Diamine oxidase is involved in H$_2$O$_2$ production in the chalazal cells during barley grain filling

Bavita Asthir$^{1,2}$, Carol M. Duffus$^{1,3}$, Rachel C. Smith$^1$ and William Spoor$^1$

$^1$ Crop Science Department, Scottish Agricultural College, West Mains Road, Edinburgh EH9 3JG, Scotland, UK

Received 6 August 2001; Accepted 26 November 2001

Abstract

The localization and activities of diamine oxidase (DAO, EC 1.4.3.6) and polyamine oxidase (PAO, EC 1.4.3.4) together with polyamine levels have been investigated in developing grains of barley (Hordeum vulgare L.). DAO (pH 7.5) is present mainly in vascular tissue and its neighbouring cells, namely chalazal cells and nucellar projection, while PAO (pH 6.0) is mainly localized in the chlorenchymatous cells of the crease and at the base of the vascular tissue. Activities of both these enzymes appear to be independently-regulated, as DAO activity increased steadily throughout grain development while PAO activity was higher during the early stages of grain filling, declined thereafter and again increased towards maturity. The maximum activities of DAO coincided with the maximum content of putrescine while the levels of PAO did not seem to be directly correlated with spermidine or spermine contents. Isoelectric focusing (IEF) of DAO and PAO activities revealed the presence of bands at 30 and 45 DPA. The possible involvement of DAO and PAO in the supply of H$_2$O$_2$ to peroxidase-catalysed reactions in the chalazal cells during grain filling is discussed.

Key words: Barley, chalazal cells, diamine polyamine oxidases, H$_2$O$_2$, lignification.

Introduction

Most of the assimilate entering the developing endosperm of cereal grains passes through the chalazal cells of the crease. Around 16–18 days post-anthesis (DPA), lignin and suberin deposition takes place in the cell walls (Cochrane et al., 2000). This begins the separation of the apoplast from the symplast and ensures that assimilate then moves into the endosperm via the symplast only. Peroxidases are present in the crease tissue at this time and it has been suggested that they may have a role in the formation of lignin/suberin in the chalazal cell walls (Cochrane, 1994). Peroxidases, however, require a source of H$_2$O$_2$ for activity and the source of this is presently unknown. That amine oxidase present in the apoplastic of pea epicotyls may account for the H$_2$O$_2$ needed in the peroxidatic coupling of lignin subunits and in wall stiffening has been suggested previously (Federico and Angelini, 1986). The objective of the work reported here was to determine whether or not such activity is present in the chalazal cells at the time of lignin/suberin deposition during grain development in barley.

Diamine oxidase catalyses the oxidative deamination of the diamines putrescine (Put) and cadaverine, producing the corresponding aminoaldehyde, ammonia and H$_2$O$_2$, while PAO preferentially cleaves the aminopropyl side-chains at secondary amino groups of polyamine substrates, such as spermidine (Spd) or spermine (Spm), producing H$_2$O$_2$, 1,3-diaminopropene and 1-pyrroline or 1-(3-aminopropyl)-pyrroline, respectively. Any H$_2$O$_2$ generated is not accumulated or if so, then only at very low levels, in healthy non-stressed plant cells (Ros Barcelo, 1998). It has been detected in xylem cell walls using cytochemical (Czaninski et al., 1993; Bestwick et al., 1997) and histochemical (Olson and Varner, 1993; Ros Barcelo, 1998) probes.

In this paper, the localization and activities of DAO and PAO during grain development in barley are reported.

---

2 Present address: Biochemistry Department, Punjab Agricultural University, Ludhiana, Punjab, India.
3 To whom correspondence should be addressed. Fax: +44 (0)131 667 2601. E-mail: c.duffus@ed.sac.ac.uk

Abbreviations: DAO, diamine oxidase; DPA, days post-anthesis; IEF, isoelectric focusing; PAO, polyamine oxidase; PCA, perchloric acid; Put, putrescine; Spd, spermidine; Spm, spermine; TLC, trichloroacetic acid.

© Society for Experimental Biology 2002
Materials and methods

Chemicals and biochemicals

The stains used in microscopy (catalase from bovine liver, horseradish peroxidase Type II) and the substrates used to determine DAO and PAO activities were all supplied by the Sigma-Aldrich Company Ltd, Poole, Dorset, UK. Isoelectric focusing markers were supplied by Pharmacia UK and TLC plates were from Merck Ltd. All other chemicals used were of analytical grade.

Plant material

Spring barley (Hordeum vulgare L.) cv. Chariot was grown (May to October 1999) in natural daylight in an unheated glasshouse, in peat-based compost. Two to three days prior to anthesis, plants were transferred to a growth cabinet set in the glasshouse, in peat-based compost. Two to three days prior to anthesis, plants were transferred to a growth cabinet set at a constant temperature of 16 °C with a daylength of 16 h.

Lighting was supplied by white tube lights and bulbs. The light intensity at ear level was approximately 120 μmol m⁻¹ s⁻¹. Uniformly-growing plants were tagged at anthesis and grain samples were collected at 7, 11, 14, 17, 25, and 45 DPA.

For all studies, fresh barley grains were extracted in triplicate (after removing the glumes and paleae and keeping the pericarp–aleurone intact) and analysed in duplicate.

Extraction and assay of DAO and PAO

Grains of barley were used as a source of enzyme. DAO and PAO activities were estimated spectrophotometrically by a method based on the colorimetric assay of Δ-pyrroline using putrescine (Put for DAO) and spermidine (Spd for PAO) as substrates (Holmstead et al., 1961). Developing grains (2 g, 26–176 grains depending on the stage of development) were homogenized (triplicate samples) at 4 °C in 100 mM K-phosphate buffer (pH 7.0) containing 5 mM dithiothreitol and the extract centrifuged at 16 000 g for 20 min at 4 °C.

In order to solubilize the DAO/PAO activities, the residue was sequentially extracted twice for 10 min with 100 mM K-phosphate buffer (pH 7.0) containing 1 mM NaCl, or 20 mM EDTA, or 1% Triton X-100. For assaying wall-bound activities of PAO, the remaining pellet was used directly. In a 2.0 ml final reaction volume, 0.1 ml of extract was combined with 50 units of catalase, 0.1% 2-aminobenzaldehyde and the reaction started with one of two different buffer and substrate combinations: 10 mM Put in 50 mM K-phosphate buffer (pH 7.5) for DAO; 10 mM Spd in 50 mM K-phosphate buffer (pH 6.0) for PAO. The reaction was carried out at 30 °C for 3 h, and then stopped with 2.0 ml of 10% (v/v) perchloric acid and the tubes centrifuged at 5000 rpm for 15 min. Formation of the Δ-pyrroline product was determined by reading the absorbance at 430 nm. Control reactions were carried out with inactivated enzyme prepared by heating for 20 min in a boiling water bath. Activities are shown as the means of six determinations and are expressed in pmol Δ-pyrroline min⁻¹ g⁻¹ fresh weight.

The conditions of the assays were optimized so as to give linear rates with respect to incubation time, optimum pH and substrate concentration.

Localization using starch–potassium iodide

In this method, the presence of H₂O₂ is inferred when iodine produced by the oxidation of KI forms a purple blue colour in the presence of starch (Smith, 1970). Unfixed, hand-cut transverse sections from developing barley grains were incubated in triplicate in a solution containing 1.3% soluble starch, 20 mM KI, 10 mM Put or Spd in 1 mM phosphate buffer pH 7.5 (for DAO) and pH 6.0 (for PAO), respectively, for periods varying from 30 min to several hours. Control incubations were carried out in the absence of Put or Spd.

Localization using peroxidase and 3-amino-9-ethyl carbazole

This method infers the production of H₂O₂ as a result of DAO or PAO activity through the development of a coloured product produced from the oxidation of an artificial substrate of per-oxidase by H₂O₂ in the presence of endogenous or exogenous peroxidase (± Put or Spd) (Angelini and Federico, 1989). Thus, fresh, hand-cut sections cut transversely through the crease region of grains at various stages of development were washed with 0.05 M K-phosphate buffer, pH 7.5 (for DAO) and pH 6.0 (for PAO) plus 0.1 M NaCl. Sections were then incubated in the same buffer (1 ml) containing 5 μl of 3-amino-9-ethyl carbazole in 0.1 M acetic buffer (pH 5.0). This artificial substrate is oxidized by peroxidase in the presence of H₂O₂, yielding pink/purple red or orange compounds. Following 5 min incubation at room temperature, 10 μl of Put or Spd solution (0.2 M in H₂O) were added, and sections incubated for 2 h at room temperature. Stain development was observed using a Zeiss photomicroscope (Axiohpt). Control sections were treated in exactly the same way and at the same time except that Put and Spd were omitted. Peroxidase activity was visualized by replacing Spd or Put with H₂O₂ using 10 μl of a 2 mM H₂O₂ solution in H₂O. Enzyme activity was found to be very sensitive to pH and substrate concentration. Thus, the pHs and substrate concentrations used were those which were found to give optimal colour development. Additional controls were carried out in the absence of exogenous peroxidase and in the absence of the artificial substrate 3-amino-9-ethyl carbazole. Incubation times were standardized in a set of preliminary experiments in order to give optimal colour development. Enzyme localization was repeated no less than 10 times for each stage of development shown in Fig. 1. Additionally, enzyme activity was localized in sections obtained over a range of stages of grain development from 10–45 d after anthesis. All photographs were taken on Kodak Ectachrome film.

Extraction and estimation of polyamines

Polyamines were extracted in triplicate using a modification of an earlier method (Goren et al., 1982). Developing grains at 4, 11, 25, and 45 DPA were extracted in 5% perchloric acid (PCA) on ice using 100 mg ml⁻¹ tissue. The homogenates were kept on ice for 60 min and centrifuged at 27000 g for 20 min. The supernatant contained free amines and the bound amines in soluble form while the pellet contained insoluble (bound) amines. The bound amines in the supernatant were released by treating the fractions with 6 N HCl at 110 °C for 18 h in a sealed ampule. After heating, the sample was filtered through glass wool, dried under a stream of air at 80 °C, and resuspended in PCA. The fractions were used for polyanamine analysis and stored in plastic tubes at −20 °C. Samples were found to remain stable under these conditions for at least 2 months, including several cycles of thawing and refreezing.

TLC analysis

PCA extracts were analysed for free polyamines following dansylation using a modification of an earlier method (Seiler and Wiechmann, 1967). The dansylation mixture consisted of 200 μl PCA extract (equivalent to about 20 mg fresh tissue),
400 \mu l dansylchloride (Sigma, 5 mg ml\(^{-1}\) in acetone), and 200 \mu l saturated Na\(_2\)CO\(_3\). After overnight incubation at room temperature and an addition of 100 \mu l proline (100 mg ml\(^{-1}\)) to remove excess dansyl chloride, dansyl polyamines were extracted in 0.5 ml of ethyl acetate by vortexing for 15–30 s. The organic layer containing the polyamines was separated by low speed centrifugation (3000 \(g\)), removed and dried under nitrogen (Smith, 1985), and 50 \mu l were used for spotting onto LK 60 Whatman silica gel TLC plates. Authentic polyamine standards (Put, Spd and Spm obtained from Sigma as hydrochlorides) were separated simultaneously by ascending chromatography in every experiment. Identification was accomplished by comparison of \(R_f\) values obtained by TLC using the solvent system: cyclohexane : ethylacetate (5:4, \(v/v\)), followed by chloroform : triethylamine (25:2, \(v/v\)). Dansyl polyamines were quantified in duplicate on silica gel plates using a densitometer with excitation 360 nm and emission at 500 nm.

**Isoelectric focusing**

Proteins were extracted in triplicate from grains (2 g, 26–176 grains) at 7, 11, 14, 17, 25, 30, and 45 DPA in approximately 5 ml distilled water (Glazmann et al., 1988) at 4 °C and centrifuged at 20,000 \(g\) for 20 min. The supernatant was used as the source of protein. The gels were focused for 90 min at 16 °C, current of 50 mA, 30 W and voltage stepped up from 800 to 1500 units during the run. The sample applicator strip was placed nearer the anode than the cathode at a ratio of 1:2 to allow the separation of cationic and anionic proteins, and the strip left on the gel for the whole run to avoid sample cross-contamination. Pharmacia isoelectric focusing calibration markers (broad range pH 3–10) were run on each gel along with the samples (20 \mu l). Gels were run in triplicate for each stage of development. After running, each gel was cut into four sections and each section was developed separately for protein or for enzyme activities. PI markers (Fig. 3, track 1) were visualized with Coomassie Blue. For peroxidase activity (Fig. 3, track 2), gels were soaked for 20 min in 1 mM 3-amino-9-ethyl carbazole and then a 0.3% (\(v/v\)) aqueous solution of H\(_2\)O\(_2\) was added to give a final concentration of 1 mM of H\(_2\)O\(_2\). For PAO and DAO activities (Fig. 3, tracks 3 and 4), gels were soaked in 0.1 M K-phosphate buffer (pH 6.0, for PAO) and (pH 7.5, for DAO), 1 mM 3-amino-9-ethyl carbazole, peroxidase (250 U in 1 ml buffer), and 10 mM Spd (PAO) or Put (DAO) as substrates.
Results

Histochemical localization of diamine and polyamine oxidase

Localization of DAO and PAO activities using the starch–potassium iodide method proved unsatisfactory in that it depends on the presence of starch near the site in the cell where H₂O₂ is being produced. Thus, if a cell does not contain endogenous starch, or the added starch does not penetrate the cells, then a negative result may be obtained even though H₂O₂ is being generated. Furthermore, endogenous catalase may decompose the H₂O₂ as quickly as it is evolved and, although catalase inhibitors such as aminotriazole could be used, not all forms of catalase are inhibited by aminotriazole. Other potential difficulties include the possibility of a negative test due to the presence of some endogenous reductant, such as ascorbic acid, which could reduce H₂O₂ and/or I₂ before enough of the starch–iodine complex had accumulated to be detected. For these reasons, the peroxidase/3-amino-9-ethyl carbazole method was developed for determining the site of DAO or PAO activity.

When transverse sections were incubated in K-phosphate buffer (pH 6.0) containing peroxidase, 3-amino-9-ethyl carbazole and Spd, a dark pinkish colour developed in the chlorenchymatous cells of the crease, and at the base of the vascular tissue (of xylem/phloem) (Fig. 1b, e, h). By contrast, those incubated in buffer (pH 7.5) containing Put and peroxidase developed a bright orange colour in the vascular tissue (i.e. xylem/phloem) and its neighbouring cells, namely chalazal cells and nucellar projection (Fig. 1c, f, i). This pattern of staining was seen in sections of all caryopses harvested at all stages between 10–45 DPA. The maximum intensity of the stain was observed at 10 DPA for PAO and at 45 DPA for DAO during grain development. By contrast, no (or only very faint) colour developed in the absence of exogenous peroxidase (results not shown). Colour was observed only in the presence of the artificial substrate of peroxidase (3-amino-9-ethyl carbazole). No colour developed in the endosperm or in any other part of the grain. Sections appeared coloured at other pHs but the intensity of the colour was faint.

Activities of diamine and polyamine oxidases

Variation in diamine and polyamine oxidase activities over the developmental period is shown in Table 1. Diamine oxidase activity increased steadily throughout grain development while PAO activity showed higher activity at 11 DPA and then declined until 25 DPA. It again increased towards grain maturity, i.e. at 45 DPA. Between 2% and 24% of total PAO activity was tightly-bound to insoluble material and could not be released with 1 M NaCl. In order to characterize the interaction between the enzyme and the cell wall, the pellet was sequentially extracted with 0.1 M K-phosphate buffer (pH 6.5) containing 1 M NaCl or 1% Triton X-100, or 0.02 M EDTA. Of total enzyme activity, 24% was released by NaCl, 9% by EDTA and 45% by Triton X-100 (results not shown).

Polyamines

The qualitative and quantitative estimations of polyamines (Put, Spd, and Spm) in barley grain expressed on a fresh weight basis at different stages of grain development are shown in Fig. 2 and Table 2. Put, Spd and Spm were the major polyamines present in the developing grains at all stages studied (Fig. 2). The content of Put and Spd increased in grain from 10–45 DPA. Spm concentrations were considerably lower in comparison. Levels rose significantly between 4 and 11 DPA but did not change significantly thereafter (Table 2).

Isoelectric focusing

Active DAO proteins were present in grains at 30 and 45 DPA (Fig. 3, track 4, left and right hand lanes, respectively). Proteins with PAO activity also showed similar bands at 30 and 45 DPA (Fig. 3, track 3, left and right hand lanes, respectively). Proteins with DAO activities exhibited pIs of 5.6 (major activity) and 6.2. PAO activity was associated with proteins with pIs of

### Table 1. Activities of DAO and PAO in developing grains of barley

<table>
<thead>
<tr>
<th>Stage (DPA)</th>
<th>Activities (pmol Δ-pyrroline min⁻¹ g⁻¹ FW)</th>
<th>Wall-bound (% of total activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAO (soluble)</td>
<td>PAO (soluble)</td>
</tr>
<tr>
<td>-------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>7</td>
<td>3.72 ± 0.06</td>
<td>1.47 ± 0.02</td>
</tr>
<tr>
<td>11</td>
<td>20.48 ± 1.14</td>
<td>7.26 ± 0.17</td>
</tr>
<tr>
<td>14</td>
<td>25.45 ± 1.66</td>
<td>6.05 ± 0.26</td>
</tr>
<tr>
<td>17</td>
<td>36.80 ± 3.12</td>
<td>5.22 ± 0.13</td>
</tr>
<tr>
<td>25</td>
<td>46.03 ± 2.42</td>
<td>4.12 ± 0.09</td>
</tr>
<tr>
<td>45</td>
<td>49.06 ± 2.04</td>
<td>6.56 ± 0.21</td>
</tr>
</tbody>
</table>

*nd, Not detected.
5.4 (major activity) and 6.1. No bands could be seen for DAO and PAO activities before 30 DPA (data not shown). The pattern of peroxidase isozyme activity at 30 DPA is shown in Fig. 3, track 2 for comparison. Many bands with peroxidase activity were present at all stages of grain development.

Discussion

The histochemical results show that DAO activity is mainly present in the vascular tissue (xylem/phloem) and its neighbouring cells, namely the chalazal cells and nucellar projection, while PAO activity is present mainly in chlorenchymatous cells of the crease region and the base of the vascular tissue (i.e. xylem/phloem). DAO activity gave intense staining at neutral pH (i.e. 7.5) while PAO gave more intense colour at acid pH (i.e. 6.0) indicating that DAO is more active at neutral pH while PAO is more active at acidic pH. The apparent absence of PAO activity from the cells of the chalazal region suggests that this enzyme may not be involved in the biosynthesis of lignin in these cells. That the observed activity is indeed due to DAO/PAO is evidenced by the fact that no, or very little, colour is produced in the absence of the substrates. Further support for the hypothesis that amine oxidases are active in the tissue of the crease comes from evidence that the substrates are present in the cells or cell walls where activity was observed. That is, using light microscopy, it was observed that some cut sections of the control (without Put or Spd), also developed slight orange coloration in the chalazal cells. This is not visible in Fig. 1 but is sufficient to indicate the presence of endogenous Put or Spd. That these substrates are present in developing barley grains over the period from 11–45 DPA has been confirmed in the present work following extraction and chromatographic separation. The spots identified as putrescine ran slightly behind the standard. The reason for this may be that the extracted putrescine has co-polymerized with other phenolics thus resulting in the observed lag. Another possibility is that these spots may be diamines other than authentic Put.

Extractable DAO activity increased steadily throughout grain development while PAO activity was higher during the early grain-filling stages. The presence of DAO activity in chalazal cells over the period from 7–45 DPA suggests that it may be involved not only in lignin/suberin deposition (which appears to be restricted to the period 16–18 DPA) but also to the general provision of

Table 2. Amounts of specific polyamines present (nmol g\textsuperscript{-1} FW) in developing grains of barley

<table>
<thead>
<tr>
<th>Stage (DPA)</th>
<th>Put</th>
<th>Spd</th>
<th>Spm</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>195 ± 4</td>
<td>84 ± 2</td>
<td>51 ± 1</td>
</tr>
<tr>
<td>11</td>
<td>526 ± 10</td>
<td>210 ± 4</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>25</td>
<td>805 ± 23</td>
<td>384 ± 5</td>
<td>89 ± 2</td>
</tr>
<tr>
<td>45</td>
<td>874 ± 11</td>
<td>486 ± 6</td>
<td>95 ± 6</td>
</tr>
</tbody>
</table>

5.4 (major activity) and 6.1. No bands could be seen for DAO and PAO activities before 30 DPA (data not shown). The pattern of peroxidase isozyme activity at 30 DPA is shown in Fig. 3, track 2 for comparison. Many bands with peroxidase activity were present at all stages of grain development.

Fig. 2. Separation of amines. Amines visualized as fluorescent spots after separation by ascending TLC in cyclohexane:ethylacetate (5:4, v/v) followed by chloroform:triethylamine (25:2). Track 1: External markers of 10 μg Put, Spd, Spm; Tracks 2, 3 and 4: amines extracted at 10, 20 and 45 DPA.

Fig. 3. Separation of DAO and PAO using isoelectric focusing. Proteins were separated by isoelectric focusing over a pH range of 3–10. External pl markers (shown in track 1) were visualized with Coomassie Blue. The pattern of peroxidase isozyme activity at 30 DPA is shown in track 2. Proteins extracted from intact grains at 30 and 45 DPA and stained for PAO activity are shown in track 3 (left and right hand lanes, respectively). Proteins extracted from intact grains at 30 and 45 DPA and stained for DAO activity are shown in track 4 (left and right hand lanes, respectively). An arrow indicates the position of PAO and DAO activity in tracks 3 and 4.
peroxidase substrate in these cells throughout grain development. It is noteworthy that activity could not be detected earlier than 30 DPA using isoelectric focusing. The reason for this is that the method used may not have been sufficiently sensitive to detect the small amount of enzyme protein present at early stages of grain development. The amount of material required to produce visible bands would have overloaded the gels.

A possible correlation between DAO and PAO activities and the endogenous content of their preferential substrates has emerged. In developing barley grain, the highest DAO and PAO activities corresponded to the highest Put and Spd contents (Tables 1, 2). These data are in agreement with those reported for bean and soybean seedlings in which DAO activity increased concomitantly with Put content (Scoccianti et al., 1990). This suggests that enzyme activity is not regulated by substrate supply. Very similar results were obtained previously at different physiological stages of *Helianthus tuberosus* tubers (Torrigiani et al., 1989) where it was shown that the increase in DAO activity paralleled the accumulation of putrescine. It was suggested that this indicates a direct correlation between the biosynthesis and oxidation of putrescine and is associated with stages of intense metabolism such as, in the present case, organ formation.

The proportion of wall-bound DAO and PAO activity was shown to vary from 2–24%. However, with the exception of PAO at 7 DPA, wall-bound activity was low. This suggests nevertheless, that there may be two forms of PAO activity present in developing barley grain, one soluble and the other cell-wall bound. However, the IEF data show only one main dark band along with light bands with DAO and PAO activities (Fig. 3). Hence it seems likely that both enzymes exist in a single form, some of which may be in association with the cell wall. In *Helianthus tuberosus* tubers it was shown (Torrigiani et al., 1989) that a greater proportion (50%) of total recovered DAO activity was associated with the resuspended pellet. It was suggested that the soluble and cell wall enzymes may play distinct roles. Thus, the apoplastic enzyme might have a direct role in wall stiffening and lignification and the cytoplasmic enzyme might have a role in polyamine regulation.

The results reported here demonstrate the presence of both DAO activity and its substrate in the chalazal cells of the crease region at a time when lignin and suberin deposition is taking place. It seems likely then that the H$_2$O$_2$ required for the peroxidase activity which may be involved in lignin/suberin synthesis is derived from DAO activity. Thus it may be that DAO is involved in the regulation of the H$_2$O$_2$ supply in the cell walls/apoplast of chalazal cells. It therefore may have a role in the control of grain-filling through an effect on lignification of the cell wall and subsequent separation of the apoplast from the symplast. Any role of PAO in the control of assimilate supply seems unlikely since no activity was detected in the chalazal cells.

**Acknowledgements**

The work was carried out during the tenure of a Commonwealth Fellowship to Bavita Asthir. BA also acknowledges support from the Royal Society of Edinburgh.

**References**


