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Purification and properties of D-Amino-Acid Oxidase from *Candida guilliermondii НП-4*

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Keywords: *Candida guilliermondii НП-4, D-amino-acid oxidase, chromatofocusing, PAAG-electrophoresis, activation energy, substrate specificity.*

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 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 [1]. 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, preparation of keto-acids, in the modification of cephalosporin C, estimation of D-amino acids, especially in biosensors for the detection of D-amino acids in various biological samples [2,3]. Despite the fact that the presence of D-amino-acid oxidases (DAAO) has been reported in many organisms [4], the only DAAO species available for commercial use in a homogenous form and in large quantities was the one from pig kidney [5]. 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 [6]. 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 [7].

We here describe for the first time the purification scheme of *Candida guilliermondii НП-4* 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. All reagents were of analytical grade and purchased from commercial sources. *C. guilliermondii* cells were from YSU 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 $MgSO_4*7H_2O$, 0.125 g $CaCl_2*2H_2O$, 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 [8]. Cells were grown at 30°C under shaking conditions (200 rpm). After 20 h cultivation (late exponential phase) cells were harvested by centrifugation at 10,000g for 20 min at 4°C and stored at -20°C prior to use.

DAAO 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. Protein concentration was determined using Lowry, Bradford or Groves-Davis protein assay according to the sample total protein content.

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 (30 seconds processing and 30 seconds of rest) at 4°C. After sonication the homogenate was centrifuged and supernatant was applied to DEAE-toyopearl 650M anion exchange column (90x2.7) 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 (30x1.5) 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 (12x1.5) 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 35,000 for 4 h. Concentrated protein probe was applied to a column (70x1.0) 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.

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

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 DAAO reaction was obtained from the slopes of the Arrhenius and the first-order inactivation plots.

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

Results and discussion. DAAO 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.

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	12 000	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.

After each step of purification PAAG electrophoresis was performed to determine homogeneity of DAAO samples (fig. 1a).

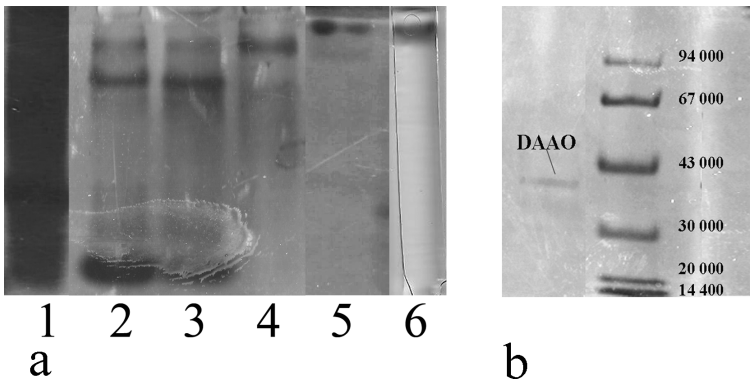

Fig. 1. a - Polyacrylamide gel electrophoresis of samples at different stages of purification. Gel was stained with Coomassie Blue R-250. (1) crude extract after sonication (supernatant); (2) after DEAE-toyopearl 650M; (3) after phenyl-sepharose chromatography; (4) after chromatofocusing; (5) after gel-filtration; (6) activity staining of enzyme after gel filtration with D-Ala as substrate, b - SDS-polyacrylamide gel electrophoresis of purified DAAO. Marker proteins and their molecular weights are shown at the right side in the figure.

On SDS-gel electrophoresis, the final preparation migrated as a main protein band, with a molecular mass of 38.4±1.2 kDa (fig. 1b). The native molecular weight was estimated to be 78.6 kDa by gel-filtration on toyopearl HW-50F (data not shown), suggesting that the DAAO from the yeast *C. guilliermondii* exists as a homodimer. The molecular mass is similar to those of DAAO

from *Rhodotorula gracilis* [6], *Rhodosporidium toruloides* [9] *and Trigonopsis variabilis* [10], which have been reported to be homodimers of 79, 72 and 80 kDa, respectively.

The pH-activity profile of the enzyme (fig. 2a) shows that the optimum pH was at 8.0. The enzyme activity was assayed in 50 mM polybuffer, containing citric acid, Tris, Hepes and boric acid in the 2.8-9.35 pH range, and DAAO 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 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. 2b) with the loss of its activity no more than 10%.

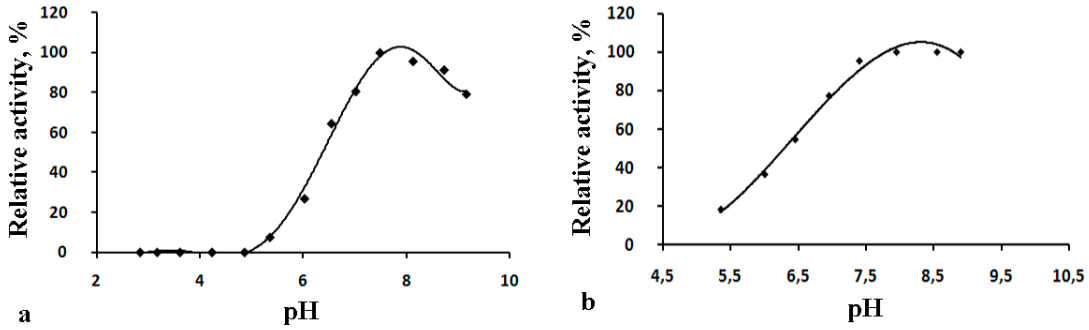

Fig. 2. pH-activity (a) and pH-stability (b) profiles of *C. guilliermondii* D-amino-acid oxidase

The optimum temperature was 33°C and the activity was above 90% of the maximum in the range from 24 to 38°C (fig. 3a). 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. 3b). The activation energy for DAAO 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 [11].

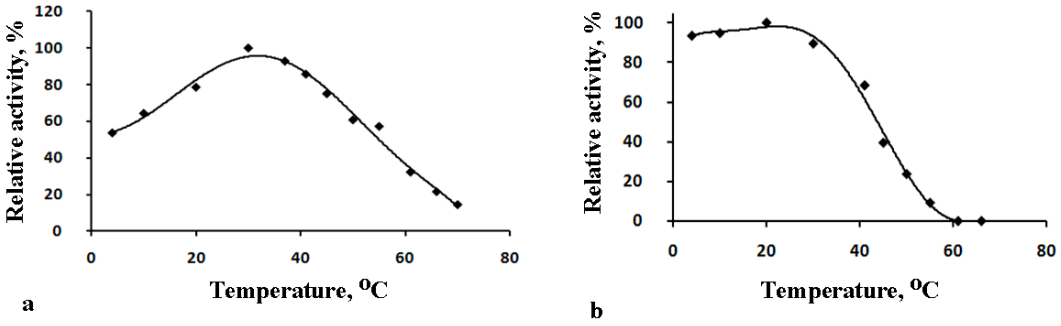

Fig. 3. An optimum temperature profile (a) and relative activity/temperature dependence (b) for *C. guilliermondii* DAAO

The strict D-isomer specificity of the enzyme is confirmed, since no reaction could be detected with L-amino acids [12]. Moreover, the presence of the L-isomer does not interfere with DAAO 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. The Km and Vmax values were calculated to be 7.9±0.9 with 22.3±3.6 for D-Pro and 8.8±1 with 16.9±3.4 for D-Ala, respectively. Table 2 summarizes the substrate specificity data 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 [12]. Similarities are observed with the *Candida boidini* DAAO in manner to oxidize neutral and hydrophobic D-amino acids which have shorter carbonic chain [13]. Substrate specificity then differs markedly from other yeast source DAAO 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.

Table 2

Substrate specificity of DAAO from *C. guilliermondii*

Substrate	Concentration, mM	Relative activity, % *	Substrate	Concentration, mM	Relative activity, % *
D-Pro	30	100	DL-Met	60	7,6
D-Ala	30	74,5	DL-Tre	60	6,1
D-Val	30	29,1	DL-Asp	60	11,2
D-Leu	30	3,9	DL-Asn	30	7,7
D-Ser	30	13,2	DL-norvaline	60	71,6
D-Lys	30	3,4	Glycine	60	5,8
D-Trp	10	3,3	L-Pro	30	0,0
D-Tyr	15	0,8	L-Tre	30	0,0
DL-Ile	60	83,6	L-Ile	30	0,0

* Activity is given relative to that measured for D-Pro; values are means for three determinations; the enzyme did not show activity toward all L-amino acids

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

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Purification and Properties of D-Amino-Acid Oxidase from *Candida guilliermondii НП-4*

D-amino-acid oxidase was purified from the yeast *Candida guilliermondii НП-4* using chromatofocusing method. The native enzyme exists as a homodimer with the molecular weight of 78.6 kDa. The optimum pH and temperature were 8.0 and 33°C. The strict D-stereospecificity of the enzyme is confirmed, 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.

Գ. Կ. Գևորգյան, ակադեմիկոս Մ. Ա. Դավթյան, Ա. Ա. Համբարձումյան

***Candida guilliermondii* НП-4 խմորասնկերի D-ամինաթթվային օքսիդազի մաքրումն ու հատկությունների ուսումնասիրումը**

D-ամինաթթվային օքսիդազն անջատվել և մաքրվել է *Candida guilliermondii* НП-4 խմորասնկերից քրոմատոֆոկուսացման մեթոդի կիրառմամբ: Նատիվ ֆերմենտն իրենից ներկայացնում է հոմոդիմեր՝ 78.6 կԴա մոլեկուլյար զանգվածով: Օպտիմալ pH և ջերմաստիճանը կազմել են, համապատասխանաբար, 8.0 և 33°C: Հաստատվել է բացարձակ ստերեոսպեցիֆիկություն D-իզոմերների նկատմամբ: Հաշվարկվել են K_M-ի ու V_{max}-ի արժեքները D-պրոլինի և D-ալանինի համար, որոնք փորձարկված 22 ամինաթթուների շարքում լավագույն սուբստրատներն էին տվյալ ֆերմենտի համար:

Г. К. Геворкян, академик М. А. Давтян, А. А. Амбарцумян

Очистка и свойства D-аминокислотной оксидазы из дрожжей *Candida guilliermondii НП-4*

Из дрожжей *Candida guilliermondii НП-4* с использованием метода хроматофокусирования выделена и очищена D-аминокислотная оксидаза. Природный фермент представляет собой гомодимер с молекулярной массой 78.6 кДа. Оптимальные значения pH и температуры – 8.0 и 33°C. Подтверждена абсолютная стереоспецифичность фермента, а также вычислены значения K_M и V max для D-пролина и D-аланина, которые оказались наиболее хорошими субстратами из 22 испытанных аминокислот.

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