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EXPERIMENTAL ARTICLES

A Simple Purification Procedure of D-Amino-Acid Oxidase from *Candida guilliermondii*[1]

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Abstract—D-amino-acid oxidase (EC 1.4.3.3) was purified about 1480-fold from the yeast *Candida guilliermondii* using chromatofocusing method. The purification procedure gave an enzyme preparation which is greater than 90% homogenous on SDS-polyacrylamide gels with a specific activity of 11.54 U/mg at 30°C with D-proline as substrate with the yield of total activity 9.3%. The molecular weights of subunit and native enzyme were determined to be 38.4 and 78.6 kDa by SDS-polyacrylamide gel electrophoresis and gel-filtration, respectively, suggesting that the native enzyme exists as a homodimer. A single molecular form with an isoelectric point of 6.85 was detected in analytical isoelectrofocusing. The optimum pH and temperature were 8.0 and 33°C. An enzyme shows stability in the pH range from 7.4 to 9.0 and at the temperature no higher than 38°C. Activation energy for D-amino-acid oxidase reaction was calculated to be 60 kJ/mol at 30°C. The strict D-isomer specificity of the enzyme is confirmed, since no reaction could be detected with L-amino acids, and a large number of D-amino acids could be substrates for this enzyme. K_m and V_{max} values were determined for D-proline and D-alanine, which, among 22 tested, were the best substrates of the enzyme. D-amino-acid oxidase from the yeast *C. guilliermondii* is a flavoprotein oxidase in which the prosthetic group is tightly, but not covalently, bound FAD. The enzyme is completely inhibited by sodium benzoate, SH-oxidizing agents, but not by sodium azide, toluene or chloroform.

Keywords: *Candida guilliermondii*, D-amino-acid oxidase, chromatofocusing, enzyme stability, substrate specificity

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INTRODUCTION

D-amino-acid oxidase (D-amino acid: oxygen oxidoreductase (deaminating); EC 1.4.3.3) is a member of the class of flavin dehydrogenase/oxidases [1, 2] and catalyzes the oxidative deamination of D-amino acids, producing the corresponding α-keto acid and ammonia with concomitant reduction of molecular oxygen to hydrogen peroxide. This enzyme has found considerable practical importance not only in basic research but also in biotechnology. D-amino-acid oxidases from porcine kidney and several fungi have been used for the separation of racemic amino acid mixtures [3], preparation of keto-acids [4], in the modification of cephalosporin C [5], estimation of D-amino acids [6], especially in biosensors for the detection of D-amino acids in various biological samples [7–9]. Despite the fact that the presence of D-amino-acid oxidases (DAAO) has been reported in many organisms [10, 11], the only DAAO species available for commercial use in a homogenous form and in large quantities was the one from pig kidney [12]. In last two decades efforts of many scientists were directed towards obtaining an alternative source among microorganism species. The attempts to purify DAAO as a flavoprotein from microorganisms, particularly from yeast, have been hampered by the low concentration of the enzyme in the cells, its instability and the impossibility of using the well-established purification procedure of DAAO from pig kidney [13, 14]. It has been shown that DAAO is constitutively present at a very low level in yeast *Rhodotorula gracilis*, but its synthesis can be selectively induced by the presence of D-amino acids in the growth medium [15].

We here describe for the first time the purification scheme of *Candida guilliermondii* DAAO, including the chromatofocusing step, without any initial induction, and represent some physicochemical and catalytic properties of the enzyme to be helpful in biotechnological applications.

MATERIALS AND METHODS

Materials. DEAE-toyopearl 650M and toyopearl HW-50F gels were from Toyo Soda MFG (Tokyo, Japan); phenyl-sepharose CL-4B, polybuffer

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exchanger (PBE-94) and molecular mass marker proteins for PAGE were from Pharmacia Fine Chemicals (Uppsala, Sweden); polyethylene glycol 35000 (PEG) was from Loba-Chemie, Wien-Fischamend (Austria); yeast extract was from Becton, Dickinson and Company (France); 4-aminoantipyrine was from Sigma-Aldrich Chemie GmbH (Germany); phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), acrylamide, bis-acrylamide, molecular mass marker proteins for gel filtration were from SERVA Electrophoresis GmbH (Germany); ultrasonic homogenizer with temperature controller 115 VAC was from Cole-Palmer (United States). All other reagents were all of analytical grade and purchased from commercial sources.

Organism and cultivation. *C. guilliermondii* cells were from Yerevan State University's microorganism culture collection. For production culture, an optimized synthetic growth medium containing 3.1 g $(NH_4)_2SO_4$, 1.23 g KH_2PO_4, 0.625 g $MgSO_4 \cdot 7H_2O$, 0.125 g $CaCl_2 \cdot 2H_2O$, 0.125 g NaCl, 0.1 g $ZnSO_4$, 8×10^{-5} g biotin, 10 g glucose and 10 g yeast extract in a total volume of 1 L, with the pH adjusted to 5.5 by HCl was used [16]. Cells were grown at 30°C under shaking conditions (200 rpm). After 20 h cultivation (late exponential phase) cells were harvested by centrifugation at 10000 *g* for 20 min at 4°C and stored at −20°C prior to use.

Enzyme assay and protein determination. D-amino-acid oxidase activity was assayed spectrophotometrically by measuring hydrogen peroxide production by monitoring an absorbance at 550 nm. The assay mixture (total volume of 1 mL) contained 30 mM D-proline, 5 mM phenol, 0.3 mM 4-aminoantipyrine in 50 mM Tris-HCl buffer (pH 8.3) with 2 U horseradish peroxidase. FAD could be omitted from assay mixtures without any effect on activity measurements. One unit of activity corresponds to the production of 1 μmol hydrogen peroxide per minute at 30°C with 30 mM D-proline as substrate. For determination of kinetic parameters and substrate specificity, assays were run under the same conditions, except for the substrate and its concentration. Protein concentration was determined using Lowry [17], Bradford [18] or Groves-Davis protein assay [19] according to the sample total protein content.

Purification of D-amino-acid oxidase. The purification procedure was carried out at 4°C. Cell paste was resuspended (300 g/L) in 20 mM Tris-HCl (pH 8.3) buffer containing 2 mM EDTA, 0.1 mM PMSF (buffer A) and homogenized by sonication with ultrasonic disrupter for 20 min (40 cycles of 30 seconds processing and 30 seconds of rest) at 4°C. After sonication the homogenate was centrifuged at 10000 *g* for 20 min at 4°C. The supernatant was applied to DEAE-toyopearl 650 M anion exchange column (90 × 2.7) pre-equilibrated with the buffer A and eluted with a linear gradient from 0 to 0.15 M NaCl. Active fractions were combined, dialyzed against 100 volumes of the same buffer and saturated with ammonium sulfate at 0.5 M saturation. The suspension was loaded on phenyl-sepharose CL-4B column (30 × 1.5) pre-equilibrated with 0.5 M ammonium sulfate saturated buffer A. The column was washed of its 3 volumes with salt saturated pre-equilibration solution at room temperature to remove unabsorbed proteins. The enzyme was eluted with a linear gradient containing from 0.5 to 0 M ammonium sulfate and from 0 to 2% Triton X-100 elution detergent in buffer A. Active fractions were combined and dialyzed against 100 volumes of buffer A pH adjusted to 8.6 and applied to PBE 94 chromatofocusing column (12 × 1.5) pre-equilibrated with Tris-acetic acid buffer pH 8.6. Elution was made with the diluted polybuffer at pH 6.5. Active fractions were combined, dialyzed against 100 volumes of buffer A pH adjusted to 8.3 and concentrated against polyethylene glycol 35000 for 4 h. Concentrated protein probe was applied to a column (70 × 1.0) of toyopearl HW-50F equilibrated with 20 mM Tris-HCl (pH 8.3) buffer containing 2 mM EDTA and 0.1 mM PMSF for gel-filtration. The enzyme was eluted with the same buffer. The molecular mass of the native enzyme was determined by gel filtration on the same column calibrated with marker proteins mentioned above.

Polyacrylamide gel electrophoresis. The homogeneity of DAAO was determined by polyacrylamide slab gel (7.5%) electrophoresis at pH 8.9. SDS-polyacrylamide slab gel (7.5%) electrophoresis was performed following a modification of the method of Laemmlii [20] with separating gels of pH 8.9; gels were stained with Coomassie Brilliant Blue R-250 for 12 h (overnight) and destained by diffusion in a mixture of 7% acetic acid and 25% methanol in water.

Analytical isoelectrofocusing. Purified samples of DAAO and marker proteins were electrofocused on polyacrylamide-gel slabs containing LKB Ampholines with the pH range of 3.5–10.0 at 10°C. Broad range IEF marker proteins (Pharmacia, Sweden) were used to calibrate the electrofocusing gels. The samples were focused at a constant current of from 20 to 2 mA for 4.0–5.0 h with the voltage limitation of not higher than 500 V. The plates were fixed with 25% trichloroacetic acid, 7% sulphosalicylic acid and 12.5% ethanol containing solution, stained with Coomassie Brilliant Blue R-250, and destained with 8% acetic acid and 25% ethanol containing solution.

Thermostability and pH-stability analyses. The time-dependence of thermal inactivation of DAAO was determined by heating enzyme solutions in tubes over the temperature range 20–60°C in 20 mM Tris-HCl (pH 8.3) buffer containing 2 mM EDTA and 0.1 mM PMSF. The activation energy (E_a) for D-amino-acid oxidase reaction was obtained from the slop of the Arrhenius plot and from the slopes of the first-order inactivation plots. The pH-stability analyses were carried out at DAAO half-inactivation temperature

Table 1. Purification of D-amino-acid oxidase from *C. guilliermondii*

Purification step	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract	93.6	12000	0.0078	1	100.0
DEAE-toyopearl 650M	76.2	924	0.0825	11	81.4
Phenyl-sepharose CL-4B	45.4	372	0.122	16	48.5
Chromatofocusing on PBE 94	28.7	6.33	4.53	581	30.6
Toyopearl HW-50F	8.7	0.75	11.54	1480	9.3

The activity was measured with D-proline as substrate. Starting material: 128 g of frozen cell paste.

(45.6°C for 20 min) in 50 mM buffers over the pH range 5.0–10.0.

The values of K_m and V_{max} together with their standard deviations were determined as a result of multidimensional linear regression [21] of the reaction rate depending on the concentration of D-amino acids using the program "GAUSS 4.0".

RESULTS AND DISCUSSION

D-amino-acid oxidase isolated by the present procedure (1480-fold purification factor) had a specific activity of 11.54 μmol/min per mg protein using D-proline as substrate at 30°C. Table 1 summarizes the results of a typical purification procedure. It has been investigated and proved experimentally that DAAO from *C. guilliermondii* cells could be absorbed on a hydrophobic support in the presence of low salt concentration (0.5 M $(NH_4)_2SO_4$) in place of 2 M KCl [22] as well as in the presence of high salt concentration (up to 3.3 M) due to its high binding affinity to phenyl-sepharose CL-4B material. The last point was demonstrated by the elution behavior of DAAO from phenyl-sepharose with linear gradient from 3 to 0 M ammonium sulfate in buffer A (Fig. 1). This behavior accompanies with the loss of total activity from 60 to 75%. The difference observed during absorption on phenyl-sepharose and further elution from it may be a result of possible existence of isoenzymes of DAAO, but this three preparations, which corresponding to three different enzyme activity maximums, demonstrated similar properties at PAGE and isoelectric focusing analyses. In all three cases there was single protein band observed during enzymatically staining process, so this phenomenon could be a result of formation of monomeric or other enzymatically active forms during hydrophobic chromatography and is currently under investigation in our laboratory. Based on these data we decided to use this step of purification because it strongly helps to eliminate the protein impurities which have no affinity to hydrophobic interactions (Fig. 2a), but to apply DAAO preparation on phenyl-sepharose column at 0.5 M ammonium sulfate saturation and elute it with the linear gradient from 0.5 to 0 M ammonium sulfate and concomitant from 0 to 2% gradient of Triton X-100 in buffer A.

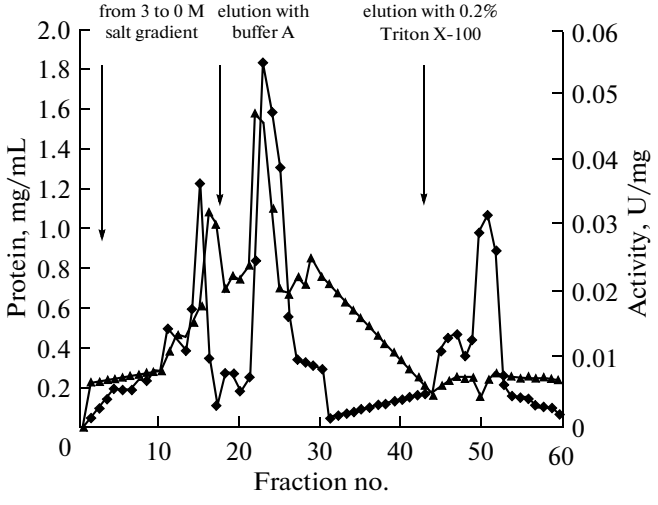

Fig. 1. DAAO elution profile from phenyl-sepharose CL-4B hydrophobic chromatography column. ▲—protein concentration, ■—enzyme activity.

The chromatofocusing on PBE 94 was particularly effective, especially followed by gel-filtration, such as this type of purification procedure is based on different isoelectric points of proteins. Followed by separation of proteins based on their molecular weight (gel filtration), this procedure yields to obtain an enzyme preparation, the sample of which migrated on polyacrylamide gel-electrophoresis and stained enzymatically as a single protein band (Fig. 2).

On SDS-gel electrophoresis, the final preparation migrated as a main protein band, with a molecular mass of 38.4 ± 1.2 kDa (Fig. 2b). The native molecular weight was estimated to be 78.6 kDa by gel-filtration on toyopearl HW-50F (Fig. 3), suggesting that the DAAO from the yeast *C. guilliermondii* exists as a homodimer. The molecular mass is similar to those of D-amino-acid oxidases from *Rhodotorula gracilis*

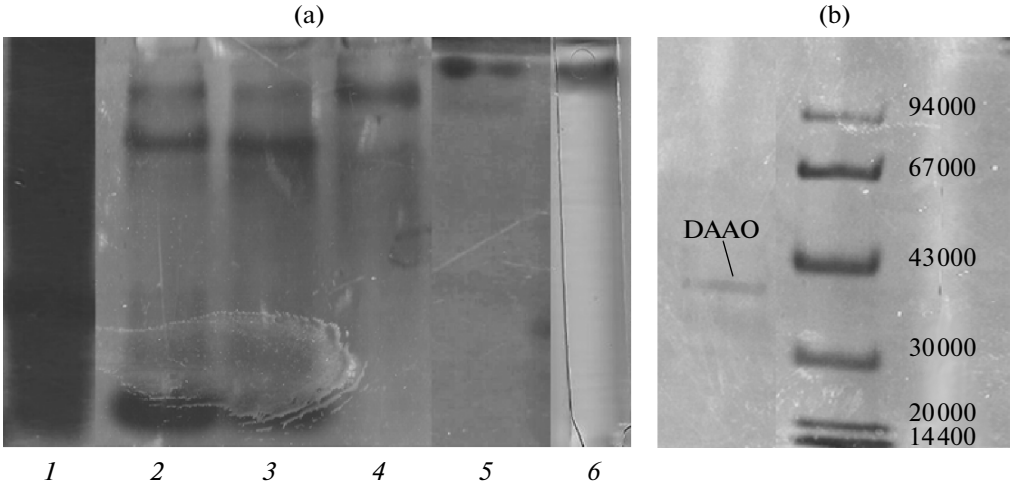

Fig. 2. a—Polyacrylamide gel electrophoresis of samples at different stages of purification. Gel was stained with Coomassie Blue R-250. *1*—crude extract after sonication (supernatant); *2*—after DEAE-toyopearl 650 M; *3*—after phenyl-sepharose chromatography; *4*—after chromatofocusing on PBE 94; *5*—after toyopearl 50F gel-filtration; *6*—activity staining of enzyme after gel filtration with D-Ala as substrate. b—SDS-polyacrylamide gel electrophoresis of D-amino-acid oxidase. Samples were incubated at 95°C for 10 min in 1% SDS and 2.5 mM 2-mercaptoethanol. Marker proteins (at the right, from up to down): phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactoalbumin; (marker proteins' molecular weights are shown in the figure).

[14], *Rhodosporidium toruloides* [23] and *Trigonopsis variabilis* [24], which have been reported to be homodimers of 79, 72 and 80 kDa, respectively.

The pH-activity profile of the enzyme (Fig. 4a) shows that the optimum pH was at 8.0. The activity was more than 90% of the maximum in a rather narrow pH from 7.5 to 8.4. The pH-stability was also determined by incubating DAAO preparations at the temperature of enzyme half inactivation (45.6°C, determined from temperature stability profile) for 20 minutes with 50 mM buffers in the pH range from 5.0 to 10.0. As it could be expected from the pH-activity profile, DAAO from *C. guilliermondii* shows stability in the pH range from 7.4 to 9.0 (Fig. 4b) with the loss of its activity no more than 10%.

The optimum temperature was 33°C and the activity was above 90% of the maximum in the range from 24 to 38°C (Fig. 5a). It is also noticeable, that this enzyme was highly unstable to heat inactivation, and a sharp decrease in activity is observed above 40°C. DAAO from *C. guilliermondii* losses 50% of its enzymatic activity starting from 45°C achieving to full inactivation at the range of temperature from 58 to 60°C (Fig. 5b). From Arrhenius equation and from the slope of activity/temperature dependence plot the activation energy for D-amino-acid oxidase reaction was calculated to be 60 kJ/mol at 30°C. It was slightly higher in comparison with *Rhodotorula gracilis* DAAO catalytic reaction activation energy, which has been reported to be 38.3 kJ/mol [25].

The strict D-isomer specificity of the enzyme is confirmed, since no reaction could be detected with L-amino acids [26]. Moreover, the presence of the L-isomer does not interfere with D-amino-acid oxidase activity, so for some amino acids DL-mixtures were used for substrate specificity determination with the concentrations two times higher than that for pure D-amino acids (60 mM DL-mixtures instead of 30 mM D-amino acids). The K_m and V_{max} values for D-Pro and D-Ala were calculated with their standard deviations. Table 2 summarizes the substrate specificity of purified DAAO. An enzyme was active toward several D-amino acids with D-proline being the best substrate for *C. guilliermondii* DAAO. This pattern is similar to that for mammalian DAAO to have the highest affinity to D-Pro and to oxidize achiral glycine [26]. Similarities are observed with the *Candida boidini* DAAO in manner to oxidize neutral and hydrophobic

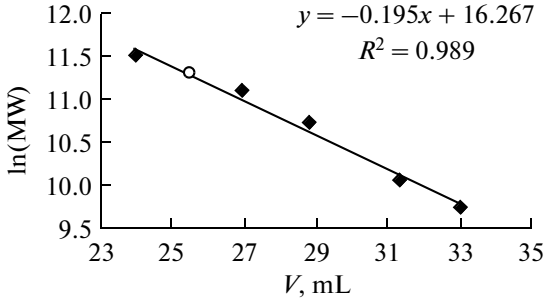

Fig. 3. Molecular weight of D-amino-acid oxidase (o) as determined by gel filtration on toyopearl HW-50F. Marker proteins (◆) were as follows (molecular weight are indicated in parentheses): alkaline phosphatase (100000); bovine serum albumine (67000); ovalbumin (45000); chymotrypsinogen A (25000); horse myoglobin (17800).

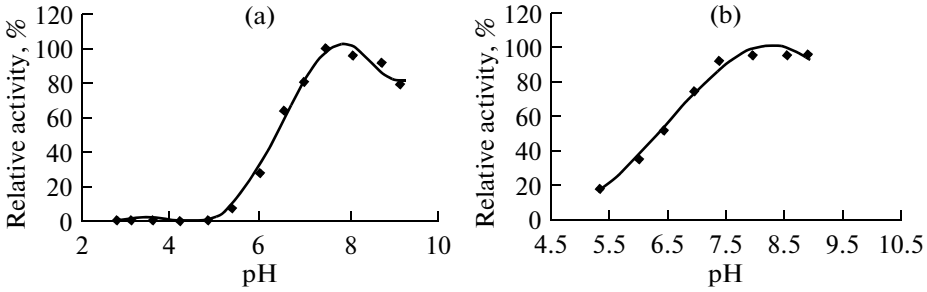

Fig. 4. pH-activity (a) and pH-stability (b) profiles of *C. guilliermondii* D-amino-acid oxidase. a—Enzyme activity was assayed in 50 mM polybuffer, containing citric acid, Tris, Hepes and boric acid in the 2.8–9.35 pH range. b—Enzyme preparations were incubated at 45.6°C for 20 min with 50 mM buffers in the pH range from 5.0 to 10.0. Data are expressed as percent of the highest activity observed. Values are means for three determinations.

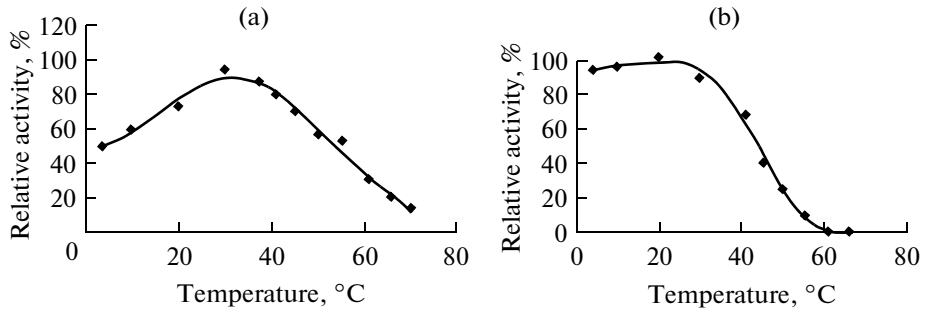

Fig. 5. An optimum temperature profile (a) and relative activity/temperature dependence (b) for *C. guilliermondii* D-amino-acid oxidase. Data are expressed as percent of the highest activity observed. Values are means for three determinations.

D-amino acids which have shorter carbonic chain [27]. Substrate specificity then differs markedly from other yeast source D-amino-acid oxidases with it more widely specification. This could be a probable result of non-inducible DAAO obtaining procedure from *C. guilliermondii* cells. It was also noticeable that *C. guilliermondii* DAAO has a significant affinity to D-isoleucine and D-norvaline 22.6 and 2.4 times higher than that for D-Leu and D-Val, respectively (see Table 2). This type of specificity, however, associated with the enzyme catalytic active site three-dimensional structure, which, in contrast to human DAAO, is not investigated clearly.

Attempts were made to stabilize the enzyme during the purification process by addition of FAD, which were, however, found to be ineffective. First of all, glycerol was found to stabilize an enzyme, especially in diluted solutions of enzyme, but the use of glycerol in association with other protectors, such as PMSF, EDTA and 2-mercaptoethanol all together yields to sharp inactivation of DAAO. From the other hand, bovine serum albumin (BSA) was found to stabilize purified enzyme, but it does not prevent from microorganism growth, so it was included in buffers during the experiments for enzyme characterization in association with 0.1% sodium azide, whenever it was appropriate. Sodium azide proved to have no negative impact on DAAO activity. Chloroform and toluene demonstrated strong protective properties against microorganism growth at the concentration of 1%, but the decrease of enzyme activity was observed after two weeks. Sodium benzoate at the concentration of 0.2% brought to complete and quick inactivation. Also the enzyme was inhibited by 1 mM 5,5'-dithiobis (2-nitrobenzoic acid) (3,3'-6) followed by reactivation after adding 10 mM 2-mercaptoethanol. These results suggest that a sulfhydryl group of the enzyme is important in the activity. Based on this data we used 10% glycerol or 2 mg/mL BSA with 0.1% sodium azide or 2 mM EDTA with 0.1 mM PMSF individually, whenever it was appropriate.

As it was stated above starting material for enzyme purification was 128 g cell paste. It is noticeable, that from this material after homogenization by sonication it was available to obtain pure supernatant with the total protein content of 12 g, which is nearly 10% of initial weight. This first step with high yield of protein extraction was also important and peculiar procedure demonstrated. Considering the fact that these results were obtained without any initial enzyme inducing

Table 2. Substrate specificity of DAAO from *C. guilliermondii*

Substrate	Concentration, mM	Relative activity, %[a]	K_m, mM	V_{max}, U/mg
D-Pro	30	100.0	7.88 ± 0.94	22.28 ± 3.59
D-Ala	30	74.5	8.77 ± 1.02	16.85 ± 3.38
D-Val	30	29.1	n.t.[b]	n.t.
D-Leu	30	3.9	n.t.	n.t.
D-Ser	30	13.2	n.t.	n.t.
D-Lys	30	3.4	n.t.	n.t.
D-Trp	10	3.3	n.t.	n.t.
D-Tyr	15	0.8	n.t.	n.t.
DL-Ile	60	83.6	n.t.	n.t.
DL-Met	60	7.6	n.t.	n.t.
DL-Tre	60	6.1	n.t.	n.t.
DL-Asp	60	11.2	n.t.	n.t.
DL-Asn	30	7.7	n.t.	n.t.
DL-Norvaline	60	71.6	n.t.	n.t.
Gly	60	5.8	n.t.	n.t.
L-Pro	30	0	n.t.	n.t.
L-Tre	30	0	n.t.	n.t.
L-Ile	30	0	n.t.	n.t.

The enzyme did not show activity toward all L-amino acids. Values are means for three determinations.
[a] Activity is given relative to that measured for D-Pro. [b] n.t., not tested.

procedures, and, in addition, the wide substrate specificity of *C. guilliermondii* DAAO, we assume that this source of D-amino-acid oxidase could be produced, purified in short order with chromatofocusing, immobilized and used for different biotechnological applications.

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