Formation of Protein-like Activator for *n*-Alkane Oxidation and Its Properties

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Received June 14, 1976

“Protein-like activator (PA)” for *n*-alkane oxidation was formed by *Pseudomonas aeruginosa* S₇B₁ from long-chain *n*-alkanes, 1-hexadecene and cetyl alcohol but not from glucose, glycerol and palmitic acid. The molecular weight and the total amino acid residues of PA were estimated at about 14,300 and 147, respectively. PA was relatively stable to low pH and high temperature, and completely inactivated upon heating at 98°C for 45 min. The cultural fluid obtained from *n*-hexadecane medium stimulated the growth of the strain on *n*-hexadecane. The degree of the growth stimulation by the fluid depended on the amount of PA and rhamnolipid (RL) in the fluid. The heat-treated PA lost the growth-stimulating effect and the emulsifying power on the *n*-hexadecane medium in the presence of RL.

Two kinds of activators for *n*-alkane oxidation formed by *Pseudomonas aeruginosa* S₇B₁ were isolated and their role on *n*-alkane oxidation was previously reported.^{1,2)} One of them, which was termed “protein-like activator (PA)” was purified to a homogeneous state as judged by cellulose acetate membrane electrophoresis.

In the preceding paper,³⁾ the authors reported that the oxidation capacity of the strain was reduced by treatment with EDTA on the oxidation of long-chain *n*-alkanes and the reduced capacity to oxidize these substrates was restored to the level of the non-treated cells with PA. In terms of the PA activity on EDTA-treated cells, it became possible to determine PA in the cultural fluid and to investigate the properties of PA.

The present paper deals with the amount of PA formed from various carbon sources, the amino acid composition and some properties of PA and the stimulating effect of PA on the growth of the strain on *n*-hexadecane.

MATERIALS AND METHODS

Cultivation of microorganisms and preparation of EDTA-treated cells. *Ps. aeruginosa* S₇B₁ was employ-

ed throughout this investigation. The basal medium, the condition of cultivation and the preparation of EDTA-treated cells were described in the previous paper.³⁾

Determination of cell growth. Absorbance of the cultures was measured at 660 nm with a Spectronic 20 spectrometer (Shimadzu Co.) according to our method.⁴⁾

Determination of PA. Method I. Thirty ml of the supernatant of the culture broth was brought to 70% saturation with ammonium sulfate with constant and slow stirring. After standing overnight at 5°C the mixture was centrifuged at 10,000 × *g* for 10 min and the supernatant was discarded. The precipitate was dissolved in a small amount of distilled water and dialyzed against running tap water for 24 hr, followed by dialysis against 0.01 M Na₂HPO₄ for 24 hr. The dialyzed solution was filled up to 5 ml with 0.01 M Na₂HPO₄ and placed on a column of DEAE-Sephadex A-50 (0.8 × 15 cm), which had been equilibrated with 0.01 M Na₂HPO₄. The elution was carried out with the same solution. The first 5 ml of the eluate was discarded and the next 20 ml of the eluate was collected. PA activity of the eluate collected was measured by the Warburg technique as described in the previous paper.³⁾ PA was determined by the calculation on the basis of the restoration effect of PA on *n*-hexadecane oxidation capacity in EDTA-treated cells.

Method II. Four ml of the supernatant of the culture broth was dialyzed against running tap water for 24 hr, followed by dialysis against 0.01 M Na₂HPO₄ for 24 hr. The dialyzed solution was placed on a column of DEAE-Sephadex A-50 (0.8 × 15 cm), which had been equilibrated with 0.01 M Na₂HPO₄. The first 4 ml of

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the eluate was discarded and the next 10 ml of the eluate was collected. PA was determined by the same method as described above.

Determination of rhamnolipid (RL). The amount of RL produced in the culture broth was colorimetrically determined by the anthrone method as follows.

Cells were centrifuged off at $10,000 \times g$ for 10 min and discarded. Two ml of the supernatant or the diluted solution was adjusted to pH 2.0 with 1 N HCl and extracted twice with 10 ml of ethyl acetate. The extract was evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of diethyl ether and was poured into a test tube. After the addition of 2 drops of methanol, 0.5 ml of distilled water and 5 ml of anthrone reagent,⁵⁾ the mixture was heated in boiling water for 3 min, cooled in running tap water. Absorbance of the solution at 630 nm was measured with a Spectronic 20 spectrometer.

Ultracentrifugal analysis. Ultracentrifugal analysis of PA was performed with a Hitachi model UCA-1 ultracentrifuge equipped with a Schlieren optical system.

Estimation of molecular weight. The molecular weight of PA was determined by the method of Andrews,⁶⁾ using a Sephadex G-75 column and several standard proteins.

Amino acid composition. The hydrolysis of PA was carried out with 6 N HCl in a sealed tube *in vacuo* at 110°C for 24, 48 and 72 hr, respectively. The hydrolyzate was subjected to chromatographic analysis with an automatic amino acid analyzer. The cysteine content was determined by analyzing cysteic acid in a sample oxidized with performic acid.⁷⁾ The tryptophan content was determined by a spectrophotometric method⁸⁾ and the *p*-dimethylaminobenzaldehyde method.⁹⁾

Determination of protein concentration. Protein concentration was determined by the method of Lowry *et al.* as described previously²⁾ and expressed as a human albumin equivalent.

RESULTS AND DISCUSSION

Effect of carbon sources on the formation of PA

Table I shows the amount of PA formed from various carbon sources. PA was formed from longer-chain *n*-alkanes and 1-hexadecene in larger quantities and from *n*-tridecane and cetyl alcohol in smaller quantities, but not from glucose, glycerol and palmitic acid. It is interesting that PA is formed not only from *n*-

TABLE I. EFFECT OF CARBON SOURCES ON THE FORMATION OF PA

A spoonful cells grown on *n*-hexadecane slant was inoculated. The cultivation was carried out until stationary phase at 30°C in a shaking flask (500 ml) containing 50 ml of the basal medium and each carbon source. The amount of PA was determined according to METHOD I.

Carbon source	Cultivation time (hr)	Growth (OD at 660 nm)	PA (μg^a /ml)
1% (w/v) glucose	14	0.74	0
2% (w/v) glycerol	32	0.65	0
1% (w/v) palmitic acid	14	0.76	0
1% (w/v) cetyl alcohol	27	0.69	30
1% (v/v) 1-hexadecene	88	0.75	80
" <i>n</i> -tridecane	88	0.72	50
" <i>n</i> -tetradecane	"	0.75	70
" <i>n</i> -pentadecane	"	0.71	100
" <i>n</i> -hexadecane	"	0.74	100
" <i>n</i> -heptadecane	"	0.75	110
" <i>n</i> -octadecane	"	0.73	120

^{a)} Human serum albumin equivalent.

alkanes but also cetyl alcohol and 1-hexadecene. As shown in Table I, the amount of PA increases as the chain-length of *n*-alkane increases. This fact indicates that the formation of PA relates to the hydrophobic property of the carbon source.

Time course of the formation of PA and RL from n-hexadecane

As shown in Fig. 1, the formation of PA was associated with the growth until the middle phase of the growth. RL isolated from the culture broth of the strain as a growth stimulant on *n*-hexadecane medium¹⁰⁾ was formed little in the initial phase of the growth.

When RL or PA is added to the medium from the start of the cultivation, RL is more effective on the growth of *Ps. aeruginosa* S₇B₁ than PA.²⁾ But from the result of Fig. 1, it can be presumed that PA plays a more important role in the initial growth of the strain on *n*-hexadecane.

Homogeneity of the purified PA

PA was purified by ammonium sulfate precipitation and chromatography on DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-75 columns according to the pre-

FIG. 1. Time Course of the Formation of PA and RL from *n*-Hexadecane.

The conditions of the cultivation were described in the legend of Table I. The amount of PA was determined according to METHOD II.

Carbon source: 1% (v/v) *n*-hexadecane.

○—○, growth; □—□, PA; △—△, RL.

^{a)} Human serum albumin equivalent.

viously outlined procedure.²⁾

Homogeneity of the purified PA was analyzed by ultracentrifugation and the patterns are shown in Fig. 2. The patterns show a single peak which reveals that the purified PA is homogeneous in ultracentrifugal analysis.

FIG. 2. Sedimentation Patterns of PA.

The sample was 1% solution of PA in 1/15M phosphate buffer (pH 7.0) containing 0.1 M of KCl. The photographs were taken at the indicated times after reaching the full speed (60,000 rpm).

Estimation of molecular weight and amino acid composition of PA

The elution patterns of standard proteins were measured from the optical extinction at

280 nm and the peak of PA was determined from its biological activity. From a plot of the molecular weight against the volume of eluates, as shown in Fig. 3, the molecular weight of PA was assumed to be 1.4×10^4 to 1.5×10^4 .

FIG. 3. Estimation of Molecular Weight by Sephadex G-75 Gel Filtration.

1. cytochrome C
2. myoglobin
3. chymotrypsinogen A
4. serum albumin
5. γ -globulin

Table II shows the amino acid composition of PA and the molecular weight of PA estimated from the amino acid composition. From the results of Table II, the molecular weight of PA and the total amino acid residues were calculated as about 14,300 and 147, respectively.

The characteristics of the amino acid composition of PA are high contents of serine and threonine residues and the absence of arginine and histidine residues. As shown in Table II, the total amino acid residues account for 102.1%. This high recovery may be due to the absence of other substances than amino acids.

Effect of pH and temperature on the stability of PA

The pH stability of PA was tested between pH 1.3 and 12.5. PA was incubated at a given pH for 20 hr and then diluted with 1/15 M phosphate buffer (pH 7.0). The residual activity of PA was calculated on the basis of the standard curve of PA concentration vs. QO_2 measured by the Warburg method. In order to measure correct activities, the assay was

TABLE II. AMINO ACID COMPOSITION OF PA

Amino acid	Amino acid (g/100 g protein)	Amino acid residues (g/100g protein)	Minimum molecular weight	Molecular weight estimated ^{a)}	Residues to nearest integer
Arg ^{b)}	0.00	0.00	—	—	0
Lys ^{b)}	7.32	6.42	1997	13979	7
His ^{b)}	0.00	0.00	—	—	0
Asp ^{b)}	13.20	11.41	993	13802	14
Glu ^{b)}	2.11	1.85	6973	13946	2
Ser ^{c)}	23.56	19.52	446	14272	32
Thr ^{c)}	15.71	13.34	758	14402	19
Tyr ^{b)}	1.32	1.19	13421	13421	1
Gly ^{b)}	6.05	4.52	1241	13651	11
Ala ^{b)}	7.41	5.91	1183	14196	12
Val ^{d)}	10.56	8.94	1109	14417	13
Leu ^{d)}	8.38	7.23	1565	14085	9
Ile ^{d)}	5.53	4.77	2372	14232	6
Pro ^{b)}	7.97	6.61	1444	14440	10
Phe ^{b)}	4.57	4.07	3552	14208	4
Try ^{e)}	2.87	2.62	7786	15572	2
Met ^{b)}	0.89	0.78	14509	14509	1
Cys (1/2) ^{f)}	3.44	2.92	3783	15132	4
Total		102.10			147
Average				14267	

^{a)} Based on a molecular weight of $(14509 + 13421/2) \approx 14,000$.

^{b)} Average value from 24, 48 and 72 hr hydrolysates.

^{c)} Extrapolated value to zero time of hydrolysis.

^{d)} Highest value from 24, 48 and 72 hr hydrolysates.

^{e)} According to the UV method and the *p*-dimethylaminobenzaldehyde method.

^{f)} Analysis of cysteic acid in a performic acid oxidized sample.

done under the conditions giving approximately a linear relationship between the amount of protein in the sample and QO_2 , although the situation is not the same as in an enzyme reaction. The results in Fig. 4 indicate that PA is quite stable in a range between pH 1.7 and 11.4 at 5°C.

The effect of temperature on the stability of PA was determined by incubating PA at various temperatures for 20 min. From the results in Fig. 5, it is evident that PA is relatively stable to heat and partially destroyed at 98°C. Figure 6 shows the relationship between the stability of PA and the heating time at 98°C. PA was completely inactivated by the heating at 98°C for 45 min.

As shown in Figs. 4~6, PA is relatively stable to low pH and high temperature as a protein. Regarding the stability of PA, it is possible that PA is hardly denatured itself or denatured PA is easily renatured.

Effect of PA, RL and cultural fluid on the growth of Ps. aeruginosa S₇B₁

A PA solution and the culture fluid obtained from the *n*-hexadecane medium by centrifugation at $10,000 \times g$ for 10 min were sterilized by filtration with a Millipore membrane filter (pore size = 0.22 μ), respectively. Twenty-five ml of the fluid blended in an equal volume of the basal medium was used for the cultivation.

As shown in Figs. 7 and 8, PA and RL cooperatively stimulated the growth of the strain, and the degree of the growth stimulation with the cultural fluid depended on the amount of PA and RL contained in the fluid. These results indicate that the growth-stimulating factors in the culture fluid are PA and RL.

Interaction between PA and rhamnolipid on the emulsification of n-hexadecane

It is worth noting that the heat-treated PA lost the restration effect on the *n*-hexadecane

FIG. 4. Effect of pH on Stability of PA.

Buffers used were 0.1 M HCl-KCl (○), 0.1 M phthalate (△), 0.1 M phosphate (●), 0.1 M $\text{NaHCO}_3\text{-Na}_2\text{HCO}_3$ (□) and 0.1 M KCl-NaOH (×). Equal volumes of a PA solution ($320 \mu\text{g}^a/\text{ml}$) and buffer were mixed and incubated at 5°C for 20 hr. After the incubation, the PA solution was suitably diluted with 1/15 M phosphate buffer, pH 7.0 and portions of the diluted solution were analyzed for the remaining activity by the Warburg technique as follows.

Each Warburg vessel contained $20 \mu\text{l}$ of *n*-hexadecane, 0.5 ml of an EDTA-treated cell suspension, 1 ml of 1/15 M phosphate buffer containing $160 \mu\text{g}$ of rhamnolipid and 0.5 ml of the diluted PA solution in the main compartment, and 0.3 ml of 10% KOH in the center well, to give a total volume of 2.32 ml. Incubation was carried out at 30°C .

The estimation of the residual activity of PA is described in the text.

^a) Human serum albumin equivalent.

FIG. 5. Effect of Temperature on Stability of PA. Two milligrams of PA was dissolved in 10 ml of 1/15 M phosphate buffer, pH 7.0 and this solution was subjected to 20 min of heat treatment at the temperatures indicated. The residual activity of PA was measured under the same conditions as described in the legend of Fig. 4.

FIG. 6. Heat Inactivation of PA.

Two milligrams of PA was dissolved in 10 ml of 1/15 M phosphate buffer, pH 7.0 and this solution was stored at 98°C for various times. The residual activity of PA was measured under the same conditions as described in Fig. 4.

FIG. 7. Effect of PA, RL and Cultural Fluid on the Growth of *Ps. aeruginosa* S₇B₁.

The cultivation was carried out in a shaking flask (500 ml). All other conditions are described in the text. Carbon source: 1% (v/v) *n*-hexadecane.

●—●, twenty-five ml of cultural fluid containing $5 \mu\text{g}^a/\text{ml}$ of PA and $0.5 \mu\text{g}/\text{ml}$ of RL, which was obtained from the broth cultivated on *n*-hexadecane for 63 hr; ○—○, $125 \mu\text{g}^a$ of PA and $12.5 \mu\text{g}$ of RL; △—△, $125 \mu\text{g}^a$ of PA heated at 98°C for 45 min and $12.5 \mu\text{g}$ of RL; □—□, $12.5 \mu\text{g}$ of RL; ×—×, none.

^a) Human serum albumin equivalent.

oxidation capacity in EDTA-treated cells (Fig. 6) and could not exert the stimulating effect on the growth of the strain on the *n*-hexadecane medium (Figs. 7, 8). Taking into account the role of PA as an emulsifier or

FIG. 8. Effect of PA, RL and Cultural Fluid on the Growth of *Ps. aeruginosa* S₇B₁.

The cultivation was carried out in a shaking flask (500 ml). All other conditions are described in the text.

Carbon source: 1% (v/v) *n*-hexadecane.

●—●, twenty-five ml of cultural fluid containing 30 µg^{a)}/ml of PA and 1.5 µg/ml of RL, which was obtained from the broth cultivated on *n*-hexadecane for 82 hr; ○—○, 750 µg^{a)} of PA and 37.5 µg of RL; △—△, 750 µg^{a)} of PA heated at 98°C for 45 min and 37.5 µg of RL; □—□, 37.5 µg of RL; ×—×, none.

^{a)} Human serum albumin equivalent.

emulsion stabilizer, the emulsifying power of native and the heat-treated PA was tested in the presence of rhamnolipid. As shown in Fig. 9, the PA heated at 98°C for 45 min almost lost the emulsifying power on *n*-hexadecane.

While the interaction among PA, fine droplets of *n*-alkanes and bacterial cell surface must be taken into account, the significant role of PA can be regarded as forming fine droplets of *n*-alkanes based on the results of this paper.

It is thought that more detailed studies on the role of PA is helpful to clarify the mechanism of transport of water-insoluble substrates in bacteria. The results will be reported in further detail in a subsequent paper.

FIG. 9. Interaction between PA and Rhamnolipid on Emulsification.

After test tubes containing 9 ml of the basal medium^{a)} and 0.1 ml of *n*-hexadecane were instantaneously sterilized at 120°C, 0.5 ml of distilled water No. 1, 0.5 ml of PA solution (1.6 mg^{a)}/ml) No. 2 and 0.5 ml of heat-treated PA solution (1.6 mg^{a)}/ml) No. 3, which were filtrated on a Millipore membrane filter (pore size=0.22 µ), were respectively added. Shaking was carried out for 18 hr on a reciprocal shaker.

^{a)} Human serum albumin equivalent.

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