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Purification and characterization of an L-amino acid deaminase used to prepare unnatural amino acids

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Abstract

L-Amino acid deaminase (L-AAD) from *Proteus myxofaciens* was cloned and over-expressed in *Escherichia coli* K12. This enzyme has a broad substrate specificity, working on both natural and unnatural L-amino acids. Of the 20 naturally occurring L-amino acids, L-AAD prefers amino acid substrates that have aliphatic, aromatic or sulfur-containing side chains; those with charged side chains ($-CO_2^-$ or $-NH_3^+$) are poor or non-substrates. Enzyme activity was monitored using a microtiter-plate-based assay, which measures the formation of phenylpyruvic acid from L-phenylalanine. The reaction has an absolute requirement for O_2, releases NH_3 and does not produce H_2O_2. Substrate comparisons were carried out by using an O_2 electrode to measure the O_2 utilization rates. Studies on partially purified enzyme show a pH optimum of 7.5 with a subunit molecular weight of approximately 51 kDa. Additional purification and characterization strategies will be presented. The use of whole cells containing L-AAD will be discussed to prepare chiral pharmaceutical intermediates.

Keywords: Biotransformation; Unnatural amino acid; Amino acid deaminase; *Proteus*

1. Introduction

The use of aminotransferases (ATs) [EC 2.6.1.x] [1–3] or amino acid dehydrogenases (dHs) [E.C. 1.4.1.x] [4,5] to prepare chiral pharmaceutical intermediates has been well documented. In the case of ATs, the unfavorable equilibrium has been overcome by coupling them with other enzymes or enzyme systems in whole cells for biocatalytic production of the desired amino acid [6,7]. With dHs, also known as reductive deaminases, the need for a cofactor recycling system is necessary due to their dependence on either NADH or NADPH to supply the reducing equivalents. Both ATs and dHs require α-keto acids as substrates and therefore, for commercial purposes, a low-cost supply of these starting materials is needed.

It has been known for quite some time that α-keto acids can be prepared by the action of amino acid oxidases [E.C. 1.4.3.x], which are also called amino acid deaminases. With the readily available supply of low-cost L-amino acids, L-amino acid oxidase would be the enzyme of choice to produce the needed α-keto acids [8]. L-Amino acid oxidases have been identified in many bacterial sources; those most stud-

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ied belong to the *Proteus* and *Providencia* genera [9]. In addition, the enzyme found in snake venom is commercially available, but too expensive for large-scale use. Since the bacterial sources are more easily genetically manipulated, we focused on them as our enzyme source.

We have developed a whole-cell *Escherichia coli*-based biotransformation to convert L-amino acids into D-amino acids [10,11]. As shown in Scheme 1, an L-amino acid is first converted to its respective α-keto acid by the action of L-amino acid deaminase (L-AAD) cloned from *Proteus myxofaciens*. This keto acid is subsequently converted into the D-isomer by the action of D-amino acid transaminase (DAT) cloned from *Bacillus sphaericus* along with a cloned alanine racemase (AR) from *Salmonella typhimurium* used to recycle the amino donor, D-alanine. To gain further understanding as regards to the versatility of this enzyme system, we present data on the L-AAD enzyme, which is critical for this biocatalytic process.

2. Materials and methods

2.1. General

Restriction enzymes were from New England Biolabs (Beverly, MA). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from Boehringer Mannheim (Germany). Cetyltrimethylammonium bromide (CTAB) was from Aldrich (Milwaukee, WI). Molecular weight protein standards and prestained protein standards, low range, were obtained from BioRad Laboratories (Richmond, CA). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated and were of the highest quality available.

2.2. Bacterial strains and plasmids

The source of the *aad* gene was *P. myxofaciens* ATCC 19692. Plasmid pPT381 (temperature inducible; λP_R *aad* 6xHis) was constructed using the primers shown in Table 1. The host for expression of this construct was *E. coli* XL1-Blue [12]. The strain carrying the plasmid pPT381 was designated NS3365.

2.3. Media

Strains were routinely grown in Lennox L-broth or L-agar (1.5% agar) solid medium from Gibco BRL (Rockville, MD) supplemented with chloramphenicol (10 μg/ml). Induction medium contained 0.5% glucose and 2.0% casamino acids.

2.4. Gene isolation and manipulation

The amino acid deaminase gene from *P. myxofaciens* was isolated by whole cell PCR using primers shown in Table 1 designed to the published *P. mirabilis* DNA sequence [13]. Ligations were carried out using a Takara Biochemicals DNA ligation kit from Panvera (Madison, WI). PCR was carried out using standard conditions in a Perkin Elmer 9600 Thermal Cycler with *Taq* or Ultma DNA polymerase from Perkin Elmer (Norwalk, CT). Oligonucleotides were prepared using an Applied Biosystems 300 B DNA synthesizer.

Table 1
Primers used to construct plamid pPT381 (*aad* 6xHis)

Primer	Sequence
5′ *aad* **MB2171**	5′-TTT GGA TCC AAA ATG AAC ATC TCT CGT CGT AAA CTG CTG TTA GGT GTT GGT GCT GCG GGC GT-3′
	*Bam*HI
3′ *aad* 6xHis **MB2280**	5′-AGC TTT GTC GAC GGG CCC TTA GTG ATG GTG ATG GTG ATG CTT CTT AAA ACG ATC CAA AC-3′
	*Sal*I

Dashed box designates the *aad* gene and the solid box designates the 6xHis insert at the C-terminus.

2.5. Enzyme assays

2.5.1. Phenylpyruvate (PPA) determination

Enzyme was incubated at 37°C with 25.0 mM L-phenylalanine (L-Phe), 50.0 mM potassium phosphate buffer, pH 7.0, in a final volume of 0.1 ml in a microtiter plate. The reaction was stopped after 3 min by the addition of 3.0 N NaOH (0.05 ml). The absorbance at 320 nm was measured using a SpectraMAX plate reader from Molecular Devices (Sunnyvale, CA) and quantitated from a standard curve with phenylpyruvic acid. One unit of enzyme activity is defined as 1 μmol PPA produced/min at 37°C.

2.5.2. Oxygen consumption

Oxygen consumption was measured as described by Pelmont et al. [14] using a Clark-type O_2 electrode in an Oxygraph system from Hansatech Instruments (Norfolk, England), which was zeroed with dithionite. Amino acid substrates (10.0 mM) were incubated in 50.0 mM potassium phosphate buffer, pH 7.5, at 30°C for 2 min in a total volume of 990 μl prior to adding enzyme. Reactions were initiated by adding 10 μl enzyme (104 μg protein) and the O_2 consumption measured for an additional 3 min. L-Phe was used as the reference substrate and the linear rate was determined and set to 100%. All other amino acids were compared to L-Phe after the buffer-only blank rate was subtracted.

2.5.3. Kinetic parameter determination

The K_m for L-AAD was determined using the O_2 consumption assay. The substrate, L-Phe, was incubated at varying concentrations in 50.0 mM phosphate buffer, pH 7.5, at 30°C and the reaction initiated by the addition of 10 μl (10.4 mg/ml) crude lysate. Rates were measured as described above and analyzed using the double-reciprocal method of Lineweaver and Burk [15].

2.6. Peroxide detection

Peroxide was determined by a modification of the method of Duley and Holmes [16] where sodium azide was added to inhibit the catalase often present in crude lysates. The reaction mixture contained the following in a final volume of 1.0 ml: 50.0 mM Tris–HCl (pH 8.0), 7.5 mM L-Phe, 0.5 mM sodium azide, 100 μg/ml *o*-dianisidine, 2 IU horseradish peroxidase (HRP) and 10.4 μg/ml crude lysate. The reaction was initiated with HRP and incubated at 30°C for up to 20 min. A Beckman DU640 spectrophotometer (Fullerton, CA) was used to monitor the reaction at OD_{440}.

2.7. pH optimum determination

The pH optimum was determined by measuring the enzyme activity based on O_2 consumption at various pH values using L-Phe as substrate as described above. The following buffers used to maintain the pH were: acetate (4.0, 4.5, 5.0, 5.5); 2-(*N*-morpholino)ethanesulfonate ([MES]; 6.0, 6.5); phosphate (6.5, 7.0, 7.5, 8.0); tris(hydroxymethyl)methylamino propanesulfonate ([TAPS]; 8.0, 8.5, 9.0); borate (9.0, 9.5); and 3-(cyclohexylamino)-1-propanesulfonate ([CAPS]; 10.0).

2.8. Protein analysis

Proteins were analyzed by 4–15% Tris-glycine SDS-PAGE from BioRad and stained using Rapid Coomassie Stain from Diversified Biotech (Boston, MA). Protein concentration was determined using the method of Bradford [17].

Western blot analysis was carried out by blotting SDS-PAGE gels onto Immobilon-P^{SQ} transfer membrane from Millipore (Bedford, MA) using a Mini-Transblot electrophoretic transfer cell from BioRad [18]. The primary antibody was an anti-poly-histidine monoclonal (Sigma, H-1092) diluted 1:1000. The secondary antibody was anti-mouse IgG alkaline phosphatase conjugate (Sigma, A-1293) diluted 1:20,000. The 6xHis proteins were detected by staining with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma, B-5655).

2.9. Enzyme isolation

2.9.1. Small scale

Induction medium (1 l) was inoculated with 10 ml of an overnight culture grown at 28°C. Cultures were incubated with shaking in 4 l baffle flasks at 28°C until the OD_{600} reached 0.5; induction was at 36°C

for 3 h. Cells were harvested by centrifugation, washed with 50.0 mM potassium phosphate buffer, pH 7.0, and stored at −80°C. This procedure yielded approximately 4 g/l of wet cell pellet. Cells were resuspended (4 g wet weight/10 ml) in lysis buffer (20.0 mM potassium phosphate, pH 7.0 containing 5.0 mM $MgCl_2$) and disrupted using a French pressure cell from SLM-Aminco (Rochester, NY) at 20,000 psi. Extracts were clarified by centrifugation at 10,000 × *g* for 20 min and stored at −80°C.

2.9.2. Large scale

Biolaffite fermentors (20 l) were used with a 10 l set volume and an inoculation volume of 1 l. Fermentation medium was AG192 minimal medium, which contains: $MgSO_4 \cdot 7H_2O$, 5.35 g/l; ferric ammonium citrate, 0.3 g/l; K_2HPO_4, 4.60 g/l; $MnSO_4$, 0.023 g/l; trace metals solution, 0.66 ml/l; yeast extract, 5 g/l; DF204 antifoam (Mazur Mazu), 0.4 ml/l. The following process variables were used: agitation, 500 rpm; growth temperature, 28°C; back pressure, 0.7 bar; pH control at 7.2 using gaseous NH_3; aeration, 1.0 vvm. Following the depletion of the initial glucose, a feed stream of sterile glucose (70%, w/v) was supplied as required to maintain the dissolved oxygen at 15%. Antibiotics were used in the seed flasks but not in the fermentors. The culture was induced at 8,000 Klett units as measured on a Klett–Summerson colorimeter from Klett Manufacturing (New York, NY) for 3 h at 36°C. When the appropriate biomass was reached, the cells were harvested using an Amicon DC10L system (Bedford, MA) with a 500 K MWCO hollow fiber cartridge from A/G Technology (Needham, MA). After concentration to approximately 4 l, the cells were diafiltered, washing with ∼20 l of 20 mM potassium phosphate buffer, pH 7.0 containing 5.0 mM $MgCl_2$. The cells were disrupted using a Microfluidizer M-110EH from Microfluidics International (Newton, MA) at 12,000 psi. The resulting extract was centrifuged at 10,000 × *g* for 20 min and drop frozen in liquid N_2 and stored at −80°C.

2.10. Enzyme purification

Preliminary purification studies were carried out with crude lysate (60.0 ml) using ultracentrifugation in a Beckman LB-70 (Fullerton, CA) at 150,000 × *g* for 2 h. Supernatant fluid and pellet were designated S150 and P150, respectively. The pelleted membrane fraction was washed once with lysis buffer, resuspended in 15.0 ml lysis buffer and stored at −80°C.

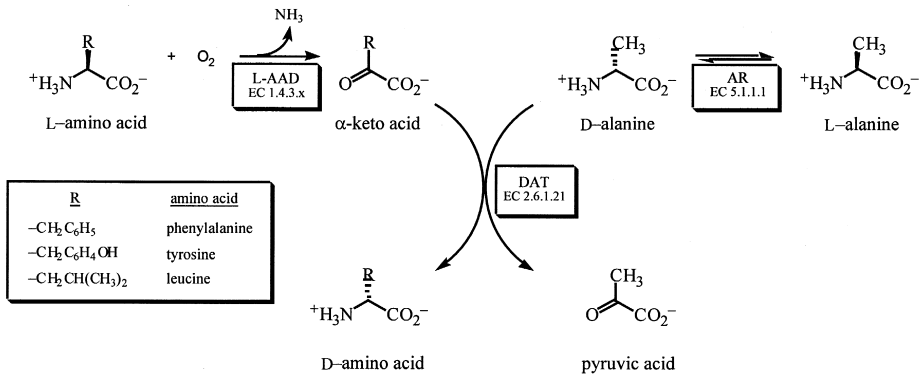
L-AAD was isolated using 1.0 ml HisTrap columns from Amersham Pharmacia Biotech (Piscataway, NJ) under denaturing, native solubilized (2.0 mM CHAPS) and native conditions from 2.0 ml aliquots of the P150 membrane fraction. Purification under denaturing conditions was carried out using 8 M urea with elution using a pH excursion to 4.5 [19]. Native solubilized purification was as follows: L-AAD (2.0 ml) of resuspended P150 fraction was diluted to 10.0 ml in start buffer (20.0 mM potassium phosphate, pH 7.5, containing 0.5 M NaCl and 2.0 mM CHAPS) with incubation on ice for 1.0 h. The solubilized extract was loaded onto a 1.0 ml HisTrap column followed by washing with start buffer until the OD_{280} was < 0.01. The column was next washed with start buffer containing 60.0 mM imidazole until the OD_{280} was < 0.01. The enzyme was eluted with 5.0 ml of start buffer containing 150.0 mM imidazole and 1.0 ml fractions were collected. Native purification was identical to the native solubilized purification except CHAPS was omitted from all buffers. Column fractions were analyzed by Western blot as described above.

3. Results and discussion

The L-AAD reaction, as shown in Scheme 1, has an absolute requirement for oxygen, releasing NH_3 and α-keto acid from an L-amino acid substrate. A typical amino acid oxidase enzyme also releases H_2O_2 upon catalysis, however when H_2O_2 was assayed for, none was detected (see below). Both NH_3 and phenylpyruvate (when L-Phe was used as the substrate) were detected (data not shown).

3.1. Oxygen dependence

A 2.0 ml enzymatic reaction was carried out both in a glove box (N_2 atmosphere) and on the bench (O_2 atmosphere). Each reaction contained 25.0 mM L-Phe in 0.1 M potassium phosphate buffer, pH 7.0,



Scheme 1. D-Amino acid production is shown using the L-amino acid deaminase (L-AAD) enzyme coupled to D-amino acid aminotransferase (DAT) and alanine racemase (AR). A strain containing these cloned enzymes has been constructed and used to prepare the following D-amino acids: phenylalanine, tyrosine and leucine.

and was incubated at 25°C. The reaction was initiated by adding 0.11 U of crude lysate. Samples (75 μl) were removed periodically, quenched with and equal volume of 3 N NaOH and the OD_{320} measured. As shown in Fig. 1, the OD_{320}, a measure of the PPA as previously described, was shown to increase dramatically in the presence of O_2, whereas in the absence of O_2, only the slightest rate was measured. This indicates that O_2 is a necessary component for the reaction to proceed.

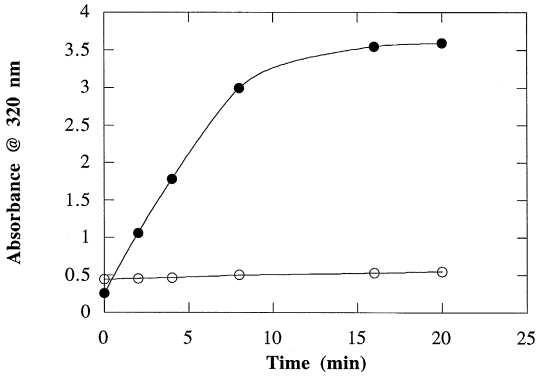


Fig. 1. The rate of phenylpyruvate (PPA) formation measured at 320 nm with or without O_2. A 2.0 ml reaction without O_2 (○) in a glove box or with O_2 (●) on the bench was incubated at room temperature and samples were removed over time, quenched with 3 N NaOH and the OD 320 nm determined.

3.2. Substrate specificity

The 20 natural L-amino acids were tested with L-AAD as described in Section 2 based on O_2 consumption rates and all amino acids were compared to L-Phe, which was set at 100% activity. Selected D-amino acids were also tested. As shown in Table 2, L-Met and L-Leu had activities greater than L-Phe. In all cases where D-amino acids were tested, the

Table 2
Substrate specificity of L-AAD with the 20 natural L-amino acids and selected D-amino acids

Amino acid	% Activity	Amino acid	% Activity
Ala	1.1	Leu	111.0 (3.4)
Arg	7.2	Lys	6.6
Asn	0.0	Met	102.2 (3.0)
Asp	1.1	**Phe**	**100.0** (3.1)
Cys	71.5 (3.5)	Pro	0.3
Gln	0.0	Ser	2.7 (1.0)
Glu	1.4	Thr	0.3 (0.4)
Gly	1.0	Trp	90.6
His	10.1	Tyr[a]	38.5
Ile	27.8	Val	7.9 (3.5)

Activity is based on the O_2 consumption assay described in Section 2 with L-phenylalanine (bold) being set at 100%. Results in parentheses are for the D-isomer.

[a]Tyr was tested at 5.0 mM due to poor solubility.

activity was less than that for the corresponding L-isomer. For good substrates (activity > 50%) such as L-Cys, L-Leu, L-Met and L-Phe, the activity of the D-isomer was < 5% of the L-isomer. The activity measured for the D-isomer is possibly due to contaminating L-isomer in the commercial preparation.

3.3. pH profile

The pH profile for the L-AAD reaction, based on O_2 consumption rates, is shown in Fig. 2, indicating that the pH optimum is 7.5. Also, a profound buffer effect was observed at pH 6.5 (MES vs. phosphate) and at pH 9.0 (TAPS vs.borate). This pH optimum is consistent with that described by others for similar deaminases [14,20,21].

3.4. Peroxide detection

As shown in Fig. 3, control reactions with HRP ± NaN_3 indicate the continuous production of H_2O_2 based on the OD_{440} as described in Section 2. For L-AAD ± NaN_3, there was no detected increase in the absorbance at 440 nm. The inclusion of NaN_3 rules out the presence of catalase, which may have utilized the peroxide produced from the L-AAD reaction [16]. Therefore, these results are consistent with L-AAD not being a classic amino acid oxidase and are similar to that described by Pelmont et al. [14].

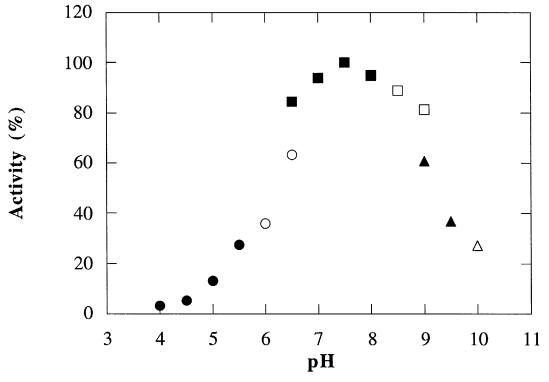


Fig. 2. The pH optimum of L-AAD was determined using the O_2 consumption assay. The pH value was determined using the following buffers: acetate (●); MES (○); phosphate (■); TAPS (□); borate (▲); CAPS (△).

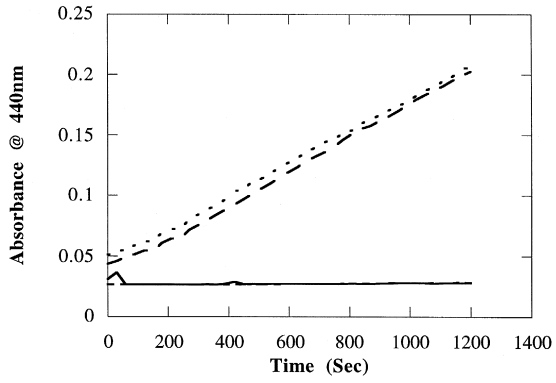


Fig. 3. The rate of H_2O_2 formation as measured by HRP and *o*-dianisidine at 440 nm. Crude lysate was incubated at 30°C with 7.5 mM L-phenylalanine in 50.0 mM Tris–HCl (pH 7.0) with (—) and without (- - -) 5.0 mM NaN_3 with 100 μg/ml *o*-dianisidine and 2 IU HRP. Controls are shown to contain 10 μg L-amino acid oxidase (known to form H_2O_2) with (· · · · · ·) and without (––) 5.0 mM NaN_3.

3.5. Kinetic parameters

As shown in Fig. 4, the Lineweaver–Burk plot indicates the cloned L-AAD enzyme has a K_m for L-Phe of 2.28 mM and a V_{max} of 0.26 μmol O_2 consumed min^{-1} mg^{-1}. The K_m value is very similar to the one described for L-Phe with Fraction I from *P. rettgeri* by Duerre and Chakrabarty [20]

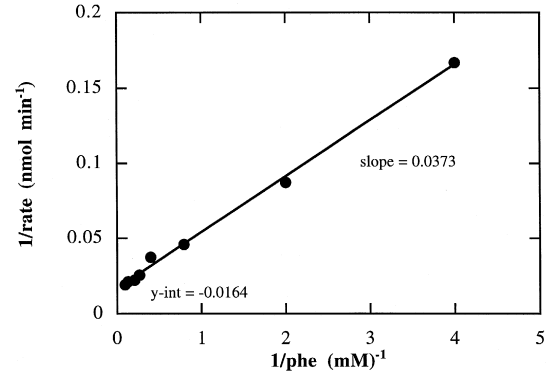


Fig. 4. Lineweaver–Burk plot for L-AAD as measured by the O_2 consumption assay. The reciprocal of the L-phenylalanine concentration is plotted vs. the reciprocal of the rate of O_2 consumption in nmol O_2 consumed min^{-1}. The calculated kinetic constants are: $K_m = 2.28$ mM and $V_{max} = 0.26$ μmol O_2 consumed min^{-1} mg^{-1}.

(3.1 mM). However, the V_{max} they report was approximately 10-fold greater than what was observed here (2.00 μmol O_2 consumed min^{-1} mg^{-1}). This is likely due to the fact that their enzyme preparation was fractionated as opposed to the crude lysate used here.

3.6. Western blot analysis

Fig. 5 (panels A and B) shows the SDS-PAGE and Western blot results, respectively, of the L-AAD purification on HisTrap columns under different conditions. Lanes 2–4 are with 8 M urea, lanes 5–7 are with 2.0 mM CHAPS and lanes 8–10 are native enzyme. In all cases, a protein band with an approximate M_r of 51,000 was detected with Coomassie Blue along with a positive Western blot for the 6xHis tag. Fraction #2 in all cases showed the highest concentration of 6xHis protein, but it was not entirely pure. The urea-purified enzyme (lane 4) contained the most contaminants, while the CHAPS-purified enzyme (lane 7) showed the fewest contaminants. When these fractions (#2) were assayed for PPA production, only those purified under native conditions showed activity, albeit the recovery was very poor. This would suggest that the enzyme is eluting from the column as a complex. An alternate assay described in the literature using synthetic electron acceptors dichlorophenol-indophenol (DCPIP) and phenazine methosulfate (PMS) will be tested to corroborate this observation [14,22].

3.7. Preliminary purification

The first step in the purification process was to separate the membrane fraction from the cytosol using ultracentrifugation as described in Section 2. As shown in Table 3, the S-150 (supernatant) fraction had no detectable activity and the activity was concentrated in the P-150 (pellet) fraction. This resulted in a 3.4-fold purification and a substantial concentration of the enzyme.

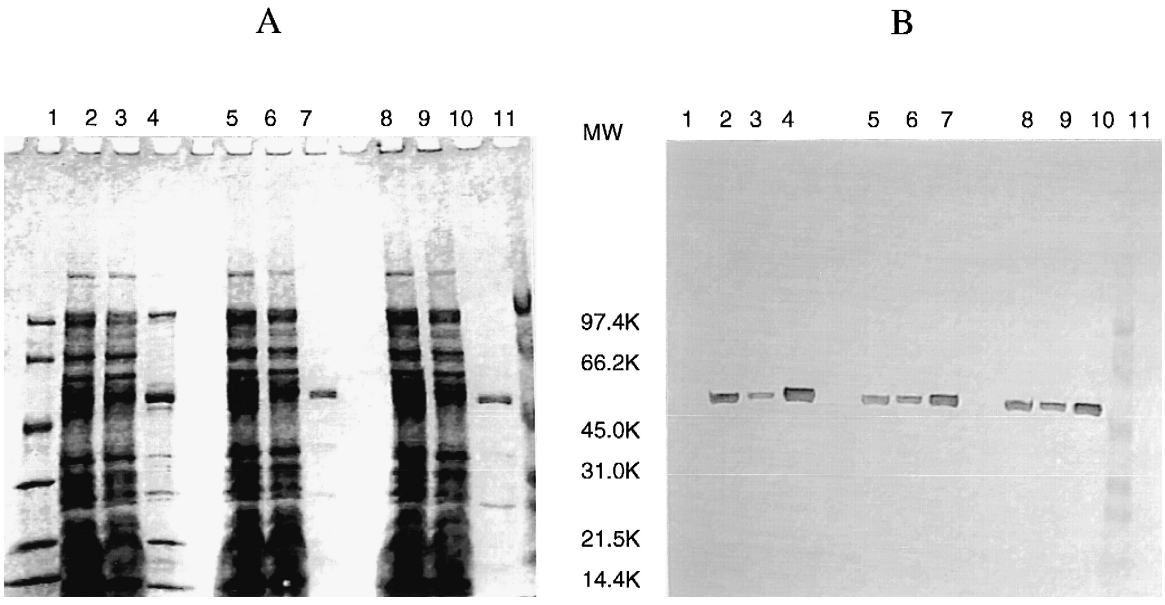


Fig. 5. (A) SDS-PAGE analysis of selected purification regimes of L-AAD on HisTrap columns. (B) Western blot analysis of the same fractions from the SDS-PAGE analysis, panel A, probed with a 1° antibody (anti-poly-histidine) and a 2° antibody (anti-mouse IgG alkaline phosphatase conjugate) and the color developed using BCIP/NBT as described in Section 2. Lanes in both panels are: (1) — molecular weight standards (BioRad, low); (2) — 8 M urea, pH 8.0; (3) — 8 M urea, pH 8.0, column flow through; (4) — 8 M urea, pH 4.5, fraction #2; (5) — 2.0 mM CHAPS, pH 7.5; (6) — 2.0 mM CHAPS, pH 7.5, column flow through; (7) — 2.0 mM CHAPS, pH 7.5, 150 mM imidazole fraction #2; (8) — native, pH 7.5; (9) — native, pH 7.5, column flow through; (10) — native, pH 7.5, 150 mM imidazole fraction #2; (11) — molecular weight standards, prestained (BioRad, low).

Table 3
Purification table of L-AAD from *P. myxofaciens* cloned into *E. coli*

Fraction	Activity[a] (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Fold
Crude lysate	59.1	85.3	0.69	1
S-150	ND	33.7	–	–
P-150	96.6	41.1	2.35	3.4

ND = not detected.
[a]Activity based on μmol PPA produced min^{-1} as described in Section 2.

Further attempts were made using the HisTrap column to recover enzyme activity from this fraction. Although the detergent CHAPS has been described in the literature to solubilize membrane proteins analogous to L-AAD, viz., L-phenylalanine oxidase [23], preliminary studies using CHAPS with L-AAD resulted in a loss of enzyme activity. A screen of other detergents such as Triton X-100, cetylpyridinium chloride, Tween 20 and CTAB were tested at various concentrations, which also led to a loss of enzyme activity (results not shown). Similar observations were made by Duerre and Chakrabarty using Triton X-100, Brij-35 or any of the Tweens [20]. The use of the detergent dodecyl maltoside has been described for the solubilization of a 6xHis-tagged protein from *Rhodobacter sphaeroides* [24]. This detergent may be useful to solubilize L-AAD without causing inactivation.

4. Summary

The enzyme L-AAD is a very important industrial enzyme used for the production of D-amino acids. We have begun to study this complex enzyme to learn more about how it might be used to prepare other bioproducts. Our preliminary studies to solubilize L-AAD were not successful and therefore our focus will be on this along with defining the substrate profile for other L-amino acids not described here. Since L-AAD appears to function differently as compared to the classical L-amino acid oxidases, further investigation of its mechanism of action will also be our target in the future.

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