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Sequencing and Expression of the L-Phenylalanine Oxidase Gene from *Pseudomonas* sp. P-501. Proteolytic Activation of the Proenzyme

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The nucleotide sequence encoding L-phenylalanine oxidase (deaminating and decarboxylating) (PAO) from *Pseudomonas* sp. P-501 was determined. The open reading frame is arranged in the order of prosequence, α subunit, dipeptide and β subunit from the 5′- to 3′-end. Expression of the gene in *Escherichia coli* showed that without the prosequence, PAO is produced in small quantity as a soluble form with no visible absorption, but with the prosequence (proPAO), PAO is highly expressed and yellow. The purified proPAO contained one mol of FAD per mol of proPAO polypeptide, but had no catalytic activity. Treatment of proPAO with a mixture of Pronase and trypsin converted the noncatalytic proPAO to the catalytic form, and the Pronase-trypsin–treated proPAO showed kinetic and spectral properties comparable to the native enzyme. These results suggest that in *Pseudomonas*, PAO is expressed as a proenzyme that is processed by proteolysis to the active form.

Key words: flavoprotein, gene expression, L-phenylalanine oxidase, nucleotide sequence, prosequence, proteolytic activation.

Abbreviations: DAO, D-amino acid oxidase; IPTG, isopropyl-β-D-thiogalactopyranoside; ORF, open reading frame; PAO, L-phenylalanine oxidase (deaminating and decarboxylating); PO, phenol oxidase; proPO, prophenol oxidase; proPAO, PAO with prosequence; recPAOp, recombinant PAO activated with Pronase; recPAOpt, recombinant PAO activated with Pronase and trypsin; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

L-Phenylalanine oxidase (EC 1.13.12.9; PAO) from *Pseudomonas* sp.P-501 catalyzes both oxidative deamination and oxygenative decarboxylation (*1–3*). The enzyme has 2 mol of noncovalent FAD and consists of 2 mol each of α and β subunits (*2*, *4*). On the basis of spectral and kinetic studies, the reaction of the enzyme with β-thienylalanine (oxygenase substrate) and L-Met (oxidase substrate) is explained by the same scheme (*5*). The scheme is essentially the same as that for DAO (*6*) and tyramine oxidase (*7*). One of the catalytically important species is the purple intermediate, which consists of the reduced enzyme and an imino acid derived from a substrate (*8*), by analogy with DAO (*9–11*) and lysine monooxygenase (*12*). Protein-chemical studies of the enzyme have shown that the α-subunit consists of 92 amino acids and contains the common characteristic sequence of AMP binding in the FAD binding domain (*4*), and that Arg-35 in the β-subunit is essential for activity (*13*). The amino acid sequence around the Arg residue is homologous to the sequence of Trp 2-monooxygenases, as discussed (*13*). Trp 2-monooxygenase from *Pseudomonas savastanoi* has been purified and characterized (*14–18*). Chemical modification of the enzyme by 2-oxo-3-pentynoate inactivates the enzyme, and Cys511 and either Cys339 or His338 are the likely sites of modification (*18*). However, we did not find the corresponding Cys- or His-containing sequences in partial sequences of PAO (*4*, *13*). It is therefore essential to determine the complete amino acid sequence of PAO.

This work describes the cloning, sequencing and expression of the gene encoding the enzyme from *Pseudomonas* sp. P-501. The results of sequencing show that the PAO gene is arranged from the 5′ to 3′ end as a prosequence, α subunit, dipeptide and β subunit. Expression of the gene in *E. coli* showed that PAO is produced as a single noncatalytic polypeptide (proPAO) consisting of propeptide-α subunit–dipeptide-β subunit, and that proPAO becomes catalytic by proteolysis.

EXPERIMENTAL PROCEDURES

Materials—The *pfx* DNA polymerase was obtained from Gibco, BRL. All restriction endonucleases were from Roche. His-Bind resin was from Novagen. The Tetra His HRP conjugate kit was from Qiagen. The thermo Sequenase fluorescent labeled primer cycle sequence kit and [α-^{32}P]dCTP (3,000 Ci/mmol) were from Amersham Biosciences. Protein markers, Broad Range and Kaleidoscope, were from Bio-Rad. Pronase and trypsin were from Sigma and Worthington, respectively. Lys endoprotease and V8 protease were from Wako Chemicals. IPTG and X-gal were from Takara Biomedicals. Oligonucleotide primers were obtained from Invitrogen or SciMedia. All

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Primer	Sequence	Location[b]
(Cloning)		
α 74-6$_f$[a]	5'-AT(C/T)TACGA(A/G)GT(C/G)GG(T/C/G)GC(C/G)ATG-3'	873-893
T7pro$_f$	5'-TAATACGACTCATCACTATAGGG-3'	c
KC10F8-2$_r$	5'-AT(C/G)GGGTACAT(C/G)GC(C/G)AC(A/G)TT-3'	1128-1147
KC10F8-3$_f$	5'-AAGGTACTGGGCGTCGTTGC-3'	1077-1096
K12-2$_r$	5'-TA(C/T)TC(C/G)GG(C/G)GC(C/T)TG(C/G)C(T/G)(A/G)TC-3'	2034-2053
K12-3$_f$	5'-AAGGCCGTCGTCTCCGACAG-3'	1971-199
F3$_r$	5'-AT(A/C/G)A(A/G)(A/C/G)CC(C/G)GC(C/G)AC(C/G)GCGTT-3'	2475-2494
F7$_r$	5'-TT(T/C/G)GA(C/G)GC(A/C/G)CC(C/G)GCGTACTT-3'	2201-2221
HGS-1$_f$	5'-GGTCGCTCGACAACCGCTTC-3'	2389-2408
HGS-2$_r$	5'-GGTCAGATGCGGTCGAAGTC-3'	3223-3242[d]
(Expression)		
PNnde1$_f$	5'-catatgGGCGTTACCGTCATTCC-3'	465-484
PANnde$_f$	5'-catatgAAGAAGATTGCGACGACCGT-3'	510-527
PCxho$_r$	5'-ctcgagCTGGCTGGTGGCCAGCTCCG-3'	2587-2606

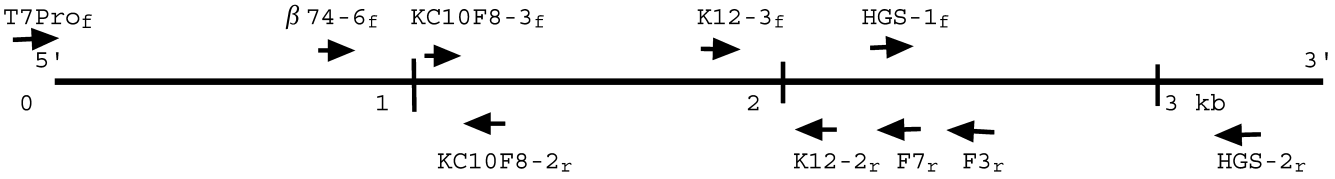

Fig. 1. **PCR primers.** Restriction enzyme sites are underlined. [a]f, forward; r, reverse. [b]Location is indicated by nucleotide numbers from the nucleotide at the 5′ end of the DNA sequence in Fig. 2. c, sequence of the T7 promoter. [d]written in bold in Fig. 2. The mixed primer for cloning were designed on the basis of the partial amino acid sequence of the peptides derived from PAO (unpublished results).

other chemicals were obtained from Wako Chemicals. Native PAO, prepared as described (*1*), was a gift from Dr. H. Koyama.

Buffers—TE: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 in 1 liter water. Buffer A: 20 mM Tris-HCl, pH 7.9, 500 mM NaCl.

Cells, Strains, and Plasmid—Pseudomonas sp. P-501 was kindly supplied by Dr.H.Koyama. The pBluescript II KS(+) was from Stratagene, and the pET vectors, 22b(+) and 31b(+) were from Novagen. Trypton peptone and Bacto yeast extract were from Difco. Two different bacterial growth media, LB and TP, were used in the present experiments. LB: 5 g Bacto yeast extract, 10 g Bactotrypton, 8 g NaCl, 2 g Na_2HPO_4, and 1 g KH_2PO_4 were dissolved in 1 liter water. TP: 15 g Bacto yeast extract, 20 g Bacto Trypton, 8 g NaCl, 2 g Na_2HPO_4, and 1g KH_2PO_4 were dissolved in 1 liter water, and 1 ml of 0.2 g/ml glucose was added just before use (*19*). *Escherichia coli* strains JM109 and BL21(DE3) were from Novagen.

Preparation of Pseudomonas sp. P-501 Genomic DNA—All common DNA manipulations were performed by standard procedures (*20*). The bacterial strain *Pseudomonas* sp. P-501 was cultivated in 1 ml medium (1% phenylalanine, 0.15% yeast extract, 0.1% KH_2PO4, 0.1% K_2HPO_4, 0.01% $MgSO_4$ and 0.001% $FeCl_3{\cdot}7H_2O$) (*1*) for 8 h at 30°C. Then the whole culture was further incubated in 100 ml of medium at 30°C. When the OD_{600} of the medium reached approx. 4, the cells were collected by centrifugation and suspended in 20 ml of 50 mM Tris-HCl (pH 8.0) containing 50 mM EDTA. The genomic DNA was prepared from the cells by the standard procedures (*20*) and dissolved in TE.

Preparation of a Genomic Library—Genomic DNA (30 μg) was incubated with 0.3 units of *Sau* 3AI (Roche) in 300 μl of M buffer (Roche) at 37°C for 1 h for the partial digestion of the genomic DNA. The whole mixture was heated for 20 min at 70°C to denature the enzyme. The size-fractionated genomic DNA fragments were inserted into a λFIX II vector (Stratagene) using a λFIXII/*Xho*I Partial Fill-in vector kit and packaged by adding the Gigapack III Gold packaging extract (Stratagene) according to the manufacturer's instructions. The phage solution thus obtained was used as a library of the *Pseudomonas* genomic DNA.

Preparation of ^{32}P-Labeled Probe—The probe was prepared by PCR using the genomic DNA as the template. PCR was performed using the degenerate primers KC10F8-2$_r$ and β74-6$_f$ (Fig. 1). The PCR product (approx. 300 bp) was labeled with [α-^{32}P]dCTP using a Megaprime DNA labeling system RPN 1606 (Amersham Biosciences). The labeled DNA was separated from [α-^{32}P]dCTP by gel-filtration.

Screening of the PAO Gene and DNA Sequencing—Screening of the PAO gene from the phage library was performed by standard procedures (*9*) using the probe as prepared above. Approx. 4×10^5 clones were screened, and 29 positive clones were selected. By several screening steps, one clone containing the longest insert size was selected. DNA was prepared from the amplified phage

and used as a template for DNA sequence analyses. Several sets of PCR and the cloning of the PCR products into pBluescript were performed using the phage DNA as template. The degenerate PCR primers were designed on the basis of the partial amino acid sequences obtained by CNBr cleavage or protease(trypsin, chymotrypsin) treatment of PAO. Figure 1 shows the sequences of the 3 sets of primers (T7pro$_f$ and KC10F8-2$_r$), (KC10F8-3$_f$ and K12-2$_r$) and (K12-3$_f$ and F3$_r$) that were successfully used for the cloning and sequencing. DNA sequencing of the insert in the plasmid was done using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit (Amersham Pharmacia Biotech) and a Shimadzu DNA sequencer DSQ-100L. Sequences were assembled, edited and analyzed using GENETYX-MAC (Software Development). Most of the PAO gene sequence was determined, but a 3′-terminal region of the PAO gene (approx. 100 bp long) was not. To determine the sequence, DNA from the phage library was digested with the restriction endonuclease *Not*I, and DNA fragments of 3 kbp were ligated into the *Not*I site of pBluescript KS(+), and transformed into *E. coli*, JM109. The transformation mixture was screened for α-complementation on plates containing X-Gal. White colonies were further selected on the basis of the formation of an approx. 300 bp fragment by PCR using the primers K12-3$_f$ and F7$_r$. One of the plasmids was found to contain the PAO gene and was named pBSNot5-4. The 5′- and 3′-sequences of the insert were determined by the Genomic Research Dept., Shimadzu-Biotech, using sequence primers M13-20, 5′-CGACGTT-GTAAAACGACGGCCAGT-3′, and M13$_r$, 5′-GGAAAC-AGCTATGACCATGATTAC-3′. The data showed that pBSNot5-4 covers the entire sequence of the PAO gene. To confirm the sequence of the 3′-region of the PAO gene, PCR was performed with the primers HGS-1$_f$ and HGS-2$_r$, and the PCR product was cloned into pBluescript. The sequence of the insert was determined and assembled into the 3′-region of the sequence of the PAO gene determined above.

Construction of the Expression Vectors for PAO—Two expression plasmids were constructed. One, pPAO$_{+15}$, contains a gene coding a prosequence consisting of 15 amino acids, α subunit, dipeptide, and β subunit, and the other, pPAO, contains the gene coding the α subunit, dipeptide and β subunit.

Using pBSNot5-4 as a template, a DNA fragment containing the prosequence was obtained by PCR using the primers PNnde1$_f$ and PCxho$_r$; a DNA fragment not containing the prosequence was obtained by PCR using the primers PANnde1$_f$ and PCxho$_r$ (Fig. 1). Amplification was performed using Platinum *Pfx* DNA polymerase (Gibco BRL) under the conditions recommended by the manufacturer. In the case of the primers PNnde$_f$ and PCxho$_r$, the first 5 min denaturation at 95°C was followed by 30 cycles consisting of 1 min at 94°C, 2 min at 50°C and 3 min at 68°C. In the case of the primers PANnde1$_f$ and PCxho$_r$, the first 5 min denaturation at 95°C was followed by 40 cycles consisting of 1 min and 20 s at 94°C, 2 min at 54°C, and 4 min and 10 s at 68°C. Two fragments were successfully amplified by these PCRs, and inserted into the *Eco*RV site of pBluescript vector. The plasmids were named pBPAO$_{+15}$ and pBPAO, respectively. Formation of restriction enzyme sites of each insert was confirmed by DNA sequencing. The plasmids were digested with *Xho*I and *Nde*I as described in the protocol of the supplier of these enzymes. The fragments (approx. 0.7 kbp and approx. 1.4 kbp) were purified by agarose-gel electrophoresis. The small fragments (approx. 0.7 kbp, one with the prosequence and the other without) were inserted into the *Nde*I–*Xho*I site of pET vectors 22b(+) and 31b(+), respectively, and the plasmids were digested with *Xho*I. The approx. 1.4 kbp fragments obtained were inserted into the *Xho*I site of the plasmid, and the vectors were transformed into *E. coli* BL21(DE3). The expression vectors obtained were named pPAO and pPAO$_{+15}$ indicating the vectors without and with the 15 amino acid prosequence, respectively. The sequence of the insert was confirmed by single sequencing conducted by the Genomic Research Dept., Shimadzu-Biotech.

Expression of Genes Encoding PAO, and the Purification and Protease Treatment of proPAO—E. coli strain BL21(DE3) cells transformed with the expression vectors pPAO or pPAO$_{+15}$ were grown in 5 ml LB medium containing ampicillin (50 μg/ml) overnight at 37°C. The culture medium (500 μl) was added to 50 ml of LB medium (50 μg/ml ampicillin) and cultured at 30°C. When the OD$_{600}$ of the culture medium reached approx. 0.5, induction was started by the addition of 1 mM IPTG. After incubation for given times, 2.5 ml of medium was withdrawn, and the cells collected from 100 μl of medium were analyzed by SDS/PAGE to estimate whole protein synthesis. The remaining cells in the medium were collected by centrifugation and lysed by sonication. The whole lysates were centrifuged, and the supernatants were collected. The suspension of cell debris in PBS and the supernatant were analyzed by SDS-PAGE to estimate the amounts of insoluble and soluble proteins, respectively.

Large scale preparation of proPAO was performed by the following procedures. *E. coli* BL21(DE3)/pPAO$_{+15}$ cells were cultured in 1 liter of TP medium for 4 h at 30°C, and the cells (16 g) obtained were washed with PBS, suspended in 50 ml of Buffer A, and lysed by sonication using a Branson sonicator (Model 250; output, 15; duty cycle, constant) below 7°C under constant mixing of the cell suspension. The cell debris was removed by centrifugation. To the supernatant, 10 ml His-Bind resin that had been pre-equilibrated with buffer A was added. The whole was mixed overnight at 4°C. The resin was packed in a glass column and washed completely with buffer A containing 5 mM imidazole. Then proPAO was eluted with buffer A containing 50 mM imidazole. The proteins in the eluate were further purified on a DEAE-Sepharose column (1.5 × 4.0 cm) equilibrated with 10 mM potassium phophate buffer (pH 8.0) containing 0.3 mM EDTA. ProPAO was eluted from the column by a linear concentration gradient of KCl. The purified proPAO was dialyzed against 10 mM phosphate buffer (pH 8.0) containing 0.3 mM EDTA and kept at 4°C for further use.

ProPAO was activated by incubation with given concentrations of proteases in 20 mM potassium phosphate buffer(pH 7.0) at 25°C for given times, and aliquots of the mixtures were taken to measure the catalytic activity and to perform SDS/PAGE. Protein was determined by measuring the absorbance at 280 nm using an absorbance coefficient of $A_{1\%/1cm}$ = 19.7 (*2*). Absorption spectra

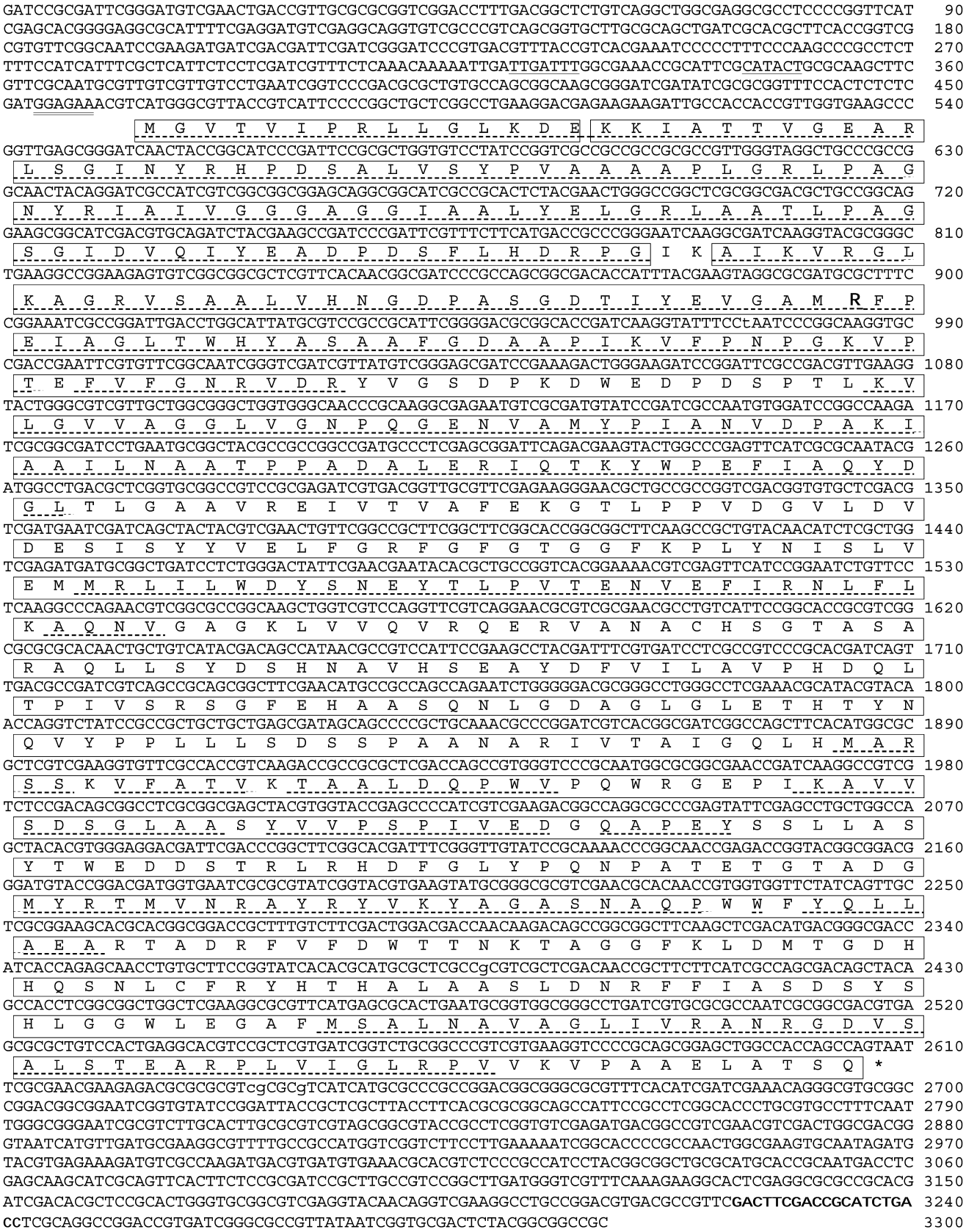

Fig. 2. **Nucleotide sequence of a 3,300 base pair *Pseudmonas* sp. P-501 DNA fragment containing the L-phenylalanine oxidase gene and the deduced amino acid sequence of the gene product.** Prosequence, α subunit, and β subunit are boxed, respectively. Regions where the deduced amino acid sequence matches the peptide sequencing data are underlined (- - -). Arg-35 in the β subunit is written in bold. A putative ribosome-binding site is indicated by double underlining.

were measured in a double beam spectrophotometer, type V-520-SR or type V-560, from Japan Spectroscopic.

Cofactor Analysis—The enzyme sample was treated with 10% trichloroacetic acid, and the supernatant was removed and rinsed with ether to remove the trichloroacetic acid. A solution was made in 0.1 M sodium phosphate (pH 5.1), then a part of the solution was analyzed by HPLC using FAD, FMN, AMP, NADH and NAD as standards. The solution was applied to an Inertsil ODS column (4.6 × 250 mm, GL Sciences Inc.), which was pre-equilibrated with 0.1 M sodium phosphate (pH 5.1), and eluted at 1 ml/min with a linear methanol gradient. The elution was monitored at 260 nm.

SDS-PAGE—Gel electrophoresis was performed according to Laemmli (*21*). Protein samples were boiled for 5 min in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 5% mercaptoethanol, and subjected to electrophoresis.

Assay of Enzyme Activity—The activity of the enzyme was assayed by two methods. In the first, oxygen uptake was measured polarographically using a Clark-type oxygen electrode (Yellow Springs Instruments, USA) in the presence of 0.5 mM L-Phe in 20 mM potassium phosphate buffer (pH 8.0) at 25°C. The rate was expressed as the mol oxygen consumed per s. The apparent kinetic parameters, k_{cat} and K_m, were determined from the rates obtained at various concentrations of L-Phe in the presence of 253 μM molecular oxygen. The second method was to measure hydrogen peroxide produced by the oxidation of L-Phe. The reaction mixture (250 μl) contained 7.2 mM L-Phe, 45 μl of the *o*-dianisidine solution, 5 μl peroxidase (1 mg/ml) in 20 mM potassium phosphate buffer (pH 8.0) containing 0.1 mM EDTA. At given times, the OD_{490} was measured using an automatic microtiter plate reader. A solution of *o*-dianisidine was prepared by dissolving 3 mg of *o*-dianisidine in 1 ml ethanol, and the solution was centrifuged for 5 min at 14,000 rpm. The supernatant was collected and diluted by the addition of a 19-fold excess deionized water.

Protein Sequence Analysis—Peptide and protein samples were purified by reverse-phase HPLC on an Inertsil ODS column (4.6 × 250 mm, GL Sciences Inc.) as described previously (*4*, *13*). The amino acid sequences of the proteins and peptide fragments were analyzed with a gas-phase sequencer(Shimadzu, PPSQ-10).

RESULTS

Sequence Analysis of the Gene Encoding PAO—The nucleotide sequence of the gene encoding PAO was determined as described in "EXPERIMENTAL PROCEDURES." Based on the amino acid sequence of the α subunit and partial amino acid sequences of the β subunit (*4*, *13*), one ORF was identified as shown in Fig. 2. A putative promoter sequence can be traced in the upstream region (single underline) of the ORF by using the "Search for Promoter Sequence" function of GENETYX-MAC (Software Development Co., Ltd.). The sequence contains a less conserved –35 hexanucleotide sequence of the *E. coli* promoter except for the last 2 bases. Within a distance of 17 bp, a possible –10 region is located (Fig. 2).

A homology search of the deduced amino acid sequence of the ORF with BLAST (*22*) found a putative Trp monooxygenase of the plant pathogen *Ralstonia solanacearum* (*23*) with 63.5% homogeneity, and Trp monooxygenases with a low scores. The amino acid sequence of the putative *Ralstonia* Trp monooxygenase is homologous with that of proPAO in their overall sequence, but sequences of other oxygenases (*24–26*) are partially homologous as shown in Fig. 3. Moreover, the corresponding Cys- or His-containing sequences of Trp 2-monooxygenase (*13*) were not found.

Comparing the deduced amino acid sequence (see Fig. 2) of the PAO gene with the protein sequence of the α subunit and partial amino acid sequences of the β subunit (*4*, *13*), the following conclusions can be drawn. That is, the open reading frame is arranged in the order of a prosequence consisting of 15 amino acid residues, α subunit, dipeptide and β subunit from the 5′- to 3′-end. This suggests that PAO is synthesized as a proenzyme (proPAO) that is processed to the mature form by proteolysis. To clarify this assumption, we constructed a PAO expression plasmid and expressed the gene in *E. coli* as described in "EXPERIMENTAL PROCEDURES."

Expression of PAO and proPAO in E. coli—*E. coli* BL21(DE3) cells haboring pPAO or $pPAO_{+15}$ were incubated in LB medium in the presence or absence of IPTG at 30°C as described in "EXPERIMENTAL PROCEUDRES." After given times, whole cell proteins were analyzed by SDS-PAGE. The gel staining pattern clearly showed that PAO and proPAO are well expressed in *E. coli*. To see if these proteins are expressed as soluble or insoluble proteins, the *E. coli* cells were collected and lysed, and the cell debris and the supernatant were analyzed by SDS-PAGE. PAO was found to be expressed at a low level as a soluble form (data not shown). To characterize the soluble PAO (recPAO), we prepared recPAO from *E. coli* grown in 1 liter LB medium for 18 h at 30°C, using the method described for the preparation of proPAO. The protein obtained was approx. 30 μg, and appeared as a single band on SDS-PAGE, but had no visible absorption (data not shown). The enzymatic activity of the protein was assayed before and after proteolytic treatment of the protein. No activity was observed by the hydrogen peroxide–peroxidase–coupled method even in the presence of added FAD.

As for proPAO, *E. coli* cells harboring $pPAO_{+15}$ were grown at 30°C for 21 h in LB or TP media in the presence of various concentrations of IPTG. As Fig. 4 shows, the amount of soluble proPAO was high in the medium without added IPTG. Moreover, more proPAO was produced as a soluble form in TP medium than in LB (compare lane 3 in Fig. 4B with that in Fig. 4A).

Proteolytic Activation of proPAO—As described above, proPAO was overexpressed, and approx. 40 mg of the purified proPAO was obtained from the cells in a 1 liter culture medium. To identify the cofactor, proPAO was treated with trichloroacetic acid and analyzed by HPLC. HPLC analysis showed that proPAO contains only FAD as a cofactor (data not shown). Moreover, the amount of FAD in proPAO was calculated to be 1.0 mol/mol, assuming that the molecular weight of proPAO is identical to that calculated from the proPAO sequence. ProPAO did not oxidize L-Phe when assayed polarographically.

Koyama (*1*) prepared that the wild type enzyme from cell extracts obtained after the autolysis of *Pseudomonas* sp. P-501, and reported that the autolysis is stimulated

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Agro      1 ----------------------MAGSSFTLPSTGSAPL-DMMLIDDSDLLQLGLQQVFSK    37
savast    1 ------------------------------------------------------------     1
Oct       1 MSASPLLDNQCDHLPTKMVDLTMVDKADELDRRVSDAFLEREASRGRRITQISTECSAGL    60

Agro     38 RYTETPQSRYKLTRRASPDVSSGEGNVHALA-FIYVNAETLQMIKNARSLTEANGVKDLV    96
savast    1 ------------------------------------------------------------     1
Oct      61 ACKRLADGRF-------PEISAGG-KVAVLSAYIYIGKEILGRILESKPWARATVS-GLV   111

                                        ## ##   #           # ##   #
PAO       1 ---------------------------------MGVTVIPRLLGLKDEKK---IATTVGEA    25
Rals      1 ---------------------------------MGITVMPGLTSMESDTRTQRISTTIGQA    28
Agro     97 AIDVPPFRNDFSRALLLQVINLLGNNRNADDDLSHFIAVALPNSARSKILTTAPFEGS-L   155
savast    1 ------------------------------------------------------------     1
Oct     112 AIDLAPFCMDFSEAQLIQALFLLSGKRCAPIDLSHFVAISISKTAGFRTLPMPLYENGTM   171

            ## #  ###############     ## ##   # #   ####### ###    #### #
PAO      26 RLSGINYRHPDSALVSYPVAAAAP-LGRLPAGNYRI---AIVGGGAGGIA---ALYELGR    78
Rals     29 RLNGRFYRHPDSALVSYPVAVDNA-LGALPDRAYNI---AIVGGGAAGIA---SLYELSR    81
Agro    156 SENFRGFPITREGNVACEVLAYGNNL--MPKACSDSF--PTVDLLY-DYGKFFESCAADG   210
savast    1 ---------------------------------MYDHFNSPSIDILY-DYGPFLKKCEMTG    27
Oct     172 -KCVTGFTITLEGAVPFDMVAYGRNL--MLKGSAGSF--PTIDLLY-DYRPFFDQCSDSG   225

            ##  # ##    #  ## ######     #  ## #######   ##### #    #
PAO      79 LAATLPAGSGIDVQIYEADPDSFLHDRP-GIKAIKVRGLKAG---RVSAALVHN-GDPAS   133
Rals     82 LAKTLSAGTMT-VTLYESDPDSFLTRLEAGTQAINVRGLKAG---RVSAARVNQ-NDT--   134
Agro    211 RIGYFPEGVTKPKVAIIGAGFSGLVAASELLHAGVDDVTVYEASDRLGGKLWSHGFKSAP   270
savast   28 GIGSYSAGTPTPRVAIVGAGISGLVAATELLRAGVKDVVLYESRDRIGGRVWSQVFDQTR    87
Oct     226 RIGFFPEDVPKPKVAVIGAGISGLVVANELLHAGVDDVTIYEASDRVGGKLWSHAFRDAP   285
                           * *                     *

                ####################   ## ################ ## # ### ## #
PAO     134 GDTIYEVGAMRFPEIAGLTWHYASAAFGDAAPIKVFPNPGKVPTEFVFGNRVDRYVGSDP   193
Rals    135 ---VYEVGAMRFPEIAGLTWHYASVVYGDGAPIKVFPNPGKVPTEFMFGDRIDRYSGSAP   191
Agro    271 NVIA-EMGAMRFPRSESCLFFYLKKHGLDSVG-L-FPNPGSVDTALFY--RGRQY-----   320
savast   88 PRYIAEMGAMRFPPSATGLFHYLKKFGISTST-T-FPDPGVVDTELHY--RGKRY-----   138
Oct     286 SVVA-EMGAMRFPPAAFCLFFFLERYGLSSMR-P-FPNPGTVDTYLVY--QGVQY-----   335
                 * ******                        ** ** * *          *

             ## #  ##  ##  ### ## #            ##   ##   #  #   #     #
PAO     194 KDWEDPDSPTLKVLGVVAGGLVG-NPQGENVA-MYPIANVDPAKIAAILNAATPPADALE   251
Rals    192 EDWLDRESPARKVFLVVASGLAG-APDSQTPIALFPIGGRDPVQVAQLLKSETAGPAQLR   250
Agro    321 -IWKAGEEPPELFRRVHH-GWRAFLQDGYLHDGV-MLAS--PLAIVDALNLGHLQQAHGF   375
savast  139 -HWPAGKKPPELFRRVYE-GWQSLLSEGYLLEGG-SLVA--PLDITAMLKSGRLEEAAIA   193
Oct     336 -MWKAGQLPPKLFHRVYN-GWRAFLKDGFHERDI-VLAS--PVAITQALKSGDIRWAHDS   390
              *     *      *   *                     *      *

             ##   ##  ###   # ## ###  ###    ##    ###  #  #    # ######
PAO     252 RIQTKYWPE-FIAQYDGLTLGAAVREIVTVAFEKGT---LPPVDGVLDVDESISYYVELF   307
Rals    251 AIQERDWPA-FIAAHDGTTLEAAVRRIVTREIGKGAASSLPPIAGL-DPAETINYYVELF   308
Agro    376 WQS---WLTYFERE----SFSSGIEKMFL-----GNHP---PGGEQWNSLDDLDLFKA--   418
savast  194 WQG---WLNVFR-DC---SFYNAIVCIFT-----GRHP---PGGDRWARPEDFELFGS--   236
Oct     391 WQI---WLNRFGRE----SFSSGIERIFL-----GTHP---PGGETWSFPHDWDLFKL--   433
                  *   *                       *      *
```

Fig. 3

by the addition of a small amount of Pronase. Therefore, we treated proPAO with Pronase for given times, and then assayed the enzymatic activity polarographically. As Fig. 5A shows, no activity was observed after 3 min incubation, but almost maximum activity was attained after 6 h incubation. The SDS-PAGE pattern shows that proPAO was cleaved into 4 main polypeptides after incubation for 3 min. The N-terminal sequences of the proteins in the bands migrating around the α and β subunits were determined as follows. The incubation mixture was applied to a reversed phase HPLC column. Two peaks, a relatively small peak eluting ealier (α subunit) and a

```
                ############# ###################### ######  #  ##   #   #
PAO      308 GRFGFGTGGFKPLYNISLVEMMRLILWDYSNEYTLPVTENVEFIRNLFLKAQNVGAGKLV   367
Rals     309 GRFGFGTGGFKPLFNISLVEMMRLILWDYSNEYTLPVEENVEFIAGLYQKALEQGGSRLT   368
Agro     419 --LGIGSGGFGPVFESGFIEILRLVVNGYEDNVRLSYEG-ISELPHRIASQVINGRSIRE   475
savast   237 --LGIGSGGFLPVFQAGFTEILRMVINGYQSDQRLIPDG-ISSLAARLADQSFDGKALRD   293
Oct      434 --MGIGSGGFGPVFESGFIEILRLVINGYEENQRMCPEG-ISELPRRIASEVVNGVSVSQ   490
                * * *** *       *  *     *                          *

                # ## ###   ###  #  #      ##         ## #### # # ##   #####
PAO      368 VQVRQERVANACHSGTASARAQLLS-YDSHNAVHSEAYDFVILAVPHD-QLTPIVSRSGF   425
Rals     369 VNVRRERVSDVCHSDAARVRQPFVVAYDRAGKLSAPNYDYVILAAPQD-QLALAVSRSGY   427
Agro     476 R-TIHVQVEQIDREEDKINIKIKGGKVEVYDRVLVTSGFANIEMRHLLTSSNAFFHADVS   534
savast   294 R-VCFSRVGRISREAEKIIIQTEAGEQRVFDRVIVTSSNRAMQMIHCLTDSESFLSRDVA   352
Oct      491 R-ICHVQVRAIQKEKTKIKIRLKSGISELYDKVVVTSGLANIQLRHCLTCDTNIFQAPVN   549
                    *

                     ###  ## # #       ####### # # #######  #### ##### #
PAO      426 EHAASQN-LGDAGLG-LETHTYNQVYPPLLLSDSSPAANARIVTAIGQLHMARSSKV-FA   482
Rals     428 AFGPASLVLGDGALG-LGTITIRDALPPLLLSDTSSAPNARIVTALNQLHMVRSSKV-FG   485
Agro     535 HAIGNSHMTGASKLFLL-TNEKFWLQHHLPSCILTTGVAKAVYCLDYDPRDPSGKGLVLI   593
savast   353 RAVRETHLTGSSKLFIL-TRTKFWIKNKLPTTIQSDGLVRGVYCLDYQPDEPEGHGVVLL   411
Oct      550 QAVDNSHMTGSSKLFLM-TERKFWLDHILPSCVLMDGIAKAVYCLDYESQDPNGKGLVLI   608
                      *   *    *         *

                   ## ##   ##   # #####  ###############         ### ######
PAO      483 TVKTAALDQPWVPQWRGEPIKAV--VSDSGLAASYVVPSPIVEDGQAPEYSSLLASYTWE   540
Rals     486 TIETARLDEPFVPRLKGQPIKAV--VSDSGLAASYVVPSPTEPGA-P-RYSSFLASYTWG   541
Agro     594 SY-TWEDDSHKLLAVPDKRERFASLQR--D-IGRAFPDFAKHLTPADGNYDDNIVQHDWL   649
savast   412 SY-TWEDDAQKMLAMPDKKTRCQVLVD--DLAAIH-PTFASYLLPVDGDYERYVLHHDWL   467
Oct      609 SY-TWEDDSHKLLAVPDKKERLCLLR---DAISRSFPAFAQHLFPACADYDQNVIQHDWL   664
                   *   *                                        *       *

                ###### # ## ##### #  ###  # ## # ### # #       #       ### #
PAO      541 DDSTRLRHDFGLYPQNPATETGTADGMYRT-MVNRAYRYVKYAGASNA---Q--PWWFYQ   594
Rals     542 DDSTRLQHNFGYYPQNPPTSDGTATAMFRT-MINRATRNVRDPAEPAA---APAVWWFSQ   597
Agro     650 TDP---HA-GGAFKLNRRGNDVYSERLFFQPFDVMHPADDKGLYLAGCSCSFTGGWVHGA   705
savast   468 TDP---HS-AGAFKLNYPGEDVYSQRLFFQPMTANSPNKDTGLYLAGCSCSFAGGWIEGA   523
Oct      665 TDE---NA-GGAFKLNRRGEDFYSEELFFQALDTAN--D-TGVYLAGCSCSFTGGWVEGA   717
                 *        *    *                                        *

                ##   #  ########## ######## ##################   # #### ####
PAO      595 LLAEARTADRFVFDWTTNKTAGGFKLDMTGDHHQSNLCFRYHTHALAASLDNRFFIASDS   654
Rals     598 LLSRVREQDRFVFDWTTNGTAGGFKLDGTGDHHQSNLCFRYHTHALNPRLGNRFFLASDS   657
Agro     706 IQTACNATCAIIYGSGHLQELIHWRHLKEGNPLAHAWKRYRYQA                   749
savast   524 VQTALNSACAVLRSTGGQ--LS-----K-GNPLDCINASYRY                     557
Oct      718 IQTACNAVCAIIHNCGGI--LA-----K-GNPLEHSWKRYNYRTRN                 755

                ############### ###### # ## #   ###  ##  ######
PAO      655 YSHLGGWLEGAFMSALNAVAGLIVRANRGDVSALSTEARPLVIGLRPVVKVPAAELATSQ   714
Rals     658 YSHLGGWLEGAFMSAINAVAGLVVAANGGNAGALSNAAREVVTGLRPIAP----------   707
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Fig. 3. **Alignment of the protein sequences derived from the nucleotide sequence coding L-phenylalanine oxidase from *Pseudomonas* sp. P-501 (PAO) with those of related genes of a putative tryptophan 2-monooxygenase of *Ralstonia solanacearum* (Rals) (*23*), tryptophan 2-monooxygenases of *Agrobacterium rhizogenes* (Agro) (*24*), *Pseudomonas savastano I* (savast) (*25*), and *Agrobacterium tumerfaciens* (Oct) (*26*).** *, residues conserved among the 5 sequences. #, residues conserved between PAO and Rals.

relatively large peak eluting later (β subunit) were observed (*4*). The peak materials were collected and their N-terminal sequences were determined. The peak material eluting earlier showed a single sequence, GVTVI-PRLLGLKDEKKI (88.3/185.5/36.6/128.9/131.8/71.4/19.4/106.6/127.1/61.1/96.8/49.2/47.7/50/47.3/56.2/12.9), while the second peak showed one major sequence IKAIKVRGLK (40.2/41.8/43.3/22.6/27.8/29.3/5.3/22.4/26.2/18.4, accounting for approx. 65% of the total PTH–amino acids in the first cycle), and 3 minor sequences. From the recovery of the PTH amino acid at each cycle, the 3 minor sequences can be traced to fragments starting at positions 70, 135, and 184 in the β subunit. After 10 h incubation, the sequences were KIATTVGEA (322/296/329/104/79.3/203/104/145/192) and AIKVRGLK (139/164/117/146/17.8/78.5/144/141), respectively (see Scheme 1). In the above

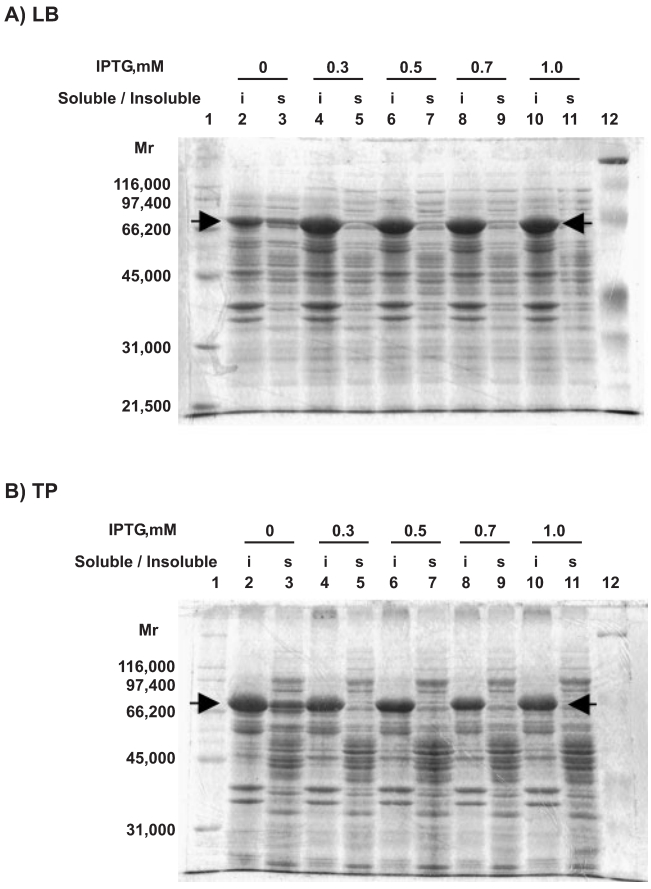


Fig. 4. **SDS-PAGE analysis of proPAO expressed in *E. coli*.** The media used were LB (A) and TP (B). At 21 h after induction with various concentrations of IPTG, cells were collected and lysed by sonication. Soluble (s) and insoluble (i) fractions were separated as described in "EXPERIMENTAL PROCEDURES" and analyzed. Molecular weight markers were Broad Range (lane 1) and Kaleidoscope (lane 12). Arrows indicate the position of proPAO.

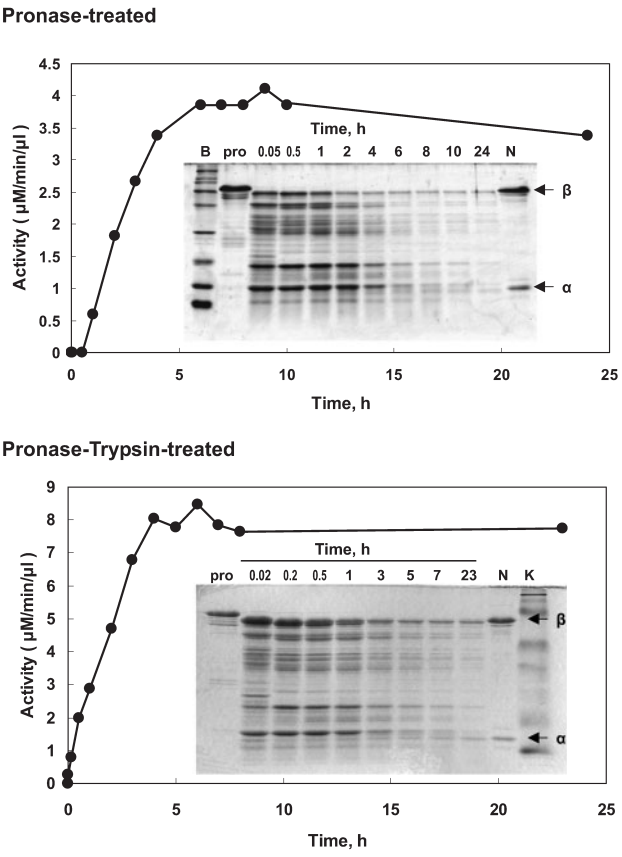


Fig. 5. **Proteolytic activation of proPAO.** A: ProPAO (0.8 mg) was treated with 14 μg Pronase in 300 μl of 20 mM potassium phosphate buffer (pH 7.0) at 25°C for the given times; 5 μl aliquots were taken to measure enzyme activity and to perform SDS-PAGE. B: ProPAO (0.7 mg) was treated with a mixture of Pronase (10 μg) and trypsin (7 μg) for the given times at 25°C in 200 μl of 20 mM potassium phosphate buffer (pH 7.0); 5 μl aliquots were taken to measure enzyme activity and to perform SDS-PAGE as described in "EXPERIMENTAL PROCEDURES." B, K, pro, and N represent Broad Range, Kaleidoscope, proPAO, and the native enzyme, respectively.

sequences, the numbers in the parentheses after each sequence are pmols of PTH amino acids recovered in each cycle of the Edman degradation starting at the N-teminal amino acid residue.

To characterize the nature of the Pronase-treated enzyme, proPAO (9.7 mg) was incubated with Pronase (136 μg) in 3.1 ml of 20 mM potassium phosphate buffer (pH 7.0) for 16 h; then, the activated enzyme was purified by column chromatography on His-Bind resin and DEAE-Sepharose columns as described for the purification of proPAO. The kinetic parameters of the activated enzyme (recPAOp hereafter) were k_{cat} 52 s^{-1} and K_m 8.2 μM. The K_m value is almost the same as that of the native enzyme, but the k_{cat} value is half the native one. Moreover, the spectrum obtained following the addition of L-Phe to recPAOp was quite different from that of the native enzyme (data not shown). Therefore, we tried to activate proPAO with various proteases, such as trypsin, Lys endoprotease, V8 protease (Lys endoprotease, then V8 protease), (Pronase and trypsin), and (Pronase and V8 protease). Among these, the most efficient activation was found following the treatment of proPAO with a mixture of Pronase and trypsin. Figure 5B shows the activation of proPAO by Pronase and trypsin by monitoring the enzyme activity and protein mobility in SDS-PAGE. To characterize the activated enzyme (recPAOpt hereafter), proPAO (9.7 mg) was incubated with 136 μg of Pronase and 97 μg of trypsin in 20 mM potassium phosphate buffer (pH 7.0) for 16 h at 25°C. The activated enzyme was purified by chromatography on His-Bind resin and DEAE-Sepharose columns as described for the purification of proPAO. The amount of recPAOpt obtained was 0.55 mg. Though the recPAOpt is His-tag free (lane 5, Fig. 6B), the recPAOpt bound to His-Bind resin pre-equilibrated with buffer A and eluted with Buffer A containing 60 mM imidazole. recPAOpt is rich in His residues (2.4%; 34 residues per molecule, $\alpha_2\beta_2$), and this may be why the protein binds to the resin.

Figure 6 shows the SDS-PAGE pattern of proPAO treated with proteases. The results in Fig. 6B clearly show that proPAO contains a His-tag, but the tag is removed by proteolytic activation (see Scheme 1). SDS-PAGE analyses of the different preparations of recPAOpt showed the molecular weight of the α subunit to be approx. 1,000 lower than that of the native protein, and

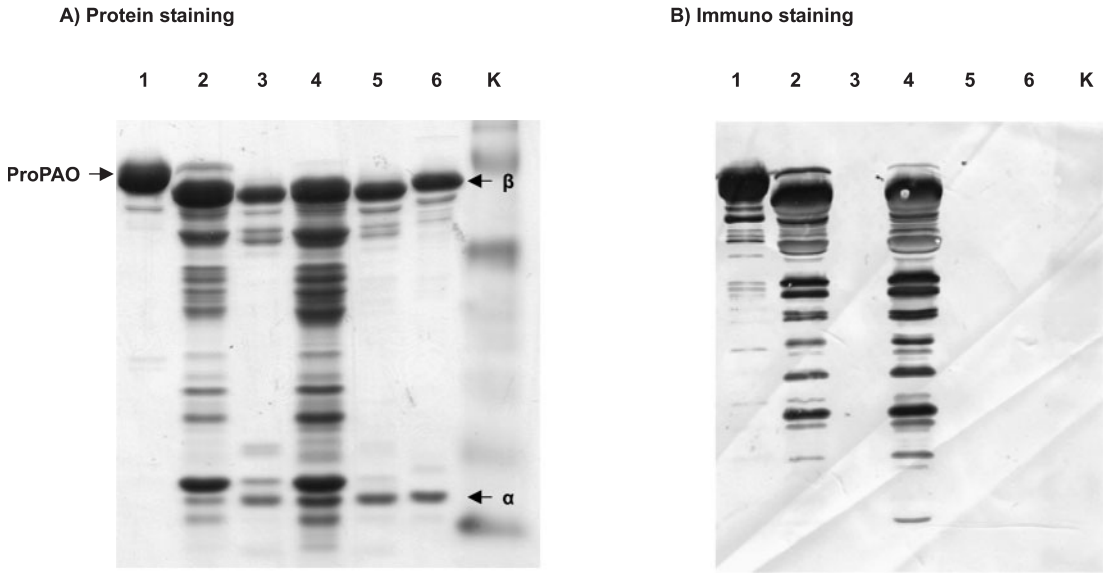


Fig. 6. **SDS/PAGE of proPAO and of the protease-treated proPAO.** A, protein staining. B, immunostaining. Lane 1, proPAO. Lane 2, Pronase treatment for 2 min. Lane 3, recPAOp. Lane 4, treatment with Pronase and trypsin for 2 min. Lane 5, recPAOpt. Lane 6, native PAO.

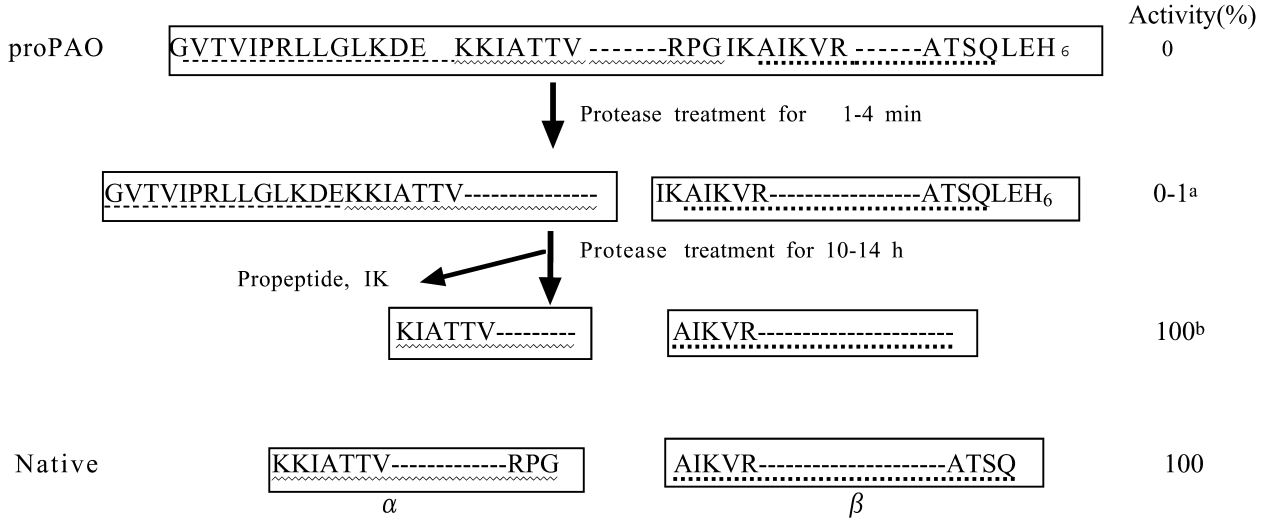
that of β to be approx. 2500 lower than the native protein. These results indicate that the α and β subunits were cut off on the C-terminal side.

Figure 7 shows the spectra of proPAO and recPAOpt in the visible region. The addition of L-Phe to proPAO changed the spectrum slightly, but the same large change was observed with recPAOpt as with the native PAO (*5*). The spectrum showing a broad peak from 500 to 600 nm is similar to that of the native enzyme (*5*).

The kinetic parameters of recPAOpt were determined at 30°C in 50 mM potassium phosphate buffer (pH 7.0) at various concentrations of L-Phe in the presence of 237 μM molecular oxygen. The apparent k_{cat} and K_m values were 103 s^{-1} and 17 μM, respectively. The apparent specificity constant, k_{cat}/K_m was calculated to be 6.0×10^6 M^{-1} s^{-1}.

DISCUSSION

L-Phe oxidase (deaminating and decarboxylating) [EC 1.13.12.9] from *Pseudomonas* sp.P-501 was isolated from soil by an enriched-culture technique with L-Phe as the sole carbon and nitrogen source to obtain an enzyme highly specific for L-Phe (*1*). Unexpectedly, the enzyme also oxidizes L-Tyr and L-Met in addition to L-Phe (*1*). However, the enzyme is unique in that it catalyzes both the oxidative deamination and oxygenative decarboxylation of L-Phe (*1*). We have been studying the enzyme kinetically and protein-chemically (*4*, *5*, *8*, *13*). To understand the structure-function relationship of the enzyme in more detail, information on the amino acid sequence is essential.



Scheme 1. **Proteolytic activation of proPAO.** Activity was assayed polarographically as described in "EXPERIMENTAL PROCEDURES," and shown as the activity relative to that of the native enzyme (104 s^{-1}). [a]Estimated from Fig. 5. [b]Activity for recPAOpt.

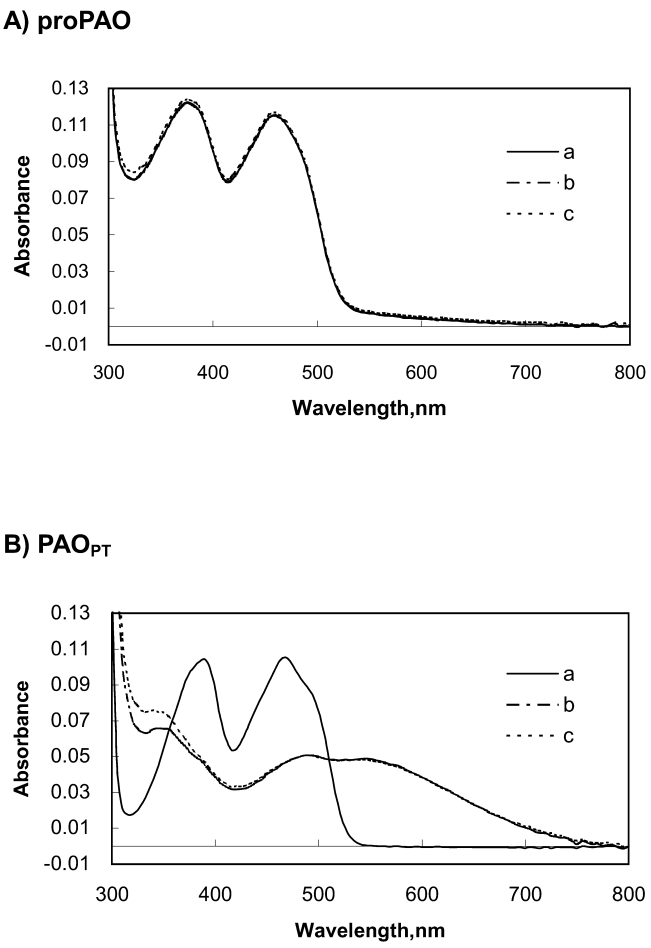


Fig. 7. **Spectral changes of proPAO (A) and recPAOpt (B) with sufficient amounts of L-Phe in 20 mM potassium phosphate (pH 8.0) under aerobic conditions.** Before addition (a), immediately (b), and 60 min (c) after the addition of L-Phe.

This work reports the deduced amino acid sequence of the nucleotide sequence of the gene encoding PAO (Fig. 2). The amino acid sequence of the ORF suggests that the enzyme is expressed as a proenzyme and processed by proteolysis to produce the mature form. Expression of the gene lacking the prosequence was successful, but the amount of soluble PAO was very low. Moreover, PAO expressed without the prosequence did not contain FAD and did not show enzyme activity before or after proteolysis. On the other hand, proPAO that was overexpressed in *E. coli* contained 1 mol of FAD per mol of proPAO and was made catalytic by proteolysis. The PAO obtained by proteolysis had k_{cat} and K_m values comparable to those of the native enzyme. These facts indicate that the prosequence is essential for the incorporation of FAD and the formation of the active conformation.

The recPAOpt prepared from the Pronase-trypsin digests of proPAO had kinetic parameters similar to those of the native enzyme. Moreover, recPAOpt showed spectral properties similar to those of the native enzyme (Fig. 7); that is, the oxidized enzyme was reduced to the intermediary form showing a characteristic broad absorption band around 500–600 nm (*5*). Although recPAOpt showed kinetic and spectral properties similar to those of the native enzyme, the electrophoretic mobilities of the recPAOpt subunits were slightly larger than those of the native protein (Fig. 6). The N-terminal sequence of the β subunit was identical to that of the native subunit, and one amino acid residue of the α subunit was removed from the N-terminus. These facts indicate that the C-terminal sequence must be partially cleaved by the Pronase-trypsin treatment of proPAO.

It is worthwhile to compare the staining patterns of SDS-PAGE gel with the increase in enzyme activity after the addition of proteases (Fig. 5, Scheme 1). In the early stages of incubation, the link between the α subunit and the dipeptide before β subunit is cleaved, but the activity of the enzyme is very low. With longer incubation, the activity of the incubation mixture increases and becomes almost constant after 5 h incubation, but the protein-staining gradually weakens, except for the appearance of a weak band near the native α subunit. These facts indicate that only part of the proPAO has a conformation that is activated by proteolysis, although proPAO is overexpressed.

The proteolytic activation of enzymes, such as zymogen activation, is widely known (*27*). As for oxidases, the activation of prophenol oxidase (proPO) to phenol oxidase (PO) by limited proteolysis is well documented (see Refs. *28–32* for reviews). PO, the enzyme responsible for the biosynthesis of melanin, is considered to be an important component of the insect immune response. Infection in insects stimulates a complex defense response that is often accompanied by proteolytic activation of the proPO that is present in the hemolymph. A specific protease designated as the proPO-activating enzyme (PAE) activates proPO. PO and PAE constitute the so-called "prophenol oxidase-activating system." Koyama (*1*) prepared PAO by autolysis of *Pseudomonas* sp. P-501. This means that these cells contain the protease to convert proPAO to PAO. However, it is not known at present whether *Pseudomanas* sp. P-501 contains the specific protease to activate proPAO as reported for the activation of prophenol oxidase.

The nucleotide sequence reported in this paper has been submitted to the DDBJ with accession number AB167410. We would like to express our thanks to Dr. H. Koyama for the kind gifts of *Pseudomonas* sp. P-501 and the native PAO, and to Mr. A. Ishizaki for technical assistance.

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