Lytic Enzymes toward Aniline-assimilating *Rhodococcus erythropolis* AN-13: Purification and Characterization[†]

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Received September 30, 1987

Three lytic enzymes, C-2, C-4 and C-5, capable of lysing cells of *Rhodococcus erythropolis* AN-13 were purified from the cultural filtrate of *Flavobacterium* species SH-548 by $(\text{NH}_4)_2\text{SO}_4$ fractionation and column chromatographies on CM-Toyopearl and SP-Sephadex. The three purified enzymes gave single protein bands on polyacrylamide gels. C-4 and C-5 were stable between pH 3.0 and 12.5, and C-2 between pH 5.5 and 11.0. The molecular weights of C-4 and C-5 were 26,000 and that of C-2 was 36,000, as judged on sodium dodecylsulfate-polyacrylamide gel electrophoresis. C-4 and C-5 also showed proteolytic activity toward casein, but C-2 did not exhibit such activity. C-2 showed higher specific lytic activity toward cells of *R. erythropolis* AN-13 than C-4 and C-5.

Rhodococcus erythropolis AN-13 can grow on aniline as a sole carbon and nitrogen source,^{1,2)} and metabolize aniline to the inorganic end products CO_2 , NH_3 and H_2O .³⁾ For investigations on enzymes related to the aniline metabolism, we needed bacteriolytic enzymes that can lyse the cell wall of this strain. As *R. erythropolis* strains have cell walls of the glycolyl type,^{1,5)} they are resistant to egg white lysozyme and other commercially available bacteriolytic enzymes.

In the previous paper,⁴⁾ we reported the isolation of a microorganism producing lytic enzymes toward aniline-grown intact cells of *R. erythropolis* AN-13. The isolated strain *Flavobacterium* species SH-548 was found to produce several lytic enzymes effective for the cells of the *R. erythropolis* strain in the cultural fluid. The present report describes the purification and characterization of these enzymes, and the spectra of the lytic activity toward various bacteria of the purified enzymes.

MATERIALS AND METHODS

Chemicals. CM-Toyopearl 650S was obtained from

Toyo Soda, Tokyo. SP-Sephadex C-25 and electrophoresis calibration kits LMW were from Pharmacia Fine Chem., Uppsala. Casein (Hammarsten) was from Merck, Darmstadt. Other chemicals were described in the previous paper.⁴⁾

Microorganisms. The strains used for lytic activity tests were generous gifts from the Central Research Lab., Ajinomoto Co., Kawasaki, from the Institute for Fermentation, Osaka, stocked cultures in our laboratory, and cultures isolated in our series of studies¹⁾ and in this work. The sources of the strains were described in the previous paper.⁴⁾

Strain and culture. *Flavobacterium* sp. SH-548 was pre-cultured at 30°C for 14 hr with shaking in 7 ml of medium containing 10 g of meat extract (Riken Vitamin), 5 g of Polypepton (Daigo Eiyo), 2.5 g of dextrin, 0.25 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g of KH_2PO_4 and 7.4 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1 l of water (pH 6.8). The culture was transferred to 150 ml of the same medium in a 500-ml shaking flask and then the cultivation was carried out at 30°C on a reciprocating shaker for 18 hr. The cells were removed by centrifugation at $10,000 \times g$ for 20 min and the supernatant was used for the purification of enzymes.

Substrate for the enzyme assay: *R. erythropolis* AN-13 was cultivated in aniline medium at pH 6.0⁷⁾ and cells at various growth phases were examined as to their sensitivity to the enzyme action. The most sensitive cells,

[†] Microbial Metabolism of Aromatic Amines. Part XI. For Part X, see *Agric. Biol. Chem.*, **52**, 113 (1988).

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obtained in the middle-exponential growth phase, were lyophilized and then the lyophilized cells were used for the lytic-enzyme assay as a substrate. Lyophilized cells of *Bacillus subtilis* IFO 3009, harvested in the late-exponential growth phase, were used as a substrate for purified C-4 and C-5 in all the experiments except for the purification procedure for these two enzymes, because the lytic activities of these enzymes toward lyophilized cells of *R. erythropolis* AN-13 were much less than that of C-2.

Enzyme assays. The lytic activities and the numbers of enzyme units were determined as described in the previous paper,⁴⁾ with the modifications that lyophilized cells of *R. erythropolis* AN-13 or *B. subtilis* IFO 3009 mentioned above and 10 mM potassium-sodium (K-Na) phosphate buffer (pH 7.1) were used in this experiment, instead of intact cells of *R. erythropolis* AN-13 and 25 mM buffer, respectively. Specific activity was defined as units per mg protein. Protein concentrations were determined by the method of Lowry *et al.*⁸⁾ Proteolytic activity was determined by the method of Reimerdes and Klostermeyer,⁹⁾ with casein as a substrate. One unit of enzyme activity was defined as the amount of enzyme that increased the optical density of a 3.5-ml reaction mixture at 570 nm by 1 per min.

Inhibitory tests. Purified enzymes were incubated with various inhibitors in 0.1 M Tris-acetate buffer (pH 8.0) at 35°C. After 30 min incubation, the residual lytic activity was assayed.

Amino acid analysis. Purified enzymes (C-2, 19 μ g; C-4, 21 μ g; and C-5, 23 μ g) were hydrolyzed with 6 N HCl at 110°C for 24, 48 and 72 hr. Amino acid analysis was

carried out with a Hitachi 835-200 amino acid analyzer. The tryptophan content of the enzymes was determined spectrophotometrically on the basis of measurements of absorbance at 280 and 288 nm in a solution of 6 M guanidine hydrochloride-0.02 M K-Na phosphate buffer (pH 6.5).⁶⁾ Half cystine was determined with the analyzer as cysteic acid after the oxidation with performic acid.

Lytic tests with various bacteria. Intact cells of various bacteria, that had been harvested in the late-exponential growth phase, were suspended in 10 M K-Na phosphate buffer (pH 7.1); the optical density of each suspension was adjusted to 0.7 at 660 nm. To 3 ml of the cell suspension was added 0.1 ml of a purified enzyme solution (0.8 μ g of C-2, 44 μ g of C-4 and 24 μ g of C-5 per 0.1 ml). After 60 min incubation, the decrease in optical density at 660 nm was determined. The control mixture contained 0.1 ml of the buffer used for enzyme preparations, instead of the enzyme solution.

RESULTS

Purification of enzymes

Step 1. Ammonium sulfate fractionation. All operations for enzyme purification were performed at zero to 7°C. To 3 l of cultural filtrate was added 1.17 kg of ammonium sulfate to 60% saturation with stirring. The mixture was stirred overnight and then centrifuged at 12,000 $\times g$ for 25 min. The precipitate was dissolved in 400 ml of 10 mM K-Na phosphate buffer (pH 6.0) (buffer A), and the resultant

FIG. 1. Column Chromatography of Lytic Enzymes on CM-Toyopearl 650S.

The enzyme solution obtained on 60% saturated ammonium sulfate fractionation was applied to a column (2 $\times$ 90 cm) of CM-Toyopearl 650S equilibrated with buffer A. The column was washed with 1 l of buffer A and then with 1 l of buffer A containing 0.05 M NaCl. Enzymes were eluted with a linear gradient of between 0.05 and 0.15 M NaCl in 2 l of buffer A at a flow rate of 60 ml/hr. Δ --- Δ , proteolytic activity; $\circ$ --- $\circ$, lytic activity (toward *R. erythropolis* AN-13); —, OD₂₈₀; ———, NaCl.

solution was dialyzed against buffer A for 6 hr with three changes of the buffer.

Step 2. First CM-Toyopearl chromatography. The dialyzed solution (430 ml) was applied to a column (2×90 cm) of CM-Toyopearl 650S that had been equilibrated with buffer A. The column was washed with 1 l of buffer A and then with 1 l of buffer A containing 0.05 M NaCl. Enzymes were eluted with a linear gradient starting with 1 l of buffer A containing 0.05 M NaCl in the mixing chamber and 1 l of buffer A containing 0.15 M NaCl in the reservoir. Fractions (10 ml/tube) were collected at a flow rate of 60 ml per hr, and then the optical density at 280 nm and enzymatic activities were assayed. Four peaks of lytic activity appeared on the chromatogram (Fig. 1). The first peak seemed to contain several proteins or enzymes, in view of the optical density at 280 nm and proteolytic activity. Then, the three other peaks of lytic activity (C-2, C-4 and C-5 enzymes) were subjected to further examination. Fractions of C-2 did not show proteolytic activity, whereas those of C-4 and C-5 showed this activity besides lytic activity. C-4 obtained in this step gave a single protein band on a polyacrylamide gel. Therefore, further purification of this enzyme was not required.

Step 3. SP-Sephadex chromatography. Fractions of C-2 were dialyzed against 10 mM acetate buffer (pH 5.5) (buffer B) for 6 hr with three changes of the buffer, and then the dialyzed solution was applied to a column (2×30 cm) of SP-Sephadex C-25 equilibrated

with buffer B. The column was washed with 500 ml of buffer B and then C-2 was eluted with a linear gradient between zero and 0.25 M NaCl in 1 l of buffer B. The effluent was collected in 5-ml fractions at a flow rate of 40 ml per hr. The highest peak of enzymatic activity was eluted with 0.13 M NaCl in buffer B.

Step 4. Second CM-Toyopearl chromatography. C-5 fractions in the first CM-Toyopearl chromatography were dialyzed against buffer

FIG. 2. Polyacrylamide Gel Electrophoresis of the Purified Enzymes.

C-2 (18 µg), C-4 (9 µg) and C-5 (10 µg) were run on 7.5% polyacrylamide gels, pH 4.3, at 2 mA/tube for 2.5 hr in a running buffer (pH 4.5) of β-alanine-acetic acid.¹⁰ The gels were then stained with 0.25% Coomassie brilliant blue in 7.5% acetic acid for 2 hr and destained in 5% methanol-7.5% acetic acid.

TABLE I. SUMMARY OF PURIFICATION OF THE LYTIC ENZYMES

Fraction	Total activity ^a (units)			Total protein (mg)			Specific activity (units/mg)			Recovery (%)		
	C-2	C-4	C-5	C-2	C-4	C-5	C-2	C-4	C-5	C-2	C-4	C-5
	× 10 ³						× 10 ³					
Cultural filtrate		8100			7100			1.1		100		
Ammonium sulfate		5900			1100			5.4		73		
CM-Toyopearl I	330	54	38	16	63	28	21	0.9	1.4	4.1	0.7	0.5
SP-Sephadex	100	—	—	0.76	—	—	130	—	—	1.2	—	—
CM-Toyopearl II	—	—	13	—	—	8.6	—	—	1.6	—	—	0.2

^a The activities of C-2, C-4 and C-5 were assayed toward *R. erythropolis* AN-13.

FIG. 3. Optimal pHs of the Lytic Enzymes.

The lytic activity of C-2 (0.8 μ g) was assayed toward *R. erythropolis* AN-13 and those of C-4 (44 μ g) and C-5 (24 μ g) toward *B. subtilis* IFO 3009. The buffers used were: $\circ$, acetate; $\bullet$, K-Na phosphate; $\triangle$, Tris-HCl; and $\times$, carbonate. The ionic strength of the buffers was 0.01.

FIG. 4. Effect of the Ionic Strength on Lytic Activity.

The lytic activities were assayed in K-Na phosphate buffer (pH 7.1) of various ionic strengths. $\circ$ — $\circ$, C-2 (1.2 μ g) toward *R. erythropolis* AN-13; and $\triangle$ — $\triangle$, C-4 (36 μ g) and C-5 (24 μ g) toward *B. subtilis* IFO 3009.

A for 12 hr with three changes of the buffer and then applied to a column of CM-Toyopearl as described in step 2. The enzyme was eluted at the same position as in the first CM-Toyopearl chromatography.

The purification procedure is summarized in

Table I. Of the three purified enzymes, C-2 showed the highest specific activity, 130×10^3 units per mg protein, with an overall recovery of 1.2%; which was a 118-fold increase over the cultural filtrate.

Purity of enzymes

Each purified enzyme obtained through this purification procedure gave a single protein band on a polyacrylamide gel (Fig. 2).

General properties of the purified enzymes

C-2 showed an optimal pH of 7.5 for lyophilized cells of *R. erythropolis* AN-13, whereas those of C-4 and C-5 were 10.0 for lyophilized cells of *B. subtilis* IFO 3009, as shown in Fig. 3. C-2 was stable in the pH range of 5.5 to 11.0 and up to 40°C. On the other hand, C-4 and C-5 were stable between pH 3.0 and 12.5 and up to 60°C.

Effect of ionic strength on the lytic activity

Ionic strength was found to be an important factor when assaying lytic activity. As the ionic strength of the enzyme reaction mixture increased, the lytic activity of C-2 decreased in proportion to the logarithmic value of the ionic strength (Fig. 4). On the other hand, the activities of C-4 and C-5 remained about con-

FIG. 5. Determination of the Molecular Weights of the Lytic Enzymes by SDS-Polyacrylamide Gel Electrophoresis.

C-2 (18 μ g), C-4 (9 μ g), C-5 (10 μ g) and markers denatured with SDS were run on 10% gels containing 0.1% SDS at 6 mA/tube for 4.5 hr in a running buffer (pH 7.2) of 0.1% SDS-0.1 M sodium phosphate. The gels were stained and destained as described in Fig. 2. An electrophoresis calibration kit LMW was used as markers: 1, phosphorylase *b* (molecular weight, 94,000); 2, bovine serum albumin (67,000); 3, ovalbumin (43,000); 4, carbonic anhydrase (30,000); 5, trypsin inhibitor (20,100); 6, α -lactalbumin (14,400); C-2, (36,000); and C-4 and C-5, (26,000).

TABLE II. EFFECTS OF VARIOUS COMPOUNDS ON LYTIC ACTIVITY

Compound	Concentration (mM)	Remaining activity (%) ^a		
		C-2	C-4	C-5
None	—	100	100	100
CuSO ₄	0.1	76	89	93
	1	45	79	69
FeSO ₄	1	105	99	101
FeCl ₃	1	105	100	99
HgCl ₂	0.1	70	99	106
	1	0	33	30
ZnCl ₂	1	64	82	75
AgNO ₃	0.1	93	94	94
	1	58	90	88
Iodoacetate	1	88	101	92
EDTA	1	86	104	90
PCMB	0.3	81	123	105
DTNB	0.5	54	97	94
NEM	1	86	100	101

EDTA, ethylenediaminetetraacetic acid; PCMB, *p*-chloromercuribenzoic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); and NEM, *N*-ethylmaleimide.

^a The activities of C-2, and C-4 and C-5 were assayed toward *R. erythropolis* AN-13 and *B. subtilis* IFO 3009, respectively.

TABLE III. AMINO ACID COMPOSITIONS OF THE LYTIC ENZYMES

The enzymes were hydrolyzed with 6N HCl at 110°C for 24, 48 and 72 hr. All the numbers of amino acid residues except those of half cystine and tryptophan were obtained by extrapolation to zero time, or are the average or maximum of three values.

Amino acid	Number of residues per molecule ^a		
	C-2	C-4	C-5
Aspartic acid	28	19	20
Threonine	19	20	16
Serine	36	28	23
Glutamic acid	32	22	26
Proline	13	8	9
Glycine	35	38	35
Alanine	36	27	27
Half cystine ^b	10	8	8
Valine	21	19	19
Methionine	3	2	1
Isoleucine	13	11	11
Leucine	23	15	15
Tyrosine	7	6	6
Phenylalanine	9	7	7
Lysine	17	7	9
Histidine	9	5	6
Arginine	12	12	11
Tryptophan ^c	12	2	2

^a The values are expressed on the basis of molecular weights of 36,000 for C-2 and 26,000 for C-4 and C-5.

^b Determined as cysteic acid after oxidation with performic acid.

^c Determined spectrophotometrically.⁶⁾

stant at ionic strengths from 10^{-3} to 0.05, and decreased rapidly at ionic strengths over 0.05.

Molecular weight

The molecular weight of C-2 was determined to be 36,000 by dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and those of C-4 and C-5 to be 26,000 (Fig. 5).

Inhibition

The effects of metal salts and some inhibitors on the lytic activity of the enzymes were examined. Table II shows that C-2 was inhibited by HgCl₂, CuSO₄, ZnCl₂, AgNO₃ and DTNB. However, C-4 and C-5 were less inhibited than C-2 by these compounds.

TABLE IV. LYTIC ACTIVITIES OF THE PURIFIED ENZYMES TOWARD INTACT CELLS OF VARIOUS BACTERIA

Incubation was performed at 35°C for 60 min in a reaction mixture containing 3 ml of an intact cell suspension in 10 mM K-Na phosphate buffer (pH 7.1) and 0.1 ml of an enzyme solution, and then the optical density of the reaction and control mixtures was monitored at 660 nm.

Strain	Percent lysis ^a			Strain	Percent lysis ^a		
	C-2	C-4	C-5		C-2	C-4	C-5
Gram-positive bacteria:				<i>Rhodococcus erythropolis</i>	5	3	2
<i>Arthrobacter simplex</i>	0	28	0	AN-1			
AJ 1420 (ATCC 6946)				AN-2	0	0	0
<i>Arthrobacter ureafaciens</i>	1	2	2	AN-5	16	3	1
AJ 1421 (ATCC 7562)				AN-12	57	35	21
<i>Bacillus circulans</i>	83	21	21	AN-13	74	27	11
IFO 13626				AN-17	0	0	0
<i>Bacillus licheniformis</i>	64	13	14	AN-18	0	0	0
IFO 12200				AN-19	0	0	0
<i>Bacillus megaterium</i>	12	27	28	<i>Staphylococcus aureus</i>	2	5	10
<i>Bacillus subtilis</i> IFO 3009	57	20	19	<i>Streptococcus faecalis</i>	3	0	0
<i>Brevibacterium linens</i>	16	21	12	AJ 3263 (ATCC 12984)			
AJ 1520 (ATCC 8377)				<i>Streptomyces rimosus</i>	0	16	5
<i>Brevibacterium testaceum</i>	0	8	12	AJ 9042 (IAM 0014)			
AJ 1464 (IAM 1537)				<i>Streptomyces venezuelae</i>	0	14	5
<i>Cellulomonas flavigena</i>	12	18	18	AJ 9154 (IFO 13096)			
AJ 1571 (ATCC 484)				Gram-negative bacteria:			
<i>Corynebacterium diphtheriae</i>	18	30	26	<i>Enterobacter aerogenes</i>	1	0	0
AJ 1414 (ATCC 11913)				IFO 13534			
<i>Corynebacterium equi</i>	18	0	0	<i>Enterobacter cloacae</i>	1	3	1
AJ 1402 (ATCC 6939)				IFO 12935			
DAB-type coryneform bacteria	28	72	77	<i>Flavobacterium</i> sp. SH-548	2	6	6
AN-3				<i>Klebsiella pneumoniae</i>	0	0	0
AN-6	34	87	89	IFO 13541			
AN-9	3	9	12	<i>Proteus mirabilis</i> IFO 3849	1	1	2
AN-11	17	46	64	<i>Proteus vulgaris</i>	3	0	0
AN-14	11	45	66	<i>Pseudomonas maltophilia</i>			
AN-15	5	19	21	AN-4	2	0	1
AN-20	6	15	29	AN-7	5	2	2
<i>Micrococcus luteus</i>	75	14	27	AN-8	3	4	4
AJ 1053 (ATCC 4698)				AN-10	31	32	26
<i>Micromonospora chalybeata</i>	0	16	12	AN-16	8	3	3
AJ 9116 (NARI 2517)				<i>Serratia liquefaciens</i>	0	0	0
<i>Nocardia asteroides</i>	57	35	21	IFO 12979			
AJ 9112 (IFO 3384)							
<i>Nocardia erythropolis</i>	7	42	77				
AJ 9126 (ATCC 4277)							

^a Percent lysis:⁽¹¹⁾ $\left(1 - \frac{\text{optical density in reaction mixture}}{\text{optical density in control mixture}^b}\right) \times 100$.

^b See the text for details.

Amino acid compositions

Table III shows the amino acid compositions of the three enzymes. Those of C-4 and C-5 were similar to each other.

Lytic activity spectra

Table IV shows the lytic activities of the three purified enzymes toward intact cells of various bacteria. C-2 well lysed intact cells of *Bacillus* strains, *Micrococcus luteus* AJ 1053,

Nocardia asteroides and *R. erythropolis* AN-12 and AN-13. On the other hand, C-4 and C-5 showed high activities toward several DAB (diaminobutyric acid)-type coryneform bacteria and *Nocardia erythropolis* AJ 9126, although higher concentrations of the two enzymes than that of C-2 were needed in the experiments. The three enzymes also exhibited lytic activities toward cells of gram-negative *Pseudomonas maltophilia* AN-10.

DISCUSSION

The three lytic enzymes C-2, C-4 and C-5 were purified to homogeneity from the cultural fluid of *Flavobacterium* sp. SH-548. Among these enzymes, only C-2 completely lysed intact cells of *R. erythropolis* AN-13 grown on aniline, although C-4 and C-5 caused incomplete lysis of the strain. In addition, C-2 showed much higher specific activity than C-4 and C-5 toward intact cells of *R. erythropolis* AN-13 and several other bacteria listed in Table IV; the specific activity of C-2 toward *R. erythropolis* AN-13 and *M. luteus* AJ 1053 was 80-150 times higher than those of C-4 and C-5.

C-2 lysed intact cells of *R. erythropolis* AN-12 and AN-13 well (Table IV). C-4 and C-5 also showed considerable lytic activity toward cells of these strains. However, the three enzyme did not attack any of the cells of *R. erythropolis* AN-17, AN-18 and AN-19, and lysed those of *R. erythropolis* AN-1 and AN-5 only a little, although all the *R. erythropolis* strains mentioned above have a similar peptide glycan structure.¹⁾ In these organisms, cells change from hyphae to oval coccoids with the growth phase.¹⁾ The sensitivity of intact cells of *R. erythropolis* AN-13 to C-2 was markedly affected by the growth phase. Therefore, the lytic activities of C-2, C-4 and C-5 toward intact cells of the *R. erythropolis* strains seem

to depend primarily on the cell development of the organisms.

C-4 and C-5 had similar features as to the molecular weight, pH- and thermo-stability, optimal pH, inhibition and amino acid composition, although they were different in the lytic activities toward some bacteria. Therefore, the two enzymes seem to be of the same origin; either C-4 or C-5, or both enzymes were presumably modified during secretion from the inside of cells or in the cultural fluid.

C-4 and C-5 showed proteolytic activity toward casein, however, C-2 did not act on this substrate. This lack of activity by C-2 will be useful for the preparation of cell-free extracts from intact cells, because some desired enzymes and proteins in the cells could be obtained without any modification by this lytic enzyme when used. We are now investigating the mechanism of the lytic action of C-2 on cell walls of *R. erythropolis* AN-13.

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