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ISOLATION AND CHARACTERIZATION OF WHEAT PEROXIDASE ISOENZYME B1

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Key Word Index—*Triticum aestivum*; Gramineae; peroxidase isoenzymes; indole-3-acetic acid; phenolic substances; ethylene.

Abstract—Two pure peroxidase isoenzymes B1 and D4 were isolated from the upper parts of 10-day-old wheat seedlings by means of gel and ion-exchange chromatography. Their MWs were 85000 and 24000 respectively. B1 was unstable and under various conditions it was converted to another isoenzyme, electrophoretically identical with D4. B1 contains about 40% of neutral sugars: 17.2% arabinose, 15.3% galactose, 5% glucose and traces of mannose. D4 is free of neutral sugars. None of the isoenzymes contained amino sugars. B1 oxidizes ferulic and *p*-coumaric acids. This oxidation has two pH optima of 4.4 and 5.4–5.6 and is inhibited by high concentrations of substrates, cyanide and azide. B1 oxidizes IAA in the presence of phenolic cofactor and Mn^{2+} ions. IAA oxidation has two pH optima of 4.5 and 5.6 and is inhibited by high substrate concentration, cyanide and azide, and by a number of indole derivatives. The main products of IAA oxidation are 3-methyleneoxindole and indole-3-methanol. *o*- and *p*-diphenols induce a lag period prior to IAA oxidation. Ferulic acid is oxidized during this lag period, probably to a dimer. B1 is able to produce H_2O_2 from oxygen. Mn^{2+} ions, a phenolic cofactor and an electron donor (IAA or NADH) are needed. B1 oxidizes α-keto-γ-methylmercaptobutyric acid to ethylene. D4 has a low peroxidatic activity and is inactive as an IAA oxidase. Thus B1 is probably an active cell wall-bound peroxidase isoenzyme, whereas D4 is its decomposition product.

INTRODUCTION

Individual peroxidase isoenzymes have been isolated from a number of plants and characterized [1]. These generally differ in MW, amino acid and sugar composition, kinetic properties and substrate specifities. Different isoenzymes oxidize various substrates, mainly of phenolic nature. Some can also oxidize IAA under certain conditions [2], and α-keto-γ-methylmercaptobutyric acid or methional to ethylene [3].

Monophenols are usually cofactors of IAA oxidase; *o*- and *p*-diphenols usually induce a lag period prior to the onset of IAA oxidation. They are oxidized themselves during the lag period [4]. The subcellular localization of some isoperoxidases is known; some isoenzymes bound to cell walls, ribosomes and mitochondria have been described [1]. The broad spectrum of substrates and the large number of isoperoxidases complicate elucidation of *in vivo* functions of individual isoenzymes.

Recently we studied properties of a crude wheat peroxidase preparation [4]. Isolation and the structural and kinetic characterization of isoenzyme B1 are described in the present paper.

RESULTS

The crude wheat peroxidase preparation was resolved into 5 peaks (A–E) on a Sephadex G-100 column, and the isoenzymes in individual peaks were separated by electrophoresis (Fig. 1). The first peak contained a small amount of material. The third and fifth peaks

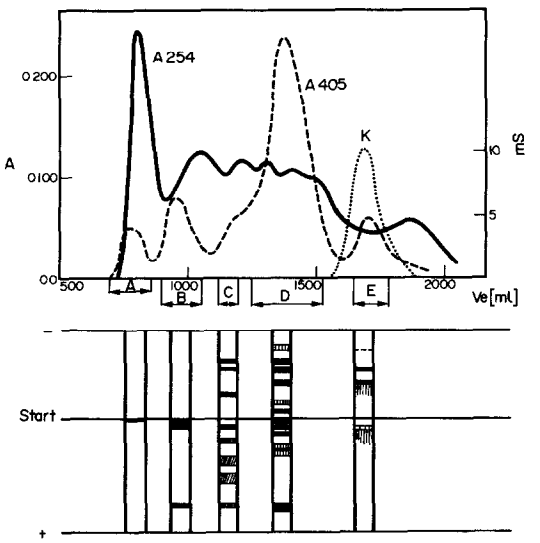

Fig. 1. Elution curve of the crude peroxidase preparation from the Sephadex G100 column (6 × 83 cm) eluted with 0.05 M KPi buffer pH 6. Absorbance of protein (A_{254nm}), of heme group (A_{405nm}) and conductivity κ were recorded. A–E are combined fractions. The lower part of the figure shows the electrophoretic spectra of the combined fractions obtained (starch gel, borate buffer pH 9, stained with benzidine reagent for peroxidase activity).

Abbreviations used: IAA: indole-3-acetic acid, KMBA: α-keto-γ-methylmercaptobutyric acid, FA: ferulic(3-methoxy-4-hydroxy-cinnamic)acid, HCA: *p*-coumaric(4-hydroxycinnamic acid), MOI: 3-methyleneoxindole, NADH: reduced nicotinamide adenine nucleotide, SDS: sodium dodecyl sulphate, HRP: horseradish peroxidase.

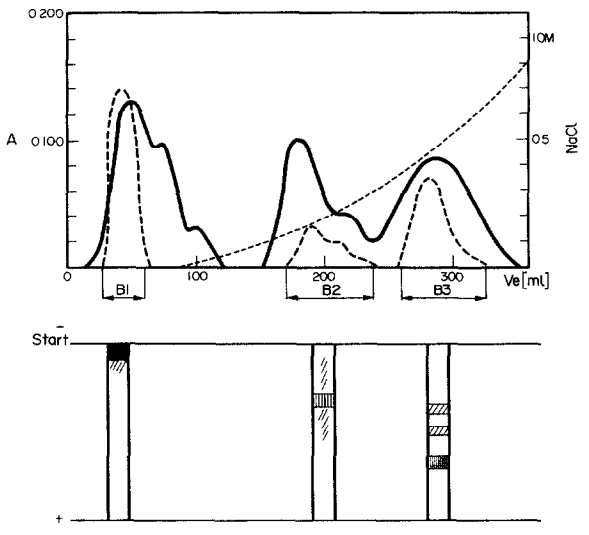

Fig. 2. Elution curve of combined fraction B of the crude peroxidase from the DEAE–cellulose column (1 × 42 cm), eluted with 10 mM Tris–HCl buffer pH 7.6 and with a concave gradient of 0–1.0 M NaCl in buffer. Absorbance of protein (A_{254nm}), of heme group (A_{405nm}) and conductivity κ were recorded. B1–B2 are combined fractions. The lower part of the figure shows the electrophoretic spectra of the combined fractions obtained (starch gel, borate buffer pH 9, stained with benzidine reagent for peroxidase activity).

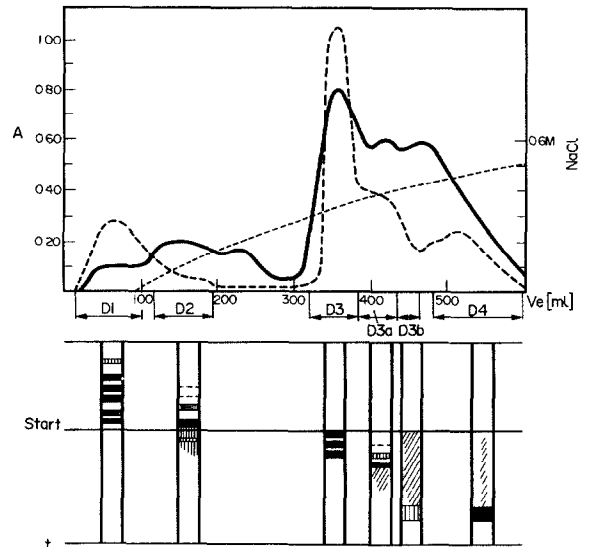

Fig. 3. Elution curve of combined fraction D of the crude peroxidase from a DEAE–Sephadex A-50 column (2 × 62 cm), eluted with 10 mM Tris–HCl buffer pH 7.6 and with a convex gradient of 0–0.6 M NaCl in buffer. Absorbance of protein (A_{254nm}), of heme group (A_{405nm}) and conductivity κ were recorded. D1–D4 are combined fractions. The lower part of the figure shows the electrophoretic spectra of the combined fractions obtained (starch gel, borate buffer pH 9, stained with benzidine reagent for peroxidase activity).

contained unstable material, which formed ill-defined bands on starch gel electrophoresis. Therefore, only peaks B and D were studied further. The fraction B was resolved on a DEAE–cellulose column into the pure isoenzyme B1 and two fractions exhibiting anodic mobility (Fig. 2).

The fraction D was resolved into 6 fractions (D1–D4) on a DEAE–Sephadex A-50 column (Fig. 3). The last fraction D4 contained the isoenzyme possessing the highest anodic mobility. This fraction was purified by rechromatography under the same conditions. The homogeneity of B1 and D4 was tested by means of starch gel and disc electrophoresis. Gels were stained for peroxidase, IAA oxidase activities and proteins. D4 did not react as an IAA oxidase.

The absorption spectrum of B1 had a sharp maximum at 405 nm and minimum at 320 nm. D4 had no characteristic spectra of hemoproteins. MWs of B1 and D4 as determined by gel filtration were 84000 and 25000 respectively. Values obtained from SDS electrophoresis were 86000 for B1 and 23000 for D4.

B1 was unstable. Overnight action of media of high and low pH (10 and 2.4) and of higher ionic strength (1 M NaCl) caused its partial decay to an isoenzyme with a higher mobility toward the anode, electrophoretically identical with D4.

The amino acid composition of both isoenzymes is presented in Table 1. It is evident that amino acids form only 32.8% of the isoenzyme B1 molecule. After digestion of the protein moiety by pepsin, the rest of the molecule has a MW of *ca* 35000. The positive reaction with Dubois' reagent [5] showed that the B1 molecule contains neutral sugars. An analysis of B1 for neutral sugars showed that it contains about 40% of neutral sugars: 17.2% arabinose, 15.3% galactose, 5% glucose and trace amounts of mannose. D4 was free of neutral sugars. Both isoenzymes were free of amino sugars.

D4 was also released directly from the cell walls by means of pectinase.

B1 possesses a high peroxidase activity. It oxidizes

Table 1. Amino acid composition of B1 and D4

Amino acid	B1		D4	
	A	B	A	B
Lys	2.31	13	6.99	13
His	0.73	4	2.43	4
Arg	1.37	7	4.44	7
Asp*	3.95	26	12.50	26
Thr	3.05	22	5.18	12
Ser	2.33	19	4.32	12
Glu*	3.02	18	9.81	18
Pro	2.80	21	4.05	10
Gly	2.27	26	5.54	26
Ala	4.55	43	4.81	15
Val	2.99	22	6.90	17
Met	0.73	4	1.18	2
Ile	1.52	10	4.61	10
Ieu	3.48	22	8.70	18
Tyr	1.32	6	3.86	6
Phe	2.04	10	5.60	10

A = g of amino acid residue per 100 g protein
B = Number of residues per protein molecule
*The values of Glu and Asp are totals of Glu and Gln and Asp and Asn, respectively. Contents of cysteine and tryptophan were not determined.

ferulic and *p*-coumaric acids in the presence of exogenous H_2O_2 with $K_m = 5 \times 10^{-3}$ and specific activity of 7 U/mg (117 nkat/mg). This oxidation has two pH optima: 4.4 and 5.4–5.6. High concentrations of both substrates inhibit the reaction. Cyanide and azide inhibit this reaction as well.

B1 oxidizes IAA in the presence of Mn^{2+} ions and a phenolic cofactor. When using HCA as a cofactor, the specific activity is 6 U/mg (100 nkat/mg). pH optima of this reaction are 4.5 and 5.6. Resorcin also acts as a cofactor. L-Tyrosine does not, and *p*-hydroxybenzoic acid only very slightly. Mn^{2+} ions cannot be replaced by any other metal ions tested, e.g. Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+} and Cd^{2+}. The reaction follows Michaelis–Menten kinetics with $K_m = 4 \times 10^{-3}$. IAA concentrations higher than 10^{-3} M inhibit the reaction, KCN and NaN_3 also inhibit. IAA oxidation is further inhibited competitively by a number of indole derivatives: tryptophan, indole, indole-3-carboxylic, indole-3-pyruvic, indole-3-propionic, indole-3-butyric, indole-3-acrylic and indole-3-aspartic acids. These compounds have no effect on FA oxidation in the presence of H_2O_2. None of them is a substrate of B1 with the exception of indole-3-acrylic acid, which is oxidized in the presence of H_2O_2. As long as IAA oxidation proceeds under optimal conditions, H_2O_2 addition is without any effect. Under suboptimal conditions H_2O_2 stimulates IAA oxidation. Catalase has no effect on the reaction. B1 is not able to oxidize IAA directly by means of H_2O_2. HCA or resorcin as a cofactor is required.

The absorption spectrum of the main product of IAA oxidation by B1 has maxima at 247 and 254 nm corresponding to 3-methyleneoxindole [6]. After extraction with ether and chromatographic analysis, MOI, indole-3-methanol and traces of indole-3-aldehyde and indole-3-carboxylic acid were found.

The presence of *o*- and *p*-diphenols (ferulic ,caffeic and 3,4-dihydroxybenzoic acids and *p*-phenylenediamine) induces a lag period prior to IAA oxidation by B1. No increase in *A* at 247 nm was detected during the lag period induced by FA, but *A* at 320 nm decreased. This indicates that the structure of ferulic acid is modified in some way. Chromatographic analysis showed that the reaction product of FA during the lag period is the same as after direct oxidation by B1 and H_2O_2 and it is probably a dimer [4]. Duration of the lag period depends on the FA/IAA concentration ratio and on HCA concentration. H_2O_2 abolishes the lag period; catalase has no effect.

In order to produce H_2O_2, the isoenzyme B1 requires Mn^{2+} ions, a phenolic cofactor and IAA. FA may serve as a cofactor too, but with a very low efficiency. The H_2O_2 formation is competitively inhibited by all indole derivatives mentioned above. None of these derivatives can replace IAA as an electron donor for H_2O_2 formation. NADH can substitute for IAA in this respect. KCN and NaN_3 inhibit H_2O_2 formation; catalase is without any effect.

B1 oxidizes α-keto-γ-methylmercaptobutyric acid or methional, forming ethylene. Mn^{2+} ions, HCA and IAA are required for this reaction.

Both peroxidase and IAA oxidase activities of B1 are lost when the heme moiety is removed. The fraction D4 is only slightly active in the FA and HCA oxidation in the presence of H_2O_2 (sp. act. 1 U/mg (16.6 nkat/mg). It is inactive as an IAA oxidase and cannot form H_2O_2 from oxygen. It dissolved with difficulty and seemed to be partially denatured and as it could not be considered as active isoenzyme, was not studied in detail.

DISCUSSION

The amino acid composition of B1 differs from that of previously described isoperoxidases. It has a relatively high content of Asp, Thr, Ser, Glu, Ala and Leu. No hydroxyproline was found; but relatively large amounts of proline could be detected.

B1 contains galactose and arabinose and minor amounts of glucose and mannose. The composition of the sugar moiety suggests that it might be an arabinogalactan of the pectic part of the cell wall [7], to which the isoperoxidase is bound. Instability of B1 under extreme conditions of pH and ionic strength, together with the fact that D4 is released by pectinase from the cell wall preparation further support this view. D4 which is released from B1 does not correspond fully to the protein moiety of B1. During the cleavage, a portion of peptidic material is lost together with the sugar moiety. D4 is only very slightly active and cannot be considered an active isoenzyme. Probably it is formed only during isolation of B1.

The cell wall-bound isoenzyme B1 is very active both as a peroxidase and as IAA oxidase. The same scheme is valid for the interaction of phenols and IAA with B1 as suggested for the crude wheat peroxidase preparation [4]. Mn^{2+} ions, a phenolic cofactor and an electron donor are required for E–H_2O_2 complex formation (we cannot suppose free H_2O_2 in view of the lack of catalase effect). This reaction is competitively inhibited by a number of indole derivatives, none of which can substitute for IAA as an electron donor. Thus, the function of IAA is very specific, but NADH can substitute for it. As far as duration of the lag period is concerned the same holds true for the crude peroxidase preparation [4].

For both its oxidizing functions, B1 requires the presence of the heme moiety. We assume that B1 acts *in vivo* as a terminal oxidase with the peroxidase mode of action [8].

B1 reacts according to Michaelis–Menten kinetics, thus differing from a number of other peroxidase preparations with allosteric kinetics [1]. High concentrations of IAA and phenols inhibit the isoenzyme activity. This indicates that both types of substrates interact sterically with the isoenzyme, and it is likely that the isoenzyme contains more binding sites for these substrates [9].

B1 contains a large amount of sugars. The fact that removal of the sugar moiety causes the loss of the IAA oxidase activity suggests that this activity depends on the presence of sugars in the molecule of the enzyme, or that presence of a sugar increases the activity. All the other isoenzymes of wheat IAA oxidase also contain sugars (unpublished results).

EXPERIMENTAL

Isolation of the isoenzyme. Isolation of crude peroxidase preparation from the upper parts of 10-day-old wheat (*Triticum aestivum* L. cv Jubilar) seedlings was recently described [4]. The preparation started with 10 kg fresh material, from which 10 g lyophilized crude enzyme were obtained. The enzyme was dissolved in 0.05 M KPi buffer pH 6 and resolved on a Sephadex G-100 column (6 × 85 cm) using the same buffer. The *A* of

effluent at 254 and 405 nm was recorded ,and peroxidase activity of the fractions was determined with H_2O_2 and benzidine. Active fractions were combined and dialysed against H_2O and lyophilized. This and all other chromatographic procedures were performed at 4 ± 1°.

The second fraction (B) from Sephadex G-100 column was further chromatographed on a DEAE–cellulose column (1 × 42 cm) in 10 mM Tris–HCl buffer pH 7.6. Elution was performed with 100 ml of this buffer and a convex gradient 0–0.6 M NaCl in the buffer.

Electrophoresis. Starch gel and disc electrophoresis were performed according to described methods [4, 10] and stained for peroxidase activity using benzidine reagent, and for proteins with amido black reagent. Staining for IAA oxidase activity was performed using a modified method of ref. [11]. The gel was incubated 30 min at room temp. in 0.1 M NaOAc buffer pH 5, 60 min in a soln of 2 mM IAA, 1 mM Mn^{2+} ions and 0.5 mM 2,4-dichlorophenol in 0.1 M Pi buffer pH 5.5 and 30 min in 0.5%, *p*-dimethylaminocinnamaldehyde in 2 M HCl.

MW determination. MWs of both isoenzymes were determined by gel filtration on a Sephadex G-100 column (0.9 × 150 cm) in 0.1 M Pi buffer pH 6 and by disc electrophoresis in SDS [12]. The MW of the non protein moiety of B1 was estimated by gel filtration on a Bio Gel A-150 column (0.9 × 150 cm) in 0.1 M Pi buffer pH 6 using blue dextran, bovine serum albumin, bovine chymotrypsinogen A and horse myoglobin as standards.

Amino acid composition. The amino acid composition was analysed by the method of [13]. Hydrolysis in 6 M HCl proceeded 48 hr at 110° in the absence of O_2. Amino acids were determined in an automatic amino acid analyser.

Sugar analysis. The content of neutral sugars was determined according to the procedure of ref. [5]. Neutral sugars were analysed in the form of their alditol acetates [14]. Alditol acetates were analysed by GLC using a 3 mm × 3 m glass column with 0.2% ethylene glycol succinate, 0.2% ethylene glycol adipate and 1% silicone XE 60 on Chromosorb W at 179°; rate of flow of N_2 carrier gas was 21 ml/min. Amino sugars were analysed after an 8 hr hydrolysis in 4 M HCl at 110°. The analysis was performed in an amino acid analyser

Release of isoperoxidases from the binding to the cell wall. The cell wall preparation obtained by means of the method of ref. [15] was digested with 2.5% pectinase (Serva) in 0.05 M NaOAc buffer pH 5 for 20 hr at 25°. The supernatant was centrifuged, dialysed and chromatographed on a Sephadex G-100 column 2 × 60 cm) in 0 1 M Pi buffer pH 6. Fractions obtained were analysed electrophoretically and the peroxidase activity was assayed.

Estimation of peroxidase activity. Ferulic and *p*-coumaric acids were used as substrates. Their oxidation was followed spectrophotometrically at 320 and 310 nm, respectively [4]. The reaction proceeded in 0.05 M Pi buffer pH 5.6 at 30 ± 0.5°

Estimation of IAA oxidase activity. IAA oxidation was followed spectrophotometrically at 247 nm [4].

Hydrogen peroxide formation by B1. The H_2O_2 production was followed indirectly by measuring spectrophotometrically at 320 nm the consecutive oxidation of FA with the formed enzyme–H_2O_2 complex.

Ethylene formation. As a substrate KMBA was used. KMBA was prepared from L-methionine by means of L-amino acid oxidase [16]. The reaction proceeded in a stoppered test-tube, and after 30 min a sample of gaseous phase was withdrawn with a Hamilton syringe. This sample was analysed for ethylene in a Perkin-Elmer F-30 gas chromatograph with a flame ionization detector and a Porapak N column. Carrier gas was N_2 (40 ml/min), temp. 130°.

Cleavage of heme moiety from the isoenzyme. Cleavage and reconstitution with crystalline oxyhemin were performed according to ref. [17].

Chromatographic analysis of FA and IAA oxidation products. Chromatographic analysis of FA oxidation products has recently been described [4]. IAA oxidation products were extracted from the acidified reaction mixture with Et_2O. After evaporation under red. pres., both fractions were analysed by TLC on Si gel (0.3 mm) in the following solvent systems: [18] CCl_4:$CHCl_3$:HOAc (19:19:2); *iso*-PrOH:NH_4OH (18 M): H_2O (8:1:1); $CHCl_3$:MeOH:CCl_4 (5:4:1); $CHCl_3$:EtOH (9:10).

Detection was performed with 1% soln of *N,N*-dimethylaminocinnamaldehyde in the mixture of HCl:EtOH (1:1) or by Salkowski's or Procházka's reagents [19]. The 3-methyleneoxindole standards were prepared from 3-bromoxindole-3-acetic acid (A. Skytt Andersen, personal communication).

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