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Affinity Chromatography of Neutral Metalloendopeptidase Produced by *Streptomyces mauvecolor* on *N*-Benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose

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A neutral metalloendopeptidase, produced by *Streptomyces*, which has anti-inflammatory activity against carrageenan-induced edema in rats was purified by affinity chromatography on *N*-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose (Z–Gly–D-Leu–AH-Sepharose) to electrophoretic homogeneity.

The enzyme was adsorbed on a Z–Gly–D-Leu–AH-Sepharose column equilibrated with phosphate buffer (pH 7.0). The column was washed with phosphate buffer (pH 5.6) containing 2 M urea and eluted with acetate buffer (pH 4.1) supplemented with 2 M urea. The caseinolytic activity of the enzyme, which was lost on treatment with ethylenediaminetetraacetate, was recovered by the addition of $ZnCl_2$ to the reaction mixture. The enzyme showed the maximum caseinolytic activity at pH between 7.0 and 8.0, and was stable in the pH range of 5.0—10.0. The molecular weight was estimated to be 59000. The enzyme preferentially hydrolyzed *N*-benzyloxycarbonylglycyl-L-leucine amide, *N*-benzyloxycarbonylglycyl-L-phenylalanine amide and *N*-benzyloxycarbonylglycyl-L-leucyl-L-tyrosine among the synthetic substrates tested in this work.

The producing microbe was identified as *Streptomyces mauvecolor* based on the results of taxonomic studies.

Keywords——affinity chromatography; *N*-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose; neutral metalloendopeptidase; *Streptomyces mauvecolor*; *N*-benzyloxycarbonylglycyl-L-leucine amide

Some of the proteinases produced by *Streptomyces* (including alkaline and neutral metalloendopeptidases and serine proteinases) are active against carrageenan-induced edema in rats.[1a-e,g] *N*-Benzyloxycarbonylglycyl-L-leucylaminohexyl-Sepharose (Z–Gly–L-Leu–AH-Sepharose) has been proved to be an effective affinity adsorbent for some of the enzymes in this group.[1f-h] Alkinonase A, an alkaline metalloendopeptidase produced by *Streptomyces violaceorectus*, was retained on this adsorbent at pH above 9.0,[1f] while a neutral metalloendopeptidase produced by *Streptomyces griseoruber* showed much higher affinity for this adsorbent, and was not eluted with any buffer tested in the pH range of 4.1—9.5.[1h] The latter was successfully eluted from the column with acetate buffer (pH 4.1) which was supplemented with 2 M urea. Another neutral metalloendopeptidase produced by a strain of *Streptomyces*, tentatively designated as H 1281 MY 13, showed anti-inflammatory activity against carrageenan-induced edema and the same adsorbent was tested to purify the enzyme. However, we met difficulty in eluting the enzyme from the adsorbent even with acetate buffer (pH 4.1) containing 2 M urea. Therefore, several adsorbents were surveyed to obtain easier elution of the enzyme, and *N*-benzyloxycarbonylglycyl-D-leucylaminohexyl-Sepharose (Z–Gly–D-Leu–AH-Sepharose) was found to be superior to Z–Gly–L-Leu–AH-Sepharose as an affinity adsorbent for this enzyme.

The details of the purification and characterization of the enzyme and taxonomic studies of the producing organism are presented in this paper.

Materials and Methods

Materials——*N*-Benzyloxycarbonyl amino acids and peptides such as Z–Gly, Z–Gly–Leu and Z–Gly–Leu–NH_2 were products of the Peptide Institute, Protein Research Foundation, and AH-Sepharose was obtained from Pharmacia Fine Chemicals Co. All other materials were commercial products of analytical grade, and all amino acids used in this work were of the L-form, unless otherwise specified.

Taxonomic Studies of Strain H 1281 MY 13——The organism was isolated from a soil sample collected in Miyazaki Prefecture. The methods and media recommended by the International Streptomyces Project (ISP)[2] were used with several supplemental tests. Color names were determined according to "Guide to Color Standard"[3] and the procedures of Becker *et al.*[4] were used for the preparation of whole cell hydrolysate and for the chromatographic detection of the isomers of diaminopimelic acid.

Cultivation of Strain H 1281 MY 13——The inoculum seed was developed by transferring spores of strain H 1281 MY 13 to a 500 ml Erlenmeyer flask containing 100 ml of a medium consisting of 1.0% soluble starch and 0.2% yeast extract (pH 7.0—7.2). The flask was incubated at 27 °C for 24 h on a rotary shaker at 170 rpm. Two ml of the seed culture was used to inoculate a 500 ml Erlenmeyer flask containing 100 ml of a producing medium with the following composition: 1.5% soluble starch, 1.0% glucose, 2.0% soybean meal, 0.5% Ebios (dried yeast, distributed by Tanabe Pharmaceutical Co., Ltd.), 0.25% NaCl, 0.00012% $CaCl_2 \cdot 2H_2O$ and 0.00008% $ZnSO_4 \cdot 7H_2O$ (pH 7.6, before sterilization). The culture was conducted at 27 °C for 4 d on the same shaker.

Isolation of Crude Enzyme——The broth filtrate (1400 ml) was brought to 70% saturation with ammonium sulfate while being adjusted at pH 7.0 by the addition of 1 N NH_4OH. The precipitate was collected by centrifugation, redissolved in distilled H_2O (30 ml) and dialyzed against distilled H_2O containing 5 ml/l of $CaCl_2 \cdot 2H_2O$ with 70% recovery of the caseinolytic activity. The crude enzyme thus obtained was subjected to affinity chromatography.

Preparation of Z–Gly–D-Leu–AH-Sepharose——A mixture of H_2O-swollen AH-Sepharose (20 ml as packed gel), Z–Gly–D-Leu (150 mg) dissolved in 20 ml of 40% aqueous dimethylformamide (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% aq. dimethylformamide, 1 M $NaHCO_3$, distilled H_2O, 1 N acetic acid and finally distilled H_2O, and stored at 4 °C until use. The preparation of Z–Gly–Leu–AH-Sepharose and Z–Phe–AH-Sepharose was conducted according to the same procedures.

Assay for Protein Determination——The protein concentration was measured by the method of Lowry *et al.*[5] with bovine serum albumin as a standard.

Assay of the Caseinolytic Activity——The proteolytic activity against casein was measured by the casein-275 nm method of Hagihara *et al.*[6] with some modification as described in the previous paper.[1a] One unit of caseinolytic activity was defined as the amount of enzyme giving an absorbance equivalent to 1 μg of tyrosine per min at 37 °C.

Hydrolysis of Synthetic Substrates——The hydrolysis rates of various synthetic substrates by the enzyme were determined according to the previous method[1f] except that 50 mM Tris–HCl (pH 7.0) was used instead of 50 mM Tris–HCl (pH 9.0).

Electrophoresis——Electrophoresis was performed in 10% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) by the method of Weber and Osborn.[7] Bovine serum albumin [molecular weight (MW), 68000], α-chymotrypsinogen (MW, 25400) and lysozyme (MW, 14300) were used as references to determine the molecular weight of the enzyme.

Results

Taxonomic Studies of Strain H 1281 MY 13

Morphological characteristics were observed by light microscopy and scanning electron microscopy. The organism produced substrate mycelium which did not fragment into spores on any of the media tested. Aerial mycelium monopodially branched and bore rather long spore chains with 10—50 or more spores arranged in open spirals on oatmeal agar and inorganic salts–starch agar media. Poor or no sporulation was observed on yeast extract–malt extract agar and glycerol–asparagine agar media. The spore surface was spiny and the cylindrical spores measured 0.36—0.52 μm × 0.75—1.03 μm. Morphological and physiological characteristics are shown in Table I. LL-Diaminopimelic acid was detected in the whole cell hydrolysate.

By consulting "Bergey's Manual of Determinative Bacteriology,"[8] "ISP reports" by Shirling and Gottlieb[9] and the report by Murase *et al.*,[10] *Streptomyces mauvecolor* was selected as the most closely related species; there was no discrepancy between the reported characteristics of *Str. mauvecolor*[10] and the observed ones of strain H 1281 MY 13.

TABLE I. Morphological and Physiological Characteristics of Strain H 1281 MY 13

	Characteristics
Spore wall ornamentation	Spiny
Spore chain morphology	Spiral
Number of spores per chain	10—50 or more
Color of substrate mycelium	Yellowish white or yellowish brown
Color of mature sporulated aerial mycelium	White or pale purple
Soluble pigments	None
Melanoid pigments	Positive on ISP-6, negative on ISP-1 and 7
Starch hydrolysis	Positive
Gelatin liquefaction	Positive
Action on skimmed milk	Coagulation followed by peptonization
Utilization of sugars	
L-Arabinose	Positive
D-Xylose	Negative
D-Glucose	Positive
D-Fructose	Negative
Sucrose	Negative
iso-Inositol	Negative
Cellulose	Negative
L-Rhamnose	Negative
Raffinose	Positive
D-Mannitol	Negative
D-Galactose	Positive
Cell wall type	I (LL-Diaminopimelic acid)

Affinity Chromatography of Neutral Metalloendopeptidase

The crude enzyme obtained by ammonium sulfate precipitation was subjected to affinity chromatography on Z–Gly–Leu–AH-Sepharose, which had been used as an effective affinity adsorbent for alkaline and neutral metalloendopeptidases of *Streptomyces* origin in previous studies.[1f,h] The enzyme could not be eluted with any buffer tested within the pH range of 4.1—9.5, although the addition of 2 M urea to 1/10 M acetate buffer (pH 4.1) gave a rather unsharp elution profile. Since the elution time is crucial to recover sufficient activity at this pH, we searched for a more convenient adsorbent, and Z–Gly–D-Leu–AH-Sepharose, which was tested on the basis of the structural resemblance to the inhibitor of this enzyme, Z–Gly–D-Leu–NH_2, was found to give easier elution of the enzyme under the same conditions as employed in Z–Gly–Leu–AH-Sepharose chromatography. However, the active fractions recovered with 1/10 M acetate buffer (pH 4.1) containing 2 M urea showed one major and three minor bands on SDS–polyacrylamide gel electrophoresis. Since all the minor components were eluted with 1/15 M phosphate buffer (pH 5.6) in the presence of 2 M urea, affinity chromatography was conducted as follows: charging of the sample with 1/15 M phosphate buffer (pH 7.0), washing out of minor components with 1/15 M phosphate buffer (pH 5.6) containing 2 M urea and elution of the major component with 1/10 M acetate buffer (pH 4.1) containing 2 M urea.

Purification

The crude enzyme (70 mg as protein) was applied to Z–Gly–D-Leu–AH-Sepharose column (7.5 cm × 0.8 cm, diam.); the elution profile is shown in Fig. 1. The eluate was immediately neutralized with 1 N NaOH to minimize inactivation of the enzyme at the pH of the final elution buffer. Thirty-two percent of the caseinolytic activity applied and 3.5% of the

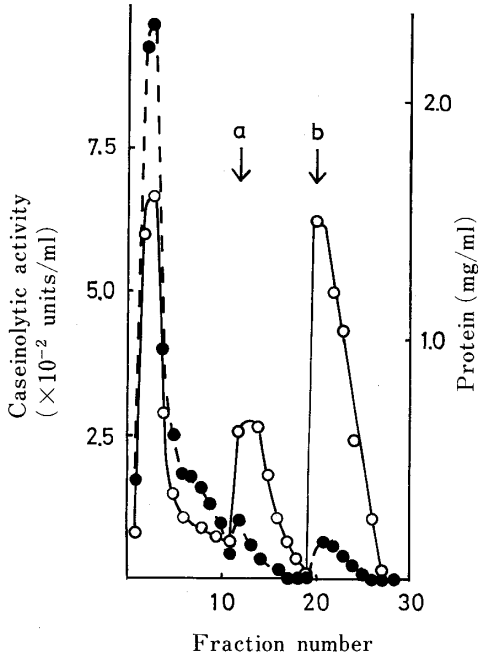

Fig. 1. Affinity Chromatography of Neutral Metalloendopeptidase Produced by *Streptomyces mauvecolor*

The crude enzyme obtained by ammonium sulfate precipitation (70 mg as protein) was applied to a Z–Gly–D-Leu–AH-Sepharose column (7.5 cm × 0.8 cm, diam.) which has been equilibrated with 1/15 M phosphate buffer (pH 7.0). The arrow a indicates a change of the buffer from the starting buffer to 1/15 M phosphate buffer (pH 5.6) containing 2 M urea, and the arrow b indicates a further change to 1/10 M acetate buffer (pH 4.1) supplemented with 2 M urea. The eluate was collected in 4.0 ml fractions. ○, caseinolytic activity; ●, protein concentration.

TABLE II. Purification of Neutral Metalloendopeptidase Produced by *Streptomyces mauvecolor*

Enzyme fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Ammonium sulfate precipitate	1000	245000	246	100
Z–Gly–D-Leu–AH-Sepharose affinity chromatography	35	7790	2200	32

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

protein were recovered in peak III of the affinity chromatography as shown in Table II. The enzyme purified by affinity chromatography showed a single band on SDS–polyacrylamide gel electrophoresis (Fig. 2) and the molecular weight of the enzyme was estimated to be 59000 by SDS–polyacrylamide gel electrophoresis and Sephadex G-75 gel filtration.

Effects of pH on Caseinolytic Activity and Enzyme Stability

The optimum hydrolysis of casein was observed at pH between 7.0 and 8.0 as shown in Fig. 3. The enzyme was stable within the pH range of 5.0—10.0.

Effects of Various Inhibitors

The effects of various chemicals on the caseinolytic activity of the purified enzyme were determined by the casein-275 nm method and the results are shown in Table III in comparison with those for peaks I and II of the affinity chromatography. All of the enzyme peaks were inactivated by ethylenediaminetetraacetate (EDTA), iodine and *N*-bromosuccinimide. No inhibition was observed by phenylmethanesulfonyl fluoride or sulfhydryl reagents. The caseinolytic activity lost on treatment with EDTA was recovered by the addition of $ZnCl_2$ to the reaction mixture (Table IV). Among the proteinase inhibitors of microbial origin, only

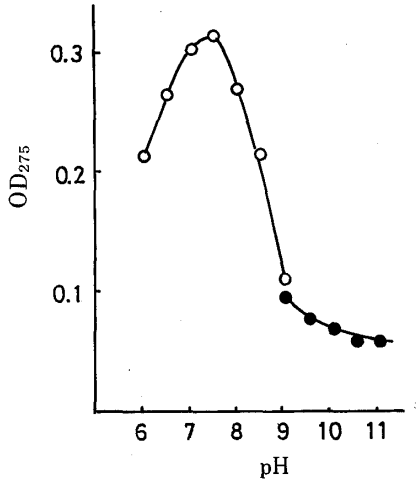

Fig. 3. Effect of pH on Caseinolytic Activity

A mixture of 5.0 μg/ml aqueous enzyme solution (1.0 ml) and 1% casein solution (1.0 ml) in 1/10 M Tris–HCl buffer (pH 6.0—9.0) or 1/10 M carbonate buffer (pH 9.0—11.0) was incubated at 37 °C for 20 min. ○, Tris–HCl buffer; ●, carbonate buffer.

TABLE III. Effects of Various Chemicals on Casein Hydrolysis

Chemical	Residual activity (%)		
	Peak I	Peak II	Peak III
Ethylenediaminetetraacetate	6	8	9
N-Bromosuccinimide	3	3	16
Iodine	0	2	0
8-Hydroxyquinoline	38	67	75
α, α′-Dipyridyl	105	98	101
Monoiodoacetate	100	109	120
p-Chloromercuribenzoate	110	106	139
Hydroxylamine hydrochloride	101	102	133
Phenylmethanesulfonyl fluoride	94	99	93
L-Cysteine	103	105	109

A mixture of the enzyme solution (0.9 ml/100 μg as protein for peak I, 20 μg for peak II or 10 μg for peak III) in 0.1 M Tris–HCl (pH 7.0) and 10 mM inhibitor solution (0.1 ml) in the same buffer was kept at room temperature for 20 min. The mixture was incubated with 1% casein solution (1 ml) in 0.1 M Tris–HCl (pH 7.0) at 37 °C for 20 min. The absorbance at 275 nm was measured against the blank after the addition of 10% cold trichloroacetic acid (TCA) (3 ml) followed by centrifugation.

TABLE IV. Effects of EDTA and $ZnCl_2$ on Casein Hydrolysis

Pretreatment	Addition of $ZnCl_2$	Residual activity (%)
None	−	100
None	+	110
1 mM EDTA	−	9
1 mM EDTA	+	109

The purified enzyme (400 μg as protein) untreated or treated with 1 mM EDTA at room temperature for 20 min was charged on a Sephadex G-10 column (85.0 cm × 1.6 cm, diam.) which had been equilibrated with 25 mM Tris–HCl (pH 7.0). The enzyme was eluted with the same buffer and the eluate was collected in 4.0 ml fractions. The active fractions were pooled and the caseinolytic activity (given as the relative value) was measured in the absence and presence of 1 mM $ZnCl_2$.

phosphoramidon showed marked inhibition (Table V).

Chromatography of EDTA-Treated Enzyme on Z–Gly–D-Leu–AH-Sepharose

The elution profile of the EDTA-treated inactive enzyme on Z–Gly–D-Leu–AH-

TABLE V. Effects of Proteinase Inhibitors on Casein Hydrolysis by the Purified Enzyme

Proteinase inhibitor	Concentration (μg/ml)	Residual activity (%)
Bestatin	10	93
	1	93
Pepstatin	10	80
	1	91
Antipain	10	81
	1	90
Phosphoramidon	10	19
	1	65
Elastinal	10	90
	1	99
Leupeptin	10	100
	1	100

A mixture of the enzyme solution (10.0 μg/0.9 ml) in 0.1 M Tris–HCl (pH 7.0) and the inhibitor solution (20.0 or 2.0 μg/0.1 ml) was incubated with 1% casein solution (1 ml) in 0.1 M Tris–HCl (pH 7.0) at 37 °C for 20 min. The absorbance at 275 nm was measured against the blank after the addition of 10% TCA (3 ml) followed by centrifugation. The inhibitor solutions were prepared in MeOH (pepstatin, antipain, phosphoramidon and leupeptin) or H_2O (bestatin and elastinal).

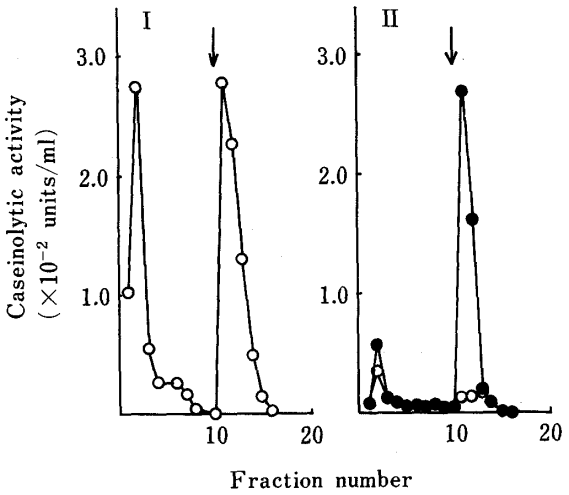

Fig. 4. Chromatography of the Native and EDTA-Treated Enzymes on Z–Gly–D-Leu–AH-Sepharose

(I) The crude enzyme obtained by ammonium sulfate precipitation (35 mg as protein) was charged on a Z–Gly–D-Leu–AH-Sepharose column (5.0 cm × 0.8 cm, diam.) which had been equilibrated with 1/15 M phosphate buffer (pH 7.0). The arrow indicates change of the buffer from the starting one to 1/10 M acetate buffer (pH 4.1) containing 2 M urea. The eluate was collected in 4.0 ml fractions. The caseinolytic activity was measured by the casein-275 nm method using 1% casein solution in 1/10 M Tris–HCl buffer (pH 7.0).

(II) The crude enzyme (35 mg) was treated with 2 mM EDTA at room temperature for 30 min and then charged on the same column equilibrated with 1/15 M phosphate buffer (pH 7.0) supplemented with 1 mM EDTA. After the column had been thoroughly washed with the starting buffer, the buffer was changed to 1/10 M acetate buffer (pH 4.1) containing 2 M urea at the point indicated by the arrow. The caseinolytic activity was measured in the absence (○) and presence (●) of 1 mM $ZnCl_2$ in 1% casein solution.

Sepharose is compared with that of the native enzyme in Fig. 4. The elution of the inactive enzyme was started with 1/15 M phosphate buffer (pH 7.0) containing 1 mM EDTA instead of 1/15 M phosphate buffer (pH 7.0) as used for the native enzyme. The enzyme activity was determined in the absence and presence of 1 mM $ZnCl_2$ in the casein solution. No activity was observed in the fractions collected with the starting buffer, although the binding ability of the EDTA-treated enzyme was suggested by the recovery of caseinolytic activity in the fractions collected with 1/10 M acetate buffer (pH 4.1) supplemented with 2 M urea.

Hydrolysis of Various Synthetic Substrates

Z–Gly–Leu–NH_2, Z–Gly–Phe–NH_2 and Z–Gly–Leu–Tyr were well hydrolyzed by the enzyme among the substrates tested in this work. These substrates were hydrolyzed at the sites indicated by the arrows in Table VI.

TABLE VI. Hydrolysis of Synthetic Substrates

Substrate	Hydrolysis rate (relative value)	Substrate	Hydrolysis rate (relative value)
Z–Gly↓Leu–Tyr	117	Z–Glu–Tyr	0
Boc–Pro–Leu–Gly–NH_2	0	Z–Gly–Phe	0
Z–Gly↓Leu–NH_2	100	Z–Phe–Tyr	0
Z–Gly↓Phe–NH_2	145	Z–Gly	0
Z–Pro–Leu–NH_2	0	Z–Phe	0
Z–Gly–D-Leu–NH_2	3	ClAc–Tyr	0
Z–Gly–Leu	2		

A mixture of 5 mM substrate solution (250 μl) in 50 mM Tris–HCl (pH 7.0) and the aqueous enzyme solution (25.0 μg/250 μl) was incubated at 37 °C for 10 min. After the addition of 1 N AcOH (250 μl) and then Moore's ninhydrin solution[15] (500 μl), the whole was kept at 100 °C for 10 min. The absorbance at 570 nm was measured against the blank and the absorbance with Z–Gly–Leu–NH_2 was defined as 100. The scissile bond of the peptide was identified by silica gel thin layer chromatography of the products. The reaction mixture, composed of the aqueous enzyme solution (50.0 μg/500 μl) and 5 mM substrate solution (500 μl) in 10 mM Tris–HCl (pH 7.0), was lyophilized after a 2 h incubation at 37 °C. The residue was dissolved in 50% aqueous MeOH (100 μl) and charged on silica gel plate (Merck, Kiesel gel 60 F_{254}) which was developed with either *n*-BuOH–AcOH–H_2O (4:2:1) or iso-PrOH–AcOEt–NH_4OH (7:9:4). The digested substrates were analyzed on thin layer plates under a UV lamp and then by spraying 1% ninhydrin solution. Boc–, *N-tert*-butoxycarbonyl; ClAc–, chloroacetyl.

Discussion

From the results presented here, the purified enzyme was concluded to be a member of the class of neutral metalloendopeptidases of microbial origin such as thermolysin[11] and neutral metalloendopeptidase *amylosacchariticus*.[12] The newly isolated enzyme showed anti-inflammatory activity against carrageenan-induced edema, and the molecular weight of the enzyme (59000) is the largest among those reported for enzymes in this group (thermolysin,[13] 37500; *Streptomyces naraensis* neutral metalloendopeptidase,[14b] 37000; neutral metalloendopeptidase from *Bacillus subtilis* var. *amylosacchariticus*,[12] 33800; alkinonase A produced by *Streptomyces violaceorectus*,[1f] 35000; neutral metalloendopeptidase from *Streptomyces griseoruber*,[1g] 52000).

As described above, the *Str. griseoruber* enzyme was adsorbed on Z–Gly–Leu–AH-Sepharose at neutral pH and could be eluted from the column with 1/10 M acetate buffer (pH 4.1) containing 2 M urea. Neither a lower pH value of the elution buffer nor the addition of urea was sufficient for the elution of the enzyme. This is also the case in the purification of the *Streptomyces mauvecolor* enzyme by Z–Gly–D-Leu–AH-Sepharose chromatography. Furthermore, NaCl could not substitute for urea in either case. Recently, Hiramatsu[14a] reported the purification of a neutral metalloendopeptidase produced by *Str. naraensis* by affinity chromatography on Z–Phe–AH-Sepharose. The *Str. naraensis* enzyme was adsorbed on the column equilibrated with Tris–HCl buffer (pH 7.2) and eluted by increasing the NaCl concentration in the starting buffer to 0.3 M. No adsorption was observed at pH lower than 7.0. Because these observations were quite different from those in our system, we applied Z–Phe–AH-Sepharose to the purification of our enzymes. Alkinonase A could not be retained on this adsorbent at any pH between 4.1 and 9.5, while no elution of the adsorbed neutral metalloendopeptidases was observed within the same pH range without the addition of NaCl to phosphate buffer (pH 7.0). From the elution profile of the *Str. naraensis* enzyme, ionic interaction seems to contribute substancially to the affinity of the enzyme for Z–Phe–AH-Sepharose. In contrast, this type of interaction has no importance in the cases of neutral metalloendopeptidases produced by *Str. griseoruber* and *Str. mauvecolor*, and a confor-

mational change induced by urea might be necessary to reduce the affinity of the enzymes for this adsorbent.

As shown in Fig. 4, the EDTA-treated enzyme retains the binding ability to Z–Gly–D-Leu–AH-Sepharose, and this result is in marked contrast with the findings of Fujiwara *et al.*[16] that modification of the active-site serine residues of α-chymotrypsin and subtilisin by treatment with diisopropylfluorophosphate or phenylmethanesulfonyl fluoride resulted in loss in their binding abilities to the affinity adsorbent, *N*-benzyloxycarbonylphenylalanyltriethylenetetraminyl-Sepharose. It remains to be elucidated whether the affinity of the *Str. mauvecolor* enzyme for Z–Gly–D-Leu–AH-Sepharose is catalytic site-specific or is simply due to a predominantly hydrophobic interaction. If the former is the case, the contribution of the metal ion in the catalytic site to the binding of the enzyme to the adsorbent seems to be negligible. The difference in the affinities of the enzyme for Z–Gly–Leu–AH-Sepharose and Z–Gly–D-Leu–AH-Sepharose suggests that the interaction between the enzyme and these adsorbents is somewhat catalytic site-specific.

While Z–Gly–Leu–NH_2 is one of the best substrates of alkaline and neutral metalloendopeptidases,[1c,f,h,11c] the ratios of the hydrolysis rate of Z–Gly–Leu–Tyr to that of Z–Gly–Leu–NH_2 by alkinonase A and neutral metalloendopeptidases produced by *Str. griseoruber* and *Str. mauvecolor* are 35, 0.01 and 1, respectively. This significant difference in values suggests the existence of heterogeneity in the substrate specificities, and it might be interesting to compare the substrate preferences of the enzymes in more detail.

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