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POLYAMINE OXIDATION BY ENZYMES FROM *HORDEUM VULGARE* AND *PISUM SATIVUM* SEEDLINGS

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; *Pisum sativum*; Leguminosae; pea; amine oxidase; putrescine; spermidine; spermine.

Abstract—The properties of the amine oxidases of barley leaves and pea seedling cotyledons have been compared using a colorimetric assay in which the hydrogen peroxide formed is utilized in the peroxidative oxidation of guaiacol. The K_m value for the barley enzyme with spermidine as substrate was 2×10^{-5} M (pH 7·5), and for spermine the K_m was 3×10^{-5} M (pH 4·5). The pH optima for the barley enzyme with spermidine and spermine were 8·0 and 4·8 respectively. The K_ms for the pea enzyme for putrescine, spermidine and spermine were 4×10^{-5}, 5×10^{-6} and 9×10^{-5} M determined at the pH optima 7·5, 8·5 and 7·8 respectively. At pHs above neutrality the barley enzyme is unstable, and at pH 7·5 50% of the activity is lost in 15 min at 25°. Activities for both spermidine and spermine are lost simultaneously. However, one fraction of the enzyme (*ca* 20% of the total activity) is stable under these conditions. Up to 5 fractions of the barley enzyme were separated by electrophoresis. At their respective pH optima spermine was oxidised 14 times faster than spermidine. Spermine oxidation was inhibited by a series of spermine analogues in which the number of methylenes in the central chain was 2, 3, 6, 8 and 10; greatest inhibition was found with analogues having 6 and 10 methylenes. None of these analogues served as substrates. At pH 4·5 spermidine inhibited spermine oxidation (K_i 10^{-4} M) and 50% inhibition of activity with spermidine as substrate (pH 7·5) was given with $3{\cdot}5 \times 10^{-6}$ M spermine. Phosphate ions inhibit the oxidation of spermine by the pea enzyme and the oxidation of spermidine by the barley enzyme. Spermine oxidation by the pea enzyme gave a stoichiometric value which approached 2. For the barley enzyme at pH 4·5, spermine oxidation was 580-fold greater than at pH 7·5. For spermidine at pH 7·5, activity was 35-fold greater than at pH 4·5.

INTRODUCTION

THE POLYAMINES spermidine and spermine, and the related diamine putrescine, appear to be ubiquitous in animals and plants,[1] and at least one of these three amines is probably present in all microorganisms.[2] Since these amines are known to be involved in the regulation of nucleic acid function[2,3] it is of considerable interest to study the enzymes which are concerned in their metabolism.

An enzyme which has been partially characterized from extracts of barley leaves[4] (E.C. 1.5.3.3) oxidizes the polyamines spermine and spermidine at the secondary amino group to give respectively 1-(3-aminopropyl)pyrroline and 1-pyrroline. 1,3-Diaminopropane and hydrogen peroxide are formed in the case of both substrates. The enzyme from barley leaves is especially active with spermine, but a similar enzyme characterized

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[3] STEVENS, L. (1970) *Biol. Rev. Cambridge Phil. Soc.* **45**, 1.
[4] SMITH, T. A. (1972) *Phytochemistry* **11**, 899.

in extracts of maize leaves has a greater activity with spermidine than with spermine.[5] Polyamine oxidases are widely distributed in the Gramineae. The amine oxidase of the Leguminosae (E.C. 1.4.3.6) is the only other plant enzyme known to be capable of oxidizing the polyamines. This enzyme, which has been studied intensively in pea seedlings,[6,7] attacks primary amino groups only, with the formation of an aldehyde, NH_3 and H_2O_2. It is particularly active with putrescine or cadaverine as substrate, but it will also oxidize primary amino groups in spermidine and spermine[8] and a variety of other mono- and di-amines.[6,7] Oxidation of putrescine by the pea seedling enzyme yields 1-pyrroline, and spermidine gives 1-(3-aminopropyl)pyrroline. Spermine is probably oxidized initially to a dialdehyde, though at 37° the dialdehyde may break down spontaneously to putrescine which is further oxidized to 1-pyrroline.[8,9]

In previous work with amine oxidase, several assay systems have been utilised[10,11] depending on oxygen uptake, and H_2O_2, NH_3 and aldehyde formation. Methods depending on the formation of H_2O_2 provide colorimetric and fluorimetric techniques for continuously monitoring enzyme activity. In the fluorimetric assays the H_2O_2 is further utilised by peroxidase to oxidize certain phenolic substrates (*p*-hydroxyphenylacetic acid, scopoletin and homovanillic acid)[12–14] The resultant change in the fluorescence provides an assay with a very high sensitivity at neutral pH and above. However, at the low pH (4–5) required by the barley leaf polyamine oxidase for optimal activity with spermine as substrate the fluorescence intensity is low.[14] In other work *o*-dianisidine has been used as the peroxidase substrate and activity estimated by the increase in absorbance at 440 nm.[15–17] On peroxidative oxidation, guaiacol (*o*-methoxyphenol) forms a colored complex,[18] and in the present work a colorimetric procedure utilizing the peroxidase/guaiacol couple has been adapted to determine the H_2O_2 formed in the course of amine oxidation.

RESULTS AND DISCUSSION

Stability of barley leaf polyamine oxidase

Enzyme (2 ml) was incubated with 0·1 M (pH 7·5) triethanolamine buffer (20 ml) at 25°, and 2·2 ml samples were removed at intervals for assay with spermidine as substrate at pH 7·5. Simultaneously, 0·2 ml samples were removed, added to pH 4·5 buffer (2 ml) and assayed with spermine. Apparently the same rate of loss of activity was found with both spermidine and spermine in two separate experiments. Half the activity was lost after about 15 min. After 30 min activity was reduced to about 20% of the original, and no further reduction in activity was observed.

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The results are consistent with the hypothesis that activity with both spermidine and spermine as substrate is due to the same enzyme. Moreover, the results suggest that the enzyme is heterogeneous, with a fraction which is progressively destroyed at pH 7·5 comprising about 80% of the activity, the residual 20% being due to a component stable at this pH. The low stability of this enzyme even at pH 7·5 is unusual and is a complication in the estimation of activity with spermidine. By contrast, the pea enzyme is stable for at least 2 hr at 37° between pH 3 and 10[7]. Additional evidence for heterogeneity was provided by electrophoresis on both starch gel and cellulose acetate strips. Up to five bands were detected, with mobilities ranging from 1 to 6 mm per hr. This is in contrast to the purified pea enzyme which is homogeneous on electrophoresis at pH 3·4, 7·4 and 9·0.[13]

pH *optimum*

Barley enzyme. The pH optimum for spermine oxidation was established using citrate buffer, and all pH values were determined after the activity measurements were completed. Activity was maximal at pH 4·8 (Table 1) and half-maximal activity occurred at pH 4 and 5·6. Activity was reduced to 10% at pH 3·5, and was barely detectable at pH 6·0 or 6·5. At pH 4·0, reduction of peroxidase concentration to 10% of the standard level caused no change in apparent activity of the polyamine oxidase.

TABLE 1. pH OPTIMUM, MICHAELIS CONSTANT, PROBABLE STOICHIOMETRY AND RELATIVE MAXIMUM VELOCITY AT OPTIMUM pH FOR THE *Hordeum vulgare* LEAF POLYAMINE OXIDASE AND THE *Pisum sativum* SEEDLING DIAMINE OXIDASE

	Substrate	Optimum pH	K_m	Standard error	Relative V_{max} (spermine = 1)
Barley leaf polyamine oxidase	Putrescine	Not oxidized			
	Spermidine	8·0	2×10^{-5} M*	10^{-5} M	0·07
	Spermine	4·8	3×10^{-5} M†	3×10^{-6} M	1·0
Pea seedling diamine oxidase	Putrescine	7·5	4×10^{-5} M	5×10^{-6} M	5·5
	Spermidine	8·5	5×10^{-6} M	10^{-7} M	3·0
	Spermine	7·8	9×10^{-5} M	4×10^{-5} M	1·0

* Determined at pH 7·5.
† Determined at pH 4·5.

The pH optimum for spermidine oxidation was determined in citrate-NaOH (pH 6–6·5), triethanolamine–NaOH (pH 6·8–7·7), Tris-HCl (pH 7·1–8·9) and bicarbonate-carbonate (pH 9·2–10·8) buffers. Since the enzyme was unstable at a pH above 7, the reaction mixture without enzyme was pre-incubated with substrate at 25° for 2 min before adding enzyme at zero time to start the reaction. Greatest activity was found at pH 8, with half-maximal activity at pH 8·4 and 7·3. Activity was negligible at pH 9·1 and 5·5. At pH 8·4 reduction of the peroxidase concentration to 10% of the standard level caused no change in apparent activity of the polyamine oxidase. The optimal pH for spermine oxidation (pH 4·8) was somewhat higher than previously found (pH 4·0) using manometry.[4] The optimal pH for spermidine oxidation was also greater than previously found (pH 6·6). In previous work to establish the optimum pH for spermidine oxidation[4] a 22 hr incubation was used during which the enzyme was continuously denatured at the higher pH values. The apparent optimum was therefore shifted to a lower pH. In previous work[4] using manometry at pH 4·5 with spermine as substrate, activity was around 30 times greater than with spermidine at pH 6·6 during the 22 hr

incubation period. In the present work using the colorimetric enzyme assay with both substrates, at their respective pH optima, spermine (pH 4·8) was oxidized 14 times faster than spermidine (pH 8·0).

Pea enzyme. The pH optimum for putrescine oxidation by the pea enzyme was investigated in Tris buffer from pH 7 to 8. Optimal activity was found at pH 7·4–7·6, confirming the results of Kenten and Mann.[19] With spermidine as substrate, Tris buffer was used for pH values from 7·2 to 8·6, citrate at less than 6·0, and carbonate–bicarbonate buffers at pH 9·3 and 10·1. Optimal activity was found at pH 8·5, and activity was reduced to half-maximal at pH 7·5 and 9·1. Activity was less than 10% maximal at pH 6·3 and 10·1. With spermine as substrate, activity was measured in triethanolamine–NaOH buffer (pH 6·8–8), Tris-HCl (pH 8–8·6) and carbonate–bicarbonate at pH 9·4 and 10·3. Activity was optimal at pH 7·8, and fell rapidly above and below this value. Half-maximal activity occurred at pH 7·2 and 8·1, and was less than 2% at pH 10·3. At low pH values (< pH 7·0) a slight protein precipitate formed on adding spermine to the enzyme, though this did not interfere with the assay. All pH values were determined after the activity measurement was completed.

Michaelis constants

Barley leaf enzyme. The K_m for spermidine, determined at pH 7·5 since the enzyme is very unstable at its optimum (pH 8·0), was 2×10^{-5} M (Table 1). With spermine as substrate the enzyme was virtually saturated at and above 10^{-3} M and activity was unchanged at a final concentration of 5×10^{-3} and 10^{-2} M spermine. However at 4×10^{-2} M spermine, activity was reduced by about 50%. The K_m was 3×10^{-5} M at pH 4·5.

Pea seedling enzyme. Yamasaki *et al.*[20] give $7{\cdot}4 \times 10^{-5}$ M as the K_m for putrescine determined at pH 8·0, and McGowan and Muir[13] give $8{\cdot}2 \times 10^{-5}$ M determined at pH 7·0. In the present work, the K_m was 4×10^{-5} M at pH 7·5 (optimum). For spermidine and spermine McGowan and Muir give $1{\cdot}9 \times 10^{-3}$ M and $1{\cdot}3 \times 10^{-3}$ M (respectively) both determined at pH 7·0. These values are considerably higher than those found in the present work, i.e. 5×10^{-6} M and 9×10^{-5} M for spermidine and spermine respectively, determined at the pH optima.

Rate of oxidation of the di- and poly-amines

Barley enzyme. At pH values close to the optima, spermine (pH 4·5) was oxidized 14 times faster than spermidine (pH 7·5). At pH 4·5, activity for spermine was 580-fold greater than activity at pH 7·5. For spermidine activity at pH 7·5 was at least 35-fold greater than at pH 4·5. The limit for this latter value was set by the stability of the spectrophotometer. None of the polyamine analogues tested in the inhibition experiment acted as substrates for the enzyme (limit 2% of activity with spermine).

Pea enzyme. Determined at their respective pH optima, initial activities with putrescine, spermidine and spermine were in the ratio of 5·5 : 3·0 : 1·0 (Table 1). In previous work in which the ratio was determined only at pH 7·0, Kenten and Mann[19] found that the ratio of activities with putrescine and spermine as substrates was 12 : 1, while Hill and Mann[21] give the ratio for putrescine, spermidine and spermine as 280 : 100 : 1,

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and McGowan and Muir[13] give 7 : 10 : 1 for this ratio. Werle *et al.*[7] found that the ratio for the oxidation of cadaverine, spermidine and spermine was 12 : 7 : 1.

TABLE 2. EFFECT OF PHOSPHATE IONS ON THE ACTIVITY OF *Hordeum vulgare* LEAF POLYAMINE OXIDASE AND *Pisum sativum* SEEDLING DIAMINE OXIDASE IN THE OXIDATION OF PUTRESCINE AND THE POLYAMINES

Enzyme	Substrate: Putrescine pH	Putrescine Activity	Spermidine pH	Spermidine Activity	Spermine pH	Spermine Activity
Barley leaf polyamine oxidase	Not oxidized		7·5	50%	4·5*	100%
Pea seedling diamine oxidase	7·5	80%	8·5	71%	7·8	23%

Activity in the presence of phosphate buffer (0·1 M) is expressed as a % of the activity using a non-phosphate buffer of the same pH. Tris (0·1 M) buffer was used for all except * in which activity in 0·1 M citrate buffer was compared with activity in 0·1 M citrate/phosphate buffers.

The lower values obtained for spermine relative to putrescine and cadaverine[7,19,21] might be explained in part by the use of phosphate buffer (see Table 2). It is also possible that the enzyme becomes modified on purification. However, an additional explanation may be proposed on the basis of product inhibition. The oxidation of spermine by the pea enzyme probably occurs at the terminal amino groups to give the mono- and di-aldehydes.[8,22–24] These are highly reactive, readily forming Schiff bases with free amino groups, and do not undergo ring closure unlike the aldehyde products of putrescine and spermidine oxidation. The amino-aldehydes derived from spermine, or the acrolein formed by the spontaneous decomposition of these aminoaldehydes,[9] may combine with the amine oxidase resulting in enzyme inhibition. Greater inactivation with spermine could have been expected in previous work with highly-purified preparations,[7,13,19,21] since the large excess of inert protein, present in the crude preparations used in the present study, was absent. Moreover, if the monoaldehyde polymerizes, or condenses with amino groups in protein or nucleic acid, potential substrate may become inaccessible to the enzyme so that loss of substrate may account for the difficulties encountered in the present work.

A decline in activity with time was found with spermine as substrate which was considerably greater than that found with spermidine and putrescine. This may be attributed both to the higher K_m and to the biphasic nature of the oxidation of spermine, oxidation of the monoaldehyde probably being slower than the oxidation of intact spermine.

Stoichiometry

The stoichiometry of the barley and pea enzymes was investigated by comparison of the increment of the absorbance obtained with the amine substrates (5×10^{-8} mol) with an equimolar amount of putrescine oxidized by the pea enzyme, which is known to produce 1 mol H_2O_2/mol putrescine.[19] The barley enzyme was incubated with spermine at pH 4·5, and the pea enzyme was similarly incubated with putrescine at pH 7·2. The absorbance increment for spermine oxidation by the barley enzyme was 92 and 94% relative to putrescine oxidation by the pea enzyme ($A_{470} = 0{\cdot}10$) in duplicate experiments.

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In a separate experiment spermidine and spermine were oxidized at pH 7·5 and 4·5 respectively by the barley enzyme. The increments of absorbance for spermidine were 103·5 and 101% (duplicate determinations) relative to spermine.

In the oxidation of spermine by the pea enzyme values of 1·45, 1·74 and 1·90 mol H_2O_2 per mol amine were obtained in replicated determinations. Variation in the stoichiometry may be attributed to the reactivity of the monoaldehyde formed. In a comparison of the stoichiometry for the oxidation of equimolar amounts of putrescine and spermidine by the pea enzyme, the absorbance with spermidine was 95, 101 and 98% of that obtained with putrescine.

Inhibition

A range of spermine analogues was tested for their ability to inhibit the barley enzyme with spermine as substrate. In these analogues the putrescine moiety in spermine was replaced by a smaller or larger number (x) of methylenes. Inhibition was least with the short chain analogues ($x = 2$). In all cases inhibition was competitive, inhibition being measured with 10^{-3} and 2×10^{-5} M spermine. Prior incubation with inhibitor for 30 min did not increase their effectiveness. Inhibition of spermine oxidation due to arcain, agmatine and guanidine was weak. Putrescine at 10^{-3} M gives no inhibition with spermine at 2×10^{-5} M; nor is putrescine a substrate. Spermine was an effective inhibitor of spermidine oxidation; with spermidine at 10^{-3} M, spermine at $3{\cdot}5 \times 10^{-6}$ M caused 50% inhibition.

Phosphate ions are known to stimulate the oxidation of benzylamine by the pea enzyme in the presence of glycine, bicarbonate and borate buffers.[13] The effect of the phosphate ion on the oxidation of putrescine and the polyamines by both the barley and pea enzymes was therefore investigated, and the results are presented in Table 2. In phosphate buffer a decrease in the rate of oxidation of putrescine, spermidine and especially of spermine by the pea enzyme was found. Oxidation of spermine by the barley enzyme was unaffected, but spermidine oxidation was reduced to 50% in the presence of phosphate ions. The apparent inhibition of the polyamine oxidation by the phosphate ion is not necessarily due to an effect on the enzyme itself, and may be due to the removal of the substrate from solution in the form of the insoluble phosphate salts.

Estimation of spermine with polyamine oxidase

A calibration curve was established for increments of spermine ranging from 5×10^{-9} mol to 10^{-7} mol. The relationship was linear and passed through the origin. This method might provide a sensitive and highly specific technique for estimating spermine in tissue extracts. However, on investigating this possibility with barley leaf amine fractions[25] a low enzyme activity was found, probably due to the high level of spermidine in the amine fractions, which acts as an inhibitor of the barley leaf polyamine oxidase.

Conclusion

The guaiacol–peroxidase method for the estimation of H_2O_2 in determining the activity of the amine oxidases in the present study appears to be sensitive and convenient. However, the guaiacol oxidation products include certain quinones[18] which generally are highly reactive. These could combine with other compounds in the reaction

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mixture, modifying the chromogen, enzyme or substrate. For example, Suzuki[26] has shown that putrescine may be deaminated by quinones. Moreover, Mann and Smithies[27] have also shown that the pyrroline formed from putrescine in the presence of diamine oxidase may be further oxidized on addition of peroxidase. For these reasons some of the results obtained with this assay in the present work may be subject to reinterpretation. Despite these potential complications, the stoichiometry attained with the various substrates suggests that the method provides a reliable estimate of enzyme activity.

EXPERIMENTAL

Enzyme preparation. Barley leaf polyamine oxidase was prepared as before[4] and normally dialysed against 0·1 M citrate buffer (pH 4·5). The pea seedling enzyme was purified by $(NH_4)_2SO_4$ precipitation (active fraction at 30–60% saturation[19]) and dialysed against 0·1 M Tris buffer (pH 7·5). Both the pea enzyme[7] and the barley enzyme are stable to freeze-drying, and freeze-dried preparations of the barley enzyme were used in investigating the inhibition due to the phosphate ion and for the inhibition of spermidine oxidation.

Assay. Air equilibrated buffer (2 ml, 0·1 M), guaiacol (0·1 ml, 25 mM), peroxidase (Sigma Type 2, 200 purpurogallin units/mg; 0·1 ml, 1 mg/ml) and the amine oxidase preparation (0·2 ml) was placed in matched cylindrical spectrophotometer tubes (1·2 × 12·5 cm). After pre-incubation at 25° for 2 min in a H_2O bath, 0·1 ml of amine substrate (2·5 μmol, unless stated otherwise) was added and the absorbance at 470 nm was determined in a spectrophotometer. The output was fed into a Vitatron VR 403 logarithmic recorder (FSD = 0·2 absorbance units). Activity was determined on extrapolation of the curve to zero time. The water-jacketed cuvette holder was at 25°. Inter-contamination of the polyamine substrates could cause strong inhibition of the barley enzyme. Moreover, in the experimental determination of the ratios of activities at pH 4·5 and 7·5 for spermidine and spermine, even small inter-contamination (< 0·2%) could significantly affect this ratio. Commercial samples of the polyamines contained varying proportions of contaminants, and these impurities were reduced by successive re-crystallisation (EtOH) before use. Under standard assay conditions with the greatest barley enzyme activity measured, the peroxidase became limiting only on reduction to 2–3% of the concentration normally used. Guaiacol became limiting (50% activity) on reduction to 5×10^{-5} M final concn, i.e. *ca* 5% of the normal concn. At 10^{-2} M guaiacol (final concn) the activity was inhibited by 20%. O_2 dissolved in the reaction mixture was calculated to be 14·5 μl at 25° (2·5 ml final vol.) which is equivalent to the amount needed to oxidize 0·65 μmol of substrate (since in the presence of peroxidase 2 atoms oxygen are equivalent to 1 mol substrate). The absorbance expected on oxidation of this amount of substrate corresponds to about 2·5 A_{470} units. The non-linearity of the reaction may be attributed to inactivation of polyamine oxidase by quinone intermediates of the peroxidative oxidation of guaiacol. Additional complications arose from loss of colour, and this was dependent on the experimental conditions. For example, in the experiments designed to determine the stoichiometry of the enzymes the A_{470} tracing obtained on oxidizing the putrescine by the pea enzyme rose to a peak and dropped as quickly by 5% before the colour stabilized. The trace obtained on oxidizing spermine with the barley enzyme rose to a peak and then declined progressively at about 1% per min. Since most assays were completed within 2 to 3 min, the error from this source was negligible.

Electrophoresis of barley enzyme. Starch gel was used in the Shandon vertical apparatus in a slab 12 × 24 × 0·6 cm with 25 mM (pH 4·5) citrate buffer. Separation was effected with 6 V/cm (25 mA) in 30–40 hr. The buffer was changed periodically during the run. The plate was developed by spraying 10 ml 2·5 mM spermine and 50 mM KI in H_2O. The blue colour, indicative of amine oxidase, developed within 15 min. The fastest component moved at 6 mm/hr. A mixture of basic fuchsin and toluidine blue was used to monitor mobility. The fastest enzyme component moved to the cathode at the speed of the basic fuchsin and the toluidine blue travelled at 2× this speed. Cellulose acetate electrophoresis was effected in 0·1 M (pH 4·5) citrate buffer on 5 × 20 cm strips. The starch/spermine/KI spray will not produce a blue colour on cellulose acetate, only the yellow iodine colour being detected, and an alternative peroxidase-based spray was utilized. The solution contained 5 ml of satd guaiacum gum in EtOH, 3 ml 0·1 M (pH 4·5) citrate buffer, 1·5 ml 25 mM spermine and 0·5 ml H_2O containing 0·5 mg peroxidase. On mixing, a small vol. of EtOH (about 1 ml) was added to dissolve the ppt. which formed. This spray gave a green colour[28] in the presence of polyamine oxidase on the cellulose acetate strips.

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