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Purification and partial characterisation of a broad-range L-amino acid oxidase from *Bacillus carotarum* 2Pfa isolated from soil

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Abstract The L-amino acid oxidase (L-aao) from *Bacillus carotarum* 2Pfa was purified to homogeneity, as judged by polyacrylamide gel electrophoresis, from crude sonicated cell extract by a combination of anion exchange chromatography and gel filtration. The purified enzyme was a dimer with a native relative molecular mass of approximately 102,000 to 115,000 and comprised two identical subunits of 54,000. The isoelectric point of the L-aao was at pH 4.8, the pH optimum was at 8.0–8.5 and the temperature optimum was at approximately 50° C. It was stable for several months at +4° C and at –20° C. The enzyme contained 2 mol flavin adenine dinucleotide (FAD)/mol enzyme and exhibited relatively broad range substrate specificity, oxidising a total of ten L-amino acids and, albeit to a much lesser extent, seven D-amino acids. Kinetic studies revealed that the three aromatic L-amino acids were the preferred substrates.

Introduction

L-Amino acid oxidases [L-amino acid:oxygen oxidoreductase (deaminating), EC 1.4.3.2; L-AAO] catalyse the oxidative deamination of L-amino acids to their corresponding oxo acids and have been reported from a variety of sources. Several exhibit broad substrate specificity such as from the red algae, *Amphiora crassissima* (Ito et al. 1987), the cyanobacteria, *Anacystis nidulans* (Pistorius and Voss 1980), the Gram negative bacteria, *Morganella morganii* (Gamati and Luong 1991), *Proteus mirabilis* (Laboure et al. 1979; Pelmont et al. 1972), *P. rettgeri* (Duerre and Chakrabarty 1975), *P. vulgaris* (Stumpf and Green 1944) and *Providencia* sp. PCM1298 (Szwajcer et al. 1982), the Gram positive bacteria, *Cellulomonas cellulans* (Braun et al. 1992) and *Corynebacterium* (Coudert and Vandercastede 1975) and the venom from two poisonous snakes, *Crotalus adamanteus* (Wellner and Meister 1960) and *Agkistrodon piscivorus piscivorus* (Singer and Kearney 1950). Others are relatively specific, such as L-cysteine oxidase from *Neisseria meningitidis* (Yu and de Voe1981), L-glutamate oxidase from *Streptomyces endus* (Bohmer et al. 1989), L-lysine oxidase from *Trichoderma viride* (Kusakabe et al. 1980) and L-phenylalanine oxidase from *Pseudomonas* sp. P-501 (Koyama 1982), although the latter two do catalyse the oxidation of other L-amino acids at low levels relative to their primary substrates.

We recently isolated a strain of the Gram-positive bacterium, *Bacillus carotarum* from soil (Brearley et al. 1994) using a chemically defined medium with L-phenylalanine supplied as the primary source of carbon and energy. The first step in the breakdown of phenylalanine was catabolised by an inducible, intracellular oxidase and the purification and partial characterisation of that enzyme are described here.

Materials and methods

Chemicals and reagents

Bicinchoninic acid (BCA) protein reagent was purchased from Pierce (Ill., USA), 3,5,-dichloro-2-hydroxybenzenesulphonic acid (DCHBS) was from BDH (Merck, Lutterworth, UK), relative molecular mass (M_r) and isoelectric point (pI) protein standards for use with the Phastsystem were obtained from Pharmacia LKB (Milton Keynes, UK). Gel filtration native M_r marker proteins, horseradish peroxidase (type II), 4-aminoantipyrine, bovine serum albumin (BSA), hydrogen peroxide (H_2O_2), blue dextran, phenylmethylsulphonyl fluoride (PMSF) and all other chemicals and reagents were from Sigma, (Poole, UK).

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Equipment

The Phast system, fast-protein liquid chromatography (FPLC) system and Mono-Q HR 16/10 column were from Pharmacia LKB. Ultragel AcA-34 and the 30 mm × 800 mm glass chromatography column were obtained from LKB. Millex filter units were purchased from Millipore (Harrow, UK). The stirred cell concentrator and PM10 ultrafiltration membranes were from Amicon (Stonehouse, UK). The densitometer was a Chromoscan 3 (Joyce-Loebl, Gateshead, UK). The Clark-type O_2 electrode was obtained from Rank Brothers (Cambridge) and the Hitachi F-2000 fluorescence spectrophotometer was purchased from Nissei Sangyo (Wokingham, UK).

Micro-organisms and cultivation

The bacterium, *B. carotarum* 2Pfa was grown in an LH 2000 series fermentor in a chemically defined medium containing L-phenylalanine as the primary source of carbon and energy as previously described (Brearley et al. 1994). Bacterial cells were harvested at the end of the exponential phase of growth by centrifugation and were washed once with chilled 100 mM potassium phosphate buffer, pH 7.0. Cells were resuspended in the same buffer at a ratio of 1 g (wet weight) of cells per 2 ml buffer prior to being disrupted. The cells were sonicated using an MSE Soniprep 150 with six cycles of 30 s sonication punctuated by cooling on ice. The cell debris was removed by centrifugation and the supernatant was used for enzyme purification.

Enzyme assay

Phenylalanine oxidase activity was measured by one of two assays. (1) A modification of a spectrophotometric method (Trinder and Webster 1984), based on the measurement of H_2O_2 was routinely used . The dye produced by peroxidase from H_2O_2, 4-aminoantipyrine and DCHBS was detected at 510 nm [$E = 28{,}000\ M^{-1}cm^{-1}$ (see below)]. One unit (U) is defined as the amount of enzyme that catalyses the formation of 1 μmol of H_2O_2 at 30° C. Unless otherwise stated, the standard assay mixture in a 1 ml cuvette contained 100 mM potassium phosphate buffer pH 7.0, 250 μM L-phenylalanine, 250 μM 4-aminoantipyrine, 5 units of peroxidase and 625 μM DCHBS, equilibrated at 30° C. The reaction was initiated by the addition of 50 μl of enzyme solution.

(2) A polarographic assay method was used to measure L-aao activity during some of the characterisation studies and used a Clark-type O_2 electrode. The instrument was calibrated with fully aerated demineralised water and dithionite-reduced (O_2–free) water at 30° C. The assay mixture contained 100 mM TRIS-HCl buffer, pH 8.0, +5 mM L-phenylalanine, equilibrated to 30° C. The reaction was initiated by the addition of 50 μl enzyme solution and the initial velocity of the O_2 consumption was measured. One unit of enzyme is defined as the amount required to consume 1 μM of O_2/min at 30° C.

Extinction coefficient

The molar extinction coefficient of DCHBS was determined under the conditions of the spectrophotometric assay by preparing a 1 mM solution of the chromogen in 100 mM potassium phosphate buffer, pH 7.0, and oxidation to the azino dye using H_2O_2 solution. The oxidised dye was scanned from 350 to 750 nm to find the absorbance maximum and the molar extinction coefficient was determined from the relative absorbances of the reduced and oxidised forms of the dye at that wavelength using the following calculation:

$$\text{Molar extinction coefficient} = \frac{\text{Absorbance (oxidised)} - \text{Absorbance (reduced)}}{\text{Concentration of dye (M) x pathlength}}$$

Protein determination

Protein concentrations were measured by a BCA method (Smith et al. 1985) using commercially available reagents with BSA as the standard.

Purification by anion exchange chromatography

Anion exchange chromatography was performed using a Mono-Q HR 16/10 column connected to an FPLC apparatus. A total of 46 ml of filtered (Millex 0.22 μM) crude enzyme (sonicated cell extract) was loaded at a flow rate of 6 ml/min via a 50-ml superloop to the column, equilibrated with 20 mM TRIS-HCl pH 7.5, +0.1 mM PMSF. The column was eluted with a 0 to 500 mM sodium chloride gradient (340 ml) added to the equilibration buffer and fractions containing the enzyme were collected, pooled and concentrated using an Amicon 8050 stirred cell fitted with a PM10 membrane.

Purification by gel filtration

The concentrated, partially purified Mono-Q column eluate was loaded to a 565 ml (30 mm × 800 mm) Ultragel AcA-34 column equilibrated with 20 mM TRIS-HCl, pH 7.5, +0.1 mM PMSF + 100 mM NaCl and run at a linear flow rate of 4.95 cm/h (35 ml/min). Fractions containing the enzyme were pooled and concentrated using the Amicon 8050 stirred cell concentrator fitted with a PM10 membrane. The FPLC anion exchange column was run at room temperature and all other operations were performed at +4° C.

Gel electrophoresis

Enzyme purity, native and sub-unit M_r and pI were all determined using the Phast system as described in the manufacturer's instructions manual (Pharmacia). Gels were stained with either Coomassie blue or a modified version of the sensitive silver-staining method (Heukeshoven and Dernick 1985) and scanned using a Joyce-Loebl Chromoscan 3 densitometer. For native M_r determination, native polyacrylamide gel electrophoresis (PAGE; 8–25% polyacrylamide gel) used Pharmacia high M_r electrophoresis calibration proteins [thyroglobulin (669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000) and BSA (67,000)]. For sub-unit relative M_r determination, sodium dodecyl sulphate (SDS)-PAGE (10–15% polyacrylamide gel) used Pharmacia low M_r electrophoresis calibration proteins [phosphorylase b (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20, 100) and lactalbumin (14, 400)]. The pI was measured by isoelectric focusing (IEF) in a broad pH range (IEF Phastgel pH 3–9) using the following protein standards: amyloglucosidase (3.50), soybean trypsin inhibitor (4.55), β-lactoglobulin A (5.20), bovine carbonic anhydrase B (5.85), human carbonic anhydrase (6.55), horse myoglobin (6.85), horse myoglobin (7.35), lentil lectin (8.15), lentil lectin (8.45), lentil lectin (8.65). For a narrow pH range (IEF Phastgel pH 4.0–6.5), glucose oxidase (4.15), soybean trypsin inhibitor (4.55), β-lactoglobulin (5.20), bovine carbonic anhydrase (5.85), human carbonic anhydrase (6.55) were used.

Gel filtration

The native M_r of the L-aao was measured using the FPLC system with a Superose 12 HR 10/30 column equilibrated with 100 mM TRIS-HCl, pH 7.5 + 0.1 mM PMSF + 0.1 M NaCl and run at 0.5 ml/min. The void volume was determined using blue dextran at 2 mg/ml and the column was calibrated with the following: β-amylase (200,000), alcohol dehydrogenase (150,000), albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000) and cytochrome C (12,400).

Prosthetic group

A 1-ml sample of the enzyme (61 μg/ml) was boiled for 5 min and the denatured protein was removed by centrifugation. The supernatant was scanned using a Perkin-Elmer 552S UV/visual spectrophotomer and the trace compared to the scans of authentic flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) dissolved in 0.1 M potassium phosphate buffer, pH 7.0. The cofactor was identified by differential fluorescence spectrophotometry, ie. the fluorescence of FAD is only about 15% that of FMN at pH 6.8 [excitation 450 nm, emission 535 nm (Keesey 1987)]. Solutions of the enzyme extract and the authentic FAD and FMN were diluted to the same concentration, determined by their respective absorbances at 450 nm and then adjusted to pH 6.8. The fluorescence of each was then measured using a Hitachi F-2000 fluorescence spectrophotometer. The prosthetic group (identified as FAD) content was determined as follows: the absorbance at 450 nm of the enzyme extract in 0.1 M potassium phosphate, pH 7.0, was measured and the FAD concentration quantified based on a molar extinction coefficient of 11300 $M^{-1}cm^{-1}$ for FAD. The concentration of enzyme was calculated based on a M_r of 108,000 (see results).

Kinetic data

Kinetic data were analysed using a non-linear regression programme (Enzfitter, Biosoft, UK). Only data from the concentration range below substrate inhibition were used.

Results

Enzyme purification

Anion exchange chromatography with the Mono-Q HR 16/10 column eluted with a 0 to 500 mM NaCl gradient resulted in a 14-fold purification over crude enzyme extract and a recovery of 73% of the total activity in the pooled fractions (Fig. 1a). The partially purified material was concentrated using a stirred cell fitted with a PM10 ultrafiltration membrane, which achieved a further purification to 21-fold over the crude extract and a recovery of 65% total activity. This material was then applied to an Ultragel AcA-34 gel filtration column (Fig. 1b) and resulted in a 67-fold purification over the

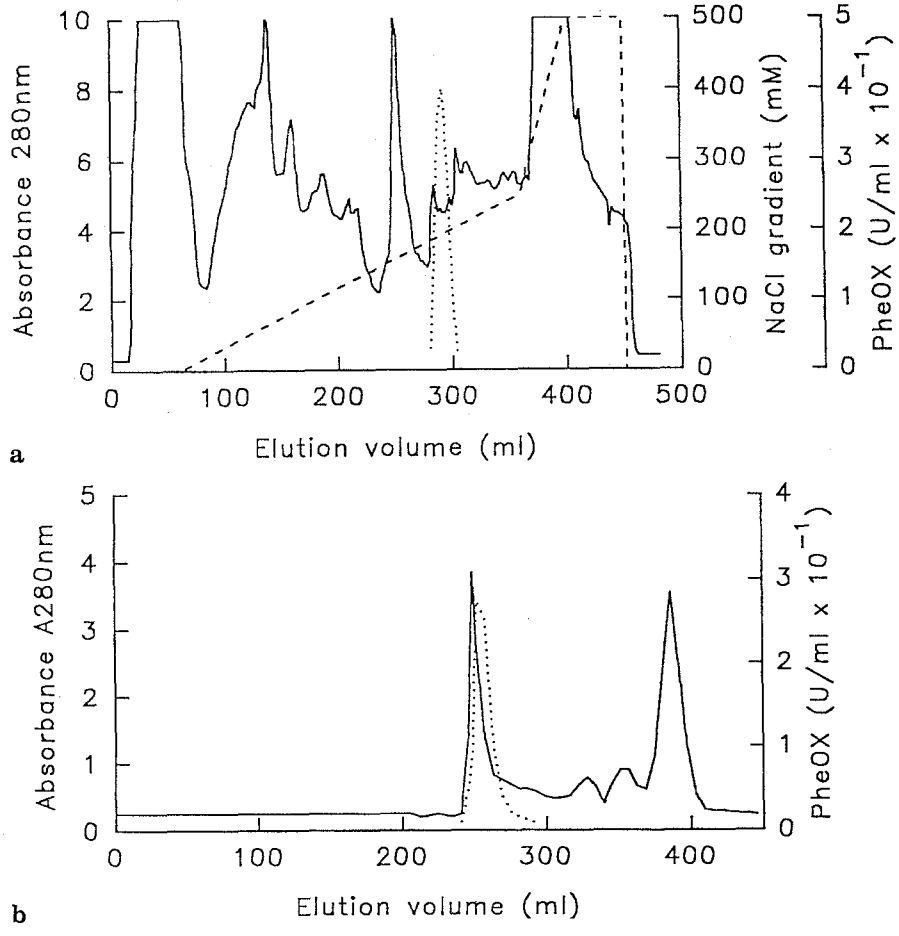

Fig. 1 **a** Purification of L-amino acid oxidase (L-aao) by Mono-Q HR 16/10 anion exchange chromatography on the fast-protein liquid chromatography (FPLC) system: 46 ml of crude extract containing 0.11 units (U)/ml L-aao (total 5.2 U) was applied to the column at a flow rate of 6 ml/min. Fractions containing L-aao activity were pooled and concentrated to 12 ml containing 0.28 U/ml (total 3.4 U) and **b** applied to a 565 ml (30 mm × 800 mm) Ultragel AcA-34 gel filtration column and run at 35 ml/min: — protein (absorbance at 280 nm *A280 nm*), - - - NaCl gradient, ... L-aao (U/ml × 10^{-1})

Table 1 Purification of L-amino acid oxidase from *Bacillus carotarum* 2Pfa (*U* units)

Step	Total enzyme (U)	Total protein (mg)	Specific activity (U/mg)	Purification (-fold)	Recovery (%)
Crude extract	5.2	138.0	0.04	1	100
Mono-Q	3.8	7.1	0.54	14	73
Concentration 1	3.4	4.0	0.85	21	65
Ultragel AcA-34	3.2	1.2	2.67	67	62
Concentration 2	2.9	0.65	4.46	112	56

crude extract and a recovery of 62% of total activity. The active fractions were pooled and a second concentration step gave a final overall 112-fold purification, with a total recovery of 56% and a final specific activity of 4.46 U/mg of protein. A summary of the purification data is presented in Table 1. The final material appeared as a single band on an overloaded Coomassie-stained SDS-Phast gel, indicating that the enzyme was homogeneous and suitable for characterisation studies.

Relative molecular mass

The native M_r was determined to be approximately 102,000 by the gel filtration method and approximately 115,000 by native PAGE. The SDS-Phast gel showed that the enzyme consisted of identical sub-units, each with an M_r of approximately 54,000.

Isoelectric point

A broad pH range (3–9) IEF gel indicated that the pI of the enzyme was in the region of 4.1 and a narrow range (4.0–6.5) IEF gel indicated a pI of 4.8.

Effects of pH and temperature

The pH dependence of the L-aao with L-phenylalanine as the substrate was determined polarographically in various buffers. The enzyme showed broad activity between pH 4.5 and 10.5, with optimal activity around pH 8.0–8.5. Enzyme activity was not significantly influenced by the type of buffer used. L-aao activity was assayed at various temperatures (Fig. 2), again by the polarographic method although this required a correction to be made because of the variation in dissolved O_2 concentration with temperature. Activity increased with rising temperature up to 50° C and then fell rapidly. An Arrhenius plot produced a straight line and the activation energy was calculated to be 70 kJ/mol.

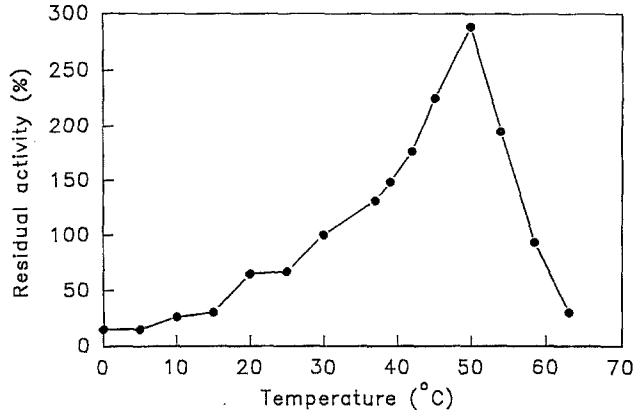

Fig. 2 Effect of temperature on the activity of L-aao with L-phenylalanine as substrate, measured by the polarographic method (see text)

Enzyme stability

The stability of the purified L-aao was measured over a range of temperatures and the half life was calculated to be 4 min at 37° C, 37 min at 30° C and 60 min at 22° C. The enzyme could be stored at +4° C and -20° C for at least 4 months without loss of activity, even at relatively low protein concentrations (<0.1 mg/ml).

Prosthetic group

Protein-free enzyme extract was scanned against authentic FAD and FMN (Fig. 3). The enzyme-factor exhibited similar characteristics to both flavins, and was identified as FAD by differential fluorescent spectrophotometry. The ratio of FAD to enzyme was calculated to be 1.8:1, indicating that the enzyme contained 2 mol FAD/mol enzyme protein.

Substrate specificity

An extensive range of L- and D- amino acids were tested as substrates for the enzyme (Table 2). Ten of the L-amino acids produced significant reaction rates, ranging from 12% (L-tyrosine) to 169% (L-leucine) compared to L-phenylalanine, whilst seven of the D-amino acids resulted in much lower rates, with D-lysine giving the highest, at 3.4% of the activity measured with L-phenylalanine.

Kinetic data

The ten L-amino acids that gave significant reaction rates when presented as enzyme substrates were studied in respect of their kinetic properties and the data obtained are presented in Table 3.

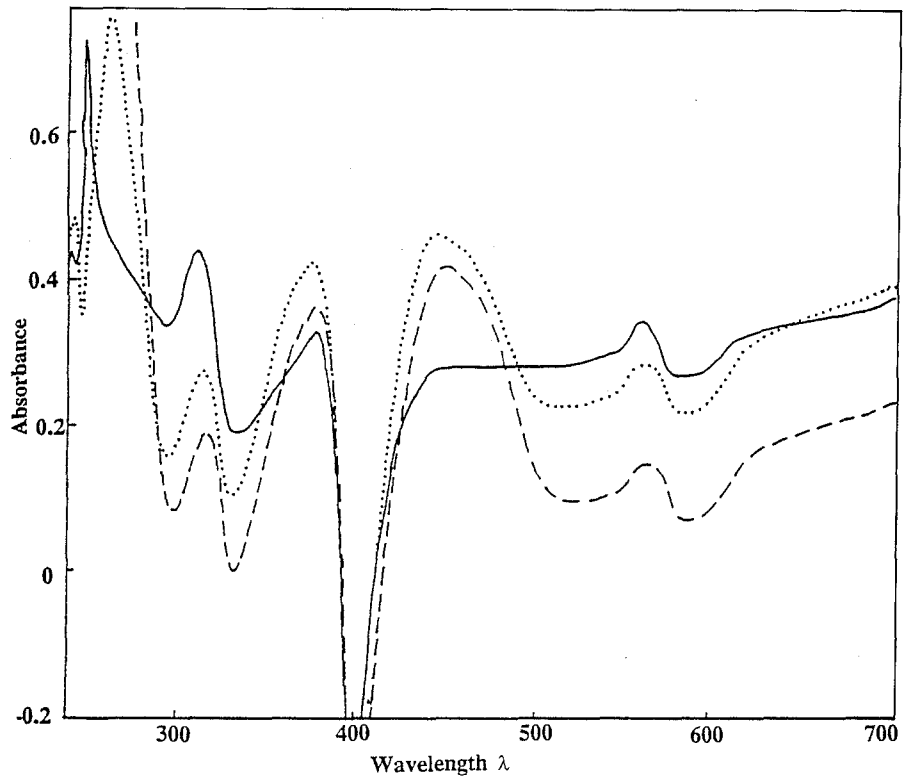

Fig. 3 Absorption spectra of flavin adenine dinucleotide (FAD) (- - - -), flavin mononucleotide (FMN) (· · · · ·) and the L-aao prosthetic group (—). Details of sample preparation are given in the text

Table 2 Substrate specificity of *B. carotarum* 2Pfa amino acid oxidase. The rate of hydrogen peroxide formation was measured by the spectrophotometric method (see text). The assay mixture contained 100 mM potassium phosphate buffer, pH 7.0, and 125 μM of the test amino acid

Substrate	Initial velocity (μmol/min/ml)	Substrate	Initial velocity (μmol/min/ml)
L-Phenylalanine	11.25	D-Phenylalanine	0.28
L-Alanine	0	D-Alanine	0
L-Arginine	14.10	D-Arginine	0.12
L-Aspartic acid	0	D-Aspartic acid	0
L-Asparagine	4.60	D-Asparagine	0
L-Cystine	0	D-Cysteine	0
L-Glutamic acid	0	D-Glutamic acid	0
L-Glutamine	3.35	D-Glutamine	0
Glycine	0		
L-Histidine	7.15	D-Histidine	0.06
L-Hydroxyproline	0		
L-Isoleucine	0	D-Isoleucine	0
L-Leucine	19.0	D-Leucine	0.16
L-Lysine	14.25	D-Lysine	0.38
L-Methionine	13.50	D-Methionine	0
		D-Norleucine	0.12
L-Proline	0	D-Proline	0
L-Serine	0	D-Serine	0.08
L-Threonine	0	D-Threonine	0
L-Tryptophan	9.75	D-Tryptophan	0
L-Tyrosine	1.35	D-Tyrosine	0
L-Valine	0	D-Valine	0

Table 3 Kinetic data obtained for *B. carotarum* 2Pfa L-amino acid oxidase with a variety of L-amino acids. Reaction velocities were measured using the spectrophotometric assay (see text) (K_m Michaelis constant, k_{cat} catalytic constant)

Substrate	K_m (μM)	k_{cat} ($s^{-1} \times 10^3$)	k_{cat}/K_m ($s^{-1} M^{-1} \times 10^6$)
L-Arginine	37.0	12.00	324
L-Asparagine	259.0	10.30	40
L-Glutamine	340.0	9.14	27
L-Histidine	67.0	7.68	115
L-Leucine	24.6	15.40	626
L-Lysine	42.8	13.70	320
L-Methionine	31.0	11.40	368
L-Phenylalanine	4.4	8.56	1946
L-Tryptophan	5.0	6.82	1364
L-Tyrosine	5.9	9.06	1536

Discussion

The purification of L-aao was relatively simple with an anion exchange separation followed by a gel filtration step. Pooled fractions from each column step were concentrated by ultrafiltration and in both cases a significant improvement in the specific activity of the material was achieved with only relatively small losses of the enzyme. A possible explanation for this would be the removal of small peptides (M_r <10,000) through the membrane, although the lack of significant amounts of protein in the ultrafiltrate would suggest that a more likely explanation is the adsorption of contaminant protein(s) to the membrane. The specific activity of the pu-

rified enzyme (L-phenylalanine oxidase activity) is approximately 4.5 U/mg protein and the enzyme constitutes about 0.5% of the total soluble cell protein when fully induced.

The native M_r was found to be 102,000–115,000 dependent upon the method and the subunit M_r was approximately 54,000, indicating that the enzyme is probably a dimer consisting of two identical sub-units. The subunits of several L-aaos have similar M_rs, for example *T. viride*, 56,000 (Kusakabe et al. 1980), *Anacystis nidulans*, 49,000 (Pistorius and Voss 1980), *S. endus*, 50,000 (Bohmer et. al. 1989) and *Cellulomonas cellulans*, 55,000 (Braun et al. 1992). Other aaos have differing subunit M_rs, such as the L-aao from *Pseudomonas* sp., 68,000 (Koyama 1983) and the D-aao from the yeast, *Trigonopsis variabilis*, 43,000 (Szwajcer and Mosbach 1985) although the majority[1] of these aaos are reported to be dimeric and composed of identical subunits.

Throughout all of the purification work and most of the characterisation study, L-aao activity was measured using the standard spectrophotometric assay. For characterisation experiments where the parameter under study was likely to have an effect on the peroxidase used in the standard assay (e.g., pH, temperature, inhibitors) a polarographic assay method was used. A spectrophotometric study of the enzyme showed that the enzyme was a flavoprotein and contained 2 mol FAD/mol enzyme, as is the case with several L-aaos (Bohmer et al. 1989; Keesey 1987, Kusakabe et al. 1980; Wellner and Meister 1960) and the D-aao from *T. variabilis* (Szwajcer-Dey et al. 1990). The L-aao from *A. nidulans* is also a flavoprotein but contains only 1 mol FAD/mol enzyme (Pistorius and Voss 1980).

The oxidase enzyme was found to exhibit a broad range of substrate specificity, accepting ten of the L-amino acids and seven of the D-amino acids as substrates. The reaction rates with the D-amino acids were relatively low compared to any of the L-amino acids and on this basis the enzyme was categorised as an L-amino acid oxidase. The bacterium was originally isolated from soil using an enrichment culture technique with L-phenylalanine as the primary carbon and energy source and had been selected for further study because of its inability to assimilate L-tyrosine when substituted for phenylalanine in the same growth medium. It had been hoped that the enzyme might be highly specific for phenylalanine with only negligible tyrosine activity. However, the enzyme's ability to utilise tyrosine as a substrate would suggest that the organism's lack of growth on the tyrosine medium must be due to some factor other than a highly specific phenylalanine oxidase, such as a block further down the pathway of tyrosine catabolism, or an inability to take up tyrosine into the cells.

[1] The native M_r for the L-aao from *C. cellulans* was not determined (Braun et al. 1992)

Most of the broad range L-aaos list phenylalanine and tyrosine amongst their substrates and indeed, most of the reported phenylalanine oxidases do in fact exhibit a broad range of activity with several amino acids (Gamati and Luong 1991; Koyama 1982, 1984; Laboure et al. 1979; Pelmont et al. 1972). The kinetic data for the ten L-amino acids that were accepted as enzyme substrates indicate that the aromatic amino acids (phenylalanine, tyrosine and tryptophan) exhibit a higher enzyme affinity, indicated by the lower Michaelis constant (K_m) values than the other amino acids. Indeed, the catalytic constant (k_{cat}/K_m) values for the aromatics, a measure of the enzyme's efficiency and a direct indication of the suitability of a given substrate, are at least an order of magnitude higher than the other substrates and hence the enzyme could be referred to as an aromatic L-aao.

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