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FURTHER PROPERTIES OF THE POLYAMINE OXIDASE FROM OAT SEEDLINGS

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Abstract—After purification, the polyamine oxidase from the leaves of oat seedlings grown in the dark appeared to be homogeneous on electrophoresis. The MW determined by density gradient centrifugation was 119 000. The enzyme would not oxidise diaminodipropylamine and neither diaminodipropylamine nor diaminopropane were inhibitors at concentrations up to 1 mM. With spermidine as substrate, the energy of activation was 19.7 kJ/mol and activity was reduced to 50% on heating for 10 min at 50°. With spermine as substrate, activity was increased up to 3-fold in the presence of M sodium chloride. This stimulation was not observed with spermidine as substrate The enzyme was also stimulated by sodium phosphate and sodium citrate at high concentrations. The pH for optimal stability was 6.5, the same as the pH for maximum activity with both spermidine and spermine as substrates. For spermidine and spermine the K_ms were 8×10^{-6} M and 2×10^{-6} M respectively. Loss of activity on storage of leaves at −15° was *ca* 5% per week and in extracts the loss was *ca* 10% per week.

INTRODUCTION

Polyamine oxidases, which occur in animals, micro-organisms and plants, oxidise spermidine and spermine by a variety of mechanisms depending on the enzyme source. In the leaves of cereals spermidine and spermine are respectively oxidized to pyrroline and 1-(3-amino-propyl) pyrroline, together with H_2O_2 and 1,3-diamino-propane in the case of both substrates. In previous work, properties of the polyamine oxidases of barley [1–4], maize [5,6] and oats [3] have been studied. By far the greatest activity was found in the leaves of oat seedlings grown in the dark, and in the present work further properties of the enzyme from oat seedlings have been studied.

RESULTS AND DISCUSSION

Purification

The enzyme was routinely purified according to the method of ref. [3]. At stage 3, on substitution of $(NH_4)_2SO_4$ (60% saturated) for acetone, the enzyme recovery was 135%. However the specific activity was increased only by 3.5 ×, compared with 11 × purification at stage 3 by acetone precipitation. In unhomogenized oat leaves, activity declined from 90 nkat/g fr. wt to 26 nkat/g fr. wt on storage at −15° for 4 months. For the purified enzyme (stage 4) loss of activity on storage at −15° was 10% per week.

Disc gel electrophoresis

The purified enzyme (stage 4) [3] (*ca* 3000 nkat/ml), migrated as a single band at pH 4.3 towards the anode at 14mm per hr (mobility 40% that of methyl green). Dialysis to remove the NaCl increased the speed of migration but did not appear to improve the resolution. Enzyme preparations (stage 4) dialysed to pH 4.3 (0.35M, β-alanine buffer) or suspended in pH 6.3 Pi buffer containing M NaCl gave similar results. Enzyme activity was always coincident with the major protein band, and in some preparations no other protein bands could be seen. Even so coincidence of bands is not proof of identity, and although it would seem likely that the preparations were homogeneous, there is as yet no direct proof. Preparations prior to Sephadex treatment (stage 3) [3] also showed one major protein band coincident with activity. In earlier work on electrophoresis using starch gels and on cellulose acetate [2], crude preparations of polyamine oxidase from barley leaves appeared to give multiple bands of activity. It is possible that these were due to an association of the enzyme with nucleic acid fragments, as this enzyme appears to be normally bound to a particulate fraction which may be nuclei [3].

Calculation of MW using density gradient centrifugation

The S_{20w} [7] was 7.26 and the MW calculated from this was 130 000. The diffusion coefficient (D_{20w}) [8] for the amine oxidase was 5.01×10^{-7} and the frictional ratio 1.3 indicating that the enzyme is not spherical. Using the data of the density gradient centrifugation (MW 130 000) and gel chromatography (MW 85 000, see ref. [3]), a corrected MW of 119 000 was obtained.

Effect of temperature

On heating to 50° for 10 min, the pH for optimal stability was 6.5. In pH 6.5 Pi buffer (0.1 M) containing M NaCl, 50% of the activity was lost on heating for 10 min at 50°. The energy of activation for the inactivation of the enzyme was 310 kJ/mol (50–55°). The energy of activation for the enzymic activity was 19.7 kJ/mol (7–36°) using the peroxidase/guaiacol assay method. In earlier work the energy of activation for the barley enzyme was 31 kJ/mol [3].

Inhibitors

Using the peroxidase/guaiacol assay at pH 6, no

activity could be detected with diaminodipropylamine as substrate (1 mM final concn), and the enzyme appears to be specific for spermidine and spermine. Neither diaminodipropylaminenordiaminopropane(1 mM)affect the oxidation of spermidine. The spermidine homologue $NH_2(CH_2)_{10}$ $NH(CH_2)_3$ NH_2 did not serve as a substrate, but it strongly inhibited spermidine and spermine oxidation. At 1.6×10^{-5} M and 4×10^{-4} M of this homologue, spermidine oxidation was inhibited 80 and 96% respectively. At these concentrations spermine oxidation was inhibited 40 and 88% respectively. From previous work with the barley enzyme the K_i for this inhibitor was 5×10^{-6} M with spermine as substrate [4].

Effect of salt

This was studied using the peroxidase/guaiacol assay, rather than the O_2 electrode, since salt affects the solubility of O_2 and as the enzyme had high K_m values for O_2. Oxidation of spermine by the oat enzyme was strongly stimulated by salt (Table 1). In equimolar concentrations (0.1 M) activity was similar in both Pi and citrate buffers. However addition of NaCl (1 M) to both systems increased oxidation 2–3-fold. Equal stimulation was found with NaPi, Na citrate or NaCl at molar concentrations, though NaPi at 2 M became inhibitory. Activity measurements were normally made from the rate of colour formation 1 min after adding substrate. Additional measurements of increase in A after 4 min indicated that the apparent salt stimulation was not attributable to the protection of the enzyme during assay. Activity declines rapidly during assay probably because reactive quinones formed from the oxidation of guaiacol cause enzyme denaturation [2]. However activity declined much faster with spermine as substrate than with spermidine.

By contrast with the effect on spermine oxidation, the rate of spermidine oxidation was not affected by the increased salt concentration up to 1 M. In optimal salt concentrations (*ca* 1 M), activity with spermidine and spermine was similar. There was no effect of salt concentration on the pH optimum (Fig. 1). The pH activity curve, with or without salt, was broader with spermine than with spermidine, but with both substrates activity declined from the optimum more quickly towards the higher than to the lower pH range.

Table 1. Effect of buffer strength and composition on activity of oat leaf polyamine oxidase, measured with the peroxidase/guaiacol method. Buffers were prepared by titrating to pH 6.5, Na_2HPO_4 with NaH_2PO_4 (Pi), or citric acid with NaOH (citrate), at the concentrations given. Solutions with NaCl were re-adjusted to pH 6.5 before use. The final substrate concentration was 1 mM

Substrate	Enzyme	Buffer		NaCl	Activity in ΔA 470 nm/min	
	µl	Pi	Citrate		Initial	After 4 min
Spermine	10	0.1 M	—	—	0.035	0.005
	10	0.1 M	—	0.5 M	0.078	0.006
	10	0.1 M	—	1 M	0.084	0.009
	10	—	0.1 M	—	0.031	0.003
	10	—	0.5 M	—	0.062	0.003
	10	—	2 M	—	0.060	0.003
	10	—	0.1 M	1 M	0.070	0.009
	10	1 M	—	—	0.093	0.002
	10	2 M	—	—	0.056	0.002
Spermidine	0	0.1 M	—	—	< 0.002	< 0.002
	1	0.1 M	—	—	0.008	< 0.002
	2	0.1 M	—	—	0.016	0.002
	5	0.1 M	—	—	0.042	0.008
	10	0.1 M	—	—	0.075	0.018
	10	0.1 M	—	1 M	0.082	0.022

For spermine oxidation the optimal concentration of salt was quite high and appeared to be in excess of 0.5 M. This salt stimulation may be ascribed to the Na^+ ion since this was the cation for all buffers used. K^+ and NH_4^+ were not tested, though NH_4^+ did inhibit the barley enzyme [1]. Stimulation of enzyme activity by salts with optima approaching 0.5 M is rare in higher plants. However, certain enzymes in yeast [9] and *Escherichia coli* [10] require 0.2 M K^+, while the halophytic bacteria have enzymes which require salts in excess of 1 M [11].

Pea seedling amine oxidase was stimulated by 0.1 M Pi in the presence of glycine, bicarbonate and borate buffers [12], and activity of the pig kidney diamine

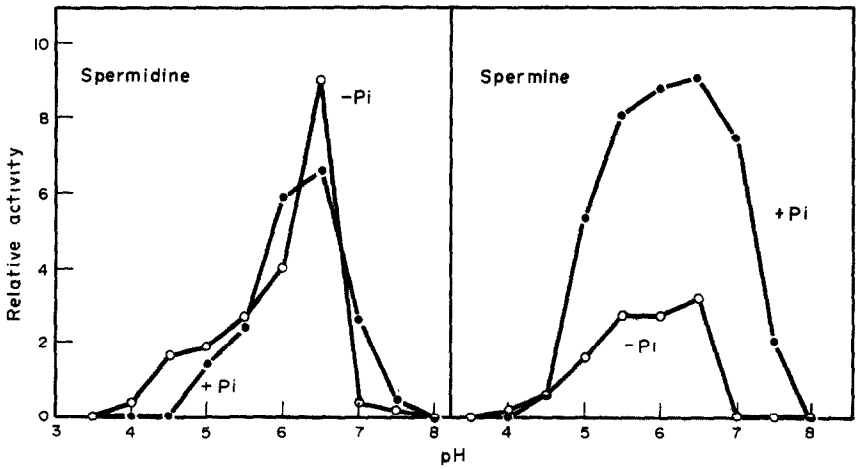

Fig. 1. Effect of pH and inorganic phosphate on the activity of oat leaf polyamine oxidase. Citrate (pH 3.5 to 6.5) or *hepes* (pH 7 to 8) buffers (0.1 M) were used in the absence or presence of mixtures of 0.5 M sodium phosphates at the appropriate pH values. The guaiacol/peroxidase assay method was used (see Experimental).

Table 2. K_m values for oat leaf polyamine oxidase determined from Lineweaver–Burk plots. Activity was estimated with the oxygen electrode at 30°, in equilibrium with air (2.4×10^{-4} M O_2) for the K_m for spermidine and spermine, and at 10^{-3} M polyamine for the K_m for oxygen

Substrate	In the presence of	K_m	Standard error
Spermidine	oxygen at 2.4×10^{-4} M	8.0×10^{-6} M	0.7×10^{-6} M
Spermine	oxygen at 2.4×10^{-4} M	2.0×10^{-6} M	0.3×10^{-6} M
Oxygen	spermidine at 10^{-3} M	1.83×10^{-4} M	0.34×10^{-4} M
Oxygen	spermine at 10^{-3} M	0.85×10^{-4} M	0.25×10^{-4} M

oxidase with histamine as substrate was stimulated × 2 on increasing the KPi buffer concentration to 0.5 M [13]. Moreover, activity of plasma amine oxidase was stimulated on increasing the Pi concentration up to 0.1 M [14].

In barley plants growing in sand it is of interest that increased salt concentration (0.1 M KCl) in the nutrient medium caused a 5-fold increase in the 1-(3-aminopropyl)pyrroline concentration in their leaves [15]. This supports the suggestion that high salt concentration stimulates spermine oxidation *in vivo* in barley leaves. Out of nine nutrient treatments ($+NH_4$, $+NO_3$, $-P$, $-S$, $-N$, $-Mg$, $-K$, $-Ca$, $+KCl$) only Ca^{2+} deficiency and high salt caused accumulation of 1-(3-aminopropyl)pyrroline. Ca^{2+} is an inhibitor of polyamine oxidase at 50 mM [3] and deficiency may relieve this inhibition. Salinity also has a marked effect on di- and polyamine metabolism in *Limonium* [16], *Oenothera* [17] and *Vicia* [18].

Effect of substrate concentration

With the peroxidase/guaiacol assay, the K_ms for spermidine and spermine (Table 2) were lower than those found for the barley leaf polyamine oxidase (2×10^{-5} M and 3×10^{-5} M respectively) [2], and for spermine oxidation by the maize enzyme (6×10^{-4} M) [5]. Increasing the spermidine concentration from 10^{-3} M to 10^{-2} M had no effect on activity. With the oxygen electrode assay the K_m for O_2 was less than the concentration of O_2 in air saturated water (2.4×10^{-4} M), unlike the K_m for the barley enzyme which was 3×10^{-4} M [4].

Effect of cysteine

Using the oxygen electrode for assay, it was shown that 10mM cysteine had no effect on the enzyme activity and it appears that sulphydryl groups are not stimulatory. However in the peroxidase/guaiacol assay cysteine appears to be oxidised in preference to guaiacol because absorbance did not increase until all the cysteine was oxidised. The effect of cysteine could therefore not be tested with the peroxidase/guaiacol assay.

EXPERIMENTAL

Purification. The enzyme purified to stage 2 (ref. [3]) was used for most of the experiments. For electrophoresis, enzyme purified to both stages 3 and 4 was used.

Electrophoresis was carried out by the method of ref. [19]. Small pore gels with 7.5% acrylamide at pH 4.3 with β-alanine as buffer were prepared by the method of ref. [20]. Sucrose (50 mg/ml) was added to enzyme preparations containing *ca* 3000 nkat/ml. Samples (10–100 μl) were added to each tube tube (anodic end) without stacking gel, together with 10μl 0.1% methyl green in 5% sucrose as marker. After stacking for 1 hr at 2 mA/tube the voltage was increased to 100 (10 mA/tube). When the methyl green had migrated to within 3 mm of the cathode end (after *ca* 30 min) the tubes were removed. The final voltage was 120 and the current 8 mA/tube. Gels were stained in Coomassie blue for protein [21]. For the detection of the enzyme, a modification of ref. [22] was used, previously applied to the detection of pea diamine oxidase after gel electrophoresis. The gel was immersed in 10 ml of pH 6.5 Pi buffer containing spermidine (5×10^{-5} M) guaiacol (5×10^{-5} M) and peroxidase (200 purpurogallin units). The enzyme showed an intense brown band within 5 min of immersion. In establishing mobility a correction was applied for the slight elongation of the gels on staining. The band of enzyme activity and the Coomassie blue stained protein bands were stable for more than 12 months in 12% TCA.

Assay. Peroxidase/guaiacol system. This was conducted at 30° by the method of ref. [2]. The assay was almost linear for up to ΔA 0.075/min (1 nkat) (Table 1). On delaying the addition of substrate for 2 hr after being left at pH 6 and 30° with the peroxidase/guaiacol assay mixture, no loss of activity could be detected. On reducing the guaiacol concentration to 1% of the normal level, the rate of increase of A was reduced to 50% of the normal rate. There was no increase in A on adding spermidine to the reaction mixture without enzyme, or on adding enzyme without spermidine. *Oxygen electrode.* This was conducted at 30° by the method of ref. [4].

Density gradient centrifugation. Sucrose gradients (5–20%) were prepared in 0,1 M Pi buffer (pH 7.5) with 10^{-3} M EDTA and 10^{-5} M FAD. Samples containing 20 μl of enzyme (30 μg of protein, 6600 nkat/mg protein) and 50 μl of 0.1% soln of catalase were applied to each gradient (total vol. 5 ml). After centrifugation for 16 hr at 0°(165000 *g*) the gradients were fractionated and assayed for polyamine oxidase and catalase.

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