Serratia Protease

Part III. Characteristics of the Enzyme as Metalloenzyme†

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Protease from a strain of *Serratia* contained one gram atom of zinc ion per mole and the zinc ion was essential for the activity. Also zinc-free apoenzyme was isolated as a crystalline form from the native-enzyme. Several metalloenzymes were prepared by the addition of corresponding metal ions to the apoenzyme. Studies on activities toward the hydrolysis of casein showed that relative activities of native- (zinc), cobalt- and manganese-enzyme were 1.0, 1.2 and 0.8, respectively. Toward the hydrolysis of hippurylleucinamide, however, specific activity of cobalt-enzyme was about 10 times that of the native- (zinc-) enzyme. Spectroscopic studies did not reveal any significant differences in conformations among native-enzyme, apoenzyme and the other metalloenzymes.

The difference in reactive function of many proteases led to the general classification of Serine-, Metallo-, Thiol- and Acid-proteases.¹⁾ Up to the present, however, *Serratia* protease has not been investigated on the reactive function in details. In the previous papers,^{2,3)} the authors reported isolation of protease from culture broth of *Serratia* sp. E-15, and the properties and the substrate specificity of the enzyme. In this paper, we present experiments to show that apoenzyme and some metalloenzymes are prepared from native enzyme, and chemical, physical and enzymatic properties of these enzymes are compared with each other. This report also includes studies on the evidence that the *Serratia* protease is a zinc enzyme.

MATERIALS AND METHODS

Assay of enzyme activity. Proteinase activity was measured by the hydrolysis of casein according to the method described previously.²⁾ Peptidase activity was measured by determining the rate of the hydrolysis of hippurylleucinamide (Mann Res. Lab.) according to the Yemm and Cocking ninhydrin method.⁴⁾ The reaction was performed with 10 mg of the substrate and 1.0 mg of enzyme in 5.0 ml of 0.02M phosphate buffer, pH 7.0 at 30°C. At intervals, 0.5 ml aliquots of the reaction mixture were withdrawn for the assay by the ninhydrin method.⁴⁾ The peptidase unit was defined as Co value (zero order velocity constant) corresponding to micromoles equivalent of leucinamide released per minute per mg of enzyme under the above condition.

Preparation of apoenzyme and metalloenzymes. Native-enzyme was prepared as previously described²⁾ and its homogeneity was electrophoretically and ultracentrifugally verified. Native-enzyme (1 g) was inactivated completely by treating with 0.01M EDTA in 50 ml of 0.1M acetate buffer, pH 5.0, for 30 min, followed by dialysis against 1 mM EDTA in 0.01M phosphate

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buffer, pH 7.0, for 3 days to give its apoenzyme. Crystallization of apoenzyme was carried out by further dialysis against distilled water for 2 weeks after the precipitation of the apoenzyme with ammonium sulfate (60%). Respective metalloenzymes were prepared from the apoenzyme (0.2 g), by the addition of corresponding metal ions as chlorides (2 mM) to the apoenzyme, followed by dialysis against distilled water for 1 day and then against 0.01 M phosphate buffer, pH 7.0, for 3 days. These procedures were carried out below 4°C. Protein concentration was measured spectrophotometrically as previously described.²⁾

Analysis of metal ions. Each enzyme prepared as described above was dialyzed against distilled water at 4°C for 3 days and further lyophilized. The enzyme (ca. 40 mg) was hydrolyzed in 1.0 ml of HNO₃ and HClO₄ mixture (1:1, v/v) by heating at 100°C for 1 hr. Metal ions in the hydrolyzate were determined by atomic absorption spectroscopy using Perkin-Elmer model 303 apparatus.

Physical measurements. UV-absorption spectrum of the enzyme was measured with Beckman DB spectrophotometer. Optical rotatory dispersion measurements were performed with both spectropolarimeters of Perkin-Elmer model 141 and JASCO model ORD/UV-5. The resulting data were interpreted by the Drude equation⁵⁾ and the Moffitt-Yang equation⁶⁾ and expressed in terms of the parameters, λ_e , a_0 and b_0 , respectively. The data of the ORD measurements in the UV region were represented in term of the reduced mean residue rotation, (m') .⁷⁾ The mean residue weight was estimated to be 115.

Gel-filtration. Gel-filtration was performed on a column (3.0 × 46 cm) of Sephadex G-75 equilibrated with 0.01 M phosphate buffer, pH 7.0, to which was added 1.0 ml of the enzyme (3 to 10 mg) solution, followed by elution with the same buffer. For the gel-filtration of apoenzyme, the column was equilibrated with 0.01 M phosphate buffer, pH 7.0, containing 1 mM EDTA, and apoenzyme was eluted with the same solution.

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RESULTS

A. Effect of various reagents

The enzyme was preincubated with various

TABLE I. EFFECTS OF VARIOUS REAGENTS ON ENZYME ACTIVITY

Reagent	Incubation time	
	1 hr	3 hr
(A)	(relative activity)	
Control (no addition)	100 (%)	100 (%)
EDTA	0.36	0.20
CyDTA ^{a)}	16	4.8
<i>o</i> -Phenanthroline	85	65
Diethyldithiocarbamate	100	95
Iminodiacetate	100	100
Nitrilotriacetate	67	56
Citrate	93	87
Oxalate	100	99
Ethylenediamine	100	100
MgCl ₂	100	98
CaCl ₂	100	100
SrCl ₂	99	95
MnCl ₂	99	98
CoCl ₂	114	120
NiCl ₂	74	55
ZnCl ₂	98	95
CdCl ₂	67	37
CuSO ₄	15	15
HgCl ₂	10	2.3
(B)		
Control (no addition)	100 (%)	100 (%)
Cysteine	100	101
Thioglycollate	100	102
Hydroquinone	100	94
Iodoacetate	102	96
PCMB ^{b)}	100	100
Azide	100	96
Arsenite	100	94
Ferricyanide	94	95
N-Ethylmaleimide	100	100
DFP ^{b)}	100	100

After the enzyme (1 mg/ml) was incubated in 0.01 M solution of a given reagent at pH 5.0 (A) or pH 7.0 (B), at 4°C for 1 and 3 hr, respectively, the residual activity was measured.

^{a)} 0.005 M, ^{b)} 0.001 M.

reagents at 4°C, and then the remaining enzyme activity was measured. The results are summarized in Table I. The enzyme was markedly inactivated by the preincubation with metal chelating agents such as EDTA and CyDTA*. While, its activity was not inhibited even in the presence of 0.1 M EDTA without the preincubation. Of various metal ions, heavy metal ions such as Ni^{2+} , Cd^{2+} and Hg^{2+} inactivated the enzyme activity, whereas Co^{3+} showed slight activating effect on the enzyme. DFP**, and SH-reagents such as PCMB*** and iodoacetate, had no inactivating effect. From these results, this *Serratia* protease was considered to be a sort of metallo-enzyme which requires an essential metal ion for the activity.

B. Effect of pH on inactivation with EDTA

Native-enzyme was preincubated at 4°C with EDTA at a given pH and the residual activity was measured. As shown in Fig. 1, the enzyme was completely inactivated with EDTA in an acidic condition, but slightly in

FIG. 1. Effect of pH on EDTA-Inactivation.

The enzyme (1 mg/ml) was incubated at a given pH in the presence or absence of 0.01 M EDTA at 4°C for 30 min and the residual activity was measured.

- , Control (absence of EDTA);
●—●, Presence of EDTA.

* CyDTA: 1,2-cyclohexanediaminetetraacetate.

** DFP: diisopropylfluorophosphate.

*** PCMB: *p*-chloromercuribenzoate.

an alkaline condition. However, when the enzyme was preincubated at an elevated temperature of 37°C and at pH 8.0, it was completely inactivated by EDTA within 3 hr.

C. Properties of apoenzyme

1) *Crystallization.* Apoenzyme was prepared from native-enzyme by treatment with EDTA as described in Methods. After apoenzyme (3%) was dialyzed against distilled water, it

FIG. 2. Crystalline Form of Apoenzyme.

was obtained as a crystalline form, as shown in Fig. 2. But native-enzyme could not be obtained as a crystalline form according to the same dialysis procedure.

FIG. 3. Reactivation of Apoenzyme with Various Metal Ions.

After apoenzyme (1 mg/ml) was supplemented with 0.001 M each metal ion as chloride at pH 5.0 and at 4°C, the restored activity was measured.

2) *Reactivation of apoenzyme with various metal ions.* The reactivation was attempted by the addition of various metal ions to the apoenzyme at 4°C. The results are shown in Fig. 3. Zn^{2+} , Co^{2+} and Mn^{2+} were effective for the reactivation. The reactivation occurred immediately by the addition of Zn^{2+} , Co^{2+} or Mn^{2+} in a pH range from 4 to 10 at 4°C.

3) *Effect of pH and temperature on the reactivation.* After apoenzyme was preincubated at a given pH and at an elevated temperature

FIG. 4. Reactivation of Apoenzyme.

Apoenzyme (1 mg/ml) was incubated at a given pH at 37°C for 10 min in the presence of 0.5mM EDTA. After incubation, the apoenzyme solution was supplemented with 1.0mM ZnCl_2 and then the restored activity was measured.

FIG. 5. Difference Spectrum of Alkali-Treated Apoenzyme *vs.* Untreated Apoenzyme.

Sample cell: apoenzyme (0.70 mg/ml) in Tris-HCl buffer, incubated at pH 9.0 at 37°C for 10 min.

Reference cell: apoenzyme (0.70 mg/ml) in Tris-HCl buffer, pH 9.0, without incubation.

of 37°C for 10 min, the reactivation was measured by the addition of Zn^{2+} . Figure 4 shows the result. The reactivation occurred in a range from pH 4 to 7, whereas, when the pH was raised from 7 to 10, the reactivation was no longer observed by the addition of any metal ions. But any ninhydrin reactive materials were not released from the alkali-treated apoenzyme. As shown in Fig. 5, the difference spectrum of the alkali-treated apoenzyme at 37°C versus the untreated apoenzyme showed a typical blue shift of negative peaks at 289, 283 and 278 mμ. Furthermore, as shown in Table IV, both ORD parameters of λ_c of the Drude equation⁵⁾ and $-b_0$ of the Moffitt-Yang equation⁶⁾ of the apoenzyme were decreased according to the treatment. These changes seem to be characteristic of the structural change of the apoenzyme from a folding form to a random coil.

D. Comparison of native-enzyme, apoenzyme and metalloenzyme

1) *Metal ion analysis of the enzymes.* Co-, Mn- and Zn-enzymes were prepared from apoenzyme by the procedure described in Methods and their metal contents were analyzed. Table II shows the metal contents. The analysis gave a value of about 1 gram atom of zinc ion per mole of native- or Zn-enzyme. Also the value of Co- or Mn-enzyme indicated the presence of about 1 gram atom of cobalt or manganese ion per mole of the respective enzyme. A trace value of zinc ion was observed in apoenzyme. This value may be caused by a contamination during the analysis procedure. On the other hand, calcium ion was found in all enzyme preparations.

2) *Enzyme activities.* The proteinase and peptidase activities were measured with casein and hippurylleucinamide as substrates, respectively. The results are shown in Table III. Native- and Zn-enzyme showed to be essentially equal in both proteinase and peptidase activities. Apoenzyme revealed neither activity.

TABLE II. METAL CONTENTS OF NATIVE-ENZYME, APOENZYME AND METALLOENZYMES

Enzyme	Content of metal ions (%)							
	Zn ²⁺	Co ²⁺	Mn ²⁺	Ca ²⁺	Ni ²⁺	Fe ²⁺	Cu ²⁺	Mg ²⁺
Native-enzyme	0.12 (1.1)	0	0	0.38	0	0.002	0.001	0.003
Apoenzyme	0.006 (0.05)	0	0	0.23	0	0.001	0.001	0.004
Co-enzyme	0.007 (0.06)	0.10 (1.0)	0	0.37	0.001	0.004	0.001	0.002
Mn-enzyme	0.004 (0.04)	0	0.075 (0.82)	0.41	0	0.001	0.001	0.002
Zn-enzyme	0.13 (1.2)	0	0	0.30	0	0.001	0.001	0.004

Number in parenthesis represents gramatoms of metal ion per mole of enzyme, respectively, based on the molecular weight of 60,000.²¹

TABLE III. ENZYME ACTIVITIES OF NATIVE-ENZYME, APOENZYME AND METALLOENZYMES TOWARD CASEIN AND HIPPURYLLEUCINAMIDE

Enzyme	Substrate			
	Casein		Hippurylleucinamide	
	PU/mg	relative activity	C ₀ (× 10 ³)	relative activity
Native-enzyme	6,330	100 (%)	2.2	100 (%)
Apoenzyme	0	0	0	0
Co-enzyme	7,600	120	26	1130
Mn-enzyme	5,070	80	1.8	80
Zn-enzyme	6,460	102	2.2	100

Relative proteinase activities of native-, Co- and Mn-enzyme were 1.0, 1.2 and 0.8, respectively. In contrast to proteinase activity, peptidase activity of Co-enzyme increased ten fold that of the native-enzyme.

3) *pH-stability*. As shown in Fig. 6, the native-enzyme and metallo-enzymes were stable in the pH range from 5 to 7. However, these enzymes were markedly inactivated in alkaline solution. Figure 7 indicates that the inactivation rate of the respective enzyme in an alkaline solution was directly correlated to the autodigestion rate of the enzyme, that is, Co-enzyme exhibited the most rapid inactivation and autodigestion rates among these

FIG. 6. pH-Stability of Native-enzyme and Metalloenzymes.

Enzyme (1 mg/ml) was incubated at a given pH at 37°C for 1 hr and the residual activity was measured by the assay of proteinase activity.

○—○, Native-enzyme; ▲—▲, Zn-enzyme; ×—×, Co-enzyme; □—□, Mn-enzyme.

enzymes. The pH-stability curve of Zn-enzyme was substantially similar to that of the native-enzyme.

4) *Thermo-stability*. Thermo-stability was measured at pH 6.0, at which these enzymes were most stable. The enzymes were incubated at 50°C and the remaining enzyme activity was assayed. As shown in Fig. 8, Mn-enzyme was the most labile, and Co-enzyme was less stable than the native-enzyme. Zn-enzyme was quite similar to the native-enzyme in the

FIG. 7. Release of Free Amino Groups during Alkali Inactivation of Native-enzyme and Metalloenzymes.

Enzyme (1 mg/ml) was incubated in 0.1 M Tris-HCl buffer, pH 8.0 at 37°C. At intervals, the residual proteinase activity (A) and the ninhydrin reactive materials (B) in the enzyme solution were assayed, respectively.

○—○, Native-enzyme; ▲—▲, Zn-enzyme; ×—×, Co-enzyme; □—□, Mn-enzyme.

FIG. 8. Thermo-stability of Native-enzyme and Metalloenzymes.

Enzyme (1 mg/ml) was incubated at pH 6.0 and at 50°C and the residual activity was measured by the assay of proteinase activity.

○—○, Native-enzyme; ▲—▲, Zn-enzyme; ×—×, Co-enzyme; □—□, Mn-enzyme.

thermo-stability.

5. *Molecular weight.* Comparison of the molecular weights of the native-enzyme, the apoenzyme and the metalloenzymes was performed on a column of Sephadex G-75. The respective molecular weights of these enzymes were calculated to be similar by the method of gel-filtration.

6) *Physical properties.* There were no differences in the UV-spectra of these enzymes, but the lyophilized Co-enzyme showed slightly pink color. The results of ORD measurements are summarized in Table IV. There were no

TABLE IV. OPTICAL ROTATORY PARAMETERS OF NATIVE-ENZYME, APOENZYME AND METALLOENZYMES

Enzyme	λ_c ($m\mu$)	a_0	b_0	$(m')_{233}$ ($\times 10^{-3}$)
Native-enzyme	230	-156	-35	-1.7 ± 0.2
Apoenzyme	230	-158	-34	-1.5 ± 0.2
Co-enzyme	233	-153	-31	-1.5 ± 0.2
Mn-enzyme	228	-155	-30	-1.7 ± 0.2
Zn-enzyme	228	-157	-28	-1.7 ± 0.2
Apoenzyme ^{a)} treated	216	-417	0	—

^{a)} Apoenzyme was treated at pH 9.0 and at 37°C for 10 min.

distinguishable differences in the values of λ_c , a_0 , b_0 and $(m')_{233}$ among native-, apo-, Co-, Mn- and Zn-enzyme. ORD curves of these enzymes were in close agreement with that of the native-enzyme, as shown in Fig. 9.

FIG. 9. Ultraviolet Optical Rotatory Dispersion of Native-enzyme.

0.25% enzyme in 0.01 M phosphate buffer, pH 7.0.
Cell length: 0.05 cm.

DISCUSSION

The native-enzyme was in agreements with Zn-enzyme in the respects of Zn^{2+} content, enzyme activity, pH-stability and thermostability, and the other metalloenzymes were different from native- or Zn-enzyme in those respects. The apoenzyme contained trace of Zn^{2+} and showed no enzyme activity. These results may introduce the conclusion that native-enzyme is zinc-enzyme. Ca^{2+} was found in all enzyme preparations. It is assumed that Ca^{2+} would bind tightly to the enzyme protein and could not be removed easily from the enzyme. *Bacillus subtilis* neutral protease,^{8,9)} a zinc-enzyme, was reported to be stabilized by Ca^{2+} , but this *Serratia* protease was not stabilized. The apoenzyme, furthermore, was not reactivated by Ca^{2+} . Therefore, Ca^{2+} may be not essential metal ion for the enzyme activity, but may be essential to maintain the conformation of *Serratia* protease. The protease was inactivated and reactivated quite reversibly at 4°C. However, when apoenzyme was

treated in an alkaline solution at 37°C, the reactivation of the apoenzyme was no longer observed as shown in Fig. 4. The non-reactivation is not due to an autodigestion of the apoenzyme, but due to a conformational change of the apoenzyme during the treatment as shown in Fig. 5 and Table IV. Apo-, Co-, Mn- and Zn-enzyme were indistinguishable from the native-enzyme by gel-filtration and the other physical measurements such as UV-spectrum and ORD-curve. These evidences suggested that the enzyme protein did not undergo marked conformational changes by the removal and the replacement of the metal ions. The helical content of this *Serratia* protease was relatively low, calculating from the values of ORD parameters such as λ_c , b_0 and $(m')_{233}$.

The inactivation of this *Serratia* protease by chelating agents and its reversal by some divalent metal ions suggest a similarity to carboxypeptidase A,¹⁰⁾ known as a zinc enzyme for which the role of metal ions has been well established. Valee *et al.*¹¹⁾ have reported that the zinc of carboxypeptidase A binds to a cysteine residue of the apoenzyme. Also the same evidence has been reported for human carbonic anhydrase.¹²⁾ *Serratia* protease,²⁾ however, was found to contain no cysteine residue. The same observation was demonstrated for bovine carbonic anhydrase¹³⁾ and *Bacillus subtilis* neutral protease.^{14,15)} The investigation of this problem is left in future.

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