Purification of an *Aspergillus oryzae* Metallo-proteinase by Talopeptin-aminohexyl-Sepharose and Its Properties

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Simple and speedy purification of *Aspergillus oryzae* metallo-proteinase was performed using Talopeptin-aminohexyl-Sepharose. The properties of the metallo-proteinase were: optimum pH 6.5; pH stability, pH 5~11; optimum temperature, 50°C; and molecular weight 42,000 (SDS electrophoresis). These results were similar to those of neutral protease I from *Aspergillus oryzae* reported by Nakadai *et al.* This metallo-proteinase was compared with others from microbes using the metallo-proteinase inhibitors FMPI, PLT, and Talopeptin. The metallo-proteinase is unique in the point at which FMPI and PLT gave nearly stoichiometrical inhibition.

A metallo-proteinase is a proteinase with zinc atoms in its catalytic residues.¹⁾ Purification and some properties of metallo-proteinases from various microbial origins have been reported by several workers.¹⁾ These reports indicated that despite the difference of their origins, these metallo-proteinases have similar properties, for example, molecular weight, amino acid composition, specificity against synthetic substrates, and splitting points against the insulin B-chain. However, the properties of metallo-proteinases from molds are less well-understood than those from bacteria, because purification of metallo-proteinases from molds is difficult. Molds produce both metallo-proteinases and serine-proteinases usually less of the latter. Moreover, metalloproteinases from molds are often inactivated by contaminating serine proteinase under purification conditions. Therefore, a new purification method to obtain metallo-proteinases from molds is necessary.

In this report we will describe a simple and speedy purification of *Aspergillus oryzae* metallo-proteinase using Talopeptin-aminohexyl-Sepharose and will describe some properties of the purified enzyme. Comparison

of the reaction of the enzyme and other bacterial metallo-proteinases with specific metallo-proteinase inhibitors, FMPI,^{2~5)} PLT,⁵⁾ and Talopeptin,^{6~8)} is also reported.

MATERIALS AND METHODS

Chemicals and materials. Sepharose 4B was purchased from the Pharmacia Japan Co., Tokyo. *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1,6-di-aminoheptane were from the Wako Pure Chem. Industry Co., Osaka.

Enzymes and substrates. Metallo-proteinase of *Asp. oryzae* was purified from Biodiastase® which was kindly supplied by the Amano Pharmacy Co., Nagoya. Metallo-proteinase of *Streptomyces griseus* was further purified from an enzyme powder of neutral proteinase from *Streptomyces griseus*,⁹⁾ Type V, the Sigma Chem. Co., St. Louis, using Talopeptin-aminohexyl-Sepharose and gel filtration. Thermolysin¹⁰⁾ (3 times crystallized) was kindly supplied by the Daiwa Kasei Co., Osaka. The crystalline metallo-proteinase of *Pseudomonas aeruginosa*¹¹⁾ IFO-3455 was kindly donated by Dr. Morihara of the Shionogi Co., Osaka. The crystalline metallo-proteinases of *Bacillus subtilis* var. *amylosacchariticus*¹²⁾ and *Bacillus amyloliquefaciens*¹³⁾ were kindly donated by Professor Tsuru of Nagasaki University. Casein according to Hammarsten was purchased from the Wako Pure Chemical Co., Osaka.

Inhibitors. The metallo-proteinase inhibitors, FMPI and

Abbreviations: FMPI, *N*-phospho-L-phenylalanyl-L-arginine; PLT, *N*-phospho-L-leucyl-L-tryptophan; Talopeptin, 6-deoxy- α -L-talopyranosyloxyphospho-L-leucyl-L-tryptophan; AH, aminoheptyl.

Talopeptin were purified from culture filtrates of *Streptomyces rishiriensis* NK-122²⁻⁴⁾ and *Streptomyces mozuensis* MK-23,⁶⁻⁸⁾ respectively. PLT was prepared from Talopeptin by alkaline hydrolysis as described previously.⁵⁾

Assay methods. Activities of enzymes were assayed by measuring caseinolysis according to the methods described previously.²⁾

Measurement of protein concentration. The protein concentrations of sample were estimated on the basis of their absorption at 280 nm.

Amino acid analysis. Purified enzyme protein was hydrolyzed with 6N HCl at 110°C in evacuated sealed tubes for 24, 48, and 72 hr. Amino acid analysis was performed by a Hitachi High Speed Amino Acid Analyzer KLA-835. The amounts of cysteine and tryptophan were determined according to the methods of Moore¹⁴⁾ and Goodwin,¹⁵⁾ respectively.

Difference spectrum. Difference spectra were measured in 0.1 M Tris-HCl buffer, pH 7.5, containing 10 mM calcium acetate using a Hitachi Spectrophotometer 320.

Preparation of Talopeptin-aminoethyl-Sepharose. Forty milliliters of Sepharose 4B was washed with 1 M NaCl and deionized water and then mixed with 20 g of CNBr in 40 ml deionized water in an ice bath. The activated Sepharose 4B was mixed with 8 g of 1,6-diaminohexane in 40 ml deionized water at pH 10.5 and stood overnight. Then, eighty-five milligrams of Talopeptin was mixed with the prepared AH-Sepharose 4B in 40 ml deionized water and 400 mg of EDC was added. The mixture was left at room temperature, pH 6 with gentle stirring overnight. The yield of immobilization of Talopeptin was 82%. The column was equilibrated with 0.5 M NaCl before use.

RESULTS AND DISCUSSION

Purification of *Asp. oryzae* metallo-proteinase

Step. 1. Affinity chromatography on Talopeptin-AH-Sepharose. Fifty grams of Biodiastase® enzyme powder was dissolved in 500 ml of 0.5 M NaCl containing 10 mM calcium acetate and centrifuged at 10,000 × *g* for 10 min at 0°C. The supernatant was applied to a column of Talopeptin-AH-Sepharose 4B (3.5 × 4 cm) at a flow rate of 150 ml/hr. The column was washed with the same eluent, and then the metallo-proteinase was eluted with 0.1 M Tris containing 0.5 M NaCl and 10 mM calcium acetate, pH 9.5. Figure 1 shows the

FIG. 1. Elution Profile of *Asp. oryzae* Metallo-proteinase on a Talopeptin-AH-Sepharose Column.

Five hundred milliliters of enzyme solution (Biodiastase 100 mg/ml) was applied to a column (3.5 × 4 cm). The vertical arrow indicates the position where the eluate solution was changed to 0.1 M Tris-0.5 M NaCl containing 10 mM calcium acetate, pH 9.5. Flow rate: 150 ml/hr (before vertical arrow) and 25 ml/hr (after vertical arrow); Fractionation: 5 ml/tube. Symbols: ○, O.D. at 280 nm; ●, proteinase activity.

elution profile. Proteinase activity of the eluted solution was inhibited completely by 10 mM EDTA; therefore, it is evident that the solution contains metallo-proteinase free from serine-proteinase. Thermolysin and *Streptomyces griseus* metallo-proteinase could also be absorbed on to the column.

Step 2. Gel filtration on Sephadex G-100. The enzyme solution obtained above was condensed to 20 ml using a Diafilter MC-50 in an ice bath. Ten milliliters of condensed sample was applied onto a column of Sephadex G-100 (2.5 × 95 cm), previously equilibrated with 10 mM calcium acetate. The sample was eluted with the same eluent and 5 ml fractions were collected. A typical elution profile is shown in Fig. 2. Three kinds of metallo-proteinases were found. Since the amounts of metallo-proteinases in peaks of A and C were smaller

TABLE I. SUMMARY OF PURIFICATION OF *Asp. oryzae* METALLO-PROTEINASE

Procedures	Volume (ml)	Total act. (PU)	Total A_{280} ($A_{280} \times \text{ml}$)	Specific act. (PU/ A_{280})	Yield (%)
Biodiastase powder	500	2,420,000	499,000	4.8	100
Talopeptin-AH-Sepharose	478	1,950,000	1,030	1,890	80.7
Sephadex G-100	49	510,000	320	1,590	21.1

FIG. 2. Gel Filtration of *Asp. oryzae* Metallo-proteinase on a Sephadex G-100 Column.

After treatment with Talopeptin-AH-Sepharose *Asp. oryzae* metallo-proteinase was applied to a column of Sephadex G-100 (2.5 $\times$ 95 cm). Fractionation: 6.5 ml/tube. Symbols: $\circ$, O.D. at 280 nm; $\bullet$, proteinase activity.

than that of peak B, the metallo-proteinase in peak B was purified hereafter. The metallo-proteinase in peak B was pooled and then ammonium sulfate was added in an ice bath until the concentration of ammonium sulfate reached 0.9 saturation. The white cloudy mixture was centrifuged at $10,000 \times g$ for 10 min at 0°C and the precipitate was collected. The precipitate was dissolved in 10 ml of 10 mM calcium acetate, and then applied onto a column of Sephadex G-100 under the same conditions as in the first gel filtration. Fractions containing metallo-proteinase were collected

FIG. 3. Polyacrylamide Gel Electrophoresis of *Asp. oryzae* Metallo-proteinase.

pH 9.5: The sample was run in the cold room (4°C) for 40 min at 4 mA per gel column.

SDS: The sample was run for 3 hr at 7 mA of per gel column (pH 7.2 $\sim$ 10% gel with SDS) after reduction and denaturation with SDS and mercaptoethanol (100°C for 3 min). Protein was stained with Coomassie brilliant blue.

and stored at -20°C (yield 21%). Summary of purification was shown in Table I.

Disc and polyacrylamide electrophoresis

Purified metallo-proteinase was homogeneous by SDS¹⁶⁾ and polyacrylamide electrophoresis¹⁷⁾ as shown in Fig. 3. Figure 4 shows a plot of R_m values against molecular weight of standard proteins and the metallo-proteinase. Molecular weight of the metallo-proteinase was estimated to be 42,000. This

FIG. 4. Estimation of Molecular Weight of *Asp. oryzae* Metallo-proteinase by SDS-Polyacrylamide Gel Disc Electrophoresis.

Standard proteins: A, bovine albumin; B, egg albumin; C, thermolysin; D, chymotrypsinogen; E, *Aspergillus oryzae* metallo-proteinase. The conditions were the same as in Fig. 3.

value is similar to that of "Neutral protease I of *Asp. oryzae*" (41,000) reported by Nakadai *et al.*¹⁸⁾

Some properties of the metallo-proteinase

The purified *Asp. oryzae* metallo-proteinase had an optimum pH of 6.5; optimum temperature of 50°C; and pH stability, pH 5~11 (at 37°C for 3 hr); $E_{280}^{1\%} = 18.2$. The optimum pH and pH stability of this enzyme are similar to those of neutral protease I of *Asp. oryzae* reported by Nakadai *et al.*¹⁸⁾

Difference absorption spectra accompanying the enzyme-FMPI complex formation

The ultraviolet absorption difference spectra caused by mixing thermolysin or *Asp. oryzae* metallo-proteinase with FMPI are shown in Fig. 5. The main peaks were seen around 295 nm and 285 nm. The difference spectra of *Asp. oryzae* metallo-proteinase and thermolysin caused by FMPI are similar. Absorbance peak height of difference spectra are plotted against amounts of FMPI and molar ratio

FIG. 5. Ultraviolet Absorption Difference Spectra Caused by Interaction of Thermolysin and *Asp. oryzae* Metallo-proteinase with FMPI.

Enzyme concentration, 3.0×10^{-5} M (based on the values of $E_{280}^{1\%}$ and molecular weight; thermolysin: 17.65 and 37,500, *Asp. oryzae* metallo-proteinase: 18.2 and 42,000); FMPI concentration, 6.0×10^{-5} M. Symbols: ----, thermolysin; —, *Asp. oryzae* metallo-proteinase.

FIG. 6. Absorbance Peak of Difference Spectra was Plotted against Amounts of FMPI and Molar Ratio (Enzyme/FMPI).

Symbols: ■, thermolysin; ●, *Asp. oryzae* metallo-proteinase (amounts of enzymes, 6×10^{-8} mol).

(enzyme/FMPI) as shown in Fig. 6. FMPI binds to the enzymes in the molar ratio of one to one.

Amino acids composition

Table II shows the amino acid composition of *Asp. oryzae* metallo-proteinase and that of thermolysin as a reference. Compared with

TABLE II. COMPARISON OF AMINO ACID COMPOSITIONS OF *Asp. oryzae* METALLO-PROTEINASE AND THERMOLYSIN

Amino acid	<i>Asp. oryzae</i> ^a	Thermolysin ¹⁹⁾
Lys	3	12
His	9	9
Arg	18	10
Try	4	55
Asp	72	43
Thr	30	23
Ser	24	23
Glu	36	20
Pro	27	8
Gly	45	36
Ala	42	28
Cys-SO ₃ H	1	0
Val	9	24
Met	9	2
Ile	12	18
Leu	30	17
Tyr	24	29
Phe	12	10
Ammonium	45	38
Total	407	317

^a Based on a molecular weight of 42,000.

thermolysin, Asp and Pro are plentiful in *Asp. oryzae* metallo-proteinase.

Interaction with inhibitors

a) *Inhibition against caseinolytic activity.* ID₅₀ values (moles of inhibitor necessary for 50% inhibition) and molar ratio of inhibitors to the enzymes at the values against caseinolytic activity is summarized in Table III. *Asp. oryzae* and *St. griseus* metallo-proteinases were most strongly inhibited by FMPI and PLT. The molar ratio in the case of *Asp. oryzae* was 0.4. This suggests that FMPI and PLT give nearly stoichiometrical inhibition of the enzyme.

b) *Effect of preincubation time on the inhibitory activity of FMPI.* The enzymes were mixed with FMPI to cause 50% inhibition, and the effect of preincubation time on restoring activity was investigated. The result is shown in Fig. 7. The proteinase from *Asp. oryzae* was not restored even after two hours. But with the other enzymes, the proteinase activity gradually increased during the elongation of pre-

TABLE III.

Metallo-proteinases	ID ₅₀ values (upper; $\times 10^{-10}$ mol) and molar ratios (bottom; inhibitor/enzyme) at the values against caseinolytic activity		
	FMPI	PLT	Talopeptin
<i>Asp. oryzae</i>			
	1	1	16
	0.4	0.4	5
	5	2	13
<i>B. thermoproteolyticus</i>			
	5.6	2.2	15
<i>B. subtilis</i> var. <i>amylosacchariticus</i>			
	800	9	1200
	440	5	660
	1000	9	1800
<i>B. amyloliquefaciens</i>			
	430	4	790
	54	13	26
<i>Ps. aeruginosa</i> IFO 3455			
	29	7	14
	1	1	11
<i>St. griseus</i>			
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FIG. 7. Effect of Preincubation Time on Reversing Inhibition by FMPI.

Various enzyme solutions (0.1 M Tris-HCl buffer, containing 2 mM calcium acetate, pH 7.5, 40 PU) and FMPI (in the same buffer solution) were mixed and incubated at 37°C, and then assayed for restored proteinase activity. Symbols: ●, *Asp. oryzae*; ▲, *St. griseus*; ■, thermolysin; □, *Ps. aeruginosa* IFO 3455.

incubation time. These results are considered as follows: in the former case, all of the FMPI had formed stable enzyme-FMPI complexes, so that FMPI was not inactivated, but in the latter case, an excess of FMPI was needed for 50% inhibition, so that free FMPI was decomposed as described previously,²⁾ and consequently the proteinase activity was restored. These results indicated that FMPI binds most tightly to *Asp. oryzae* metallo-proteinase and supported the results in Table III.

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