Purification and some properties of an amine oxidase from soybean seedlings

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Dedicated to the memory of the late Dr. I. Nikolov.

Summary. An amine oxidase was purified 447-fold from soybean seedlings and some of its properties were investigated. The molecular weight of the enzyme was estimated to be 25,000. It was most active towards putrescine, followed by spermidine and spermine. K_m-values for these substrates were relatively close. The enzyme was strongly inhibited by carbonyl reagents, such as semicarbazide and aminoguanidine.
Key words. Glycine max; soybean seedlings; amine oxidase; putrescine; spermidine; spermine.

Amine oxidases are widely distributed enzymes in mammals, plants and microorganisms [1–3]. Some of the amine oxidases in plants, such as pea seedling diamine oxidase, barley seedling polyamine oxidase and oat seedling polyamine oxidase have been well investigated [2, 4]. In the present paper we describe a method for purification of an amine oxidase from soybean seedlings, and some of its properties, since this enzyme has not been studied in detail [5, 6]. Our aim was to give more details about its molecular weight, substrate specificity, apparent kinetic constants, pH optimum and effects of inhibitors.

Methods

The activity of soybean seedling amine oxidase (SSAO) was tested photometrically by a modification of the method of Emerson [7]. The enzyme was added to the reaction mixture (final volume 1.65 ml) containing: 0.3 M Tris-HCl, 0.01 % 4-amino-antipyrine, 0.015 % phenol and 1 mM substrate (spermidine). The final pH-value was around 8.0. After incubation at 37 °C for 60 min, the optical density of the red color developed was determined at 505 nm in a cuvette of 1 cm light path against a blank containing all components except substrate.

One unit of enzyme corresponded to 1 μmol H_2O_2, formed within 60 min under the assay conditions used. Specific activity was expressed in units/mg protein.

Enzyme activity was also determined polarographically [8], using an oxygen electrode of the type designed by Clark, attached to Universal Polarograph, type OH-105 (Hungary). The standard reaction mixture (final volume 1 ml) contained 10 mM Tris-HCl, 0.1 mM EDTA, 75 units of catalase, and enzyme. The final pH-value of the reaction mixture was 7.8. The reaction was started by addition of a small volume of substrate solution after at least 10 min pre-incubation, at 25 °C, using air as the gaseous phase.

The molecular weight of the enzyme was estimated on a Sephadex G-200 column in 0.05 M Tris-HCl, pH 7.6, containing 0.1 M KCl.

To check the purity of the purified enzyme and to estimate the molecular weight, polyacrylamide gel electrophoresis as described by Davis [19] and SDS-polyacrylamide gel electrophoresis according to Laemmli [10], were carried out.

Apparent kinetic constants were determined graphically from Lineweaver-Burk plots, using 6–8 substrate concentrations, assayed in triplicate.

The enzyme protein was determined by the method of Lowry [11], using bovine serum albumin as a standard.

Results

Soybean *(Glycine max)* seeds were germinated on a moist straw mat at 20 °C for 6 days in the dark [6]. The seedlings were washed in tap water. 200 g of seedlings were blended in 4 volumes of cold bidistilled water. The macerate was squeezed through muslin and the residue was reextracted with 2 volumes of cold water. The combined extracts were adjusted to pH 4.0 with a saturated solution of citric acid. The precipitate was collected by centrifugation at 2500 × g for 15 min and extracted in 0.1 M Na_2HPO_4-citrate buffer, pH 7.8, containing 1 M NaCl at 0 °C. On recentrifugation at 3000 × g for 15 min the precipitate was discarded. The supernatant was cooled to 0 °C and 1 volume of acetone at −15 °C was added. The precipitate collected by centrifugation was extracted with 1 M NaCl in 0.1 M Na_2HPO_4-citrate buffer, pH 7.8; on recentrifugation, the precipitate was discarded. Solid ammonium sulfate was added to the supernatant to 70 % saturation. The precipitate was collected by centrifugation at 3000 × g for 15 min and extracted in 0.1 M sodium-phosphate buffer, pH 7.8. On recentrifugation at 18,000 × g for 15 min the precipitate was discarded. The supernatant was applied to a column of Sephadex G-200 (2.5 × 100 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.8. Fractions (5 ml each at 20 ml/h) eluted with the same buffer, were collected. The active band was pooled and concentrated by Millipore filtration (UM 10 filter).

On polyacrylamide gel electrophoresis the final preparation yielded a major band and a few minor bands. The enzyme activity was coincident with the major protein band.

Table 1. Purification of soybean seedling amine oxidase*

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
Water extraction	314	923.00	0.34	1	100
Citric acid purification (pH 4.0)	266	140.00	1.90	6	85
Acetone precipitation	256	11.74	21.80	64	82
Ammonium sulphate precipitation (70%)	159	3.47	45.80	135	51
Sephadex G-200 chromatography	75	0.49	152.00	447	24

*The enzyme activity was tested photometrically under standard assay conditions.

Table 2. Substrate specificity of soybean seedling amine oxidase (polarographic assay)

Substrate (1 mmol/l)	Relative activity* (%)
Putrescine	100.00
Spermidine	90.55
Spermine	37.67
Agmatine	2.80
L-lysine	3.86
Histamine	4.50
Tryptamine	1.40
Tyramine	3.12
Serotonine	2.80
Ethylamine	1.15
Noradrenaline	0.00
n-Propylamine	0.00
L-ornithine	0.00
Glutamine	0.00

*100% of the relative activity corresponded to 5.6 µmol O_2/min/mg protein.

Table 3. K_m, V_{max} and pH optima of soybean seedling amine oxidase (polarographic assay)

Substrate	K_m* (µmol/l)	V_{max}* (µmol O_2/min/mg protein)	pH optima
Putrescine	25.4 ± 1.5	6.54 ± 0.10	7–7.5
Spermidine	36.8 ± 1.4	5.40 ± 0.12	8.0
Spermine	37.1 ± 1.8	1.02 ± 0.08	8.5

*Mean ± SE of 3 independent tests.

Table 4. Effect of inhibitors on enzyme activity of soybean seedling amine oxidase (polarographic assay)*

Inhibitor	Concentration (mol/l)	Inhibition (%) Putrescine**	Spermidine**
Pargyline	10^{-4}	0.0	0.0
	10^{-3}	0.0	0.0
Semicarbazide	10^{-4}	93.0	95.6
	10^{-3}	94.2	96.0
Aminoguanidine	10^{-4}	80.6	82.2
	10^{-3}	82.6	85.0
Sodium cyanide	10^{-4}	54.0	55.5
	10^{-3}	60.3	63.4
Sodium azide	10^{-2}	11.6	10.0

*The enzyme was preincubated with inhibitors at 25 °C for 10 min.
**The substrates used were 1 mmol/l.

The lyophilized enzyme was confirmed to be stable for more than one year at −20 °C.

The purification results are summarized in table 1. The enzyme was purified 447-fold from soybean seedlings with a recovery of 24%. The molecular weight of the purified enzyme is 25,000, determined by gel-filtration and SDS-polyacrylamide gel electrophoresis.

The oxidation of a number of substrates by the enzyme was investigated, as shown in table 2. The enzyme was most active toward putrescine, followed by spermidine and spermine.

The apparent K_m values were estimated as shown in table 3. The K_m values for putrescine, spermidine and spermine as substrates were relatively close. The elongation of the chain of the substrate (from putrescine to spermine) led to decrease of V_{max} (table 3). The pH optima of SSAO, determined with 0.1 M Tris-HCl and 0.1 M sodium phosphate buffer solutions, (covering pH 6.0–11.0) were somewhat different for putrescine, spermidine and spermine (table 3).

Table 4 shows inhibition of SSAO activity by various compounds. The inhibition was independent of the substrate (putrescine or spermidine). Pargyline, a specific inhibitor of flavin-containing monoamine oxidase (EC 1.4.3.4), preduced no inhibition. The carbonyl reagents semicarbazide and aminoguanidine, inhibitors of pyridoxal-containing amine oxidases such as diamine oxidase (EC 1.4.3.6), strongly inhibited the enzyme. Copper-ligands such as sodium cyanide and sodium azide inhibited the enzyme to different extents.

Discussion

In the present paper we describe a purification method for soybean seedling amine oxidase and some of its properties. Our results (table 1) suggest that this purification procedure is eminently suitable for large-scale preparation of SSAO; soybean is an easily obtainable starting material.

The substrate specificity of the enzyme (table 2) is generally similar to that of pea seedling diamine oxidase. However, SSAO shows considerable activity towards spermine, a good substrate for all polyamine oxidases[1,2], whereas pea seedling diamine oxidase shows almost no activity towards this polyamine[12]. Nevertheless, our study of enzyme inhibitors (table 4) suggests that SSAO can be classified as a typical diamine oxidase (EC 1.4.3.6).

The SSAO purified 447-fold is strongly sensitive to the action of the carbonyl reagent semicarbazide, which according to Yamada and Yasunobu[13] is sufficient evidence for considering pyridoxal (phosphate) as a cofactor of the enzyme. It is known that amine oxidases which have pyridoxal (phosphate) as a cofactor generally attack only terminal primary amino groups of amines[14], and that as a result of the reaction aldehydes are produced.

Experiments to purify the SSAO to homogeneity and to identify the cofactor of the enzyme by mass spectrometry are still in progress in our laboratory.

The SSAO obtained by our method was found to be useful in the enzymatic determination of the total free polyamines in blood and in assays for diagnosis and the monitoring of therapy in cancer patients[15].

1 Morgan, D. M. L., Biochem. Soc. Trans. *13* (1985) 322.
2 Smith, T. A., Biochem. Soc. Trans. *13* (1985) 319.
3 Weaver, R., and Herbst, E., J. biol. Chem. *231* (1958) 647.
4 Smith, T. A., A. Rev. Plant Physiol. *36* (1985) 117.
5 Suzuki, Y., Plant Cell Physiol. *14* (1973) 413.
6 Matsumoto, T., Suzuki, O., Katsumada, Y., Oya, M., Suzuki, T., Nimura, Y., and Hattori T., Cancer Res. clin. Oncol. *100* (1981) 73.
7 Emerson, E., J. org. Chem. *8* (1943) 417.
8 Rinaldi, A., Floris, G., and Finazzi-Agro, A., Eur. J. Biochem. *127* (1982) 417.
9 Davis, B., Ann. N.Y. Acad. Sci. *121* (1964) 404.
10 Laemmli, U. K., Nature *227* (1970) 680.
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randal, R. J., J. biol. Chem. *193* (1953) 265.
12 Hill, M., Methods in Enzymology, vol. XVII B, p. 730. Academic Press, New York 1971.
13 Yamada, H., Yasunobu, K. T., Biochem. biophys. Res. Commun. *8* (1962) 387.
14 Onada, M., Kawashima, S., and Imahori, K., Protein nucl. Acid Enzyme *26* (1981) 1471.
15 Damyanov, D., Stoychkov, J., and Nikolov, I., Acta med. bulg. *133* (1986) 60.

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Oxytoxins, bioactive molecules produced by the marine opisthobranch mollusc *Oxynoe olivacea* from a diet-derived precursor

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Summary. The ethereal extract of the mucous secretion from the opisthobranch mollusc *Oxynoe olivacea* was examined and found to contain two novel ichthyotoxic compounds, named oxytoxin 1 and 2 (**1**, **2**). The structures of **1** and **2** are closely related to the metabolites previously isolated from the alga *Caulerpa prolifera*. The activity of the most stable compound was studied in order to investigate the possibility of a further biological role for these metabolites, which represent an uncommon example of bioactive molecules produced in vivo from a dietary precursor.

Key words. Opisthobranch molluscs; defense mechanisms; marine toxins; dialdehydes.

The opisthobranch molluscs of the genus *Oxynoe* utilize a series of defensive mechanisms to avoid predation[1]. First, they live camouflaged on the green algae of the *Caulerpa* genus on which they feed. When disturbed, these ascoglossan molluscs secrete an extremely toxic mucus capable of deterring various predators, including fish. Finally, if molested further, these molluscs also exhibit a defensive mechanism known as autotomy, which consists of the spontaneous detachment of the tail, which then continues to twitch for several minutes, thus distracting the predator and allowing the mollusc to escape to safety. The missing part is usually regenerated within a few days[2,3].

It has been suggested[4] that the mollusc mucous secretion owes its toxicity to substances of dietary origin, probably derived from the algae of the genus *Caulerpa* on which it feeds. However, no chemical characterization of these metabolites, and therefore no definite proof of this hypothesis, has yet been reported. In this paper we describe the results of experiments aimed at isolating and characterizing the substances responsible for its toxicity from the mucus of an *Oxynoe* species living in the Mediterranean Sea, *O. olivacea* (Rafinesque, 1819), and investigating the origin and other possible biological roles of the toxic substances by examining their gross distribution within the organism and their biological activity.

Oxynoe olivacea is a common species in littoral sheltered and shallow habitats, either marine or lagoon, where it is abundant and is always found associated with *Caulerpa prolifera*[5] ((Forsskal) Lamoureux, 1809). The animals studied were caught in the Bay of Naples.

Silica gel TLC analysis of the ethereal extract of fresh mucous secretion showed the presence of two spots visible in UV light (Rf = 0.45 and 0.55, C_6H_6 : Et_2O 8:2). A