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Purification of amine oxidase from *Pisum sativum* for the construction of amine biosensors

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Abstract. Copper-containing amine oxidase was purified from the extract of pea seedlings (*Pisum sativum*) for a possible application as a bioselective compound of amine biosensors. The specific activity of the purified enzyme at different purification steps was characterized with the help of a Clark-type oxygen sensor by measuring the dissolved oxygen consumption during the oxidation of amine compounds. This sensing system was characterized with a steady-state output parameter calculated on the basis of the dynamic model of biosensors. Among the studied amine compounds pea seedlings amine oxidase (PSAO) was found to have the highest catalysing activity for cadaverine. The three-step purification procedure of PSAO revealed 35-fold purification and an enzyme preparation ready for application in biosensors.

Key words: pea seedlings amine oxidase, purification, catalytic activity.

INTRODUCTION

The copper-containing amine oxidases (monoamine oxidase, semicarbazide-sensitive amine oxidase, histamine deaminase, amine oxygen oxidoreductase) are ubiquitous metalloenzymes as they have several essential functions in the metabolism of primary amines. They have been purified and characterized from the majority of living organisms, such as mammals [1], plants [2], fungi [3], yeasts [4], and bacteria [5]. The most extensively studied amine oxidases are from *Escherichia coli*, *Arthrobacter globiformis*, *Hansenula polymorpha*, and *Pisum sativum* [4, 6–8].

Pea seedlings (*Pisum sativum*) have been found to contain a substantial amount of amine oxidase (EC 1.4.3.6), which catalyses the oxidation of amines

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by dissolved oxygen. There is about 0.6% of this enzyme in the crude extract of germinated seeds [9]. The enzyme can be used as a selective component in biosensor systems for the determination of amines, but before application it has to be purified to eliminate possible side reactions and to ensure a fast and reliable analytical signal.

It has been shown that amine oxidases are homodimeric enzymes that contain two active centres per one molecule. Each subunit contains also a cofactor, 2,4,5-trihydroxy-phenylalanine quinone (TPQ) and a single copper(II) ion in the active site [10].

The molecular weight of pea seedlings amine oxidase (PSAO) has been found to be between 150 and 180 kDa [2, 7].

Amine oxidase catalyses the oxidative deamination of amines to the corresponding aldehyde, ammonia, and hydrogen peroxide [8, 11]:

$$R–CH_2–NH_2 + O_2 + H_2O \longrightarrow R–CHO + NH_3 + H_2O_2. \quad (1)$$

This bisubstrate (an amine and oxygen) reaction proceeds in several steps and can be characterized by ping-pong reaction mechanism [12, 13]. This reaction forms the basics of the application of this enzyme, as the rate of oxygen consumption in the reaction depends directly on the concentration of the amine. The change of oxygen concentration can be easily followed with several electrochemical or optical devices.

So, in combination with amperometric transducers, the copper-containing amine oxidases have been used as a biosensor bioselective material for the determination of amines in foods and drinks [14, 15]. There exist also biosensors for the determination of amines with optical (chemiluminescent) transducing systems that detect hydrogen peroxide [16] and potentiometric transducing systems based on the detection of ammonium concentration [17].

Besides the measurement of amine concentration, all these biosensor devices enable the characterization of the enzyme's catalytic properties [3].

In the present study we investigated application possibilities of PSAO as the biocomponent of Clark type oxygen sensor based biosensors for the determination of amines.

EXPERIMENTAL

Purification of PSAO

Pea seedlings were washed, germinated at 25°C in the dark, and harvested after 7 days. The germinated seeds were homogenized with a mincer and the coarse pellet was removed by centrifugation at 5000 *g* for 60 min. The supernatant fraction was collected, ammonium sulphate added (30% w/w), and after stirring for 30 min centrifuged at 5000 *g* for 60 min. The supernatant fraction was collected for further purification and the pellet discarded. The concentration of $(NH_4)_2SO_4$ in the solution was increased to 70% (w/w). The mixture was

incubated by stirring for 30 min at 4 °C. The precipitated fraction was collected by centrifugation at 5000 *g* for 60 min and dissolved in 20 mL of 0.1 M potassium phosphate buffer solution (pH 7.0).

The solution was dialysed first for 12 h against 800 mL 0.1 M potassium phosphate buffer and then for 12 h against 800 mL 0.02 M potassium phosphate buffer (pH 7.0) [13]. The dialysed solution was mixed with triethylaminoethyl cellulose (Reachim in Russia, 0.02 M potassium phosphate buffer, pH 7.0) and the mixture was loaded onto a column (Ø 2.5 cm, h = 40 cm). The column was washed with 600 mL 0.02 M potassium phosphate buffer (pH 7.0) and the enzyme was eluted with 0.1 M KCl in 0.02 M potassium phosphate buffer (pH 7.0). The eluate was collected into fractions of 11.5 mL. The enzyme-containing fractions were combined and dialysed overnight against 800 mL 0.02 M potassium phosphate buffer (pH 7.0). At the next step, the obtained fraction was carried onto a Sephadex G-200 (Pharmacia, Sweden) column (Ø 1.5 cm, h = 90 cm), equilibrated with 0.02 M potassium phosphate buffer (pH 7.0), eluted with the same buffer at the rate 0.15 mL/min, and collected into fractions of 2 mL. All purification procedures were performed at 4 °C.

Protein concentration and molecular mass assay

The protein pattern in chromatographic steps was determined by absorption at λ = 280 nm using an optical monitor UV-1 (Pharmacia, Sweden). The protein concentration in separate fractions was determined spectrophotometrically by the Lowry–Hartree method using bovine serum albumin as a standard [18].

Kinetic measurements and data acquisition for the determination of PSAO activity

All kinetic measurements were carried out under continuous stirring in an airtight thermostatted glass cell at 25 °C in air-saturated 0.1 M phosphate buffer solution (pH 7.0) [5, 19]. The reaction was started by the injection of the enzyme sample into the reaction cell after the output of the oxygen sensor had stabilized in the reaction medium. The concentration of dissolved oxygen in the reaction medium was determined with a cylindrical membrane-covered oxygen sensor (Elke Sensor LLC) and registered by an automated data acquisition system.

The sensor output signal was recorded automatically with the interval of 1 s, the data were normalized ($I(t)/I_0$), debugged, and the parameters characterizing the reaction were calculated with the dynamic biosensor model by nonlinear least-squares regression analysis. The statistical significance of different fittings for curves was determined by F-test. Deviations of experimental data from the model were estimated by the distribution of the residuals and the P value from the runs test.

For the comparison of different PSAO samples 0.12 mM cadaverine solution was used as the standard.

The basic principles of the dynamic biosensor model

A dynamic model for amperometric biosensors was designed to take into account the kinetics of the ping-pong mechanism enzyme reaction, substrate diffusion, and the inertia of diffusion-limited sensors. It enables the prediction of steady-state parameters from the biosensor transient response with errors less than 3% with no need for additional determination of the system's geometrical, diffusion, and partition parameters [20].

According to this model, the normalized biosensor output current $I(t)/I_0$ can be expressed as a 3-parameter function of time *t*:

$$\frac{I(t)}{I_0} = A\exp(-Bt) + (1-A) - 2A\sum_{n=1}^{\infty}(-1)^n \frac{\tau_s}{n^2/B - \tau_s}\left[\exp(-Bt) - \exp\left(-n^2\frac{t}{\tau_s}\right)\right], \quad (2)$$

where $I(t)$ is the biosensor output current at the time moment *t*; I_0 is the output current at the start of the reaction; t is time. The parameters A and B are complex coefficients, both depending hyperbolically on substrate concentration. The parameter A corresponds to the total possible biosensor signal change when time $t \to \infty$ (steady-state signal) and the parameter B to the kinetic parameter (the initial maximal slope of the enzyme-catalysed process curve); τ_s is the time constant of internal processes taken together of oxygen transducer and characterizing the inertia of the transducer's response [20]. All these three parameters *A*, *B*, and τ_s are independent of each other.

RESULTS AND DISCUSSION

Copper-containing amine oxidase is an enzyme that can possibly be used as a selective sensing system of oxygen sensor-based biosensors for the determination of amines. Seedlings of pea (*Pisum sativum)* are suggested as a good source for the extraction of amine oxidase as according to earlier studies amine oxidase extracted from this source has a relatively high specific enzymatic activity. At the same time, it is also the cheapest and most comfortable material for the extraction of the enzyme.

To study the selective activity of PSAO towards different amines, we screened eight compounds, including primary, secondary, and tertiary amines, by using the crude homogenate of pea seedlings as the catalyst of the oxidation reaction of amines (Table 1). Among the studied amines there were several so-called biogenic amines, which are products of the degradation of organic matter and may be a cause of allergenic reactions [21–23]. All the eight studied compounds caused relevant changes in the sensor output, although the effective concentrations to cause a detectable response differed about 1000 times for different amines. According to the effective concentration ranges, the studied amines formed two clearly distinguishable groups – primary amines (cadaverine,

1,7-diaminoheptane, histamine, and dopamine) and secondary plus tertiary amines (Table 1).

The dependence of the response of the biosensor as the calculated steady-state output (parameter *A*, Eq. 2) on the cadaverine concentration is presented in Fig. 1. Dependences of this kind for all studied amines allowed the calculation of the values of the parameters *A* and K_S, which characterize the maximal responses and optimal substrate concentrations of the studied reactions, respectively (Table 1). Only for diethylamine and aniline the dependences had no clear hyperbolic character at concentrations up to 400 mM and therefore reliable data for these substrates could not be obtained.

Table 1. Electrochemically determined catalytic properties of amine oxidase-containing crude *Pisum sativum* extract (0.1 M phosphate buffer, pH 7.0, 25 °C). Total signal change *A* is a complex parameter, corresponding to the total possible biosensor signal change when time $t \to \infty$ (steady-state signal). K_S is the dissociation constant for the enzyme–substrate complex, which was calculated according to the biosensor dynamic model [20]

Substrate	Total signal change *A*	K_S, mM
Cadaverine	0.90	0.29 ± 0.10
Histamine	0.43	0.15 ± 0.02
Dopamine	0.27	0.043 ± 0.012
Methylamine	0.12	2.1 ± 2.3
Diethylamine	0.35	329 ± 183
1,7-Diaminoheptane	0.71	0.43 ± 0.11
N, N-Diethylaniline	0.19	5.0 ± 2.8
Aniline	0.37	1660 ± 2350

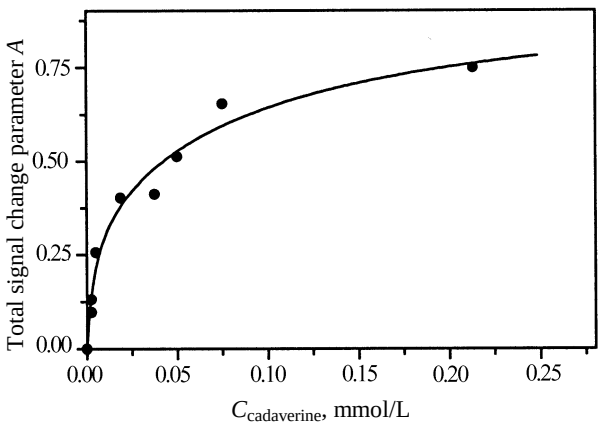

Fig. 1. The dependence of sensor response as the total signal change parameter (calculated as described in Methods) on cadaverine concentration. Experiments were carried out under constant stirring in air-saturated solutions at 25 °C, pH 7.0 (0.1 M phosphate buffer) with the crude extract of *Pisum sativum* (c_{PSAO} = 59.6 nkat/mL).

Among the studied compounds, cadaverine caused the greatest change in the total signal parameter. The response of the used biosensor system to cadaverine was 90% of its maximal working range and it was at least 2 times as high as in the case of other primary amines (Table 1). High sensitivity of the system makes cadaverine a useful tool for the characterization of amine oxidase activity in different enzyme probes as a reference substrate.

The value of K_S for cadaverine was 0.29 ± 0.10 mM (Table 1). This constant varies in a broad range for amine oxidases from different sources, having the values for amine oxidase of plant origin from 0.038 mM (*Trifolium subterraneum*) [19] to 0.7 mM (*Lens esculenta*) [24].

For the purification of PSAO we followed the three-step protocol [13], which revealed 35-fold purification with the yield of 52%. The specific activity of the final enzyme probe was 1561.1 nkat/mg protein (Table 2). For the purification of amine oxidase from pea seedlings the highest purification yield achieved has been 42% and specific activity 1.63 μkat/mg [8]. The purification efficiency has been substantially lower when other sources of the enzyme (e.g. pig kidney) were used [25].

The greatest loss of the enzyme was during the first fractionation steps. However, precipitation with 70% $(NH_4)_2SO_4$ was very effective, revealing 5-fold purification with 65% yield (Table 2).

After fractionation the enzyme was bound to TEAE resin and the probe eluded from the column as a single peak with 0.1 M KCl according to the electrochemical determination of the specific activity of PSAO (Fig. 2). Amine oxidase activity was detected in a quite large elution volume with a well-expressed maximum and it was on average 557.7 nkat/mg protein in fractions collected between 246 to 316 mL and used for further purification. A substantial amount of the protein was eluded at the beginning of the application of 0.1 M KCl, and the second peak of the total protein eluded just before the enzyme activity began to rise in the elution volume. The purification efficiency of ion exchange chromatography was 2.4 times.

Table 2. Characterization of purification steps of amine oxidase from pea seedlings and the efficiency of different steps

No	Purification step	Total protein, mg	Specific activity, nkat/mg protein	Purification	Yield of enzyme activity, %
1	Crude extract	1198	44.9	1	100
2	Extract, after centrifugation	926	49.8	1.1	85.7
3	Soluble faction in 30% $(NH_4)_2SO_4$	534	67.6	1.5	67.1
4	Pellet in 70% $(NH_4)_2SO_4$	152	230.8	5.1	65.2
5	TEAE-cellulose chromatography	53	557.7	12.4	55.0
6	Sephadex G-200 chromatography	18	1561.1	34.8	52.2

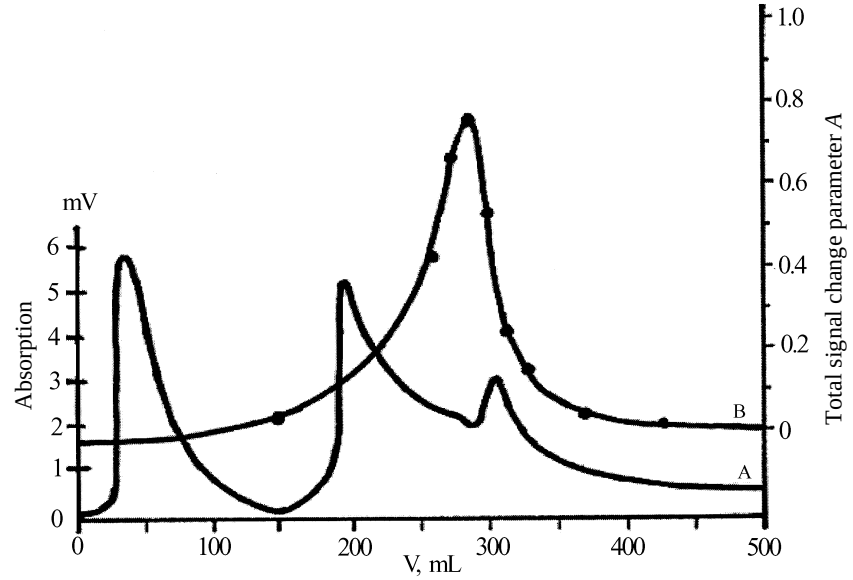

Fig. 2. Ion exchange chromatography of PSAO-containing extract on TEAE cellulose, elution with 0.1 M KCl in phosphate buffer (pH 7.0). The protein concentration was determined spectrophotometrically as absorption at λ = 280 nm (A). Amine oxidase (PSAO) activity was determined electrochemically by 0.12 mM cadaverine (B).

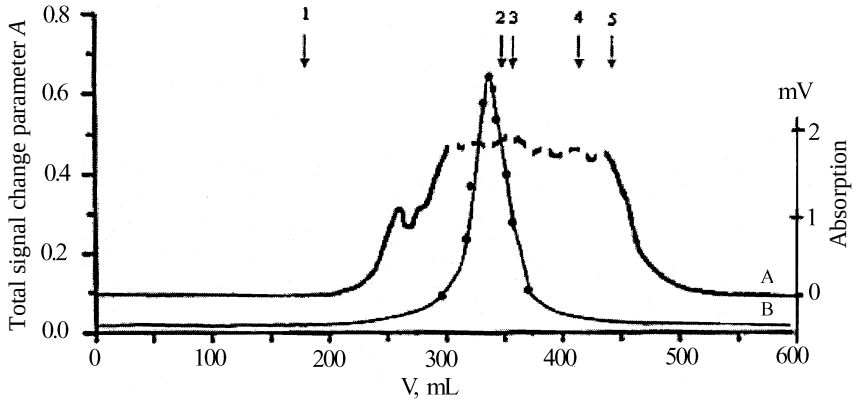

Fig. 3. Gel permeation chromatography of PSAO-containing extract on Sephadex G-200 column. The protein concentration was determined spectrophotometrically as absorption at λ = 280 nm (A). The amine oxidase (PSAO) activity was determined electrochemically at 0.12 mM cadaverine (B). Arrows show the eluation volume of the molecular weight markers: 1 – alcohol oxidase (600 kDa), 2 – catalase (232 kDa), 3 – glucose oxidase (158 kDa), 4 – bovine serum albumin (66 kDa), and 5 – cytochrome c (12.6 kDa).

The collected PSAO-containing fraction was finally carried onto a Sephadex G-200 column (Fig. 3). The PSAO activity was detected in a single narrow peak

of the elution volume. The molecular weight of PSAO, determined by gel permeation chromatography, was 220 ± 25 kDa, which is slightly greater than 150–180 kDa found by other authors [2, 7]. The yield of the purification process with ion exchange and gel permeation chromatographic methods combined was 78%, which is an excellent result compared with earlier studies of amine oxidase purification [8, 13, 25].

The specific activity of the purified enzyme towards cadaverine was detected electrochemically. The catalytic properties of PSAO did not change during the purification process.

In conclusion it can be said that copper-containing amine oxidase from *Pisum sativum* is a promising bioselective material for the construction of amine biosensors. PSAO has high selectivity towards primary amines and it can be efficiently purified with simple ion exchange and gel permeation chromatographic methods.

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Pisum sativum'i amiini oksüdaasi puhastamine ensüümi kasutamiseks biosensoris

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On uuritud *Pisum sativum*'i amiini oksüdaasi (PSAO, E.C 1.4.3.6) puhastamise võimalusi selle ensüümi kasutamiseks biosensori bioselektiivse elemendina. PSAO selektiivset aktiivsust erinevate amiinide suhtes mõõdeti hapnikuanduri abil. Ensüümi puhastamise erinevate etappide efektiivsust iseloomustati hapnikuanduri väljundsignaali dünaamika alusel arvutatud PSAO poolt katalüüsitavate reaktsioonide parameetrite abil. Kolmeetapilisel PSAO puhastamisel suurenes ensüümi eriaktiivsus 35 korda, mis loob eelduse saadud ensüümpreparaadi kasutamiseks biosensorites.