Crystallization and Characterization of Polyamine Oxidase from *Penicillium chrysogenum*[†]

Kimiyasu ISOBE,* Yoshiki TANI and Hideaki YAMADA

Department of Agricultural Chemistry, Kyoto University,
Kyoto 606, Japan

Received June 3, 1980

Polyamine oxidase was purified and crystallized with an overall yield of 35% from mycelial extract of *Penicillium chrysogenum* by a procedure involving ammonium sulfate fractionation, and DEAE-cellulose and Sephadex G-200 column chromatographies. The crystalline enzyme was homogeneous, as judged by disc gel electrophoresis and ultracentrifugation. The sedimentation coefficient ($s_{20,w}^0$) of the enzyme was determined to be 6.9S, and diffusion coefficient ($D_{20,w}$) to be 4.2×10^{-7} cm² sec⁻¹. The enzyme showed a molecular weight of about 160,000 by gel filtration method and ultracentrifugal analysis, and it was composed of two identical subunits. The enzyme was a flavoprotein with absorption maxima at 275, 375 and 450 nm. The prosthetic group was identified to be FAD. The enzyme oxidized spermine, and slightly oxidized spermidine. Diamines and monoamines were not oxidized.

It was reported that *Pseudomonas aeruginosa*,¹⁾ *Mycobacterium smegmatis*,²⁾ *Neisseria perflava*³⁾ and an unspecified pseudomonad were able to catalyze oxidation of polyamines. The enzymes of these bacteria which attacked only the secondary amino group of polyamines, have been studied using intact cells, freeze-dried cells or cell-free extract. The detailed properties of the enzyme have not been reported.

In the course of investigation on polyamine oxidase for practical use, we found the strong activity of polyamine oxidase in many fungi when they were grown on a medium containing polyamines as sole nitrogen source.⁵⁾ The enzymes from *Penicillium chrysogenum* and *Aspergillus terreus* were produced in a large quantity and characterized to be a new type of polyamine oxidase.

The present paper describes crystallization and properties of polyamine oxidase from *P. chrysogenum*.

MATERIALS AND METHODS

Chemicals. Reference proteins used for molecular weight determination, chymotrypsinogen, albumin (egg and bovine serum), catalase, ferritin, phosphorylase *b*, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor, and peroxidase were purchased from Boehringer Mannheim GmbH. Spermine tetrahydrochloride, spermidine trihydrochloride and all other chemicals were obtained from usual commercial sources.

Microorganism and cultivation. *P. chrysogenum* IFO 4626 was cultivated in 3 liters of a nitrate medium, which consisted of 3 g glucose, 0.1 g NaNO₃, 0.1 g KH₂PO₄, 0.05 g KCl and 0.05 g MgSO₄·7H₂O in 100 ml of tap water, pH 5.7, at 28°C for 24 hr with reciprocal shaking. The culture was in turn inoculated into a jar fermentor containing 30 liters of the nitrate medium and cultivated at 28°C for 48 hr with aeration (15 liters per min). The culture were transferred into a jar fermentor containing 220 liters of a spermine medium consisted of 0.1 g glucose, 0.025 g spermine tetrahydrochloride, 0.1 g KH₂PO₄, 0.15 g K₂HPO₄ and 0.02 g MgSO₄·7H₂O in 100 ml of tap water, pH 5.5. The cultivation was carried out at 28°C for 48 hr with aeration (125 liters per min). The mycelia were harvested by filtration, washed with distilled water and suspended in 0.01 M potassium phosphate buffer, pH 6.0.

Assay of polyamine oxidase.

i) **Colorimetric method.** The enzyme activity was determined by a colorimetric method measuring the

[†] Polyamine Oxidase of Fungi. Part II. For Part I, see ref. 5.

* On leave from Amano Pharmaceutical Co., Nagoya.

amount of quinoneimine dye based on the formation of H_2O_2 as described previously,⁵⁾ except for the change of pH from 7.2 to 6.5 by considering enzyme stability.

ii) *Oxygen consumption method.* The mixture (0.69 ml) containing 0.2 ml (2 μmol) of spermine or spermidine and 0.49 ml of 0.2 M indicated buffer, was stirred and the enzyme solution (0.01 ml) was injected. Oxygen consumption was measured with a Clark type of oxygen electrode at 35°C and the enzyme activity was calculated from the initial rate of oxygen consumption. The reaction mixture without a substrate or an enzyme was used as a blank.

Disc gel electrophoresis. Analytical polyacrylamide gel electrophoresis was performed according to the method of Davis.⁶⁾ After being run with a constant current of 2 mA per gel, the gels were stained with Amido black 10B, electrophoretically destained and stored in 7% acetic acid. Sodium dodecyl sulfate electrophoresis was carried out according to the method of Weber and Osborn.⁷⁾ The enzyme was treated with 1% sodium dodecyl sulfate and 2% 2-mercaptoethanol for 5 min at 100°C. The calculation of the molecular weight was based on the migration of marker proteins which were treated similarly.

Isoelectric focusing. Isoelectric point of the enzyme was determined with an LKB (Bromma, Sweden) isoelectric focusing apparatus using an enzyme preparation purified 1,267-fold. LKB ampholines of pH 3~10 range was used at 1% concentration with sucrose gradient. The focusing was continued for 3 days at 170 V at 4°C. Fractions of 2 ml were collected, and the pH was measured at 4°C.

Ultracentrifugal analysis. The sedimentation coefficient was measured with a Spinco model E analytical ultracentrifuge operated at 59,780 rpm and 20°C. Diffusion coefficient was measured with the same apparatus operated at 7,447 rpm and 20°C, with the boundary conditions at the meniscus in a sector-shaped centrifugal cell.

Molecular weight determination. The molecular weight was determined using molecular sieving according to the method of Andrews.⁸⁾ The molecular weight was also calculated from the sedimentation and diffusion coefficients according to the equation of Svedberg and Pederson.⁹⁾

Determination of protein concentration. Protein concentration was determined by measuring the absorbance at 280 nm, where $E_{1\text{cm}}^{1\%}$ value of 22.97 was used after dry weight determination.

Thin-layer chromatography for identification of prosthetic group. Solvent mixtures used for the thin-layer chromatography of flavin derivatives have the following composition (by volume): (solvent A) *n*-butanol-acetic acid-water (40:10:50); (solvent B) 5% Na_2HPO_4 . The

ascending thin-layer chromatography on cellulose (Kodak) was carried out under dark conditions.

RESULTS

Purification of polyamine oxidase

Step 1. Preparation of mycelial extract. The washed mycelia (about 1.7 kg as wet weight) were suspended in 5 liters of 0.01 M potassium phosphate buffer, pH 6.0, and disrupted at a constant flow rate of 8 liters/hr with Dyno-mill (Willy A. Bachofen GmbH). The mycelial extract was obtained by centrifugation.

Step 2. Acid treatment. The extract (8,200 ml) was adjusted to pH 3.8 with acetic acid and stood overnight at 4°C. The precipitate formed was collected by centrifugation and suspended in 0.01 M sodium acetate buffer, pH 3.8 (1,540 ml). Solid ammonium sulfate, 86 g, was then added to 10% saturation to dissolve the active protein. After stirring for 3 hr, insoluble substances were removed by centrifugation and discarded.

Step 3. Dialysis and ammonium sulfate fractionation. The resultant supernatant was dialyzed for 36 hr against three changes of 0.01 M sodium acetate buffer, pH 3.8. Precipitate formed during the dialysis was collected by centrifugation and suspended in the same buffer. Solid ammonium sulfate was then added to 10% saturation to dissolve the active protein. After stirring for 3 hr, insoluble substances were removed by centrifugation and discarded. To the supernatant, ammonium sulfate was further added to 90% saturation, adjusting to pH 3.8. After standing at 4°C for 3 hr, precipitate formed was collected by centrifugation and dissolved in a minimal volume of 0.01 M sodium acetate buffer, pH 3.8, containing 5% saturation of ammonium sulfate.

Step 4. First gel filtration on Sephadex G-200. The dissolved enzyme (10 ml) was placed on a Sephadex G-200 column (5.4 × 80 cm) which was previously equilibrated with 0.01 M sodium acetate buffer, pH 3.8, containing 10% saturation of ammonium sulfate, and eluted with the same buffer at a flow rate of 50 ml/hr.

Step 5. First DEAE-cellulose column chromatography. The active fractions (160 ml) were combined and applied to a DEAE-cellulose column (2.5×15 cm) which was previously equilibrated with 0.01 M sodium acetate buffer, pH 3.8. The enzyme was eluted with the same buffer. The active fractions (200 ml) were pooled.

Step 6. Second ammonium fractionation. To the eluate, solid ammonium sulfate was added to 70% saturation. After stirring for 3 hr, the precipitate formed was removed by centrifugation and discarded. To the supernatant, ammonium sulfate was further added to 90% saturation adjusting to pH 3.8. After standing overnight, the precipitate was collected by centrifugation and dissolved in a small amount of 0.01 M sodium acetate buffer, pH 4.0. The solution was dialyzed against three changes of the same buffer.

Step 7. Second DEAE-cellulose column chromatography. The dialyzed enzyme solution was applied to a DEAE-cellulose column (2.5×4 cm) equilibrated with 0.01 M sodium acetate buffer, pH 4.8. The enzyme was eluted with the same buffer. The active fractions were combined and concentrated to 3 ml by ultrafiltration (Sartorius-Membranefilter GmbH).

Step 8. Second gel filtration on Sephadex G-200. The concentrated enzyme solution was passed through a column (2.2×80 cm) of

FIG. 1. Elution Pattern of Second Gel Filtration on Sephadex G-200.

The flow rate was 20 ml per hr and 5 ml fractions were collected. Absorbance at 280 nm, (—○—). Polyamine oxidase activity, (—●—).

Sephadex G-200 equilibrated with 0.05 M sodium acetate buffer, pH 3.8. As shown in Fig. 1, one symmetric protein peak was eluted and the enzyme activity coincided exactly with the protein peak. A summary of the purification procedure is shown in Table I.

Step 9. Crystallization. The purified enzyme obtained from Sephadex G-200 gel filtration was used for crystallization of the enzyme. The enzyme solution was concentrated to 1.0 ml by ultrafiltration. To the concentrated enzyme solution, solid am-

TABLE I. SUMMARY OF PURIFICATION OF POLYAMINE OXIDASE FROM *Penicillium chrysogenum*

Enzyme activity was measured by following H_2O_2 formation, using spermine as the substrate. Specific activity is given as units per mg protein.

Fraction	Total activity (unit)	Total protein (mg)	Specific activity	Purification (fold)	Yield (%)
Mycelial extract	887,000	122,800	7.2	1	100
Acid treatment	837,800	6,477	129	18	94
Dialysis and ammonium sulfate (10~90%)	622,300	653	953	132	70
1st Sephadex G-200	477,200	101	4,720	656	54
1st DEAE-cellulose	475,500	80	5,940	826	54
Ammonium sulfate (70~90%)	475,500	52	9,140	1,270	54
2nd DEAE-cellulose	312,500	17.3	18,060	2,510	35
2nd Sephadex G-200	311,100	17.2	18,090	2,510	35

FIG. 2. Photomicrograph of Crystalline Polyamine Oxidase of *Penicillium chrysogenum*.

FIG. 3. Polyacrylamide Gel Electrophoresis of Crystalline Polyamine Oxidase in the Presence (B) or Absence (A) of Dodecylsulfate.

The crystalline enzyme was applied to electrophoresis with 7.5%.

monium sulfate was added gradually until a faint turbidity appeared. On standing at 4°C for 2 days, needlelike crystals were formed without increase in the specific activity (Fig. 2).

Homogeneity of the crystalline enzyme

The crystalline enzyme gave a single band on polyacrylamide gel electrophoresis (Fig. 3). The enzyme sedimented as a single and symmetric peak in the ultracentrifuge in 0.01 M sodium acetate buffer, pH 3.8 (Fig. 4). The sedimentation coefficient in water at 20°C, extrapolated to zero protein concentration, $s_{20,w}^0$, was determined to be 6.9 S and the diffusion coefficient ($D_{20,w}$) was $4.2 \times 10^{-7} \text{ cm}^2 \text{ sec}^{-1}$.

Molecular weight

Molecular weight of the enzyme was determined to be about 160,000 by gel filtration on Sephadex G-200 (Fig. 5). A value of 160,000 was also calculated from $s_{20,w}^0$ and $D_{20,w}$ values. As shown in Fig. 6, SDS-polyacrylamide gel electrophoresis gave a value of 80,000 for molecular weight of the subunit. This showed that the polyamine oxidase from *P. chrysogenum* was composed of two identical subunits.

Isoelectric point

A single peak of the enzyme activity at a pH value 5.5 was observed after the isoelectric focusing.

Identification of a cofactor of the enzyme

Absorption spectrum of the crystalline en-

FIG. 4. Sedimentation Patterns of Crystalline Polyamine Oxidase.

The photographs were taken from A to D at 22, 30, 38 and 50 min after reaching 59,780 rpm. The protein concentration was approximately 0.34% in 0.01 M sodium acetate buffer, pH 3.8.

FIG. 5. Molecular Weight Estimation of Polyamine Oxidase by Sephadex G-200 Gel Filtration.

A Sephadex G-200 column (0.9×115 cm) equilibrated with 0.1 M sodium acetate buffer, pH 3.8, containing 0.1 M NaCl was run with the crystalline enzyme and the reference proteins. The elution volume is plotted *versus* the molecular weight of the protein.

FIG. 6. Molecular Weight Determination of Polyamine Oxidase by SDS-Polyacrylamide Gel Electrophoresis.

The crystalline enzyme (about 15 μ g) and the reference proteins were denatured as described in MATERIALS AND METHODS and subjected to SDS-polyacrylamide gel electrophoresis. The molecular weight of the denatured proteins are plotted *versus* the relative mobility.

FIG. 7. Absorbance Spectrum of Crystalline Polyamine Oxidase.

Absorbance spectra were recorded with 0.0296% solution of the crystalline enzyme in 0.01 M sodium acetate buffer, pH 3.8, in the ultraviolet region and 0.269% solution in the visible region.

zyme showed a typical three-banded spectrum for flavoprotein. One absorption maximum in ultraviolet range at 275 nm and two maxima in visible range at 375 and 450 nm were observed for the native enzyme (Fig. 7). Extinction coefficient of the enzyme at 280 nm ($E_{1\text{cm}}^{1\%}$) and the ratio of $E_{280\text{nm}}/E_{450\text{nm}}$ was 22.97 and 14.4, respectively. A resolution of the crystalline enzyme by trichloroacetic acid was performed by the method of Swoboda and Massey.¹⁰⁾ The enzyme solution containing 0.25 mg of protein was first subjected to a short heat treatment (pH 4.0, 1 min, 100°C) and then trichloroacetic acid was added to the solution to a final concentration of 5%. Visible spectrum of the clear yellow supernatant coincided with that of flavin derivative. The flavin extracted from the enzyme migrated at same distance as FAD in

TABLE II. THIN-LAYER CHROMATOGRAPHY OF FLAVIN FROM THE ENZYME

Flavin derivative	R _f value	
	Solvent A	Solvent B
Extract of the enzyme	0.07	0.52
FAD	0.07	0.52
FMN	0.19	0.75
Riboflavin	0.44	0.03

FIG. 8. pH Optimum of Polyamine Oxidase Activity. Crystalline enzyme (80 units) was used and the enzyme activity was measured by following H_2O_2 formation with spermine (●) or spermidine (■) as the substrate under the standard assay conditions except for sodium acetate buffer (pH 3.0~5.5), potassium phosphate buffer (pH 5.5~8.0) and NH_4Cl-NH_4OH buffer (8.0~10.5).

FIG. 9. Effect of Temperature on Polyamine Oxidase Activity.

Enzyme activity for spermine (●) or spermidine (○) was measured as same as Fig. 8 except for reaction temperature at pH 6.5 (a, b) and 7.0 (c).

both thin-layer chromatographic system (Table II). From these results, polyamine oxidase from *P. chrysogenum* was characterized to be a flavoprotein containing FAD as a cofactor. On assumption that the enzyme-bound FAD had the same extinction coefficient as free FAD,¹¹⁾ the FAD content was tentatively calculated to be 2 mol per mol of the enzyme.

Effect of pH and temperature on the enzyme

The enzyme activity was measured at various pHs (Fig. 8) and at various temperatures (Fig. 9). When spermidine was used as the substrate, the optimum pH was found to be at 5.0 and a small peak was found at 9.5, and the optimum temperature was around 25°C at pH 6.5. Whereas, using spermine as the substrate, the optimum pH was found to be at 9.5 and a second peak was found at 6.0. The optimum temperature was 30 to 35°C at pH 6.5 and around 40°C at 7.0, respectively. Thermal stability of the crystalline enzyme was measured in 0.1 M sodium acetate buffer, pH 4.0, and 0.1 M potassium phosphate buffer, pH 6.0 and 7.0, by heating at various temperatures for 10 min. The residual activity at 35°C was about 97%, 19% and 0% at pH 4.0, 6.0 and 7.0, respectively. The enzyme was very unstable in the alkaline pH and higher temperature. When the enzyme activity was assayed with spermidine as the substrate, the tendency was strengthened.

Oxidation of various amines

Relative rates of oxidation of various monoamines, diamines and polyamines by the crystalline enzyme are listed in Table III. Spermine was rapidly oxidized, but monoamines and diamines were not oxidized at all under the standard assay conditions employed. Spermidine was slightly oxidized under the conditions and the oxidation rate changed by pHs.

Effect of compounds on the enzyme activity

The enzyme activity was measured with a Clark oxygen electrode at pH 5.0 for sper-

TABLE III. RELATIVE RATE OF OXIDATION OF VARIOUS AMINES BY POLYAMINE OXIDASE FROM *Penicillium chrysogenum*

Enzyme activity was measured by following H_2O_2 formation under the standard assay conditions with 5 μ mol of the substrate and 51 units of the crystalline enzyme in 2.5 ml.

Substrate	Relative rate (%)
Monoamine	
Methylamine	0
Ethylamine	0
Propylamine	0
Butylamine	0
Phenethylamine	0
Tyramine	0
Dopamine	0
Tryptamine	0
Serotonin	0
Histamine	0
Agmatine	0
Benzylamine	0
Diamine	
Cadaverine	0
Putrescine	0
Polyamine	
Spermidine	7
Spermine	100

midine and at pH 9.5 for spermine as the substrate (Table IV). Sulfhydryl reagents such as Ag^+ , Hg^{2+} , iodoacetate and *p*-chloromercuribenzoate inhibited the enzyme activity. Therefore, the polyamine oxidase could be characterized to contain sulfhydryl group essential for the activity. Isoniazid and iproniazid, inhibitors of pyridoxal phosphate enzymes, did not markedly inhibit the enzyme activity. Carbonyl reagents and chelating reagents had no significant effect on the enzyme activity. Besides Ag^+ and Hg^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} and Pb^{2+} were clearly inhibitory when the enzyme activity was assayed with spermine as the substrate. Mg^{2+} and Pb^{2+} inhibited the enzyme activity for spermidine oxidation.

DISCUSSION

Polyamine oxidase was crystallized from

TABLE IV. INHIBITOR SPECIFICITY OF POLYAMINE OXIDASE FROM *Penicillium chrysogenum*

Enzyme activity was measured under the standard assay conditions with a Clark oxygen electrode at pH 5.0 for spermidine and at pH 9.5 for spermine as the substrate with addition of each compound and crystalline enzyme (51 units).

Inhibitor	Concentration ($\times 10^{-3}$ M)	Relative rate (%)	
		[SPM]*	[SPD]**
None		100	100
<i>p</i> -Chloromercuribenzoate	0.14	1.1	68.4
Iodoacetate	1.4	9.6	65.4
Hydroxylamine	1.4	100	91.9
Hydrazine	1.4	100	100
Phenylhydrazine	1.4	100	94.6
Semicarbazide	1.4	100	91.4
Isoniazid	1.4	98.6	70.3
Iproniazid	1.4	98.6	83.4
α, α' -Dipyridyl	1.4	77.7	100
8-Hydroxyquinoline	1.4	91.5	100
<i>o</i> -Phenanthroline	1.4	86.2	94.6
Sodium azide	1.4	88.3	100
EDTA	1.4	95.7	95.7
Ag^+	1.4	2.4	0.0
Li^+	1.4	94.1	97.2
Ni^{2+}	1.4	45.9	85.7
Cu^{2+}	1.4	36.4	108.6
Zn^{2+}	1.4	24.4	97.1
Mg^{2+}	1.4	80.0	65.7
Hg^{2+}	1.4	0.0	40.0
Pb^{2+}	1.4	47.6	62.9

* SPM: Spermine.

** SPD: Spermidine.

mycelial extract of *P. chrysogenum*, which was cultivated on spermine as sole nitrogen source. The crystalline enzyme oxidized both spermine and spermidine. In previous paper,⁵⁾ it was shown that oxidized products of spermine were 3-aminopropionaldehyde, putrescine and hydrogen peroxide. Since the reaction velocity for spermidine was faster than that for spermine at pH 5.0 as shown in Fig. 8, spermine formed rapidly putrescine as the oxidized product. However, at pH 7.5 where the reaction velocity for spermidine was slower than that for spermine, a large quantity of spermidine and a slight amount of putrescine were accumulated by oxidation of

TABLE V. VARIATION OF REACTION PRODUCTS BY SPERMINE OXIDATION AT pH 7.5

Spermine (500 nmol) and the crystalline enzyme (190 units) were incubated at pH 7.5 for 0, 2, 5 and 20 min at 35°C. The reaction was stopped by addition of trichloroacetic acid, and aliquot (50 nmol) was applied to the amino-acid analyzer as described in previous paper.⁵⁾

Reaction time (min)	Reaction products (nmol)		
	[SPM]*	[SPD]**	[PUT]***
0	50.0	0.0	0.0
2	31.4	17.4	0.0
5	20.5	28.7	0.0
20	2.0	35.8	11.1

* SPM: Spermine.

** SPD: Spermidine.

*** PUT: Putrescine.

spermine (Table V). Therefore, it became apparent that oxidation of spermine occurs according to the following pathway.

A similar cleavage of spermidine and spermine at the secondary amino group was shown

to occur in a *pseudomonad*⁴⁾ and in *Mycobacterium*,²⁾ respectively. However, these studies were carried out with crude cell-free extract, and the identity of oxidation products has not been well established. Recently, Hölttä reported existence of polyamine oxidase capable of forming putrescine from spermidine and spermidine from spermine in rat liver.¹²⁾ The polyamine oxidase of *P. chrysogenum* differed from that of rat liver in terms of molecular weight, behavior for inhibitor and so on. Further studies on the mechanism to regulate the enzyme activity for spermine and spermidine are of interest.

Acknowledgments. The authors thank Dr. Hidehiko Kumagai, Associate Professor of Kyoto University, for his valuable advice during the course of this investigation.

REFERENCES

- 1) S. Razin, I. Gery and U. Bachrach, *Biochem. J.*, **71**, 551 (1959).
- 2) U. Bachrach, S. Persky and S. Razin, *Biochem. J.*, **76**, 306 (1960).
- 3) R. H. Weaver and E. J. Herbst, *J. Biol. Chem.*, **231**, 647 (1958).
- 4) R. Padmanabhan and K. Kim, *Biochem. Biophys. Res. Commun.*, **19**, 1 (1965).
- 5) H. Yamada, K. Isobe and Y. Tani, *Agric. Biol. Chem.*, **44**, 2469 (1980).
- 6) B. J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
- 7) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
- 8) P. Andrews, *Biochem. J.*, **96**, 595 (1965).
- 9) T. Svedberg and K. O. Pederson, "Ultracentrifuge," Oxford University Press, 1940.
- 10) B. E. P. Swoboda and V. Massey, *J. Biol. Chem.*, **240**, 2209 (1965).
- 11) L. G. Whitby, *Biochem. J.*, **54**, 440 (1953).
- 12) E. Hölttä, *Biochemistry*, **16**, 91 (1977).