those of neutral metalloendopeptidases of microbial origin such as thermolysin[5] and subtilopeptidase.[6] Some enzymatic and physicochemical properties, as well as the anti-inflammatory activity, of alkinonases A and AF were reported in the previous paper.[4] Various affinity adsorbents were surveyed to purify the enzymes and *N*-carbobenzoxy–glycyl–leucyl–aminohexyl-Sepharose (Z–Gly–Leu–AH-Sepharose) was found to be an efficient adsorbent. The purified enzyme showed a single band on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and a single peak on Sephadex G-75 gel filtration. The purified enzyme was considered to be alkinonase A from its molecular weight.

The purification and a more detailed characterization of alkinonase A are reported in this paper.

Materials and Methods

Materials——1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was purchased from Sigma Chemical Co., *N*-carbobenzoxy-peptides, such as Z–Gly–Leu–NH_2 and Z–Gly–Leu, were obtained from the Peptide Institute, Protein Research Foundation, and all amino acids used were of L-form, unless otherwise specified. Aminohexyl-Sepharose was a product of Pharmacia Fine Chemicals Co. All other materials were commercial products of analytical grade.

Production of Alkinonase A——A seed inoculum was cultured in a 500 ml Sakaguchi flask containing 100 ml of a medium composed of 1.0% soluble starch and 0.2% yeast extract (pH 7.0) at 27 °C for 24 h on a reciprocal shaker

(amplitude 7 cm, 135 strokes/min). The seed culture (2 ml) was inoculated into a 500 ml Sakaguchi flask containing 100 ml of a production medium consisting of 1.5% soluble starch, 1.0% glucose, 2.0% soybean meal, 0.25% NaCl, 0.3% $CaCO_3$, 0.0007% $CuSO_4 \cdot 5H_2O$, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0008% $MnCl_2 \cdot 4H_2O$ and 0.0002% $ZnSO_4 \cdot 7H_2O$ (pH 7.6 before sterilization). The culture was conducted at 27 °C for 4 d on the reciprocal shaker.

Isolation of Crude Enzyme——The culture broth filtrate (1300 ml) was brought to 70% saturation with ammonium sulfate and adjusted to pH 7.0 by the addition of 1 N NH_4OH. The precipitate was collected by centrifugation, redissolved in a small volume of distilled H_2O (10 ml) and dialyzed against distilled H_2O containing 10 μg/ml of $Ca(OAc)_2 \cdot H_2O$. The dialyzed solution was lyophilized to give crude alkinonase A (750 mg) with recovery of 65% of the caseinolytic activity. As mentioned below, the crude enzyme was treated with a small amount of DEAE-cellulose to remove melanoid-like contaminants prior to affinity chromatography.

Preparation of Z–Gly–Leu–AH-Sepharose——A mixture of H_2O-swelled AH-Sepharose (5 g/20 ml), Z–Gly–Leu (150 mg) dissolved in 40% aqu. dimethylformamide (20 ml, adjusted to pH 5.0) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (456 mg) was kept overnight at room temperature with gentle shaking. The resulting gel was washed successively with 1 M NaCl in 40% aqu. dimethylformamide, 1 M $NaHCO_3$, distilled H_2O, 1 N acetic acid and finally with distilled H_2O, and stored at 4 °C until use.

Assay for Protein Determination——The protein concentration was measured by the method of Lowry *et al.*[7] using bovine serum albumin as a standard.

Assay of Caseinolytic Activity——Proteolytic activity against casein was measured by the casein-275 nm method[8] with some modifications as described in the previous paper.[4]

One unit of caseinolytic activity was defined as the amount of enzyme giving an absorbancy equivalent to 1 μg of tyrosine per min at 37 °C.

Hydrolysis of Synthetic Substrates——Moore's method[9] was modified as follows to determine the rates of hydrolysis of synthetic substrates. The aqueous enzyme solution (250 μl) was added to the substrate solution (250 μl, dissolved in 50 mM Tris-HCl, pH 9.0, unless otherwise stated), and after a 10-min incubation at 37 °C, 0.1 N acetic acid (250 μl) was added to terminate the enzyme reaction. Ninhydrin solution[9] (0.5 ml) was added to the mixture and the whole was kept at 100 °C for 10 min. The absorbance at 570 nm was measured against the blank to determine the rate of hydrolysis.

Electrophoresis——Electrophoresis was performed in 10% polyacrylamide gel in the presence of sodium dodecyl sulfate according to the method of Weber and Osborn.[10] Bovine serum albumin (MW 68000), α-chymotrypsinogen (MW 25700) and lysozyme (MW 14300) were used as reference proteins to determine the molecular weight of the purified enzyme. Electrophoresis on a cellulose acetate film was conducted under the conditions previously described to compare the migration rate of the enzyme with those obtained for alkinonases A and AF in the former work.[4]

Results

Conditions for Affinity Chromatography

The effect of pH on adsorption of the enzyme on Z–Gly–Leu–AH-Sepharose was tested in the range of pH 5.0—10.0 using 1/15 M phosphate (5.0—9.0), 1/15 M Tris-HCl (7.0—9.5) and 1/15 M carbonate (9.0—10.0) buffers. The enzyme was adsorbed on Z–Gly–Leu–AH-Sepharose at alkaline pHs (8.5—10.0) in all the buffers used. However, rapid inactivation of the enzyme was observed over pH 9.5. The enzyme was eluted efficiently from the adsorbent with 1/15 M phosphate buffer (pH 7.5—5.0), but Tris-HCl buffer gave a retarded elution profile even at pH 7.0. On the basis of these results, the enzyme was adsorbed on the affinity adsorbent from 1/15 M phosphate buffer (pH 9.0) and eluted with 1/15 M phosphate buffer (pH 7.0). The affinity adsorbent, Z–Gly–Leu–AH-Sepharose, showed no detectable decrease in its capacity after more than 50 cycles of use.

Purification of Alkinonase A

The crude enzyme (750 mg) was dissolved in 25 mM Tris-HCl (30 ml, pH 8.0). The melanoid pigments in the crude powder were easily removed by passing the preparation through a DEAE-cellulose column (10.0 cm × 1.6 cm, i.d.) buffered with 25 mM Tris-HCl buffer (pH 8.0). The active eluate from the DEAE-cellulose column was dialyzed against 1/15 M phosphate buffer (pH 9.0) and adsorbed on a Z–Gly–Leu–AH-Sepharose column (10 cm × 1.0 cm, i.d.) equilibrated with 1/15 M phosphate buffer (pH 9.0). The column was thoroughly washed with the same buffer, and the enzyme on the column was eluted with 1/15 M phosphate buffer (pH 7.0) with a recovery of 75% of the original activity as shown in

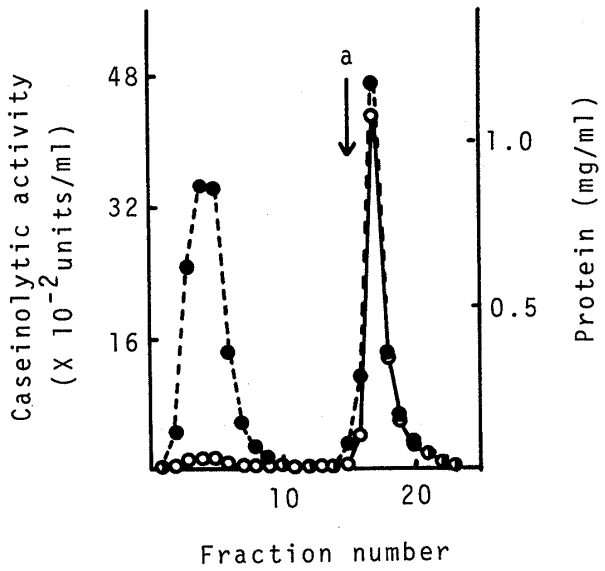

Fig. 1. Affinity Chromatography of Alkinonase A on Z–Gly–Leu–AH-Sepharose

The crude enzyme treated with DEAE-cellulose (20 mg as protein) was dialyzed against 1/15 M phosphate buffer (pH 9.0) and charged on a Z–Gly–Leu–AH-Sepharose column (10.0 cm × 1.0 cm, i.d.) equilibrated with the same buffer. Arrow a indicates the change from 1/15 M phosphate buffer (pH 9.0) to 1/15 M phosphate buffer (pH 7.0). The eluate was collected in 4.2 ml fractions. ○, caseinolytic activity; ●, protein concentration.

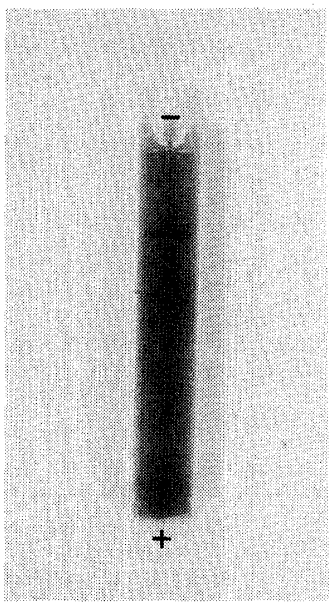

Fig. 2. SDS–Polyacrylamide Gel Electrophorogram of the Purified Enzyme

TABLE I. Purification of Alkinonase A

Enzyme fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
$(NH_4)_2SO_4$ Precipitate (750 mg)	182	428376	2354	100
DEAE-cellulose chromatography	103	419067	4069	98
Z–Gly–Leu–AH-Sepharose affinity chromatography	58	321330	5540	75

Fig. 1. The purification of alkinonase A is summarized in Table I.

Estimation of Molecular Weight

The purified enzyme showed a single band on SDS–polyacrylamide gel electrophoresis as shown in Fig. 2 and the molecular weight was estimated to be 35000. The same value was obtained by gel filtration on a Sephadex G-75 column. Since alkinonase AF was eluted near the void volume of a Sephadex G-75 column,[4] the newly purified enzyme was considered to be alkinonase A. Electrophoresis of the above purified enzyme on a cellulose acetate film gave the same mobility as found previously for alkinonases A and AF.[4]

Substrate Specificity

The relative rate constants for various synthetic substrates were measured quantitatively by the ninhydrin method of Moore.[9] As previously noted,[4] alkinonases A and AF cleaved peptide bonds involving the amino group of leucine, phenylalanine or tyrosine. Among the

Table II. Substrate Specificity

Substrate	Hydrolysis rate (Relative value)	Substrate	Hydrolysis rate
Z–Gly↓Leu–NH_2	100	Z–Gly–Leu	0
Z–Pro↓Leu–NH_2	2	Z–Gly–Phe	0
Z–Trp↓Leu–NH_2	28	Z–Glu–Phe	0
Z–Gly↓Leu–Tyr	3538[a]	Z–Phe↓Tyr	7
Boc–Pro–Leu–Gly–NH_2	0	Z–Gly	0
Z–Gly–Pro–Leu–Gly	0	Z–Phe	0
Z–Gly↓Phe–NH_2	107	ClAc–Tyr	0
Z–Gly↓D-Leu–NH_2	2		

All amino acids have the L-form, unless otherwise specified. Boc-, *tert*-butoxycarbonyl; ClAc-, chloroacetyl-.

A mixture of 5 mM substrate solution (250 μl) in 50 mM Tris-HCl (pH 9.0) and the aqueous enzyme solution (2.5 μg/250 μl) was incubated at 37 °C for 10 min. After the addition of 0.1 N AcOH (250 μl) and then Moore's ninhydrin solution (500 μl), the mixture was kept at 100 °C for 10 min. The absorbance at 570 nm was measured against a blank and the absorbance with Z–Gly–Leu–NH_2 was defined as 100.

a) Aqueous enzyme solution, 0.0625 μg/250 μl, was used in stead of 2.5 μg/250 μl.

Table III. Inhibition by Various Amino Acids and Peptides of Hydrolysis of Z–Gly–Leu–NH_2 by Alkinonase A

Amino acid or peptide	Inhibition I[a]	Inhibition II[c] (%)
Z–Gly–D-Leu–NH_2		16
Z–Gly–Leu		48
Z–Gly–Phe		48
Z–Glu–Phe		31
Z–Glu–Tyr		0
Z–Gly		0
Z–Phe		53
Gly–Leu–Tyr	[b]	

All amino acids have the L-form, unless otherwise specified.

a) Method I: A mixture of 10 mM inhibitor solution (500 μl) in 10 mM Tris-HCl (pH 9.0), 2 mM substrate solution (500 μl) in the same buffer and the aqueous enzyme solution (6 μg/50 μl) was incubated at 37 °C for 2 h. The mixture was lyophilized and the residue was dissolved in MeOH (100 μl) and subjected to silica gel thin layer chromatography developed with *n*-BuOH–AcOH–H_2O (4:2:1). The digested substrate was detected by spraying 1% ninhydrin solution.

b) Inhibited.

c) Method II: A mixture of 10 mM inhibitor solution (250 μl) in 25 mM Tris-HCl (pH 9.0), 2 mM substrate solution (250 μl) in the same buffer and the aqueous enzyme solution (5 μg/250 μl) was incubated at 37 °C for 10 min. After the addition of 0.1 N AcOH (250 μl) and then Moore's ninhydrin solution (500 μl), the mixture was kept at 100 °C for 10 min. The released Leu–NH_2 was determined by measuring the absorbance at 570 nm against a blank.

dipeptides containing these amino acids as the C-termini, only fully blocked peptides such as Z–Gly–Leu–NH_2 and Z–Gly–Phe–NH_2 were hydrolyzed effectively, as shown in Table II. The rate of hydrolysis of Z–Gly–Leu–NH_2 was defined as 100. In Z–Gly–Leu–NH_2, replacement of the glycine moiety with tryptophan or proline greatly decreased the rate of hydrolysis. On the other hand, Z–Gly–Leu–Tyr was hydrolyzed 35 times faster than Z–Gly–Leu–NH_2.

TABLE IV. Kinetic Parameters for Hydrolysis of Z–Gly–Leu–NH_2 by Alkinonase A

Buffer	S (mM)	E ($\times 10^8$ M)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
25 mM Tris-HCl (pH 9.0)	0.83—5.0	143.0	2.1	3.9	1.86
25 mM Tris-HCl (pH 7.0)	0.83—5.0	14.3	3.4	57.0	16.76
25 mM Phosphate (pH 7.0)	0.83—5.0	42.9	5.9	19.0	3.22
26 mM Phosphate (pH 6.0)	0.83—5.0	42.9	11.9	19.3	1.62

A mixture of 1.66—10.0 mM substrate solution (250 μl) in 50 mM buffer as indicated and aqueous enzyme solution (0.25, 0.5 or 2.5 μg/250 μl) was incubated at 37 °C for 10 min. After the addition of 0.1 N AcOH (250 μl) and then Moore's ninhydrin solution (500 μl), the mixture was kept at 100 °C for 10 min. The released Leu–NH_2 was determined by measuring the absorbance at 570 nm against a blank.

S, substrate concentration; E, enzyme concentration.

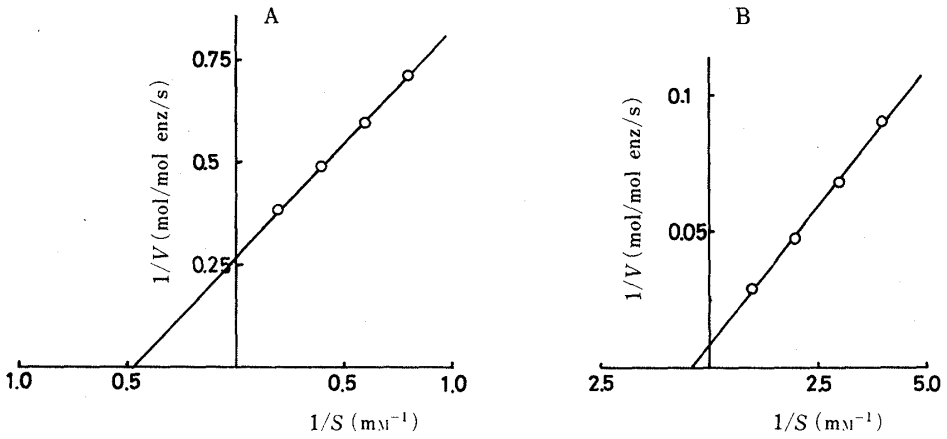

Fig. 3. Double Reciprocal Plots of Alkinonase A-Catalyzed Hydrolysis of Z–Gly–Leu–NH_2 and Z–Gly–Leu–Tyr

A: A mixture of 1.25—5.0 mM Z–Gly–Leu–NH_2 (250 μl) in 50 mM Tris-HCl buffer (pH 9.0) and the aqueous enzyme solution (2.5 μg/250 μl) was incubated at 37 °C for 10 min. After the addition of 0.1 N AcOH (250 μl) and then Moore's ninhydrin solution (500 μl), the mixture was kept at 100 °C for 10 min. The released Leu–NH_2 was determined by measuring the absorbance at 570 nm against a blank.
$E = 1.43 \times 10^{-6}$ M; $S = 1.25$—5.0 mM; $K_m = 2.1$ mM; $k_{cat} = 3.9\ s^{-1}$.

B: A mixture of 0.25—1.0 mM Z–Gly–Leu–Tyr (250 μl) in 50 mM Tris-HCl buffer (pH 9.0) and the aqueous enzyme solution (0.25 μg/250 μl) was incubated at 37 °C for 10 min. The released Leu–Tyr was determined by the same procedure as described in A.
$E = 0.143 \times 10^{-6}$ M; $S = 0.25$—1.0 mM; $K_m = 2.2$ mM; $k_{cat} = 115\ s^{-1}$.

Effect of Various Derivatives of Amino Acids and Peptides on Enzymatic Activity

The effect of various derivatives of amino acids and peptides on the enzymatic activity of alkinonase A was examined with Z–Gly–Leu–NH_2 as a substrate. The *N*-carbobenzoxy derivatives containing leucine or phenylalanine as the C-terminus reduced the enzymatic activity significantly, as shown in Table III. Glycyl-leucyl-tyrosine inhibited the enzymatic activity, exceptionally among peptides having a free amino group at the N-terminus.

Kinetics

The kinetic parameters of hydrolysis of Z–Gly–Leu–NH_2 by alkinonase A were calibrated under various conditions using Lineweaver–Burk plots, based on a molecular weight of 35000. Depending upon the hydrolysis rate, the enzyme concentration was adjusted as indicated in Table IV. The k_{cat}/K_m value obtained in 25 mM Tris-HCl buffer (pH 7.0) was 9 times as high as that in 25 mM Tris-HCl buffer (pH 9.0). At the same pH (7.0), Tris-HCl buffer

was found to facilitate the hydrolysis of Z–Gly–Leu–NH_2 as compared with phosphate buffer, giving a lower K_m value and a higher k_{cat} value.

Double reciprocal plots of the hydrolysis of Z–Gly–Leu–Tyr in 25 mM Tris-HCl buffer (pH 9.0) are shown in Fig. 4 together with those for Z–Gly–Leu–NH_2 in the same buffer. The remarkably high k_{cat} value appeared to be the basis of the markedly enhanced relative rate constant shown in Table II (3538), even though the K_m value was higher than that for Z–Gly–Leu–NH_2.

Discussion

Since Morihara *et al.*[11] reported the inhibitory activity of *N*-carbobenzoxy-phenylalanine (Z–Phe) on the hydrolysis of Z–Gly–Leu–Ala by the neutral metalloendopeptidase of *Bacillus subtilis*, affinity adsorbents containing phenylalanine as a ligand have been used for the purification of this group of enzymes. Pangburn *et al.*[12] applied acetyl-D-phenylalanine covalently linked to Sepharose 4B with various spacers to purify thermolysin and neutral subtilopeptidase. Fujiwara *et al.*[13] reported the purification of thermolysin and neutral subtilopeptidase using *N*-carbobenzoxy-phenylalanyl-triethyltetraminyl-Sepharose. Z–Phe–AH-Sepharose was used by Hiramatsu[14] to purify the neutral metalloendopeptidase from *Streptomyces naraensis*. Though alkinonase A could not be adsorbed on Z–Phe–AH-Sepharose at pH below 9.0, the enzyme was adsorbed on Z–Gly–Leu–AH-Sepharose at pH 9.0 and could be easily eluted from this adsorbent with 1/15 M phosphate buffer (pH 7.0). In general, neutral metalloendopeptidases showed higher affinity to various adsorbents at neutral pH and were eluted with either the buffer (pH 9.0) or the buffer containing 1.0—1.5 M guanidine hydrochloride. The reciprocal K_m value was generally proportional to the affinity of the enzyme to the substrate, as suggested by other investigators,[13,15] and the finding that the affinity of alkinonase A to Z–Gly–Leu–AH-Sepharose is higher at pH 9.0 than at pH 7.0 can be explained in terms of the results shown in Table IV. Furthermore, the failure of elution with 25 mM Tris-HCl buffer (pH 7.0) can be ascribed to the smaller K_m value than that obtained in 25 mM phosphate buffer (pH 7.0).

Each of the neutral metalloendopeptidases from *Bacillus subtilis, Bacillus thermoproteolyticus* and *Pseudomonas aeruginosa* has a large active site which can be divided into 6 "subsites," each accommodating one amino acid residue.[1b] The results shown in Table II (the replacement of glycine with tryptophan or proline greatly decreased the rate of hydrolysis of Z–Gly–Leu–NH_2) suggest that alkinonase A also accommodates at least two amino acids in its active center, even though the size of the active center remains to be determined.

It is interesting that the tripeptide glycyl–leucyl–tyrosine completely inhibited the hydrolysis of Z–Gly–Leu–NH_2 by alkinonase A, since no inhibition of the hydrolysis of Z–Gly–Leu–Ala by neutral subtilopeptidase was observed in the presence of tripeptides structurally related to the substrate.[11]

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