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Purification and properties of amine oxidase from pea seedlings

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Abstract. Amine oxidase (EC 1.4.3.6) was purified from pea (*Pisum sativum*) seedlings with the aim of characterizing its catalytic properties. The specific activity and selectivity of the enzyme were studied with an oxygen sensor by following the kinetics of the amine oxidation reaction, catalysed by the enzyme. The enzyme catalytic constants were calculated from the transient signal of the oxygen sensor with the help of a model proposed earlier for amperometric biosensors. The pea seedlings amine oxidase had the highest activity towards putrescine and cadaverine; a very low or zero activity was registered towards other studied amines. To characterize different steps of the purification process, we compared the activity of enzyme preparations towards 0.15 mM cadaverine. The molecular mass of the purified enzyme was 184.0 ± 2.6 kDa.

Key words: amine oxidase, *Pisum sativum*, purification, enzyme activity, oxygen sensor.

INTRODUCTION

Amine oxidases present a class of enzymes, which are divided into two main groups based on the chemical nature of the attached cofactor. The enzymes whose molecules contain flavin adenine dinucleotide belong to the group of flavin adenine dinucleotide (FAD) containing amine oxidases (FAD-AOs, EC 1.4.3.4); copper-containing amine oxidases have a tightly bound copper ion in their molecule (CuAOs, EC 1.4.3.6). Amine oxidase from pea seedlings is reported to belong to the second group [1]. The two groups of amine oxidases are also distinct in terms of their substrates, inhibitors, subcellular distribution, and biological functions [1–3].

Copper-containing amine oxidases can be found in all kinds of organisms such as bacteria, yeasts, mushrooms, various plants, and animals [2–10]. This enzyme

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catalyses the oxidative deamination of mono- and polyamines [11, 12]. The products of the CuAO-catalysed oxidative deamination of amines are various aldehydes, ammonia, and hydrogen peroxide. The summarized reaction scheme is as follows, although the reaction follows a classical ping-pong mechanism [3]:

$$RCH_2NH_2 + O_2 + H_2O \xrightarrow{CuAO} RCHO + H_2O_2 + NH_3.$$

The available data about the enzyme substrate specificity towards different substrates vary widely and are different for enzyme preparations from different sources. The best substrates for all preparations are 1,4-diaminobutane (putrescine) and 1,5-diaminopentane (cadaverine), which in nature are produced along with histamine and other biogenic amines in protein hydrolysis during putrefaction of tissues both in living and dead organisms.

Pea seedlings amine oxidase (PSAO) has been found to have a 100% relative specific activity towards 1,4-diaminobutane, 111% towards 1,5-diaminopentane, 56% towards agmatine and spermidine, 44% towards 1,6-diaminohexane, 30% towards histamine, 8% towards spermine, and no activity towards 1,3-diamino-propane [13]. It has also been reported that PSAO has at least 3 times higher specificity than other plant CuAOs towards histamine [9]. According to the literature, 1,3-diaminopropane is not a substrate for plant CuAOs, its catalytic oxidation has been found on a scant range only in CuAO from the broad bean (*Vicia faba*) [14].

Generally the amine oxidase activity and the K_m values for different substrates are studied with spectrophotometrical methods [14, 15], but also electrochemical methods are used. In the latter case, the amine oxidase activity is assayed by a sensor, monitoring either the liberation of NH_3, H_2O_2, or aldehyde [16], or by the consumption of oxygen [17]. The electrochemical methods are easy to employ. Their main drawback is the comparatively great experimental noise and difficulties in determining the exact reaction characteristics from the sensor signal, as the signal approaches its final value asymptotically. The available catalytic constants are quite dispersed and finding comparable data is problematic.

The aim of the present study was to extract and purify amine oxidase from pea seedlings, to determine the activity and selectivity of the purified enzyme towards different amine substrates with a Clark-type oxygen sensor, and to examine the possible application of the enzyme as a bioselective element in amine biosensors.

MATERIALS AND METHODS

Enzyme purification and estimation of the molecular weight

Pea (*Pisum sativum*) seeds were washed with 0.5% $KMnO_4$ solution to keep mould and bacteria from growing and germinated in dark at room temperature. After 3–16 days of germination, the seedlings were collected, homogenized, and centrifuged at 27 000 *g* for 30 min (4°C). The supernatant was brought to 30%

saturation in ammonium sulphate solution, stirred at 4 °C for 30 min and centrifuged again at 27 000 *g* for 20 min. Ammonium sulphate was added to the supernatant up to 70% saturation, the precipitate was separated by centrifugation at 27 000 *g* for 20 min, and dissolved in 0.1 M potassium phosphate buffer (pH 7.0). The solution was dialysed against 0.1 M and 0.2 M potassium phosphate buffer (pH 7.0) for 24 h. The dialysed solution was stored at −18 °C and used as a crude extract for kinetic measurements.

For further purification, the dialysed solution was applied to a diethylaminoethyl (DEAE) cellulose column (Ø = 2.5 cm, l = 20 cm). The column was washed with 0.02 M potassium phosphate buffer (pH 7.0) at a flow rate of 9 mL/h and eluted with 0.1 M potassium phosphate buffer (pH 7.0) containing 0.1 M KCl (flow rate 9 mL/h). The enzyme eluted as a single peak, and the PSAO activity in the probe was determined amperometrically. The PSAO containing mixture was loaded onto a column of Sephadex G-200 (Ø = 1.5 cm, l = 53 cm) and washed with 0.02 M potassium phosphate buffer (pH 7.0) at a flow rate of 9 mL/h. All purification steps were carried out at 4 °C and after every step the enzyme activity was estimated. The amine oxidase preparation was stable at −18 °C and there was no detectable loss of activity in 6 months at this temperature.

The molecular mass of PSAO was determined by gel filtration at 4 °C using a Sephadex G-200 column (Ø = 1.5 cm, l = 53 cm). The probe was eluted with 0.1 M potassium phosphate buffer (pH 7.0) at a flow rate of 9 mL/h. Dextrin blue (2000 kDa), alcohol oxidase (600 kDa), catalase (232 kDa), glucose oxidase (158 kDa), bovine serum albumin (66 kDa), and cytochrome C (12.6 kDa) were used for column calibration.

Enzyme assay

An amperometric oxygen sensor was used to follow the decrease in the oxygen concentration in the course of amine oxidation in the reaction medium. We calculated the maximum signal change parameter A (corresponding to the steady state) from the normalized transient sensor signal as described earlier in [18, 19]. This parameter A was used for the characterization of amine oxidase activity. According to the applied model, parameter A is the difference between the initial (at time moment $t = 0$) and the steady state ($t \to \infty$) values of the sensor signal and it depends hyperbolically on the substrate and enzyme concentrations. This parameter A does not depend on the inertia of the oxygen sensor [18]. When operating with normalized experimental data, it has no dimension and its value is between 0 and 1. In its physical meaning, parameter A is a function of the catalytic constant k_{cat}^{*}, binding constants of an amine K_m and oxygen K_{O_2} and oxygen diffusion constant $k_{diff}^{O_2}$ [18].

The kinetic measurements were carried out under continuous stirring in an airtight and thermostatted glass cell (volume 38 mL) in air-saturated 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C. Injecting amine oxidase into the reaction medium started the reaction. The sensor output was registered at 1 s

intervals until the average signal change was less than 1% in 200 s. Each experimental curve consisted of 1000–2000 data points.

The PSAO specific activity in enzyme preparations was determined as IU per 1 mg protein. The total protein amount was determined spectrophotometrically using the Lowry method.

RESULTS AND DISCUSSION

First, the specific activity of PSAO in the extract of pea seedlings towards 1,5-diaminopentane was studied during the germination of pea seeds to find the optimal time for harvesting. The specific activity per 1 mg enzyme extract increased steadily along with the increase in the cotyledon mass during the first week, reached the maximum on day eight, and then began to drop (Fig. 1). The maximum specific activity was always achieved on day eight, although the absolute values of the specific activity were different in different experimental lots. So, for further purification and kinetic measurements we used seedlings harvested on day eight of the germination, when the enzyme content was the highest. At the maximal value of $A = 1$ (or the whole possible 100% working range of the sensor), the enzyme activity equaled 0.0413 IU PSAO in 1 mL reaction medium at 1,5-diaminopentane concentration of 0.15 mmol/L.

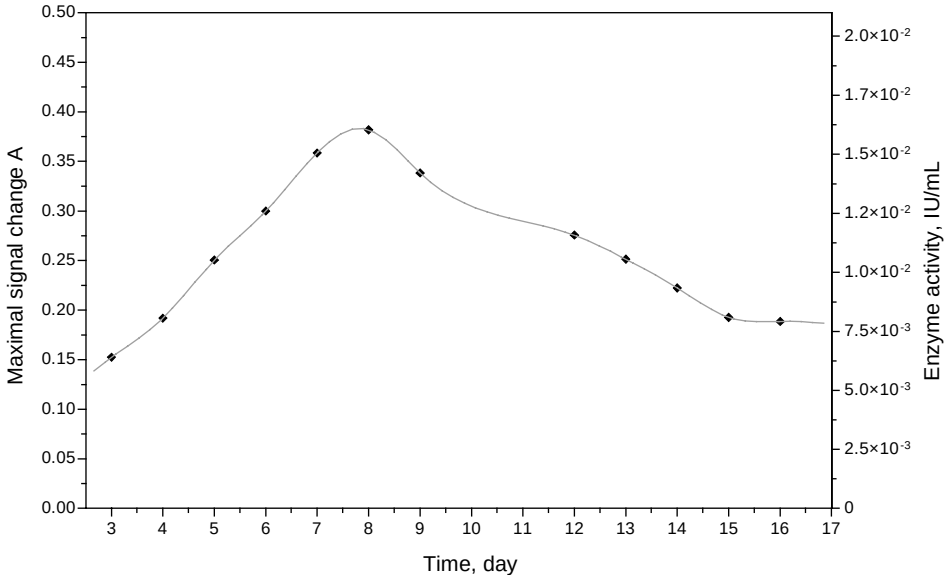


Fig. 1. Specific activity of pea seedlings amine oxidase towards 0.15 mM 1,5-diaminopentane in pea seedlings extract during the germination period. Kinetic measurements were carried out in air-saturated 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C. The enzyme activity is given per 1 mL reaction medium.

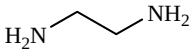
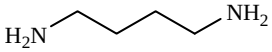
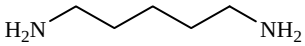
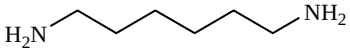
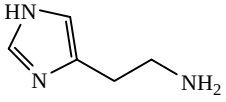
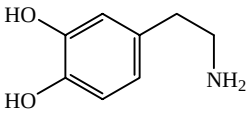
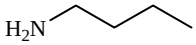
The efficiency of purification at different steps was characterized as PSAO specific activity per 1 mg enzyme preparation and the percentage of the recovery of total PSAO activity. The efficiency of different amine oxidase purification steps is shown in Table 1.

The specific activity of PSAO towards various substrates (Table 2) was studied both in crude and purified enzyme preparations and characterized with the calculated K_m values. There was no difference in the qualitative catalytic

Table 1. Characterization of the steps of amine oxidase purification process from pea seedlings

Fraction	Total activity, IU	Specific activity, IU/mg	Recovery, %
Centrifuged homogenate	84.55	0.51	100
Ammonium sulphate fractionation (30%)	79.40	0.52	93.9
Ammonium sulphate fractionation (70%)	68.66	0.75	81.2
DEAE-cellulose column	48.44	0.80	57.3
Sephadex G-200 column	43.83	0.83	51.8

Table 2. PSAO specific activity and K_m values towards different amines

Substrate		Specific activity, %*	K_m, mmolL^{-1}
1,2-Diaminoethane		6.4	0.022 ± 0.007
1,3-Diaminopropane		8.2	0.368 ± 0.143
1,4-Diaminobutane		86.2	1.150 ± 0.161
1,5-Diaminopentane		100.0	1.919 ± 0.421
1,6-Diaminohexane		42.6	0.321 ± 0.067
1,7-Diaminoheptane		37.7	0.204 ± 0.026
Histamine		9.8	0.052 ± 0.015
Dopamine		11.6	0.032 ± 0.001
1-Aminobutane		9.8	0.159 ± 0.064

* The activity of PSAO is expressed as a percentage of the signal of the most efficient substrate.

properties of different PSAO preparations. 1,5-Diaminopentane and 1,4-diaminobutane caused the largest changes in signal: the maximum oxygen sensor signal change in the case of the most efficient substrate, 1,5-diaminopentane, was 0.76 or 76% of the whole working range of the sensor. The limiting value for parameter *A* for diamines is shown in Fig. 2. Taking the PSAO specific activity towards 1,5-diaminopentane as a standard, we calculated the PSAO relative activity towards other amines and diamines as a percentage of the limiting value of parameter *A* of 1,5-diaminopentane (Table 2). The PSAO specific activity was clearly dependent on the number of methylene groups in diamine compounds and the binding of smaller and longer than 1,4-diaminobutane and 1,5-diaminopentane compounds was hindered, which may be caused by the steric hindrance in the enzyme active centre. The PSAO activity towards 1-aminobutane, which has the same number of methylene groups as 1,4-diaminobutane, was ten times smaller than for 1,5-diaminopentane, indicating the importance of chemical interaction during substrate binding. The activity towards the studied aromatic amines – histamine and dopamine – was around 10% of the activity with 1,5-diaminopentane, although in earlier studies quite high activity has been found [9, 13]. The high selectivity of PSAO towards 1,4-diaminobutane and 1,5-diaminopentane enables the application of the enzyme as a bioselective element for the detection of these diamines.

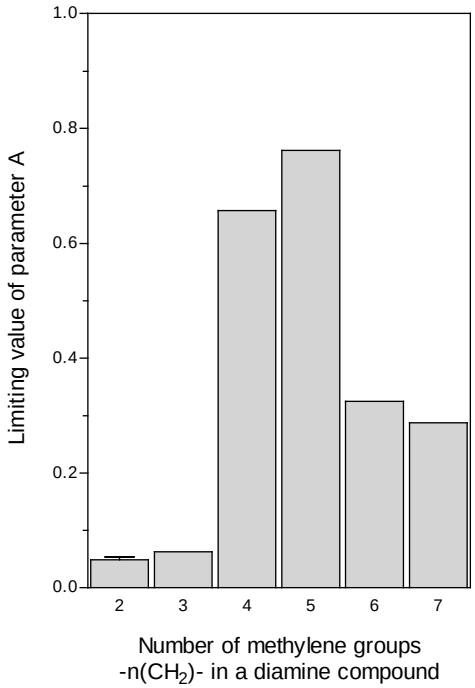


Fig. 2. The limiting value of parameter *A* as a function of the number of methylene groups of polymethylene diamines.

The K_m values were calculated using the same biosensor signal model, relating parameter A with substrate concentration [20]. The values of K_m for 1,4-diaminobutane and 1,5-diaminopentane, 1.15 ± 0.16 and 1.92 ± 0.42 mmol/L respectively, were similar to earlier results, obtained with an oxygen sensor [16], but larger in comparison with spectrophotometric measurements [15, 16, 20, 21]. These higher values indicate that there may be additional diffusion barriers for oxygen molecule to reaching the enzyme–substrate complex, which may become a limiting stage in fast processes. For less active amines, the calculated K_m values were similar to the spectrophotometrically obtained results presented in the literature.

The molecular mass of amine oxidase was determined with the method of gel filtration, indicating the value of 184.0 ± 2.6 kDa. This result is in a good correlation with earlier X-ray studies, which have reported that the protein is normally in a dimeric form with extensively interacting subunits of 70–90 kDa [8–10, 20–23].

In conclusion we can say that amine oxidase could be efficiently extracted from pea seedlings and the catalytic properties of the enzyme, determined with an amperometric oxygen sensor, were the same in crude and purified enzyme preparations and did not change during the purification process. The PSAO was very selective towards 1,4-diaminobutane (putrescine) and 1,5-diaminopentane (cadaverine) and the reaction signal could be perfectly registered with an oxygen sensor. The obtained results form a good basis for the application of this enzyme, also as a crude extract, for a rapid amperometric detection of putrescine and cadaverine, which are indicators of the freshness of fish and meat products.

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Amiini oksüdaasi puhastamine ja omadused

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Amiini oksüdaas (EC 1.4.3.6) on eraldatud herne idudest ja seda on iseloomustatud amperomeetrilise hapnikuanduri abil. Katalüütilised konstandid erinevate amiinide ja diamiinide suhtes on leitud varem välja pakutud biosensorite mudelit kasutades hapnikuanduri normaliseeritud tasakaalueelsest väljundsignaalist. Eraldatud amiini oksüdaasil on suurim aktiivsus putrestsiini ja kadaveriini suhtes; teiste uuritud amiinide suhtes on ensüümi aktiivsus väga madal või puudub hoopis. Ensüümi puhastamise erinevaid etappe on iseloomustatud amperomeetriliselt mõõdetud eriaktiivsuse muutumise alusel. Puhastatud ensüümi molekulmass on 184,0 ± 2,6 kDa.