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SOME PROPERTIES OF AMINE OXIDASE FROM SOYBEAN SEEDLINGS

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ABSTRACT

The purpose of the present study was to clarify the properties of amine oxidase partially purified from soybean seedlings, which the authors used as an enzyme sample for the assay of diamines (putrescine, cadaverine) and polyamines (spermidine, spermine). The enzyme activity was highest with cadaverine followed by putrescine, spermidine, 1,7-diaminoheptane and spermine. The optimum pH was 7.8 for putrescine and cadaverine and 8.0 for spermidine and spermine. Carbonyl reagents, such as semicarbazide, and chelating reagents, such as cuprizone, were effective inhibitots of soybean seedling amine oxidase (SSAO), suggesting that Cu^{++} and pyridoxal may be cofactors for SSAO. Therefore, SSAO appeared to be a diamine oxidase (EC 1.4.3.6). It was stable at 50°C for at least 2 hours, and the apparent K_m values for all diamines and polyamines were extremely low. Since SSAO is present in great quantities in soybean seedlings, which can be easily obtained in every season in Japan, it is very suitable as an enzyme sample for the assay of diamines and polyamines.

Key words: Soybean seedling, Amine oxidase, Diamine, Polyamine

INTRODUCTION

Diamines (putrescine, Put; cadaverine, Cad) and polyamines (spermidine, Spd; spermine, Spm) are aliphatic amines of widespread biological occurrence. These amines have received much attention, since polyamine concentrations in serum (1,2), urine (3,4,5,6), and cerebrospinal fluid (7) are elevated in certain types of cancer, in pregnancy, (8) and in the regenerating liver (9,10).

Recently, the authors succeeded in developing a new enzymatic method for the determination of total diamines and polyamines in the urine of cancer patients, using soybean seedling amine oxidase (SSAO) as a reagent (5). Although its occurrence in soybean seedlings was reported (11), SSAO has not been characterized. The present study clarifies some properties of a partially purified enzyme preparation of SSAO.

MATERIALS AND METHODS

1. Preparation of SSAO

Seeds of *Glycine max* were germinated on moist vermiculite at 20°C for 7 days in the dark.

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The etiolated seedlings were then excised and washed in tap water. Two hundred grams of the germinating seedlings were homogenized in 150 ml of cold saline in a Waring blender for 15 min. The homogenate was passed through washed gauze or muslin. The resulting filtrate was brought to 45% saturation with ammonium sulfate followed by centrifugation at 18000g for 15 min. The supernatant was then brought to 70% saturation with ammonium sulfate and recentrifuged at 18000g for 15 min. The precipitate was dissolved in 15 vols of 0.1 M sodium phosphate buffer at pH 7.8 and dialyzed with stirring against 50 vols of the same buffer solution with 3—5 changes for 24h, followed by centrifugation at 18000g for 15 min to remove inactive precipitates. By the above procedure, the enzyme was purified 2.1-fold with an activity of 3.4 μmol/60 min/mg protein when 1.0 mM Put was used as the substrate. This preparation was used as the enzyme sample. One unit of enzyme activity was defined as the amount of enzyme that produces one μmol of peroxide per 60 minutes at 37°C.

2. Assay for amine oxidase activity

The assay was carried out photometrically by a modification of Emerson's method (12). The incubation mixture contained 0.5 ml of 0.5 M Tris solution, 1.0 ml of substrate solution, 0.05 ml of 4-aminoantipyrine solution (4 mg/ml), 0.05 ml phenol solution (0.5 %v/v, aqueous solution), 0.05 ml of peroxidase solution (2 mg/ml) and 0.05 ml (2 unit) of SSAO solution. After incubation at 37°C for 60 min, the red color formed was determined at 505 nm in a cuvette of 1.0-cm light path using a Hitachi-Perkin Elmer 139 UV-VIS spectrophotometer.

3. Protein determination

Protein was measured by a modification of the conventional biuret method (13).

4. Chemicals

Put-2HCl, Cad-2HCl, Spd-3HCl, Spm-4HCl, aminoguanidine, semicarbazide, pargyline, cuprizone and acriflavin were obtained from Nakarai Chemicals, Ltd., Kyoto; horseradish peroxidase (Type II) and quinacrine from the Sigma Chemical Company, St. Louis, MO; 4-aminoantipyrine, phenol, *o*-phenanthroline, sodium azide, bathocuproine disulfonic acid, sodium diethyldithiocarbamate, phenylmercuric acetate, *p*-chloromercuric benzoic acid and iodoacetate from Wako Chemicals, Ltd., Osaka. Other common chemicals used were of the highest purity commercially available.

RESULTS

1. Substrate specificity

SSAO activities with a number of substrates were tested at pH 8.0 with a substrate concentration of 1 mM and 0.1 unit of enzyme (Table I). Of the amines (monoamines, diamines and polyamines), Cad was most readily oxidised, followed by Put and Spd. 1,7-diaminoheptane, 1,6-hexamethylenediamine and Spm were oxidised more slowly than Spd. Histamine, 1,2-ethylenediamine and 1,3-propanediamine were virtually not oxidised.

Of the monoamines, tryputamine, *n*-propylamine, benzylamine were oxidised slowly, but most of the other monoamines tested were hardly oxidised.

Table 1. Substrate specificity of SSAO

Substrate (1.0 mM)	Relative activity
Cad	100
Put	60.8
Spd	41.7
Spm	10.8
Histamine	0.68
1,2-Ethylenediamine	0
1,3-Propanediamine	0
1,6-Hexamethylenediamine	16.0
1,7-Diaminoheptane	17.6
Agmatine	1.1
Ethylamine	0.45
n-Propylamine	7.1
n-Butylamine	1.6
Monoethanolamine	0
Octopamine	0
Serotonin	1.1
Tryptamine	10.3
Benzylamine	6.9
β-phenylethylamine	2.2
Tyramine	0.5
Synephrine	0.5

The assay method is described in the text.

2. Effect of inhibitors

The effects of inhibitors on the oxidation of Put, Cad, Spd and Spm were tested (Table II). Carbonyl reagents, such as semicarbazide, aminoguanidine and KCN, strongly inhibited the oxidation of all substrates. Pargyline did not inhibit the oxidation of diamines, but it slightly inhibited the oxidation of polyamines. Acridine compounds such as quinacrine and acriflavin partially inhibited the oxidation of all the above amines. Sulfhydryl reagents such as iodoacetate did not inhibit the oxidation of any of the substrates.

Of the chelating reagents, EDTA, bathocuproine disufonic acid and sodium diethyldithiocarbamate at the concentration of 1 mM did not affect the oxidation of any of the above amines, but marked inhibition was observed with cuprizone, which is known as a typical reagent for Cu^{++}.

3. Michaelis constants

The K_m values for Put, Cad, Spd and Spm determined from the Lineweaver-Burk plots were 6.94, 6.94, 14.7 and 4.72 μM, respectively; the corresponding V_{max} values were 1.51, 3.49, 1.25 and 0.31 μmol/mg protein/30 min, respectively.

Table II Effect of inhibitors on SSAO

Inhibitor (1 mM)	Percent inhibition			
	Put	Cad	Spd	Spm
Aminoguanidine	93.0	86.6	92.7	90.9
Semicarbazide	97.7	96.6	98.0	100.0
KCN	92.6	96.4	92.0	90.0
Pargyline	0	0	10.4	12.0
o-Phenanthroline	92.2	91.7	92.8	90.0
Cuprizone	80.4	83.2	88.4	65.8
Sodium azide	73.6	71.2	77.2	83.5
EDTA	0	0	0	0
Bathocuproine disulfonic acid	0	0	0	0
Sodium diethyldithiocarbamate	0	0	0	0
Phenylmercuric acetate	97.5	99.0	100.0	100.0
p-Chloromercuric benzoic acid*	3.2	10.8	17.7	12.2
Iodoacetate	0	0	0	0
Quinacrine	27.5	13.4	32.8	39.7
Acriflavin	34.2	38.3	22.0	26.4

Each mixture was preincubated for 20 min. * Final 0.5 mM

4. PH optima

Dependence of the enzyme activity on pH was examined with 0.5 M Tris HCl buffer at varying pH values and with the substrates at the concentration of 1 mM. The enzyme was found to have maximal activities at pH 7.8 for Cad and Put, and at pH 8.0 for Spd and Spm (Fig. 1).

5. Stability against heat and aging

Heat stability of SSAO was tested at 37°C, 50°C, 70°C and 80°C for 5 to 120 min (Fig. 2). After the incubation of the enzyme at 50°C for 120 min, no significant loss of activity was observed. All activity was lost within 5 min by incubation of the enzyme at above 80°C.

The enzyme precipitated with ammonium sulfate state did not lose the activity at 4°C for at least 6 months. The dialyzed enzyme was found to be stable for at least one week at 4°C. The lyophilized enzyme was stable for more than two months at −20°C, though a 36% loss of activity was noted during lyophilization.

6. Oxidation of diamines and polyamines by a large amount of SSAO

Various amounts of diamines or polyamines in the ranges from 10.0 to 150 nmoles (incubation mixture was 1.65 ml) for Put, Cad and Spd and from 10.0 to 75.0 nmoles for Spm were subjected to the enzymatic reaction with 2 units of SSAO at the end point of the reaction was measured. Linear relationships between the amounts of the amines and the absorbances were observed (Fig. 3). The absorbance obtained with Spm was twice as high as that obtained with the other three amines.

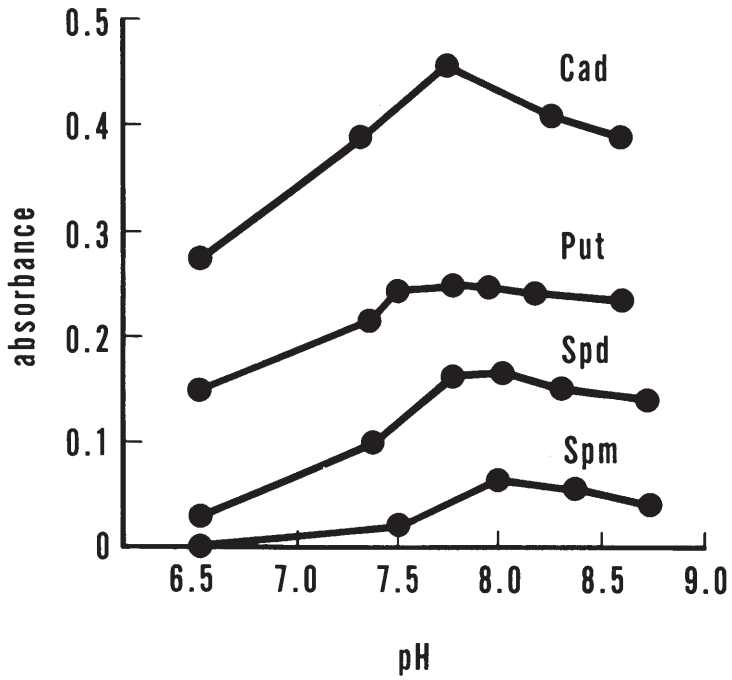

Fig. 1 Effect of pH on SSAO activity with various substrates. The assay procedure was descibed in the text (standard assay) except that the substrate concentration was 1.0 mM, and 0.1 unit of ;the enzyme was used.

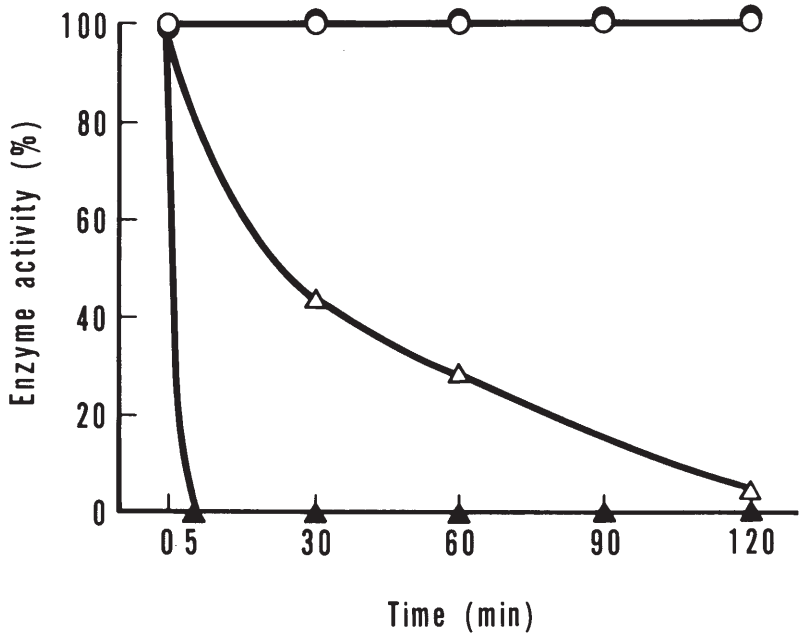

Fig. 2 Effect of heating on SSAO activity. The assay procedure was as described in the text (standard assay) except that putrescine was used as the substrate at the concentration on 1.0 mM; 0.1 unit of the enzyme was employed.
Key: 37°C (○—○); 50°C (●—●); 70°C (△—△); 80°C (▲—▲).

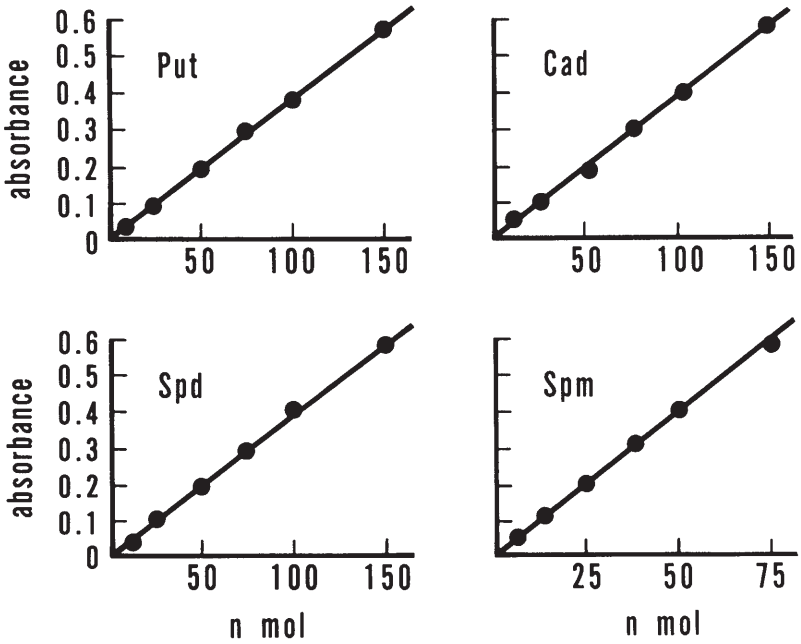

Fig. 3 Oxidation of diamines and polyamines by large amount of SSAO. The assay procedure was as described in the text (standard assay) except that 2 units of the enzyme and a varying amount of the substrate were used. The final volume of reaction mixture was 1.65 ml.
Key: Put (○—○); Cad (●—●); Spd (△—△); Spm (▲—▲).

DISCUSSION

Amine oxidases, which metabolize diamines or polyamines, occur widely in various animals, plants and microorganisms. In animals, beef plasma amine oxidase has been especially well characterized. This enzyme oxidizes the primary amino groups of propylamine (14,15). Placental diamine oxidase (16), hog kidney diamine oxidase (17,18), human seminal diamine oxidase (19) and rat liver polyamine oxidase (20) have also been characterized. In microorganisms, amine oxidases from *Pseudomonas aeruginosa* (21), *Hemophilus parainfluenzae* (22), *Microbacterium smegmatis* (23), *Neisseria perflava* (24) and *Serratia marcescens* (25) have also been studied. In plants, pea seedling amine oxidase (26,27,28) has been well characterized; lupine seedling amine oxidase (29), zea may shoot amine oxidase (30), barley seedling amine oxidase (31) and oat seedling amine oxidase (32) have been characterized to some extent, but the properties of soybean seedling amine oxidase have not been studied extensively (11).

The present enzyme from soybean seedlings perhaps attacks primary amino groups only with the formation of an aldehyde, NH_3 and H_2O_2. This enzyme readily attacks Cad, Put, Spd, Spm and a variety of monoamines. Its substrate specificity resembles that of pea seedling amine oxidase. The consumption experiments showed that the oxidation of Spm resulted in twice the optical density of the other three amines. This result suggests that one mole of Spm generates two moles of H_2O_2, while the other three amines generate equimolar H_2O_2. Thus SSAO may deaminate only one terminal amino group of Put, Cad and Spd, while it deaminates both terminals of Spm. Amine oxidases appear to be either particulate enzymes

with a flavin prosthetic group or soluble enzymes with Cu^{++} and pyridoxal phosphate as cofactors (33,34). Since this enzyme was markedly inhibited by cuprizone but not by bathocuproine disulfonic acid (Table II). Cu^{++} appears to be a cofactor (34). Quinacrine is usually used as a specific inhibitor of flavin-containing enzymes (35). The SSAO was not markedly inhibited by acridine compounds such as quinacrine and acriflavin, but was strongly inhibited by carbonyl reagents such as semicarbazide and aminoguanidine (Table II), suggesting that pyridoxal phosphate is a SSAO cofactor. Therefore, SSAO may be classified as a diamine oxidase (EC 1.4.3.6). This enzyme also seems relatively resistant to heat (Fig. 2) and aging. Since the linear relationship between the absorbance, resulting from the enzyme reaction, and amounts of amines was obtained for all Put, Cad, Spd, and Spm (Fig. 3), SSAO is very suitable for the enzymatic assay of total diamines and polyamines.

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