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PEROXIDASE ISOZYMES FROM WHEAT GERM: PURIFICATION AND PROPERTIES

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Key Word Index—*Triticum aestivum*; Gramineae; wheat germ; peroxidase isozymes; spectroscopic and molecular properties; pyrogallol peroxidation; indole acetic acid oxidation; ascorbic acid peroxidation.

Abstract—Several cationic peroxidase isozymes were identified in wheat germ. Three of them were purified to homogeneity. The pure enzymes share many properties: they show near identical spectroscopic properties and they are glycoproteins with very similar M_r values of *ca* 35000. They differed, however, in their catalytic behaviour: two of them, C_1 and C_2, oxidized pyrogallol, ascorbic acid and indole acetic acid with the same requirements, turnover number and optimum pH. The other enzyme, C_3, showed in every case a higher activity. The oxidation of indole acetic acid by C_3 was not influenced by Mn^{2+} or resorcinol. C_3-catalysed oxidation of ascorbic acid was inhibited by hydroxylamine as has been reported for ascorbate peroxidases.

INTRODUCTION

Peroxidases (EC 1.11.1.7) are haemoproteins that catalyse the oxidation of a wide variety of substrates, using H_2O_2. Their *in vitro* catalytic properties have been extensively studied: the typical peroxidase reaction involves a well established cycle in which many electron donors, mainly phenols, can be oxidized [1,2]. Some peroxidases catalyse the aerobic oxidation of indole acetic acid (IAA) with different requirements [3,4], and some can oxidize ascorbic acid constituting a special group known as ascorbate peroxidases [5]. They are ubiquitous in higher plants where they are thought to be involved in lignification, auxin metabolism and an increasing number of other functions [6], although their true physiological functions and control remain unclear. Several peroxidase isozymes occur in each species so far studied, differing in molecular and catalytic properties [7,8]. Due to the variety of reactions they catalyse *in vitro* and the large number of isozymes found, it has not yet been possible to assign an *in vivo* function to a particular isozyme.

We have begun to study the peroxidases from wheat. Except for a report on a cell wall peroxidase from wheat seedlings [9], no attempt has been made to purify and characterize the enzymes of this species. In this paper, we describe the identification of several soluble cationic peroxidases from wheat as well as the purification to homogeneity and some molecular and catalytic properties of three of these enzymes. We have worked with wheat germ, where the expression of peroxidases has not yet been influenced by environmental factors or the development of the plant.

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RESULTS AND DISCUSSION

Purification of peroxidase isozymes

Table 1 summarizes the purification steps of three peroxidase isozymes. It must be stated that, although a significant amount of peroxidase activity is lost by ammonium sulphate fractionation, none of the isozymes present in other fractions is different from those in the fraction used, which is enriched in the enzymes we could so far purify (not shown). After the DEAE column, we chose to work with the cationic fraction since by independent experiments we could determine that the cationic activity represents *ca* 85% of the total activity present in the crude extract. The anionic fraction was not further analysed. The cationic fraction was separated into three peaks on a CM-Trisacryl column (Fig. 1). Each peak contained a different number of isozymes as seen in a gel stained with benzidine/H_2O_2 after native PAGE (Fig. 2B). Each CM fraction was further purified using a mono S FPLC column. From each CM fraction, one isozyme could be purified by FPLC. Figure 2C shows the enzymes which were obtained pure as seen by benzidine staining after native PAGE, indicated as C_1, C_2 and C_3. Coomassie staining of these gels showed no other protein

Table 1. Purification of wheat peroxidases

Step	Volume (ml)	Protein (mg ml^{-1})	Sp. act.* (μkat mg^{-1})	Total act.* (μkat)	Purification (fold)	Yield (%)
Crude extract	1820	4.20	0.025	191	—	100
Acid precipitation	1680	2.80	0.043	202	1.7	105
$(NH_4)_2SO_4$ fractionation	140	3.20	0.152	68	6	36
DEAE-cellulose	190	0.22	1.29	54	52	28
CM-Trisacryl fraction a	25	0.13	2.43	7.9	97	4
FPLC isozyme C_1	11	0.12	2.95	3.9	118	2
CM-Trisacryl fraction b	38	0.30	2.72	31	109	16
FPLC isozyme C_2	19	0.22	3.35	14	134	7
CM-Trisacryl fraction c	12	0.69	2.17	18	87	9
FPLC isozyme C_3	5	0.10	12.20	6.1	488	3

*Assayed under optimum conditions using pyrogallol as substrate.

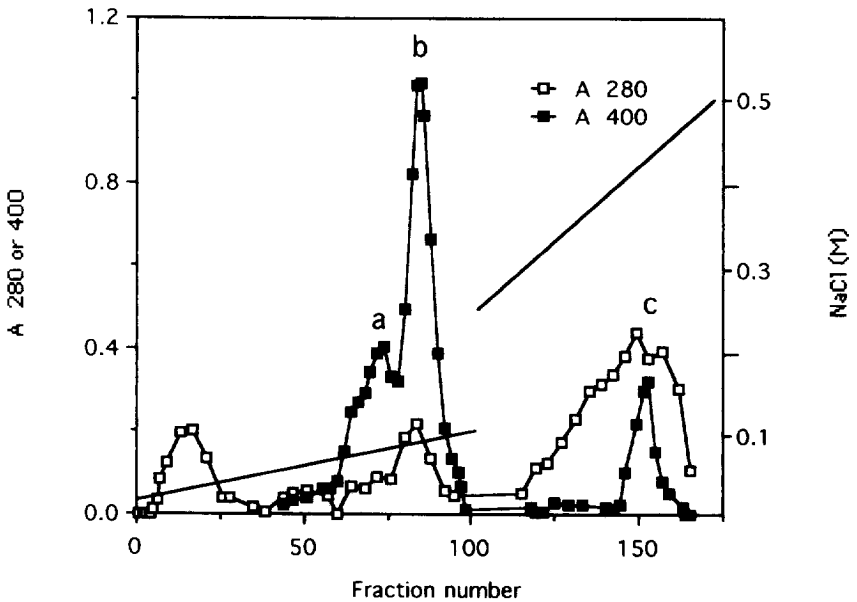

Fig. 1. Elution profile of cationic wheat preoxidases on a CM-Trisacryl column. Tubes with absorbance at 400 nm, due to the Soret band of haeme, were collected in three fractions indicated as a, b and c.

contaminating the purified enzymes. The 13 bands with peroxidase activity present in the ammonium sulphate precipitate (Fig. 2A) represent all the soluble cationic peroxidases present in the wheat germ, since every fraction discarded was carefully examined by PAGE, and no other peroxidase could be observed (not shown).

C_2 is the major cationic isozyme. Purified by us before using a different procedure, it has been characterized as a porphobilinogen oxygenase with peroxidase activity [10] and has been studied in terms of its active site structure [10, 11]. It was subsequently considered a peroxidase since its properties are those of a typical peroxidase, and the oxygenase activity towards porphobilinogen was also found in horse-radish peroxidase (HRP) [12].

Molecular properties

SDS-PAGE was performed with the three fractions that contained one isozyme. A single band was revealed in each case by Coomassie staining in the presence or absence of 2-mercaptoethanol (Fig. 3), thus verifying that the enzymes were pure and consisted of single polypeptide chains. The M_r of the enzymes were 36 000, 36 500 and 35 000 for C_1, C_2 and C_3, respectively.

As most peroxidases are glycoproteins, the presence of sugars was investigated. The glycoprotein nature of the enzymes was revealed by staining a gel after native PAGE with periodic acid–Schiff reagent. The three peroxidases appeared as pink–red bands (not shown). The phenol/sulphuric acid reaction showed contents of 7.5,

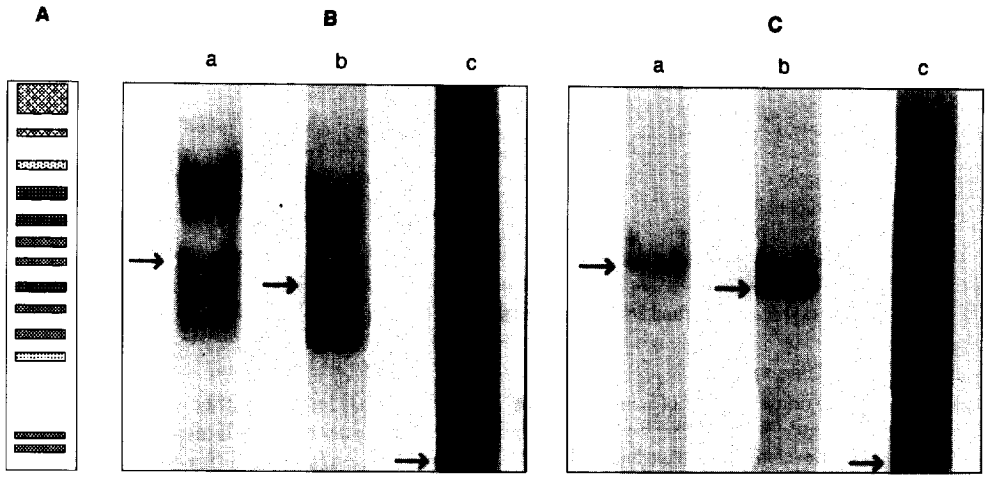

Fig. 2. Native PAGE of wheat peroxidases stained with benzidine. (A) scheme of the peroxidases present in the 50–70% $(NH_4)_2SO_4$ fraction. (B) peroxidases present in the CM-Trisacryl column peaks: (a) peak a; (b) peak b; (c) peak c. The arrows indicate the enzymes purified by FPLC. (C) peroxidase isozymes purified by the FPLC mono S column: (a) isozyme C_1; (b) isozyme C_2; (c) isozyme C_3.

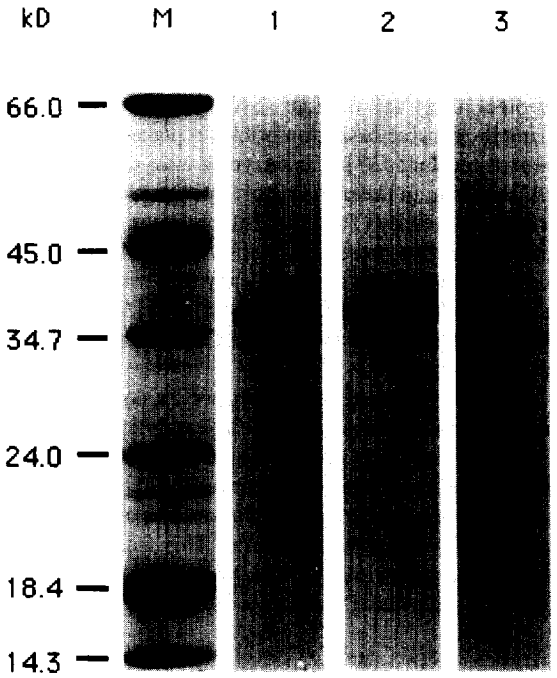

Fig. 3. SDS-PAGE analysis of wheat peroxidase isozymes stained with Coomassie blue. Lane 1, isozyme C_1; lane 2, isozyme C_2; lane 3, isozyme C_3. The position of M_r markers (M) are indicated by arrows on the left: bovine serum albumin, 66000; ovalbumin, 45000; pepsin, 34700; trypsinogen, 24000; β-lactoglobulin, 18400; lysozyme, 14300.

7.9 and 4.1% neutral sugars for C_1, C_2 and C_3, respectively.

The three enzymes contain 1 mol of iron–protoporphyrin IX mol^{-1} of protein according to the pyridine haemochromogen derivative.

Though we still do not know the structural difference among the enzymes, we could verify that they do not interconvert. A mixture of the three enzymes resulted in the same three bands after native PAGE, and no changes in electrophoresis mobility or pattern was observed upon ageing, freezing or heating (not shown).

Spectroscopic properties

The three enzymes showed a typical high spin haeme electronic absorption spectrum. The absorption maxima of the oxidized, reduced and low spin complexes of the enzymes are listed in Table 2. The Soret band of C_3 was shifted to a longer wavelength as compared to that of C_1 and C_2. The R_z values (A_{400}/A_{270}) were 4.1 for C_1, 3.7 for C_2 and 2.9 for C_3. The visible spectra of the compulsory intermediates of the peroxidase catalytic cycle, Compounds I and II, could not be obtained with any of the enzymes, under conditions in which they are obtained with HRP [13].

Table 2. Electronic absorption maxima of wheat peroxidases

Enzyme	Derivative	Absorption maxima (nm)
C_1	Oxidized	399, 499, 634
	Dithionite-reduced	433, 555
	Oxidized + cyanide	415, 530
C_2	Oxidized	398, 496, 635
	Reduced	433, 555
	Oxidized + cyanide	415, 531
C_3	Oxidized	407, 500, 630
	Reduced	435, 556
	Oxidized + cyanide	420, 530

Table 3. Peroxidase activity of wheat isozymes

Substrate	Turnover number* (kat mol^{-1}haeme)			
	C_1	C_2	C_3	HRP
Pyrogallol	107	120	393	1440
Ascorbic acid	28	32	87	4
Ratio				
Ascorbic acid/pyrogallol	0.26	0.27	0.22	0.0028

*Assayed as described in Experimental at the optimum PH for each enzyme.

Table 4. Indole acetic acid oxidase activity of wheat isozymes

Cofactors added	Turnover number* (kat mol^{-1} haeme)		
	C_1	C_2	C_3
None	0.107	0.093	0.262
Mn^{2+}	0.135	0.132	0.262
Resorcinol	0.073	0.067	0.237
Mn^{2+} + resorcinol	0.222	0.178	0.290
H_2O_2	0.143	0.183	0.470

*Assayed as described in Experimental at the optimum PH for each enzyme.

Catalytic properties

The catalytic properties of the enzymes are reported in Tables 3 and 4. Pyrogallol was oxidized in the presence of H_2O_2, with C_3 showing the highest activity (Table 3). The optimum pH for this reaction was 8.5 for C_3 and half maximal activity was obtained at pH 4.8. At higher pH, pyrogallol was oxidized non-enzymically at very high rates. C_1 and C_2 activity was independent of pH between 3.5 and 8.5.

The three enzymes oxidized ascorbic acid, but with different turnover numbers (Table 3), with C_3 again showing the highest value. The optimum pH for this reaction was 6.5 for C_3 with half maximal activity at pH 5.0 and 8.0, while for C_1 and C_2 the optimum pH was 4.6 with half maximal activity at pH 3.6 and 5.4. The ratio of ascorbate to pyrogallol peroxidase activities was very similar in the three isozymes and consistent with that of ascorbate peroxidases [14, 15]. When compared to a classical peroxidase, like HRP, the three isozymes show a much higher ratio of ascorbate to pyrogallol peroxidase activities. This resulted from the fact that these enzymes showed only 10 to 30% of the HRP activity towards pyrogallol, but 10 to 25 times higher activity towards ascorbic acid (Table 3). Ascorbate peroxidases are generally inhibited by hydroxylamine [16]. However, C_1 and C_2 were insensitive to hydroxylamine treatment, while C_3 showed a 75% inhibition when it was preincubated for 5 min with 2 mM hydroxylamine and 0.5 mM H_2O_2. These results suggest that the ascorbate to pyrogallol peroxidase activities ratio is not a sufficient characteristic to classify an enzyme as an ascorbate peroxidase, but must be considered together with other properties such as sensitivity to hydroxylamine.

The three enzymes oxidize IAA in the absence of any added cofactor, as does HRP [3], but differing from other plant peroxidases [4] (Table 4). They differed, however, in their IAA oxidation properties when cofactors were added. Divalent Mn^{2+} and resorcinol acted as non-essential activators for C_1 and C_2 (Table 4), but showed no effect on C_3 activity over a wide range of concentrations (not shown). Resorcinol, in the absence of Mn^{2+}, acted as an inhibitor of C_1 and C_2, while Mn^{2+} alone stimulated the reaction (Table 4). Catalase did not inhibit this reaction as is the case of HRP, where it is accepted that an organic hydroperoxide generated during the aerobic oxidation of IAA replaces H_2O_2 as an essential reactant [17]. The enzymes were also tested for IAA oxidation in the presence of H_2O_2, with C_3 showing the highest activity (Table 4). The optimum pH for IAA oxidation in the presence or absence of Mn^{2+} and/or resorcinol was 4.5 for the three enzymes, with half maximal values at pH 3.2 and 6.0. In the case of IAA peroxidation, the optimum pH for C_1 and C_2 was also 4.5, while that of C_3 was 6.5.

It is apparent from all the properties described here that C_1 and C_2 show identical catalytic behaviour, suggesting that they might not be true isozymes, but different forms of the same enzyme differing in their carbohydrate pattern of glycosylation. It is also apparent that C_3 behaves quite differently. This isozyme shares some properties with cytosolic ascorbate peroxidases, but differs from them in being a glycoprotein [5].

EXPERIMENTAL

Enzyme purification. Wheat germ (600 g) was extracted with water (2.4 l) by stirring 1 hr at room temp. After filtration and centrifugation (10 min at 15 000 rpm) the supernatant was adjusted to pH 5.0, with 1 N HOAc and centrifuged 10 min at 15 000 rpm. The supernatant was pptd with 0–30%, 30–50% and 50–70% $(NH_4)_2SO_4$. The 50–70% fr. was applied to a DEAE-Cellulose column equilibrated with 20 mM Tris, pH 7.4. More than 85% of peroxidase activity was found in the cationic fr. that eluted with the same buffer. This was dialysed against 20 mM K-Pi buffer, pH 5.9, and chromatographed on

a CM-Trisacryl column equilibrated with the same buffer. The peroxidases were eluted with linear gradients of 20–100 mM and 250–500 mM NaCl in the same buffer. The peaks with peroxidase activity or, alternatively, with absorbance at 400 nm due to the Soret band of haeme, were purified using a mono S FPLC column equilibrated with 25 mM NaOAc buffer, pH 5.0, and eluted with a linear gradient of 0–200 mM NaCl in the same buffer.

Enzyme assays. Pyrogallol peroxidase activity was determined by measuring the increase in absorbance at 430 nm due to the formation of purpurogallin (ε = 2.47 mM^{-1} cm^{-1}), using 5 mM pyrogallol, 0.6 mM H_2O_2, 50 mM K-Pi buffer, pH 7.4, and 10 nM enzyme in a final vol. of 1 ml, at 25°.

Ascorbate peroxidase activity was measured by following the disappearance of substrate at 290 nm (ε = 2.8 mM^{-1} cm^{-1}) using 0.4 mM ascorbic acid, 0.6 mM H_2O_2, 50 mM K-Pi buffer, pH 6.5, and 40 nM enzyme in a final vol. of 1 ml, at 25°.

IAA oxidase activity was determined by incubating 0.8 mM IAA, 0.5 mM resorcinol, 0.5 mM Mn^{2+}, 25 mM NaOAc buffer, pH 4.5, and 180 nM enzyme in a final vol. of 0.5 ml, for 75 min at 32°. Remaining IAA was determined by measuring the absorbance at 530 nm after addition of Salkowski reagent (1 ml $FeCl_3$ 500 mM + 50 ml $HClO_4$ 35%). Cofactors were also tested at other concns as described in text.

IAA peroxidase activity was measured as described for IAA oxidase, but using 1 mM H_2O_2 instead of Mn^{2+} and resorcinol.

When the optimum pH of the reactions were measured, NaOAc buffer was used for the range 3–5.5, K-Pi for 5.5–7.5 and Tris–HCl for 8–9.

Electrophoresis. Cationic PAGE was carried out according to ref. [18]. SDS-PAGE was performed according to ref. [19]. Native gels were stained for peroxidase activity according to ref. [20]. When gels were stained for glycoproteins, they were fixed 1 hr in 7.5% HOAc and subsequently incubated for 45 min in 0.2% HIO_4 and 45 min in Schiff reagent (0.5% fuchsin, 5% $NaHSO_3$ in 1 N HCl). HOAc (10%) was used for destaining. Proteins were detected in gels by Coomassie staining.

Other analyt. methods. Neutral sugars were determined by the PhOH–H_2SO_4 method [21]. Haeme content of the enzymes and enzyme concns were measured by the pyridine haemochromogen method [22]. Protein concns were determined according to ref. [23].

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