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Isolation, Purification, and Further Characterization of an L-Phenylalanine Oxidase from *Morganella morganii*

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ABSTRACT

A L-amino acid oxidase was isolated, purified, and characterized from *Morganella morganii* 53187, a bacterium formerly known as *Proteus morganii*. The synthesis of the enzyme by this bacterial strain was growth-associated and decreased sharply when the culture just reached the stationary phase. Based on this finding, the preparation of spheroplast by lysozyme-ethylene-diaminetetra-acetic acid (EDTA) disruption was carried out using the cells harvested during the exponential growth phase. Among several detergents tested, at the detergent-to-protein ratio of 2.5, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) was very effective in solubilizing most of the enzyme attached to the membranes while still preserving the activity of the solubilized enzyme. The resulting enzyme solution was then purified by hydrophobic interaction chromatography, followed by ion exchange chromatography and gel permeation.

The enzyme was purified 19-fold with an overall recovery yield of 12%, corresponding to a specific activity of 252.2 U/mg protein. The selectivity of the purified enzyme toward L-amino acids was pH-dependent. At pH 6.35, the enzyme was very specific to L-leucine, whereas the selectivity for L-phenylalanine could be improved at pH 7.4. The enzyme exhibited a wide optimum temperature range 35–43°C and exhibited 1, 1'-dimethylferricinium reductase capability in the presence of L-phenylalanine.

Index Entries: L-Amino acid oxidase; *Morganella morganii*; L-phenylalanine; purification; characterization.

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INTRODUCTION

Although L-amino acid oxidases have been isolated from several sources, including microorganisms such as *Pseudomonas* sp. (*1*), *Proteus* (*2*), *Neurospora* (*3,4*), and so on, to date few of these have been obtained in a state of high purity. Cell envelopes of *Proteus mirabilis, P. vulgaris,* and *P. morganii* are rich in L-amino acid oxidases that catalyze the oxidative deamination of several amino acids, including phenylalanine (*5*). Like *Providencia, Proteus* has been known to oxidize L-phenylalanine to phenylpyruvate in the presence of oxygen (*6*). The cytochrome components of the respiratory chain were shown to be involved in the electron transfer to oxygen and L-phenylalanine was used by the bacterial envelopes for the reduction of dichlorophenolindophenol (DCPIP). The enzyme catalyzing this reaction was termed as L-phenylalanine DCPIP-reductase, and this enzyme was also able to reduce DCPIP with several other amino acids, including methionine, leucine, isoleucine, tryptophan, and various analogs (*5*). Unfortunately, the L-amino acid oxidases from *Proteus* were associated with high-speed sedimentable particles, and several attempts to dissociate the enzyme from the particles by sonification or detergents were not successful (*7*).

Recently, an enzyme fraction, acting somewhat significantly on L-phenylalanine, was isolated from *M. morganii,* previously known as *P. morganii*. Cell envelopes of *P. morganii* were reported to be rich in L-amino acid oxidases that were present as integral proteins and associated with sedimentable particles. The activity of one of these oxidases for various L-amino acids in decreasing order was reported to be phenylalanine, methionine, leucine, and tryptophan (*8*).

The present paper is an extension of our preliminary work (*8*) and is concerned with the factors influencing the activity and stability of the L-amino acid oxidase isolated and purified from *Morganella morganii* and a protocol for solubilizing the enzyme attached to the membranes. Some of the properties of this flavoprotein preparation, such as selectivity, specific activity, and stability, were investigated and discussed in detail.

MATERIALS AND METHODS

Microorganism and Culture Medium

Morganella morganii strain 53187 was obtained from the Pasteur Institute Collection (Paris, France). This bacterium (*9*), formerly known as *Proteus morganii,* has been reclassified to a new genus of *Morganella* since its DNA contains 50 mol% G + C, which is similar to the DNA base composition of other genera of *Enterobacteriaceae.* The culture medium (PYG) consists of (g/L) bacto-peptone (Difco, Detroit, MI), 20; BBL yeast extract,

5; and β-D-glucose, 4; in distilled water. *M. morganii* was stored in liquid PYG containing 10% (v/v) glycerol at −80°C.

Production of L-Phenylalanine Oxidase

M. morganii was grown in an automated 19-L bioreactor (Bioengineering, Wald, Switzerland) with an operating capacity of 10 L at 37°C. The culture medium was agitated and aerated at 250 rpm and 10 L/m, respectively. The medium was inoculated with a 10% (v/v) inoculum grown overnight in shake flasks at 37°C and 250 rpm. Sterilized antifoam (2.5 mL, MAZU DF 10 P MOD 11, Mazer Chemicals, Gurnee, IL) was added to the culture medium prior to inoculation to minimize the development of foam during the course of cultivation.

The cells were harvested at the end of the exponential phase (7–8 h culture) by concentrating the resulting cell slurry to 1200 mL using microfiltration on hollow fibers (0.1 μm, polysulfone, 0.45 m^2, DC 10 L apparatus, Amicon, Danvers, MA) equipped with a gear pump. The concentrated cell suspension was then centrifuged at 10,000*g* for 15 min at 4°C, and the collected cells were treated for spheroplast formation.

Purification of L-Phenylalanine Oxidase

Unless otherwise indicated, all purification steps were performed at 4°C.

Spheroplast Formation and Isolation of Membranes

The harvested cells were treated by lysozyme (Sigma L-6876, St. Louis, MO) for spheroplast formation essentially as described by Osborn and Munson (*10*), except that after adding ethylene-diaminetetra-acetic acid (EDTA), the suspension was left overnight at 4°C. The spheroplasts were then harvested by centrifugation at 10,000*g* for 15 min and resuspended in lysis buffer "LB" [20 m*M* Tris/HCl, pH 7.6 containing 2 m*M* EDTA, 1 m*M* 1,4-dithioerythreitol (DTT), and 1 m*M* phenylmethane-sulfonyl fluoride (PMSF)] to obtain 90–95% of lysis as determined by microscopy. For comparison, the spheroplasts were also lysed by ultrasound as described by Osborn and Munson (*10*); three repeated bursts of 15 s with 1 min rest between each burst. The unlysed cells and spheroplasts were removed by centrifugation of the lysate at 1200*g* for 20 min. The supernatant was then centrifuged at 105,000*g* for 1 h to collect the membranes that were suspended in the washing buffer "WB" (20 m*M* Tris/HCl, pH 7.4 containing 1*M* NaCl, 2 m*M* EDTA, 1 m*M* PMSF, and 1 m*M* DTT) at a final concentration of 5 mg protein/mL, stirred at 4°C for 1 h and recentrifuged as above. The washed membranes were finally suspended in the lysis buffer at 20–30 mg protein/mL using a 22-gage needle fitted to a syringe. This suspension was stored at −80°C for at least 12 mo with no loss of activity.

Solubilization of the L-Phenylalanine Oxidase

Solubilization of L-phenylalanine oxidase was carried out in the lysis buffer containing 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma) and 5% (v/v) glycerol (referred to as solubilization buffer or "SB") at a detergent/protein ratio of 2.5. After incubation at 4°C for 1 h, the mixture was centrifuged at 131,000*g* for 1 h. The collected sediment was suspended in SB and assayed for the enzyme activity. The solubilized enzyme was diafiltered against 5 vol of SB containing 2 m*M* CHAPS, pH 8, using an Amicon apparatus equipped with a YM-30 membrane. At −20°C, this preparation was stable for at least 3 mo.

The release of membranous POD by trypsin was also attempted as outlined by Groleau and Forsberg (*11*) with the exception that the mixture was incubated 16 h and then centrifuged for 1 h at 131,000*g*, 4°C. The supernatant was then collected and assayed for the POD activity.

Hydrophobic Interaction Chromatography (HIC)

The soluble enzyme solution (363 mg, protein) was brought to 4*M* NaCl and applied to a Phenyl-Sepharose CL-4B column (Pharmacia, Uppsala, Sweden), 2.6 × 35 cm, equilibrated in 20 m*M* Tris/HCl, pH 8 containing 4*M* NaCl, 2 m*M* CHAPS, 5% glycerol, and 2 m*M* EGTA. The column was first washed with the equilibration buffer and then eluted with 20 m*M* Tris/HCl, pH 8 containing 2 m*M* CHAPS, 5% glycerol and 2 m*M* EGTA at 28 mL/h. Fractions of 7 mL were collected and the active fractions were pooled and concentrated by ultrafiltration in an Amicon stirred cell equipped with the YM-30 membrane at 38 psi under nitrogen.

Ion Exchange (IEC) and Gel Filtration Chromatography

The anion exchanger DEAE-Sephacel (Pharmacia) was equilibrated in 20 m*M* Tris/HCl, pH 8 containing 2 m*M* CHAPS, 5% glycerol, and 2 m*M* EGTA before adding the HIC purified enzyme (22 mg protein). The column (1.5 × 10 cm) was washed with the equilibration buffer and then eluted with a linear gradient of NaCl (0–1*M*) in the equilibration buffer at 9.6 mL/h. Fractions of 2.7 mL were collected and the active fractions were then pooled and concentrated.

For gel filtration, Sephacryl S-200 HR (Pharmacia) was equilibrated in 20 m*M* Tris/HCl, pH 8 containing 2 m*M* CHAPS, 5% glycerol, and 2 m*M* EGTA before adding the IEC purified enzyme. The enzyme was eluted at 10.6 mL/h, fractions of 5 mL were collected and the active fractions were pooled and concentrated.

Protein Determination and Polyacrylamide Gel Electrophoresis

Protein concentrations were measured by the method of Bradford (*12*) using the Bio-Rad protein microassay (Bio-Rad, Richmond, CA) according to the supplier's instructions. Bovine plasma γ-globulin supplied with the kit was used as the standard. The protein content of chromatography column eluates was monitored at 280 nm. The gels silver stained after

slab gel electrophoresis in the presence of SDS was performed as outlined by Laemmli (*13*) using Pharmacia's "Phast system" apparatus and gels according to the recommended specifications.

Assays of Amino Acid Oxidation

Oxygen consumption by POD in the presence of amino acids was determined by using a Clark oxygen electrode as described by Gamati and Luong (*8*). Measurement of the rate of reduction of dichlorophenolindophenol (DCPIP), an electron acceptor, by POD in the presence of amino acids was performed according to Labouré et al. (*14*). In this procedure, the POD activity was determined by following the absorbance decrease at 600 nm (Beckman DU-7, spectrophotometer) at 37°C and the reaction mixture (final vol of 0.5 mL) consisted of 37.5 m*M* L-phenylalanine, 0.85 m*M* phenazine methosulfate, and 0.2 m*M* DCPIP in 20 m*M* phosphate buffer pH 6.5. CHAPS (9 m*M*) was added to all reaction mixtures using the DCPIP reductase assay. One unit of activity is defined as 1 μmol of DCPIP reduced in 1 min.

The oxidase activity for L-phenylalanine was also measured by determining the rate of reduction of 1,1′-dimethylferricinium ($DMFeCp_2^+$), a blue dye that was electrochemically prepared from a yellow 1,1′-dimethylferrocene/2-hydroxypropyl-β-cyclodextrin inclusion complex (*15*). Ferrocene and its derivatives have been studied extensively to develop mediated amperometric biosensors since its cation counterpart, i.e., ferricinium is able to react efficiently with several FAD-containing oxidases in the reduced form (*15*). The reaction mixture (0.5 mL) consisted of 50 m*M* L-phenylalanine and 9 m*M* ($DMFeCp_2^+$) in 100 m*M* borate pH 9.4. The assay was carried out at 37°C and was linear over the range of 5–100 μg protein. The reduction of $DMFeCp_2^+$ to 1,1′-dimethylferrocene by the enzyme in the presence of L-phenylalanine resulted in an absorbance decrease that was monitored at 650 nm.

The protocol developed by Luong et al. (*15*) was essentially followed for the preparation of the blue dye. A solution (25 mL) of 1,1′-dimethylferrocene (ca. 90 m*M*, Polysciences, Warrington, PA) solubilized in 2-hydroxyproply-β-cyclodextrin (Aldrich, Milwaukee, WI; 250 m*M* in 100 m*M* KCl, 100 m*M* acetate, pH 5.2) was electrochemically oxidized at a platinum foil electrode poised at +450 mV vs an Ag/AgCl reference electrode in a three-electrode system. The counter and working electrodes were separated by a 2*M* KCl bridge and the electrochemical oxidation was performed using a polarographic analyzer/stripping voltammeter (Princeton Applied Research, Princeton, NJ). As reported (*15*), this is a one-electron transfer process and the ferricinium cation formed exhibited an absorption peak at 650 nm. The concentrated $DMFeCp_2^+$ was stable for at least 4 mo at 4°C and its absorption characteristics were insensitive to a wide pH variation (pH 2–11). $DMFeCp_2^+$ could also be reduced easily by various reducing agents such as ascorbic acid, uric acid, and sulfite, and

possessed an absorption coefficient of 325 $cm^{-1}M^{-1}$. One unit of activity is defined as 1 μmol of $DMFeCp_2^+$ reduced in 1 min.

RESULTS AND DISCUSSION

Isolation and Solubilization of the Membranous POD

The measurement of the POD activity by either oxygen consumption, $DMFeCp_2^+$ or DCPIP reduction reconfirmed that the synthesis of POD by *M. morganii* was growth-associated (data not shown). After 7–8 h of inoculation, the cells reached the stationary growth phase corresponding to an absorbency of 2.8 (at 600 nm). The production of POD then decreased sharply when the culture reached the stationary growth phase. Such behavior was also reported by Gamati and Luong (*8*) for the production of POD when the bacterium was grown in shake flasks.

As reported by Pelmont et al. (*5*), L-phenylalanine oxidase activity in *M. morganii* was associated with the membranes as compared to the cytosolic POD of *Pseudomonas* sp. P-501 (*1*). To isolate the enzyme, the first step was to recuperate the bacterial envelopes, and this could be achieved by disrupting the bacteria using the French pressure cell followed by ultracentrifugation (*8*). In this study, the lysozyme-EDTA disruption procedure was used for the preparation of spheroplast. The procedure resulted in more than 95% conversion to spheroplast as monitored by phase contrast microscopy. The spheroplasts were either lysed by osmotic shock or by sonication, and these two procedures were equally efficient in terms of the percentage of lysis and enzyme activity recovered.

The procedure suggested by Tanford (*16*) that has been successfully used for the solubilization of several membrane bound proteins (*11,17*) was attempted in this study. In this protocol, the membranous enzyme was released using trypsin, a proteolytic enzyme, instead of a detergent. Although POD was released from the membranes by the addition of trypsin, no activity of this enzyme was detected in the supernatant (Fig. 1). Further investigation may be necessary to decipher this behavior, however, one could anticipate the release of either an inactive peptide from the membrane or an active peptide followed by the hydrolysis of the peptide resulting in small inactive peptides.

Among several detergents tested, deoxycholate and to a lesser extent Triton X-100 exhibited a deleterious effect on the membranous enzyme activity (data not shown). Pelmont et al. (*5*) already presented evidence suggesting that Triton X-100 inhibited the oxidase by interfering in the electron transfer between a flavoprotein and the chain of cytochromes. CHAPS is generally less detrimental to various enzymes and therefore it was used for the solubilization of POD in this study. All of the following results were obtained with the $DMFeCp_2^+$ reductase assay because of the following reasons. First, any detergent including CHAPS added to the

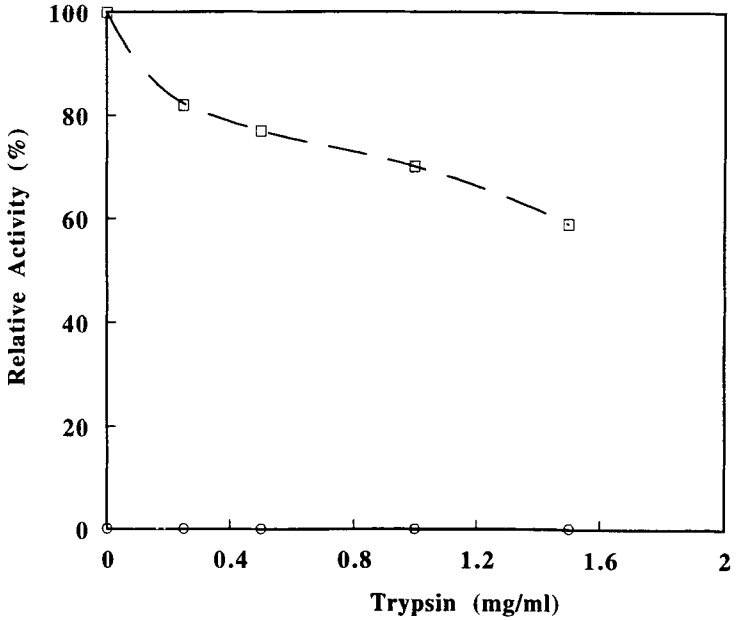

Fig. 1. Release of membranous POD by trypsin. Membranes were incubated at 25°C for 16 h in the presence of trypsin at various concentrations. (○) The POD activity in supernatant. (□) The POD activity in pellet.

membranes would totally inhibit oxygen consumption, i.e., the oxygen measurement procedure could not be used. Second, CHAPS also affected the DCPIP reductase assay, albeit to a lesser extent, and this effect was time-dependent (data not shown). Third, the enzyme activity vs pH experiment could not be performed using DCPIP since this dye is very sensitive to pH.

A series of experiment was first carried out to examine the effect of the detergent-to-protein (D/P) ratio on the solubilization of the enzyme as well as its activity. A detrimental effect on the enzyme activity was observed when the D/P ratio was higher than 5, although more protein was solubilized from the membrane. A D/P ratio of 2.5 was considered as the best compromise between the amount of activity solubilized and the specific activity of the soluble enzyme (Fig. 2). Using this ratio and 5% (v/v) glycerol at pH 7.6, most of the activity attached to the membranes was solubilized.

Enzyme Purification

The chromatographic profile obtained by HIC revealed that two peaks of enzyme activity were eluted from the HIC column (Fig. 3). The first peak (A) eluted during washing (4*M* NaCl) exhibited 3% of the activity applied and implied that most of the enzyme was bound to the column. The second peak (B) was eluted without NaCl and contained 52% of the activity applied to the column. The purification factor (9.3-fold) achieved

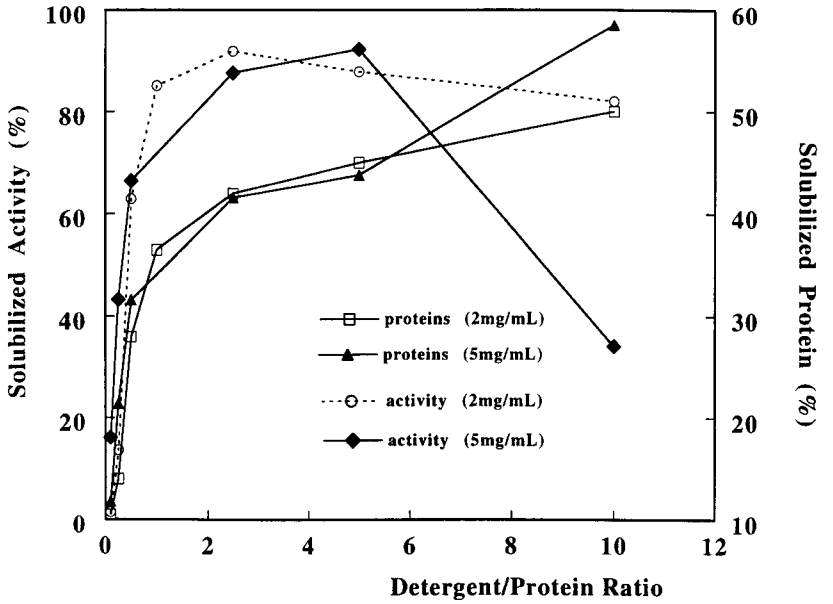

Fig. 2. The effect of the detergent/protein ratio on the solubilized activity and solubilized protein. Membranes (2 mg membrane protein/mL or 5 mg membrane protein/mL) were incubated in the presence of CHAPS for 2 h at 4°C in 20 m*M* Tris/HCl (pH 7.6), 1 m*M* PMSF, 1 m*M* DTT, and 2 m*M* EDTA.

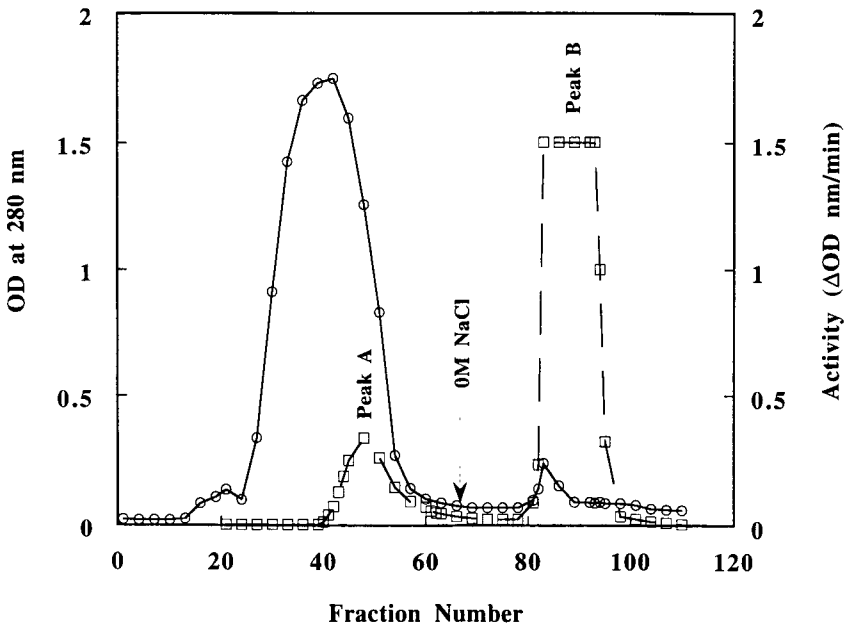

Fig. 3. Hydrophobic interaction chromatography of the soluble POD. (○) Absorbency of the column eluate monitored at 280 nm. (□) $DMFeCp_2^+$ reductase activity.

Table 1
Summary of the Purification of POD

Step	Total protein, mg	Total activity, U	Specific activity, U/mg	Yield	Purification factor
Intact cells	1455	19409	13.3	100	1
Lysate	1519	14557	9.6	75	0.7
Membranes	1102	9300	8.4	48	0.6
Washed membranes	639	8835	13.8	46	1
Soluble enzyme	363	7393	20.4	38	1.5
Hydrophobic interaction	22.1	4106	185.8	21	14
Ion exchange	12.7	2473	194.7	13	14.6
Gel filtration	9.5	2398	252.4	12	19

by hydrophobic chromatography was most efficient in comparison to the other techniques used in the protocol (Table 1).

The enzyme was further purified on the anion exchanger (DEAE-Sephacel). The activity was eluted with a linear gradient of NaCl at 0.28*M* as a single peak. About 62% of the applied activity was recovered with a purification factor of 1.1. This preparation was finally applied on a gel filtration column and the elution profile illustrated that the activity was eluted in the inclusion volume of Sephacryl S-200HR (Fig. 4). This result combined with the fact that the activity remained in the supernatant after centrifugation for 1 h at 131,000*g* indicated that the enzyme was essentially soluble. The enzyme recovered after this step was purified another 1.3-fold and 97% of the total activity applied was recovered. The enzyme was purified 19-fold with an overall yield of 12%; corresponding to a specific activity of 252 U/mg protein (Table 1). SDS polyacrylamide gel electrophoresis revealed that only two major protein bands remained after the gel filtration step compared to several protein bands observed from the starting material (figure not shown).

Characterization of the Purified Enzyme

With respect to the $DMFeCp_2^+$ reductase activity, the optimum range for pH was narrow and a maximal activity was observed at 9.4 (Fig. 5A). The enzyme exhibited a wide optimum temperature range, 35–43°C, and beyond this optimal range, the activity declined sharply as shown in Fig. 5B. It is worth noting that $DMFeCp_2^+$ has been known as an efficient mediator in the development of mediated amperometric biosensors because

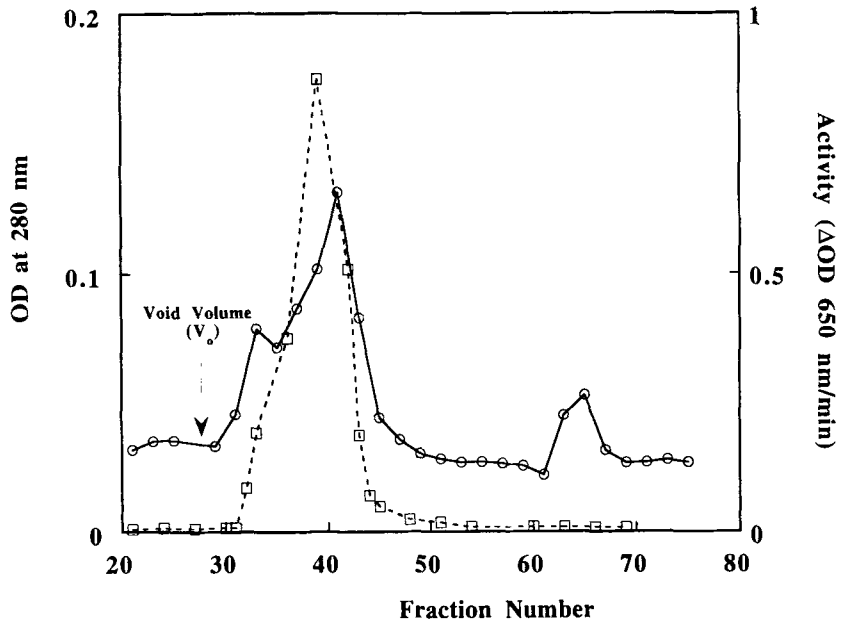

Fig. 4. Gel permeation chromatography of the ion exchange chromatography purified POD. (○) Absorbency of the column eluate monitored at 280 nm. (□) $DMFeCp_2^+$ reductase activity.

of its efficient electron exchange property with various reduced FAD-containing oxidases. In this study, the FAD moiety (if present) was dissociated from the POD enzyme by incubating the enzyme preparation (1 mL of the partially purified enzyme, 2 mg protein/mL) in a solution containing 4*M* $CaCl_2$ and using a protocol developed by Komai et al. (*18*). The fluorescence increase owing to the release of FAD was followed using a Gilson fluoro IV fluorometer (Ciba Corning Diagnostics, Ontario, Canada) with the excitation and emission wavelengths of 450 and 530 nm, respectively. The experimental data revealed that the relative fluorescence of the mixture increased until it reached a plateau after 7 h of incubation (figure not shown). This result combined with the fact that the enzyme was mediated by $DMFeCp_2^+$ thus demonstrated that POD is a FAD containing enzyme. A pH optimum could not be established for the DCPIP-reductase assay because the absorption characteristics of DCPIP are strongly dependent on pH as mentioned earlier.

A plot of the initial reaction rates of $DMFeCp_2^+$ reduction vs L-phenylalanine concentrations followed Michaelis-Menten kinetics with K_m and V_{max} values of 30.8 m*M* and 0.65 δOD/min/m*M*, respectively (figure not shown). Either borate or glycine-NaOH buffer could be used, whereas 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) buffer inhibited the enzyme activity. The $DMFeCp_2^+$ reductase activity of the POD was very sensitive to increasing concentrations of sodium chloride. At 0.5*M* NaCl, only 60% of the activity was measured.

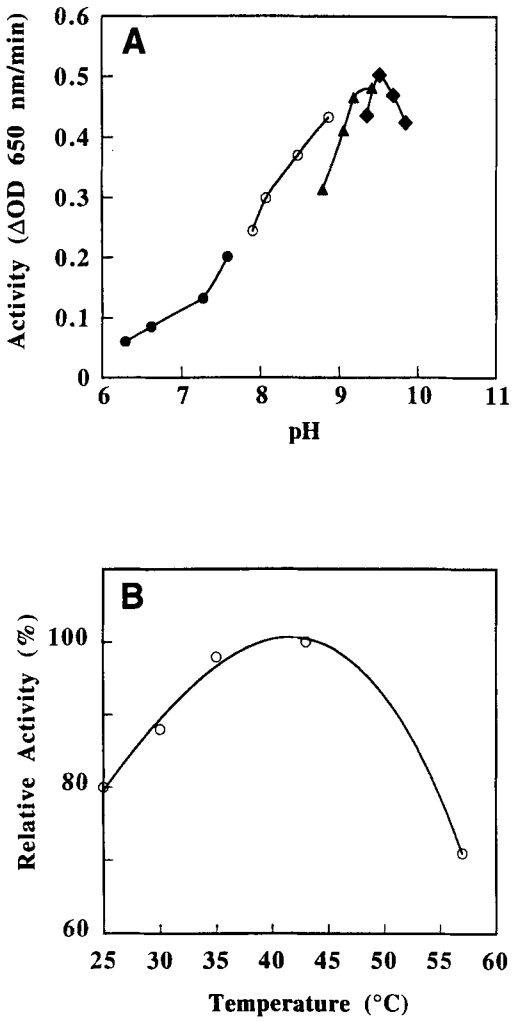

Fig. 5. A. The effect of pH on $DMFeCp_2^+$ reductase activity of the purified POD. Phosphate (●), Tris (○), Borate (▲), Glycine-NaOH buffer (◆). B. The effect of temperature on the $DMFeCp_2^+$ reductase activity of the purified POD.

Like other amino acid oxidases (*4,19*), the selectivity of the purified enzyme toward L-amino acids was pH-dependent (Table 2). At pH 6.35, the enzyme was very specific for L-leucine and the selectivity for L-phenylalanine could be improved by using pH 7.4. The activity of the enzyme toward other L-amino acids was negligible (data now shown). Ca^{2+}, Mn^{2+}, Cu^{2+}, and Mg^{2+} (1 m*M*) exhibited no noticeable effect on the activity of the enzyme. Experiments performed at higher levels of these cations (5 m*M*) or with Co^{2+} (5 m*M*) and Zn^{2+} (10 m*M*) effected precipitation in the $DMFeCp_2^+$ reductase assay mixture. Hg^{2+}, benzoic acid, iodoacetate, and 5,5′-dithiobis(2-nitrobenzoic acid) exhibited a pronounced inhibitory effect on the enzyme (Table 3). Such a result was not completely unexpected

Table 2
Substrate Specificity of POD Toward Various L-Amino Acids as a Function of pH[a]

L-Amino acids[b]	Relative activity to L-phenylalanine, %		
	pH 6.3	pH 7.4	pH 9.4
Leucine	431	160	186
Methionine	39	39	90
Phenylalanine	100	100	100
Tryptophan	57	68	182
Tyrosine	37	24	80

[a]As measured by the $DMFeCp_2^+$ reduction assay.
[b]Final concentration of the amino acid in the assay was 2 m*M*.

Table 3
Effect of Some Inhibitors on the Activity of L-Phenylalanine Oxidase

Inhibitors	Concentration, m*M*	Residual activity, %
$HgCl_2$	1	86
	10	5
Benzoate	10	82
	40	15
5,5′-Dithiobis(2-nitrobenzoic acid)	10	61
	40	42
D-Vinylglycine	1	42
	10	4
Iodoacetate	10	67

since these compounds have been known as L-amino acid oxidase inhibitors (*20–22*) and are indicative of the presence of reactive thiol groups. D-vinylglycine strongly inhibited the enzyme at $10^{-2}M$; this compound was reported to be a "suicide substrate" for the L-amino acid oxidase isolated from *Crotalus adamanteus* venom. Evidence was presented to support the fact that a covalent modification at the active site was the main reason for the loss of enzyme activity (*23*).

At 37°C, the enzyme retained only 10% of its activity after 24 h. The inactivation was somewhat less pronounced at 22°C and the enzyme was stable at 4°C. Stabilization of the enzyme using flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and EDTA up to 10 m*M* was also not effective. Stabilization was attempted using lactitol and DEAE-dextran for the enzyme during the storage at high temperatures, using the protocol described recently by Gibson et al. (*24*). Lactitol or DEAE-dextran, when used separately, was not effective and the enzyme lost its activity within 1 day at 37°C (Fig. 6). When a mixture of lactitol and DEAE-

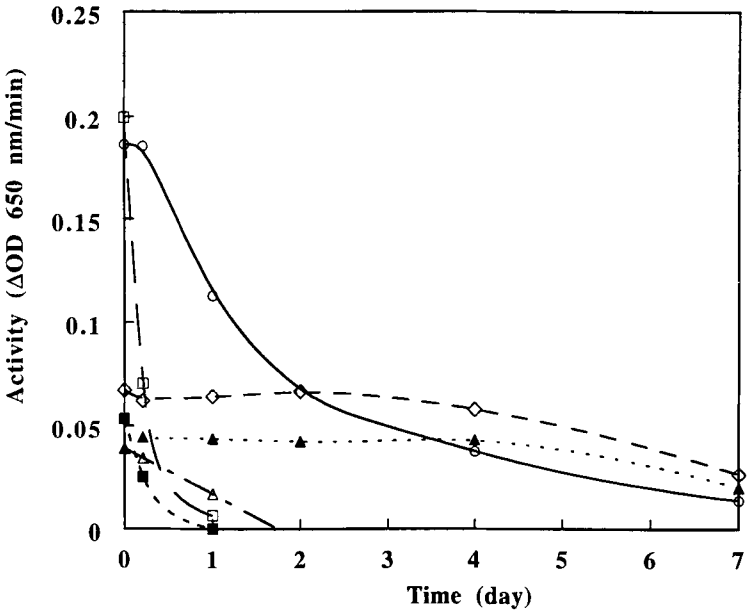

Fig. 6. Thermal stability of the purified POD; without DEAE-dextran:lactitol at 22°C (○) or 37°C (□); with DEAE-dextran:lactitol at a ratio of 10:1 at 22°C (◇) or 37°C (■); with DEAE-dextran:lactitol at a ratio of 20:1 at 22°C (▲) or 37°C (△).

dextran was added to the enzyme, there was an initial decrease in the activity and this effect became more noticeable at the high lactitol:DEAE-dextran ratio (20:1). However, the remaining enzyme activity was stable over a 4-d period at 22°C.

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