Purification of Lytic Enzymes for Radioresistant Bacteria Produced by *Achromobacter lunatus*

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The crude enzyme fraction of precipitates resulting from the addition of 70% alcohol to the culture filtrate of *A. lunatus* was separated by CM-Sephadex and Sephadex G-75 chromatography into 13 fractions having lytic activity for *M. radiodurans*, *M. lysodeikticus* and *P. radiora*. Five of the fractions showed similar lytic activity spectra, but the other fractions were separated by the specificities of their lytic activities. This result indicates that the wide lytic spectrum of the crude enzyme against microorganisms is attributable to the action of many lytic enzymes. All fractions, except for P2-2 fraction (designated as the P2-2 enzyme), contained at least two proteins as determined by disc gel electrophoresis. The P2-2 enzyme was purified 34-fold by rechromatography on Sephadex G-75, and appeared to be homogeneous on disc gel electrophoresis. The enzyme was able to lyse intact cells of *M. radiodurans* and *M. lysodeikticus* without detergent, and those of *P. radiora* with detergent, but was not able to digest casein.

Most radioresistant bacteria are not lysed easily by egg white lysozyme (EC 3.2.1.17), which is widely used to disintegrate bacterial cell walls. Therefore, it was difficult to study and compare the mechanisms of radioresistance of these bacteria. From some of the experiments carried out for this purpose, the "R1 fraction" of *Streptomyces albus* G¹⁾ and a peptidase of *Achromobacter lyticus*²⁾ were found to lyse the cells of *Micrococcus radiodurans* in the presence of detergents. In addition, the cell walls of *M. radiodurans* were rendered sensitive to decomposition by lysozyme after an extraction with *n*-butanol-saturated buffer.³⁾ However, all these treatments require the presence of alcohol and detergent for lysis by the enzyme. Recently, Yoshinaka *et al.*⁴⁾ and our group⁵⁾ reported that the lytic enzymes of *Cytophaga* sp. and of *Achromobacter lunatus* isolated by Tsumura *et al.*^{6~9)} were available for lysis of radioresistant bacteria without alcohol and detergent.

In the previous paper⁵⁾ it was shown that the crude enzyme from *A. lunatus* was able to lyse various strains of bacteria, including radioresistant bacteria. This paper aims to elucidate whether or not the wide lytic spectrum of crude enzyme is attributable to one enzyme and to purify the lytic enzyme.

MATERIALS AND METHODS

Enzyme. Crude enzyme was kindly supplied by Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). The enzyme preparation is a lyophilized powder of the precipitate which is produced by adding enough alcohol to make a final concentration of 70% to the culture filtrate of *A. lunatus*. *A. lunatus* was grown aerobically at 28°C in a culture medium (pH 6.5) containing 1% glucose, 0.5% ammonium phosphate, 0.1% yeast extract and 0.05% magnesium sulfate.

Microorganisms. *Pseudomonas radiora* O-1, *M. radiodurans* R₁ and *M. lysodeikticus* IAM1056 were used for the assays of the lytic activities of enzymes. All bacteria were cultured with shaking at 30°C for two days in medium containing 1% polypeptone, 1% meat extract, 0.5% glucose, 0.3% yeast extract and 0.2% NaCl (pH 7.0). When the cells reached the stationary phase of growth, they were harvested by centrifugation, washed, resuspend-

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ed in distilled water and stored in a freezer at -20°C .

Lytic activities.

(i) *Bacteriolytic activity.* The cells of *M. radiodurans* and *M. lysodeikticus* were suspended in the reaction mixture to give an initial absorbance of 0.9 at 660 nm with an Hitachi 239 spectrophotometer. The reaction mixture contained 0.03 M Tris-HCl buffer (pH 8.4) and 0.2 ml of enzyme solution in a total volume of 5 ml. Water replaced the enzyme solution in the control. In the case of *P. radora*, Triton X-100 was added to the reaction mixture at final concentration of 0.5 mM. The mixture was incubated at 50°C for 10 min with *M. radiodurans* and *M. lysodeikticus*, and with *P. radora* at 50°C for one hour. Reduction of turbidity of the reaction mixture was measured by optical density at 660 nm. One unit of lytic activity was defined as the amount of enzyme causing a change of 0.001 optical density units per min.

(ii) *Proteolytic activity.* The activity was determined from the digestion of casein by the method of Tsumura *et al.*⁷⁾

Analytical methods. The amount of protein present was estimated by absorption at 280 nm assuming $E_{1\text{ cm}}^{1\%} = 10$. Disc gel electrophoresis of the enzymes was carried out with a pH 6.6 polyacrylamide gel using the method of Reisfeld.¹⁰⁾

RESULTS AND DISCUSSION

Exactly 2.0 g of the crude enzyme was dissolved in 20 ml of 0.01 M citrate buffer (pH 6.4). The solution was sonicated at 0°C for 5 min to decrease the viscosity, and applied to a column ($5 \times 52\text{ cm}$) of CM-Sephadex which had been equilibrated with the same buffer.

The purification was performed at 5°C unless otherwise stated. The lytic activities were eluted with approximately two liters of the citrate buffer. The elution pattern is shown in Fig. 1. The absorbances of F1 and F3 at 280 nm, as seen in the figure, corresponded well to the absorbances at 400 nm. Absorbance at 400 nm was not detected in fractions after No. 45. The F1 and F3 peaks contain the brown compounds which are seemed in the culture filtrate, and the F1 and F2 peaks have the lytic activities for *M. radiodurans* and *P. radora*. These activities were lower than those found in other fractions, as is shown later, so these peaks were not fractionated further.

FIG. 1. Elution Pattern of Lytic Activities from CM-Sephadex C-25 with 0.01 M Citrate Buffer (pH 6.4).

●, optical density at 280 nm; ○, *M. radiodurans*-lytic activity; ⊙, *P. radora*-lytic activity.

FIG. 2. Chromatogram of Bacteriolytic Enzymes on a Column of CM-Sephadex C-25.

The elution was carried out using a 0~0.5 M gradient of NaCl in 0.01 M citrate buffer (pH 6.4).

×, NaCl concentration. The other symbols in this figure are the same as those in Fig. 1.

FIG. 3. Gel Filtration of P1 Fraction on Sephadex G-75.

⊙, *M. lysodeikticus*-lytic activity; △, proteolytic activity. The other symbols are the same as those in Fig. 1.

FIG. 4. Gel Filtration of P2 Fraction on Sephadex G-75.

All symbols are the same as those in Fig. 3.

Another sample of crude enzyme as prepared above was absorbed on CM-Sephadex and eluted by a 0~0.5 M gradient of NaCl in 0.01 M citrate buffer (pH 6.4). A typical chromatographic pattern is shown in Fig. 2. The enzymes were recovered in the 0.07~0.25 M NaCl region, and were separated into protein fractions P1, P2 and P3. All of these fractions include lytic activities for *M. radiodurans* and *P. radiora*. The P1 fraction has a higher activity for *P. radiora* than for *M. radiodurans*. On the contrary, the P2 and P3 fractions have higher activities for *M. radiodurans* than for *P. radiora*. Further purification of each fraction

was accomplished by the following procedure. The P1 fraction, consisting of 20 tubes from fraction number 57 to 76, was concentrated to approximately 5 ml by membrane filtration under pressure using a UM-2 filter in an AMICON model 202 filtration apparatus, and applied to a Sephadex G-75 column (2.5 × 85 cm) equilibrated with 0.01 M citrate buffer (pH 6.4). Figure 3 shows the gel filtration profiles of the P1 fraction, which was separated into three additional protein fractions designated P1-1, P1-2 (seen as a small shoulder) and P1-3. The P1-1 and P1-2 fractions easily lysed the cells of *P. radiora*. The

P1-1 protein also includes a proteolytic activity and a low lytic activity for *M. lysodeikticus*. The P1-3 protein has no proteolytic activity. This fraction includes the lytic activities for *M. radiodurans* and *M. lysodeikticus*, and may also have an activity for *P. radora*. The P2 (tubes 85~98) and P3 (tubes 105~116) fractions were concentrated by membrane filtration, and fractionated by Sephadex G-75 according to the same pro-

cedure used for the P1 fraction. The gel filtration patterns of the P2 and P3 proteins are shown in Figs. 4 and 5, respectively. The P2 peak was separated into two protein fractions, P2-1 and P2-2, and two active fractions, A2-1 and A2-2, which did not correspond to protein peaks. The P2-1 protein has a proteolytic activity and a low lytic activity for *M. lysodeikticus*, while the P2-2 protein contains all bacteriolytic activities, but is free from proteolytic activity. As shown in Fig. 5, the P3 peak was separated into a main protein fraction, P3-1, and a smaller fraction, P3-2. Two active fractions, A3-1 and A3-2, were also detected. The properties of the eleven fractions as described above are summarized in Table I. Fractions A2-2 and A3-2 show similar lytic activity, and fractions P1-3 and P3-2 show almost the same lytic activity as P2-2. However, the other fractions differ from each other in proteolytic and bacteriolytic activities. The highest lytic activity for *P. radora* was detected in P1-2, and the highest proteolytic activity was detected in P3-1. In addition, only the P2-2 fraction contained high lytic activities for *M. radiodurans* and *M. lysodeikticus*.

The culture filtrate of *A. lunatus* is capable of lysing the cells of bacteria, yeast and fungi.⁹⁾ The crude enzyme from the culture filtrate is also able to lyse various strains of bacteria,

FIG. 5. Gel Filtration of P3 Fraction on Sephadex G-75.
All symbols are the same as those in Fig. 3.

TABLE I. LYTIC ACTIVITIES OF EACH FRACTION SEPARATED BY SEPHADEX G-75

Fraction	Bacteriolytic activity			Proteolytic activity
	<i>P. radora</i>	<i>M. radiodurans</i>	<i>M. lysodeikticus</i>	
P1	P1-1	+	—	+
	P1-2	++	+	+
	P1-3	±	+	—
P2	A2-1	+	—	—
	P2-1	—	—	+
	A2-2	+	+	—
	P2-2	+	++	+
P3	A3-1	+	—	—
	P3-1	—	+	++
	A3-2	+	+	—
	P3-2	±	+	—

++, most active; +, active; —, negative; ±, not distinguished.

FIG. 6. Disc Gel Electrophoretic Patterns of Each Fraction Separated by Sephadex G-75.

The electrophoresis was carried out using a pH 6.6 polyacrylamide gel with a current of 2 mA/tube for one hour.

FIG. 7. Rechromatography of the P2-2 Fraction on a Sephadex G-75 Column.

The symbols are the same as those in Fig. 3.

TABLE II. PURIFICATION OF THE P2-2 ENZYME FROM A CRUDE ENZYME PREPARATION OF *A. lunatus*

Fraction	Total protein (mg)	Total activity ^a (units $\times 10^4$)	Specific activity (units/mg)	Recovery (%)
70% Alcohol precipitate	394.5	36.1	915	100
CM-Sephadex I	21.4	12.9	6028	36
CM-Sephadex II	16.0	10.5	6563	29
Sephadex G-75 I	2.7	8.5	31481	24
Sephadex G-75 II	2.0	6.3	31500	17

^a One unit of lytic activity is defined as a change in absorbance of 0.001 at 660 nm of a suspension of *M. radiodurans* cells in one minute at 50°C.

including radioresistant bacteria such as *M. radiodurans*, *M. radioproteolyticus*, *M. sp. H55* and *Arthrobacter radiotolerans* P-1.⁵⁾ In addition, Tsumura *et al.*⁷⁾ has determined the activities of protease and glucanase in the culture filtrate. These observations and our results indicate that the wide lytic spectrum is attributable to the action of many lytic enzymes.

Disc gel electrophoretic patterns of each of the fractions at pH 6.6 are shown in Fig. 6. Only the P2-2 fraction showed a single band of protein, while the other fractions consisted of at least two protein bands. Figure 7 shows the rechromatography of P2-2 on Sephadex G-75.

At this step, the P2-2 eluted as a single symmetrical peak, and the lytic activities for *M. radiodurans* and *P. radora* corresponded to the protein peak. The over-all sequence of the purification procedures is summarized in Table II. Since the crude enzyme included many lytic enzymes, only the degree of purification of P2-2 was estimated. The specific activity of the P2-2 protein was purified 34-fold from that of the crude enzyme, as estimated by *M. radiodurans*-lytic activity. The P2-2 fraction (designated as the P2-2 enzyme) has no proteolytic activity. Therefore, we examined the digestion of peptidoglycan from the cell walls of *M. lysodeikticus* by the P2-2 enzyme,

but observed no liberation of reducing groups during digestion. This indicates that the P2-2 enzyme is not a glycosidase. The detailed properties and lytic action of the P2-2 enzyme will be described in the following paper.

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