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# POLYAMINE OXIDASE FROM BARLEY AND OATS

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**Key Word Index**—*Hordeum vulgare*; barley; *Avena sativa*; oats; cereals; Gramineae; spermine; spermidine; polyamine oxidase.

**Abstract**—The pH optimum for the stability of the barley leaf polyamine oxidase is 4.8, which is also the pH optimum for its activity with spermine as substrate. Zonal centrifugation indicates that the enzyme is associated with a particle which is slightly more dense than chloroplasts, and the peak of activity corresponds with the peak of nucleic acid. Neither DNase nor RNase released the enzyme from the particles, despite the hydrolysis of more than 50% of the nucleic acid. The enzyme from the leaves of oat seedlings grown in the dark was purified 900-fold. $Mg^{2+}$ and $Ca^{2+}$ inhibited both barley and oat enzymes by *ca* 50% at 50 mM. The optimum pH for both spermine and spermidine oxidation by the oat enzyme was 6.5. The MW of the enzyme from both sources determined by gel chromatography was *ca* 85 000.

## INTRODUCTION

The polyamine oxidases of the Gramineae, first described in extracts of barley leaves [1], provide the only known specific pathway of spermine and spermidine degradation in higher plants. Polyamines interact with nucleic acids and stimulate growth in several microbial and tissue culture systems [2]. Moreover, rapidly growing cells contain increased levels of spermidine [3]. The possibility that the polyamine oxidase influences the growth rate by regulating the level of the polyamines makes further study of this enzyme of particular interest. In previous work, some properties of the polyamine oxidase of barley [4] and maize [5] leaves have been investigated. This paper describes further properties of this enzyme in barley, and partially characterises the enzyme from oats. In addition, the enzymes from both sources have been purified.

## RESULTS AND DISCUSSION

Activity was *ca* six times greater in oats (Table 1) than in barley (Table 2) or in maize (Table 1) under optimal assay conditions. Activity was low in extracts of the shoots of rice, rye, wheat and millet. In barley, activity in the leaves of seedlings grown in the dark (dark-grown) was greater in the nitrate medium, and activity in the leaves of seedlings grown in the light (light-grown) was greater with the ammonium medium (Table 2). Light-grown barley shoots, contained more putrescine (×10) and polyamines (×2) in the ammonium than in the nitrate medium [6].

### *Inability of nucleases to release enzyme from particles*

The nucleic acid content of the precipitate was reduced by 50% within the first 30 min on incubation with RNase and DNase, and a corresponding increase in $A_{260\,nm}$ was found in the supernatant. This loss of nucleic acid from the particles which presumably contain the chromatin, also occurred without adding nucleases, probably because nucleases occur naturally in barley and other members of the Gramineae [7, 8]. No significant loss of nucleic acid occurred after the first 30 min and no further increase could be detected in $A_{260\,nm}$ of the supernatant. A progressive loss of total enzyme activity was found in the particles, and after 4 hr incubation at 50° only 25% of the initial activity remained. Throughout this 4 hr period no activity could be detected in the supernatant (detection limit 2% of initial activity) and it therefore appears that 50% of the nucleic acid may be released from the particles without solubilization of the enzyme. In chromatin structures from other

Table 1. Polyamine oxidase activity of the shoots of various cereals

| Plant | Age (days) | Conditions | Assay pH | Substrate | nkat/g fr. wt |
|---|---|---|---|---|---|
| Rice | 14 | dark | 5 | spermine | <0.3 |
| | 14 | light | 5 | spermine | <0.3 |
| Rye | 14 | dark | 5 | spermine | <0.3 |
| | 14 | light | 5 | spermine | <0.3 |
| Wheat | 14 | dark | 5 | spermine | 2.2 |
| | 21 | light | 4.5 | spermine | <0.3 |
| Millet | 14 | dark | 5 | spermine | <0.3 |
| | 14 | light | 5 | spermine | <0.3 |
| Maize | 7 | dark | 6.3 | spermine | 3.3 |
| | | | | spermidine | 5.0 |
| | 8 | dark | 6.5 | spermine | 2.3 |
| | | | | spermidine | 11.3* |
| | 16 | dark | 6.0 | spermine | 7.3 |
| | 21 | light | 6.0 | spermine | 0.7 |
| Oats | 14 | dark | 6.5 | spermine | 43† |
| | 15 | dark | 6.0 | spermine | 22 |
| | 19 | dark | 6.0 | spermine | 22 |
| | 21 | dark | 6.5 | spermine | 67‡ |
| | 21 | light | 4.5 | spermine | <0.3 |

Plants were grown in the dark at 22° or in the greenhouse with natural light, with the nitrate medium only.

* Calculated from the data of ref. [5]. † in Pi buffer 0.1 M. ‡ in Pi buffer 0.5 M.

Table 2. Effect of growing conditions and age on polyamine oxidase activity in barley shoots

| Age in days | Source of Nitrogen | nkat/g fr. wt Grown in dark | Grown in greenhouse |
|---|---|---|---|
| 14 | Nitrate | 2.3 | 1.8 |
| | Ammonium | <0.3 | 12 |
| 21 | Nitrate | 5.5 | <0.3 |
| | Ammonium | 0.12 | 6.2 |

Plants were grown in the dark at 22° or in a greenhouse with natural illumination. The nitrogen source in the nutrient medium was either nitrate only or ammonium only. Values are means of 2 independent estimates with spermine as substrate obtained by the method of ref. [4] in pH 5 citrate buffer using M NaCl for elution.

organisms the accessibility of DNA to enzymic degradation has been found to be either complete [9] or only partial [10]. The inability to release the polyamine oxidase from the particulate fraction of the barley leaves does not preclude the association of the enzyme with nucleic acid because not all the nucleic acid was removed.

*Zonal centrifugation*

The particles bearing the polyamine oxidase were significantly more dense than the chloroplasts in extracts of light-grown barley leaves (22 days old). The peak of the chlorophyll was found in fraction 26, and the peak of the polyamine oxidase occurred in fraction 23. The enzyme activity coincided closely with the peak of nucleic acid determined by $A_{260\,nm}$. In a further separation of the particulate fraction from dark-grown barley leaves (19 days old) the peak for nucleic acid and the polyamine oxidase activities again coincided (in fraction 15). These experiments provide additional support for the association of the enzyme with nucleic acid, and in the case of the light-grown barley leaf extracts eliminate the possibility of an association with chloroplasts. Although these results would be consistent with an association of the enzyme with chromatin, this is not yet established.

*Optimum pH for stability of the barley leaf enzyme*

The stability of the barley leaf enzyme was determined over a range of pH values from 3 to 7.5 by heating at 50° for 1 hr. Activity was greatest in the incubates held at pH 4.5 and 5.25 (75% of the activity of the unheated control). Less than 5% of initial activity was retained at pH 3, 3.5, 6.8 and 7.6. At pH 4.1, 5.5 and 6.0 activity was respectively 65, 55 and 25% of the activity of the original sample. The optimum pH for stability is therefore *ca* pH 4.8, which is also the optimum pH for spermine oxidation [11]. Moreover, 50% of the activity of the barley leaf polyamine oxidase is lost on incubation at pH 7.5 for 15 min at 25°, though the optimum for the oxidation of spermidine is at pH 8.0 [11]. Spermine is therefore more likely to be the natural substrate of the barley enzyme.

*Effect of temperature*

Measuring the energy of activation for the barley leaf polyamine oxidase (NaCl eluate, see Experimental) using the peroxidase/guaiacol assay gave 7350 cal/mol over the range 0° to 23° for barley. Previous work [1], using an assay which necessitated a 3 hr incubation, gave a value of 4850 cal/mol. The energy of activation for the polyamine oxidase appears to be significantly less than for the pea seedling diamine oxidase for which a value of *ca* 14 000 cal/mol (4°–26°) was obtained (unpublished).

Enzyme activity was unchanged after 15 freezing and thawing cycles during one week. On freeze drying, 60% of the activity was recovered. Barley leaf material stored for 2 months at −15° lost 20% of enzyme activity, and the NaCl eluate of the particulate fraction of barley leaves stored at −15° for 2 months lost 10% of activity.

*Purification*

(a) *Barley enzyme.* Ammonium sulphate at 50, 60 and 70% saturation precipitated 60, 90 and 100% respectively of the enzyme (NaCl eluate), and purification in terms of protein was resp. ×3.1, ×2.1 and ×1.7. However, $Me_2CO$ precipitation (50% final concn) gave better purification (×4–×6) and good recoveries (90–100%). To eliminate nucleic acid from the acetone precipitate, the enzyme dissolved in 0.5 M NaCl was dialysed in the presence of cellulose phosphate against NaCl free buffer (pH 4.5). The enzyme was recovered from the cellulose phosphate on washing in 0.5 M NaCl. The efficiency of this step in removing nucleic acid determined by $A_{260\,nm}$ was high, only 1% of the nucleic acid remaining in the preparation. On removal of the NaCl by dialysis in the absence of cellulose phosphate the enzyme precipitated within the dialysis membrane, probably due to the reassociation with the nucleic acid. The cellulose phosphate may provide an alternative polyanion with which the enzyme can associate, and the nucleic acid remains in the supernatant when the cellulose phosphate onto which the enzyme is adsorbed is collected by centrifugation.

Chromatography on Sephadex G-100 of the acetone precipitate dissolved in NaCl (0.5 M) showed the enzyme as a sharp band with a retention volume corresponding to a MW of 85 000. At least 70% of the contaminating protein appeared at the exclusion volume of the column.

Using this procedure the NaCl eluate of the particles obtained from light-grown barley leaves was purified 60- to 70-fold with a recovery of 50–100%, corresponding to an overall purification of about 1000-fold in terms of protein. However, activity of the starting material was low and the activity of the final preparation (2 ml) was only 32 nkat/ml.

(b) *Oat enzyme.* On extraction of oat leaves in water, the activity remained in the supernatant, but could be recovered in a precipitate obtained on acidification to pH 4. Also the enzyme from oats did not adhere to the cellulose phosphate (at pH 6) in the step used to eliminate the nucleic acid.

Initial activity in the oat leaves was considerably higher than in the barley leaves. The final preparation in Table 3 (66 ml) contained 20 nkat/ml at a sp. act. of 6020 nkat/mg. On concentration to 2 ml (630 nkat/ml) the preparation showed only weak shoulders at 350 nm and 450 nm. Maximum A was at 275 nm, though a peak at 265 nm indicated residual nucleic acid (probably less than 5%).

The spermidine oxidase of *Micrococcus* [12] in which the final preparation possessed 250 nkat/ml (sp. act. 150 nkat/mg) showed a spectrum characteristic of FAD. Moreover, the polyamine oxidase of maize [5] in which the activity of the final preparation was 360 nkat/ml (sp.

Table 3. Purification scheme for the polyamine oxidase from oat leaves using 150 g of 3-week-old dark-grown shoots as starting material

| | Volume (ml) | Protein (mg) | Total activity (nkat) | Specific activity (nkat/mg protein) | Purification | Yield (%) |
|---|---|---|---|---|---|---|
| 1. Aqueous extract | 550 | 803 | 5600 | 6.8 | 1 | (100) |
| 2. pH 4 precipitate (NaCl eluate) | 22 | 34 | 4740 | 140 | 20 | 85 |
| 3. 50% acetone ppt. | 2.4 | 2.9 | 4580 | 1620 | 235 | 82 |
| 4. Sephadex G-100 chromatography | 66 | 0.22 | 1340 | 6020 | 885 | 24 |

The enzyme was assayed by the peroxidase/guaiacol method with spermine as substrate at pH 6.

act. 257 nkat/mg) showed an absorbance peak attributed to FAD at 450 nm.

*pH optimum of the oat enzyme*

Using the peroxidase/guaiacol assay the oat enzyme (NaCl eluate) was shown to be optimally active at pH 6.5 with both spermine and spermidine as substrates, unlike the barley enzyme which oxidizes spermine optimally at pH 4.8 and spermidine at pH 8.0 [11]. The oat enzyme therefore more closely resembles the enzyme from maize for which the optima are respectively 5.5 and 6.3 [13].

*Inhibition*

The $(NH_4)_2SO_4$ inhibition of the barley enzyme using the peroxidase/guaiacol assay (Table 4) is similar to that obtained in earlier work using the Warburg assay [1]. Of particular interest is the inhibition of the polyamine oxidases from both barley and oat leaves by relatively low concentration of $Mg^{2+}$ and $Ca^{2+}$ ions, in each case 50% inhibition being obtained with concentrations of 40–50 mM. This inhibition may be related to the known interaction between $Mg^{2+}$ and the polyamines in processes involving nucleic acid [2, 14]. The inhibition of the barley leaf enzyme by $Na_2SO_3$ (50% at 0.1 mM) is consistent with the possibility that the enzyme contains FAD as the prosthetic group [15]. In the maize leaf polyamine oxidase which appears to require FAD as the cofactor no inhibition could be detected; on the contrary, a stimulation (×2.3) was found with 20 mM $Na_2SO_3$ [5]. However, the maize enzyme may function as a dehydrogenase [5] and flavoprotein dehydrogenases do not react with sulphite [15].

In the case of the $MnCl_2$ inhibition, formation of guaiacol oxidation products during the assay was slow initially and maximum activity which indicated 50% inhibition was achieved only after 4 to 6 min incubation. This phenomenon may be related to the known oxidation of $Mn^{2+}$ by peroxidase systems [16].

For the oat enzyme the $K_m$'s for spermine and spermidine were less than $10^{-5}$ M ($O_2$ electrode). Activity was rapidly lost on oxidation of spermine and less quickly with spermidine as substrate. Activity was reduced to 50% on oxidation of 0.25 μmol of spermine. This loss was not due to oxygen depletion since it could not be restored on re-oxygenation, not could it be attributed to the instability of the enzyme in the assay in the absence of added substrate. This loss in activity on oxidation of substrate has also been observed with the barley leaf enzyme [1].

An attempt was made to establish whether the oat enzyme would reduce redox dyes as does the maize polyamine oxidase [5]. On adding spermine to a solution containing oat enzyme and dichlorophenolindophenol (DCPIP) a loss of $A$ at 600 nm was detected, but this loss was reduced by adding catalase, and this suggested that the DCPIP is decolorized by $H_2O_2$ formed in the course of amine oxidation, possibly catalysed by traces of peroxidase in the enzyme preparation. No reduction of DCPIP with or without *p*-benzoquinone, with or without catalase, could be detected for the barley enzyme.

## EXPERIMENTAL

*Plants.* Barley (*Hordeum vulgare* L. cv. Zephyr), maize (*Zea mays* L. cv. Golden Cross), millet (*Setaria italica* Beauv.), oats (*Avena sativa* L. cv. Black Supreme), rice (*Oryza sativa* L.), rye (*Secale cereale* L. cv. Lovaszpatonai) and wheat (*Triticum aestivum* L. cv. Atle) were grown in the dark at 22° or in a greenhouse at 20°–30° (May & June 1975). Plants were grown in sand culture, normally with a medium containing $NO_3$. For the experiment cited in Table 2 barley was also grown in a medium containing $NH_4$. Both media contained

Table 4. Effect of inhibitors on activity of the barley and oat leaf polyamine oxidase

| Inhibitor | Final concentration (M) | % Inhibition |
|---|---|---|
| Barley enzyme | | |
| $(NH_4)_2SO_4$ | 0.025 | 50 |
| NaCl | 0.50 | 50* |
| $MgCl_2$ | 0.01 | 9 |
| | 0.04 | 57 |
| | 0.10 | 87 |
| $CaCl_2$ | 0.01 | 0 |
| | 0.10 | 93 |
| $Na_2SO_3$ | 0.0001 | 50 |
| EDTA | 0.02 | 57 |
| $CuSO_4$ | 0.004 | 57 |
| $FeCl_3$ | 0.01 | 50 |
| $MnCl_2$ | 0.01 | 50 |
| Oat enzyme | | |
| 2,4-D | 0.001 | 0 |
| $MgCl_2$ | 0.05 | 70 |
| $CaCl_2$ | 0.05 | 59 |
| | 0.10 | 82 |

The activity of the barley enzyme was determined using the peroxidase/guaiacol assay, and the activity of the oat enzyme was determined using the oxygen electrode. NaCl eluates of the particles were used as the enzyme preparation (see Experimental). The pH was 5 and 6 for barley and oats respectively, with spermine as substrate.

* Inhibition compared with activity in soln containing 0.05 M NaCl.

(in m eq/l.) $K_2SO_4$(4), $MgSO_4$ (3), $CaCl_2$ (8) together with FeEDTA and micronutrients. In addition, the $NO_3$ medium contained $NaH_2PO_4$ (4) and $NaNO_3$ (12) and the $NH_4$ medium contained $Na_2HPO_4$ (1) and $NH_4Cl$ (12). The initial and final pH values of the $NO_3$ medium were resp. 3.8 and 4.2, and for the $NH_4$ medium 5.4 and 4.6. *The enzyme was assayed* at 30° by the peroxidase/guaiacol couple which estimates the $H_2O_2$ produced [11], or by the oxygen electrode (Table 4) [4]. Activities are expressed in nkat (the amount of enzyme catalyzing the oxidation of 1 nmol of substrate per sec). The activity of the enzyme in the cereal species (Tables 1 and 2) was obtained by the method of Ref. [4], using M NaCl at the pH stated in the Tables.

*Effect of nucleases on the enzyme in the particles.* Crude green ppt. obtained from frozen extracts of light-grown barley leaves prepared in $H_2O$ [1] was washed in buffer (pH 4.5, 0.1 M citrate) 3 × and resuspended in 12 ml of this buffer with a glass homogenizer. Samples (2 × 1.5 ml) were removed and RNase (670 nkat/mg; Boehringer) and DNase II (3.3 nkat/mg; Koch-Light) (500 μg of each) in 1 ml of buffer was added to the remainder. Further duplicate 1.5 ml samples were withdrawn at intervals on incubation at 50°. One set of samples was centrifuged and the nucleic acid estimated in the supernatant by $A_{260\,nm}$. Nucleic acid in the ppt. was released by a modification of the method of Ref. [17]. The ppt. was suspended in 3 ml M NaCl and 3 ml of a mixture of octanol–$CHCl_3$ (1:8) was added and dispersed by vortex mixing. On centrifugation at 11 000 *g* the $A_{260\,nm}$ of the supernatant was measured to estimate the nucleic acid in the particles.

*Zonal centrifugation.* Barley leaves were homogenised in unbuffered sucrose (0.5 M, 2 ml/g fr. wt), squeezed through muslin and layered on a gradient of sucrose (1430 ml) (0.5–2.2 M) in a zonal rotor. After equilibration for 18 hr at 6000 *g*, the gradient was displaced with $H_2O$ and collected in 20 ml fractions. Chlorophyll was estimated by $A_{450\,nm}$ and nucleic acid by $A_{260\,nm}$. The enzyme was determined by the peroxidase/guaiacol assay.

*pH for optimal stability of barley enzyme.* Enzyme preparation (NaCl eluate) (0.2 ml) was incubated for 1 hr at 50° with 5 ml of citrate of Pi buffers covering the range pH 3–7.5. The solns were cooled to 0° and the pH values recorded. Each soln was then adjusted to pH 4.5 with 0.1 M NaOH or HCl and the vols made up to 10 ml with pH 4.5–0.1 M citrate buffer. Samples (2 ml) were used for the assay by the peroxidase/guaiacol method.

*Enzyme purification.* (a) *Barley enzyme.* The particulate fraction of the leaves was extracted with 0.5 M NaCl and used undialysed (NaCl eluate) [4]. After cooling to 0° an equal vol. of $Me_2CO$ at −15° was added and the ppt. collected by centrifugation at 3000 *g* for 5 min at 0°. The ppt. was redissolved in 0.5 M NaCl (pH 4.5). Cellulose phosphate (Whatman P 11) was added (2 mg/ml) and the soln dialysed for 18 hr against NaCl-free citrate buffer at pH 4.5. Cellulose phosphate was collected by centrifuging and washed in NaCl-free buffer. The enzyme was recovered by washing the cellulose phosphate in 0.5 M NaCl in pH 4.5 citrate buffer. The enzyme (17 nkat in 2 ml) was chromatographed (8 ml fractions, 3/hr) on a column of G-100 Sephadex (3.5 × 87 cm) equilibrated with 0.5 M NaCl in pH 4.5 buffer. The column was calibrated with blue dextran, bovine serum albumin (monomer and dimer) and cytochrome *c*.

(b) *Oat enzyme.* Oat leaves were extracted in 4 vol. cold $H_2O$, the macerate was squeezed through muslin and the residue re-extracted in 2 vols cold $H_2O$. Extracts were combined and the pH adjusted to 4 with saturated citric acid. The ppt. was collected by centrifuging at 2500 *g* for 15 min and extracted in pH 6 citrate buffer containing 1 M NaCl. The supernatant obtained after centrifuging at 3000 *g* for 15 min (NaCl eluate) was cooled to 0° and 1 vol. of $Me_2CO$ at −15° was added. The ppt. collected by centrifugation was extracted in 1 M NaCl in pH 6 citrate buffer and chromatographed on the Sephadex G-100 column used for the barley enzyme purification, equilibrated in 1 M NaCl (pH 6). The column was recalibrated in this buffer for the MW estimation. After elution the enzyme was concentrated by Millipore filtration (UM10). The pH optimum was determined in citrate (pH 3.5–6.5) or HEPES (pH 7–8) buffers. Protein was estimated by the method of ref. [18] with BSA as standard.

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