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Purification and Properties of Alcohol Oxidase from *Pichia putida*

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Abstract—Alcohol oxidase (AO) was extracted from the methylotrophic yeast *Pichia putida* and purified using various methods. AO purified by crystallization was homogeneous based on analytical centrifugation with subsequent gel filtration and SDS-PAGE. The molecular weight of the enzyme was around 600 kDa. SDS-PAGE revealed a single protein band (74 ± 4 kDa), and 8-9 bands of native protein with similar specific AO activities and substrate specificities were identified by PAGE without SDS. Electron microscopy of a single molecule revealed eight subunits located on the top of a regular tetragon with dotted symmetry of 422D4 providing evidence that AO consists of eight subunits. Apparently, each molecule of AO has two types of subunits with very similar molecular weights and differing from each other by the number of acidic and basic amino acid residues. Each subunit includes one molecule of FAD and 2-3 cysteine residues. The pH optimum was within 8.5-9.0. Specific activity of the enzyme varied from 10 to 50 μmol methanol/min per mg protein from batch to batch depending on separation methods and had linear relationship with protein concentration. The AO was quickly inactivated at 20°C and seemed to be stable in phosphate-citrate buffer with 30-50% (w/v) of sucrose. Different forms of 0.1-1 mm crystals of the enzyme were obtained. However the crystals did not yield X-ray reflections, apparently as a result of their molecular microheterogeneity.

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Key words: alcohol oxidase, subunits, quaternary structure, crystallization

Alcohol oxidase (AO) (alcohol:dioxygen-oxidoreductase, EC 1.1.3.13) was first purified in 1965 from basidiomycetes [1-3]. AO is a component of peroxisomal crystalloids from yeast cells, which have been characterized in detail in *Hansenula polymorpha* [4-7]. The enzyme has been obtained in pure form from a number of yeasts, and its properties have been described [8-20]. This is the key enzyme of methanol metabolism of methylotrophs *Hansenula*, *Torulopsis*, *Candida*, *Kloeckera*, *Pichia*, etc. The AO concentration was found to be 10-20% of all cell proteins in wild type yeasts. AO catalyzes the oxidation of methanol and low molecular weight α-alcohols as described by the following scheme: $R\text{-}CH_2OH + O_2 \rightarrow R\text{-}CH{=}O + H_2O_2$. It has a significant practical role in analytical determination of alcohols [21-27] and yielding aldehydes, hydrogen peroxide [28], and various heterologous proteins [29-34].

Alcohol oxidase from *P. putida* was never before purified and studied. The purpose of the present work was to develop the methodology of large-scale purification and investigation of catalytic and molecular properties of this enzyme.

MATERIALS AND METHODS

Chemicals. The following chemicals were used in this study: benzamidine, acrylamide, N,N-bis-acrylamide, ammonium persulfate, SDS, NaN_3, DEAE-cellulose, FAD, thiamin, and biotin from Sigma (USA); Sephadex G-25, Sepharose 2B, and standards for gel filtration and electrophoresis from Pharmacia, LKB Biotechnology (Sweden); Coomassie Blue G-250 and R-250, *o*-dianisidine, $(NH_4)_2SO_4$, NaCl, KCl, KH_2PO_4, Na_2HPO_4, *p*-chloromercuribenzoate, and 5,5′-ditiobis-2-nitrobenzoate (DTNB) from Merck (Germany); methanol, ethanol, propanol, butanol, pentanol, formaldehyde,

Abbreviations: AO, alcohol oxidase.

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NH_4Cl, $MgSO_4$, $CaCl_2$, $FeSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot H_2O$, $MnSO_4$, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ from Khimmed (Russia), all of "chemically pure" grade. Buffers were prepared in twice distilled water.

Cultivation of the methylotroph *P. putida* and methods of AO isolation. Pure protein from *H. polymorpha* and culture of methylotrophic yeast *P. putida* were kindly provided by the Research Institute for Protein Synthesis (Moscow). The culture was cultivated on agar plates and grown in the flow-type fermenter ANKUM-2M in volume of 8 liters with air aeration in the following saline medium (g/liter): KH_2PO_4 (0.047), NaH_2PO_4 (0.013), NH_4Cl (2.5), $(NH_4)_2SO_4$ (0.4), $MgSO_4$ (0.07), NaCl (0.7), $CaCl_2$ (0.11), $FeSO_4 \cdot 7H_2O$ (0.017), $ZnSO_4 \cdot 7H_2O$ (0.0005), $CuSO_4 \cdot H_2O$ (0.00004), $MnSO_4$ (0.0004), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.0002), thiamin (0.001), biotin (0.0005), methanol (no more than 2%, w/v), pH 5.0, at a flow rate of 0.05 fermenter volume per hour and temperature 35°C. During growth the pH of medium in the fermenter was maintained by addition of 0.2 M NaOH. Cells (100-150 g by dry weight) were concentrated by centrifugation for 20 min at 12,000*g* (r_{av} 10 cm), washed twice in 0.02 M phosphate-citrate buffer, pH 8.0, and broken either using a planetary disintegrator with glass beards (d = 0.2-0.5 mm) for 10 min at 1000 rpm (4°C) or by lysis in the presence of 1.2% (w/v) methylene chloride [13] for 28 h at 30°C (in the latter case cellular suspension was supplemented with 0.02% NaN_3 [35], 0.1 mM benzamidine, and 0.05% Tween 60). The pH of the cell-free preparation was adjusted to 8.0 using 0.1 M NH_4OH, and then $(NH_4)_2SO_4$ was added to 25% (pH 7.0) and the mixture was centrifuged for 20 min at 20,000*g* (r_{av} 10 cm). The supernatant fraction was supplemented with $(NH_4)_2SO_4$ to 60% of saturation (pH 7.0) and kept for 2 h at 5°C. The precipitate obtained by centrifugation of this fraction for 10 min at 20,000*g* (r_{av} 10 cm) was dissolved in 0.01 M phosphate-citrate buffer (pH 8.0) and separated from salts by ultrafiltration through a membrane from Vladipor (Russia) with dilution with water and phosphate-citrate buffer, pH 6.2, then 6-12% (w/v) polyethylene glycol (6000 Da), 0.1 mM benzamidine, and 0.02% NaN_3 were added and the mixture was stored for two day at 5°C. The resulting crystals of AO were separated by centrifugation for 10 min at 10,000*g* (r_{av} 10 cm) and dissolved in 0.02 M phosphate-citrate buffer (pH 8.0) containing 0.02% NaN_3 and 0.1 mM benzamidine. The enzyme was recrystallized in a similar manner.

The alcohol oxidase from cell-free preparation was also purified by sequential use of traditional methods: 1) a step-wise fractionation by $(NH_4)_2SO_4$ (25-60% saturation) in 0.02 M phosphate-citrate buffer with 0.01 mM benzamidine; 2) dialysis overnight against 1000-fold volume of 0.01 M phosphate-citrate buffer, pH 8.0; 3) fractionation on DEAE-cellulose by a 0-0.4 M linear gradient of NaCl; 4) gel-filtration chromatography on Sepharose 2B. The fractions received after steps 3 and 4 were concentrated by ultrafiltration using a membrane from Vladipor. All purification steps were performed in 0.02 M phosphate-citrate buffer, pH 8.0, containing 0.1 mM benzamidine.

All AO preparations obtained were supplemented with sucrose (50% w/v), frozen in liquid nitrogen, and stored at −20 or −80°C. Before use, AO was thawed and, if necessary, separated from sucrose by gel filtration on a Sephadex G-25 column (3 × 20 cm) equilibrated with 0.02 M phosphate-citrate buffer, pH 8.0.

Determination of enzyme activity. Enzymatic activity of AO was determined by the initial rate of oxidation of α-alcohols at 30°C using a KFK-3-01 electrocolorimeter (ZOMS, Russia). Oxidation rate of α-alcohols was measured by the reaction of formed H_2O_2 with *o*-dianisidine yielding a colored product in the presence of one enzymatic unit of horseradish peroxidase (1 μmol H_2O_2/min per mg protein). The extinction coefficient ε_{460} = 28,800 $M^{-1} \cdot cm^{-1}$ was used for oxidized *o*-dianisidine. One unit of AO enzymatic activity was equivalent to the amount of enzyme producing during oxidation of α-alcohol 1 μmol H_2O_2/min per mg protein at 30°C.

Activity of portions of the purified enzyme and kinetic parameters were measured by initial rate of O_2 consumption via amplitude of a polarographic oxygen record at a direct current of 0.65 V using the closed type of Pt-Ag electrode (analog of a Clark's electrode) as a part of the device designed and manufactured by Biosensor AN (Russia). (It should be noted that activity of AO determined by reaction of H_2O_2 with *o*-dianisidine and peroxidase using purified samples was identical to the activity determined by consumption of O_2 polarographically. The same result was observed using unpurified specimens at the initial stage of reaction when the concentration of consumed O_2 was negligible and was significantly lower than K_m of catalase for H_2O_2.) Further, the activity of AO will be taken as the amount of methanol oxidized per minute per mg of protein because the quantity of consumed O_2 is equal to the quantity of oxidized α-alcohol and of formed H_2O_2.

Physicochemical methods of analysis. Native AO was electrophoresed without SDS in a 5-10% polyacrylamide gradient gel according to Ornstain and Davis [36]. AO in the gel was identified by enzymatic activity: the gel was placed into saturated solution of *o*-dianisidine in 0.02 M phosphate-citrate buffer, pH 8.5, containing 3 units of horseradish peroxidase and 0.1 M methanol. The reaction was carried out for 20 min in volume of 5 ml at 30°C and terminated by acidification with acetic acid to a final concentration of 7% (v/v). The molecular weight of AO peptides was determined by SDS-PAGE [37]. Protein bands in the gel were stained with Coomassie Blue G-250 or R-250 during electrophoresis in the absence or presence of SDS, respectively.

Protein content was evaluated by reaction with bromophenol blue or by the biuret method [38]. BSA was

used as a standard, the solution of which was desalted by gel filtration using a column (3 × 20 cm) with Sephadex G-25 and dried by lyophilization in vacuum (0.05 mm Hg); water vapor was frozen in the glass trap by liquid nitrogen.

Content of FAD in AO was determined spectrophotometrically at 339 nm ($\varepsilon_{339} = 6220\ M^{-1}\cdot cm^{-1}$). For this purpose the enzyme was denatured by 3% TCA or by heating for 3 min at 100°C, the denatured protein was separated by centrifugation (5 min at 10,000*g*, $r_{av} = 8$ cm), and the supernatant was neutralized with 0.1 M NH_4OH and the concentration of FAD in the supernatant was determined. Concentration of oxidized FAD was additionally measured spectrofluorimetrically with an SPF-500 spectrofluorimeter (Aminco, USA) using a standard curve ($\lambda_{ex} = 450$ nm, $\lambda_{em} = 520$ nm).

Sedimentation coefficient ($s^0_{20,w}$) and diffusion coefficient ($D^0_{20,w}$) of AO were determined by ultracentrifugation on an Optima L-90K analytical ultracentrifuge (Beckman, USA). Molecular weight was calculated on the basis of $s^0_{20,w}$ and $D^0_{20,w}$ values at the assumed partial density of protein 0.73 using Svedberg's equation [39]: $M = sRT/D(1 - \nu\rho)$, where M is molecular weight, s is the sedimentation coefficient, R is the gas constant, T is absolute temperature, D is the diffusion coefficient, ν is partial specific volume of protein, and ρ is density of solution. $s^0_{20,w}$ was determined based on sedimentation rate using schlieren optics and $D^0_{20,w}$ on sedimentation equilibrium at 6000 and 12,000 rpm registered at 280 nm.

In addition, molecular weight was determined using the method of gel chromatography in a column with Sepharose 2B (2 × 90 cm) calibrated with proteins with different molecular weights.

Free SH-groups were quantitatively determined after 30-min incubation of 0.005 mM enzyme solution at 20°C with 1 mM dithiothreitol and 0.5 mM EDTA. The reagents were eluted by anaerobic gel filtration on a column (2 × 30 cm) with Sephadex G-25. The quantity of free cysteine residues of the enzyme was determined by reaction with DTNB by rate of DTNB oxidation at 412 nm using coefficient of molar extinction of 5-thio-2-nitrobenzoate (14,150 $M^{-1}\cdot cm^{-1}$ at pH 8.0).

Electron microscopy of AO was carried out using an EM 400 electron microscope (Phillips) (magnification 50,000 times at 80 kV) as described earlier [40].

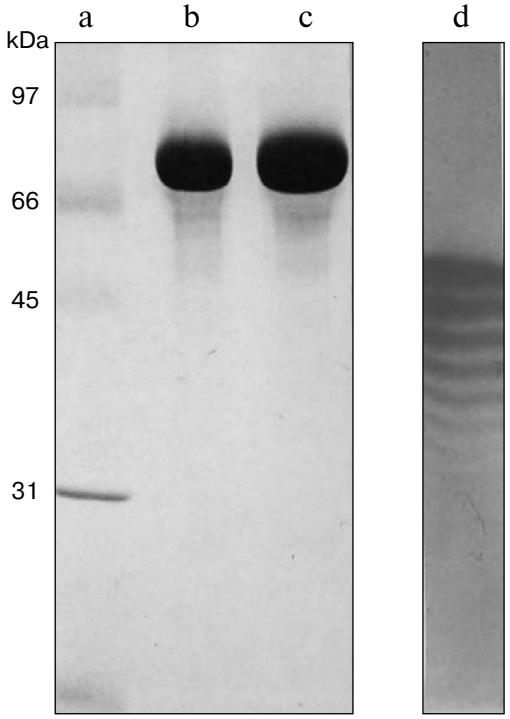

Fig. 1. Electrophoresis of alcohol oxidase in polyacrylamide gel. a) Protein standards in presence of SDS; b) 100 μg of AO with SDS; c) 200 μg of AO with SDS (a single protein band was observed in SDS-PAGE of AO with 6-15 mg protein); d) activity of AO in polyacrylamide gel without SDS in 0.02 M phosphate-citrate buffer, pH 8.5, at 30°C (activity-based staining as a result of the enzymatic reaction with methanol in the presence of *o*-dianisidine and horseradish peroxidase).

RESULTS AND DISCUSSION

Results of AO purification are presented in the table. Apparent purification efficiency was equivalent to 5.6-fold (in various experiments it varied from 4 to 7). Specific activity was about 50 units/mg. In different experiments it varied from 15 to 50 units/mg. This could be explained by differences in yeast growth conditions and in methods of AO purification. Based on separation efficiency the contents of AO in *P. putida* cell was 10-20% of total cell protein, and this is close to the contents of AO in other methylotrophic yeasts [4-12].

The AO preparations isolated by different methods in SDS-PAGE contained a single band (Fig. 1, b and c). In SDS-free PAGE, preparations of native AO (purified by different methods) possessed 8-9 protein bands (Fig. 1d). The amount of protein was determined by densitometry of the gel. Intensity of bands decreased from band to band as R_f increased by 2-3 times. Therefore intensity of bands with higher R_f was weak. Protein bands with high R_f could not be detected in general because of low band intensity in the gel.

Alcohol oxidase enzymatic activity in polyacrylamide gel without SDS in the presence of methanol (or other alcohols; the bands were not detected without alcohols) was estimated as intensity of formed color of bands and was detected as AO activity and corresponded to protein bands observed in the same gels with Coomassie R-250. All observed protein bands had similar specific activity and substrate specificity. These proteins were able to oxidize methanol (100 ± 2%), ethanol (92 ± 2%), propanol (75 ± 3%), butanol (40 ± 2.5%), pentanol (3 ±

Purification of *P. putida* methanol oxidase by salting out with ammonium sulfate with subsequent crystallization

Purification step	Fraction volume, ml	Protein amount, g	Specific activity*, unit/mg	Yield by activity, %
Fraction above pellet (25% $(NH_4)_2SO_4$ saturation)	1187	53.4 ± 1	8 ± 7	100
Pellet (60% $(NH_4)_2SO_4$ saturation)	273	19.1 ± 0.1	21 ± 6	92
Crystallization (dissolved pellet)**	76	7.6 ± 0.1	46 ± 5	80

* AO activity was determined in four parallel samples. Before determination, salts were removed from the AO preparation by gel filtration on a Sephadex G-25 column equilibrated with 0.02 M phosphate-citrate buffer, pH 8.5.

** Before crystallization, the AO preparation was ultrafiltered in the presence of water and then of 0.02 M phosphate-citrate buffer, pH 6.2.

1%), and formaldehyde (35 ± 3%). Several active AO bands in gel was shown earlier in other yeast [25].

One symmetric peak of protein was detected during gel filtration of AO on a column with Sepharose 2B (1.2 × 0.02 m; elution with 0.02 M phosphate-citrate buffer, pH 8.0, containing 0.1 M NaCl and 0.1 M benzamidine). The electrophoresis of AO from a peak maximum under the conditions specified in the legend to Fig. 1d showed the same set of bands. The molecular weight of the AO determined by gel filtration is 590 ± 50 kDa.

One symmetric peak (Fig. 2) with sedimentation coefficient $s^0_{20,w}$ = 19.2 S and diffusion coefficient $D^0_{20,w}$ = 3.1 cm^2/sec was observed during analytical ultracentrifugation. The presence of one symmetric peak during gel filtration and analytical ultracentrifugation indicates molecular homogeneity of the preparation. The molecular weight of the AO according to sedimentation (at suppose partial volume of protein, 0.73) is 580 ± 40 kDa. Approximately the same molecular weight was obtained by gel filtration through Sepharose 2B. The molecular weight of the subunit of the AO determined by electrophoresis in a 5-15% linear gradient polyacrylamide gel with SDS was 74 ± 4 kDa. Thus, the AO from *P. putida* is an octamer and contains eight peptides with identical (or very similar) molecular weight.

The cause of multiple bands during SDS-free PAGE with identical molecular weight of AO can be presence in yeasts used in the experiments of two genes which participate in a synthesis of two peptides, apparently, depending on concentration of methanol in the culture medium. These peptides should have similar molecular weights, but they should differ in the number of acidic and basic amino acids. Such assumption is based on experiments [41] that revealed in other methanol-oxidizing yeast strain two genes whose content in a cell depends on methanol concentration in the culture medium.

The number of FAD molecules determined by different spectral methods is to 8 ± 0.8 per molecule of the enzyme. This suggests that each AO subunit contains one FAD molecule.

The dependence of activity of AO on concentration of protein is approximately linear and extrapolates to zero. Such dependence is characteristic of undissociated polysubunit enzymes or enzymes whose dissociation to separate subunits or conformational changes in separate subunits do not affect enzymatic activity.

The maximum activity of the enzyme is observed at pH 8.5-9.0 (Fig. 3), which is close to the optimum pH observed for AO from other sources [8-13].

The Lineweaver–Burk dependence for O_2 under conditions of excess of oxidizable substrate was linear over the studied range of molecular oxygen concentrations. Value of K_m for O_2 determined of the dependence is 0.25 mM.

Enzyme activity was inhibited completely by substances reacting with sulfhydryl groups (*p*-chloromercuribenzoate and DTNB, molar excess of reagents in relation to AO 20 : 1) whose content in AO, according to DTNB titration, is equal to 17 ± 0.5. It is possible to

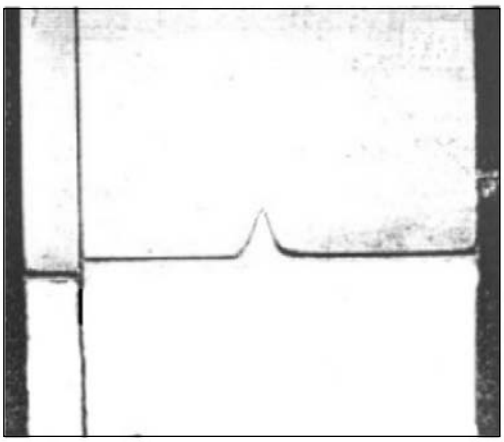

Fig. 2. Ultracentrifugation of alcohol oxidase from *P. putida*. Concentration of protein was 8 mg/ml in 0.01 M citrate-phosphate buffer, pH 8.5; 50,000 rpm (140,000*g*), 30 min, 5°C. (A similar pattern was observed for all methods of AO purification; concentration of protein was varied from 3 to 10 mg/ml.)

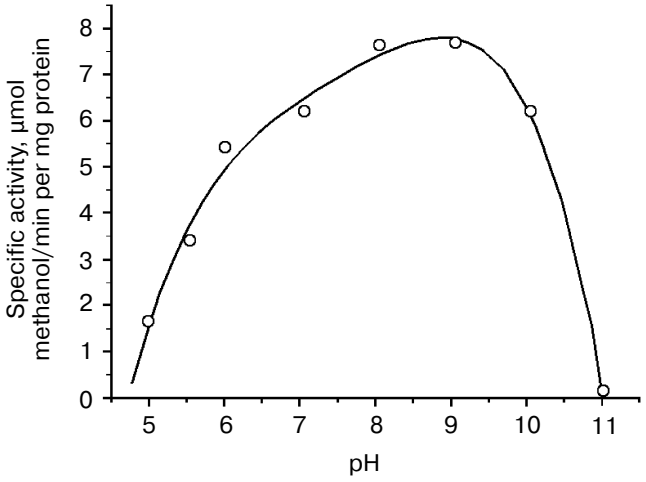

Fig. 3. The pH dependence of activity of alcohol oxidase from *P. putida*. Concentration of protein, 0.05 mg/ml; 0.02 M phosphate-citrate buffer, pH 8.5; 30°C.

assume that these SH-groups are evenly distributed between subunits of the enzyme (two SH-groups per each subunit).

The AO from *P. putida* possesses relatively low thermostability: 10% of activity is lost on storage within 24 h at 20°C, and the enzyme is completely inactivated within 10 min at 60°C. The AO has increased stability in phosphate-citrate buffer and low stability in Tris-HCl and phosphate buffers. Its stability is increased tenfold in the presence of sucrose, mannitol, polyethylene glycol, polyvinyl alcohol, polyvinylpyrrolidone, and EDTA. Sucrose at 50% concentration (w/v) enhances stability of the enzyme tenfold at 50°C. Supposedly, stability of AO strongly depends on viscosity of the medium and the presence of mild reducing agents and compounds chelating transition metal ions, which are able to catalyze oxidation of functional groups of protein such as Cys SH-groups. The enzyme can be stored without change in activity for four years in 0.02 M phosphate-citrate buffer, pH 8-9, in presence of 30-50% (w/v) sucrose at temperatures from −20 to −80°C.

General views of negatively contrasted specimens of AO from *P. putida* (Fig. 4a) and *H. polymorpha* (Fig. 4b) show separate molecules. Separate molecules are observed in negatively contrasted AO specimens from *P. putida* (Fig. 4a) and *H. polymorpha* (Fig. 4b). Different variants of molecular images are observed. The first type is represented by square projections with side of 12 ± 0.5 nm consisting of four protein spots. There are also some types of particles in projections of which two parallel protein layers are separated by a fine layer of contrasting material. The width of these peaks of images is 11 nm, but they differ in form: there are right-angled particles with length about 11-12 and 13-13.5 nm, respectively, and also trapezoidal particles. Analysis of the images shown in Fig. 4a indicated that a molecule of AO from *P. putida* consists of eight subunits with a dotted symmetry $422D_4$ in vertexes of an angle of two squares. The molecular size is 12 × 12 × 11 nm. Comparison of separate types of images showed the first type is attributed to head-on projection (about an axis of fourth order), and images on which two protein layers are lateral projections (perpendicular to axes of fourth order). The view of the lateral projection particle images of trapezoidal form indicates that two square tetramers not completely cover each other along an axis of the fourth order, and are slightly turned about the axis. Comparative electron microscope studies of AO from two sources, lead to the present work, showed that the packaging of subunits of AO from these sources is identical. These conclusions are consistent with electron microscopy of single molecules in two-dimensional crystals of AO from *H. polymorpha* [42, 43].

Enzyme crystals with different sizes and forms were obtained in a hanging drop and in a volume with polyethylene glycol as precipitating agent. None of these forms yielded X-ray diffraction, which could be an indication of

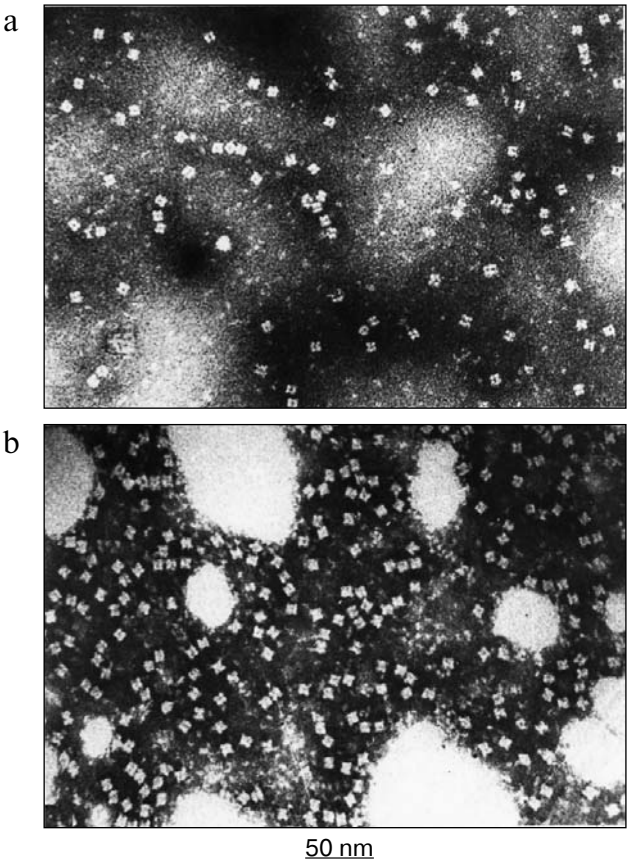

Fig. 4. Electron microscopy of single molecules of alcohol oxidase from *P. putida* (a) and *H. polymorpha* (b). AO on a carbon emulsion carrier was negatively contrasted by 1% uranyl acetate and was desiccated in vacuum. Magnification, ×300,000.

their intramolecular heterogeneity. The result of such heterogeneity could be the presence in an enzyme preparation of different combinations of two various subunits (theoretical estimation of the number of combinations is about nine). We also cannot exclude observed earlier post-translational modifications of FAD depending on culture conditions [15, 17, 18, 44]. They could also affect peptide structure of AO subunits.

Crystals of AO from *P. pastoris* have been prepared earlier and only parameters of the unit cell were determined by X-ray crystal analysis with resolution only 6 Å [45, 46]. However, spatial structure of the enzyme was not further refined. The possible cause of failures could be associated with the suggested intermolecular microheterogeneity of the enzyme. Preparation of mutants lacking one of the genes responsible for synthesis of peptides of AO could be used to avoid these difficulties, as has been done earlier [20], or by selection of conditions of growing the yeast under which mainly one peptide is synthesized.

Our studies show that AO from *P. putida* is similar in macromolecular structure to AO from other sources, and has a dotted symmetry $422D_4$. The enzyme probably occurs as an octamer like the AO from *P. pastoris* [41], from two genetically determined polypeptides with similar molecular weight, containing one molecule of FAD, including possibly modified FAD [15, 17, 18, 44]. As follows from the data of SDS-free PAGE, these subunits slightly differ from each other by quantitative content of amino acids but have a different ratio of acidic and basic amino acids. The enzyme isolated in this study differs from AO from other sources by a number of kinetic parameters and stability.

A large-scale method for enzyme isolation and purification was developed and conditions for its long-term storage (more than four years without loss of activity in presence of sucrose at temperatures from −20 to −80°C) were established, which significantly reduces its cost. Therefore AO from *P. putida* can be used for practical semiquantitative and quantitative determination of low molecular weight alcohols by various methods including methods of dry chemistry.

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