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PEROXIDASE FROM THE GREEN ALGA *ENTEROMORPHA LINZA*

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Abstract—An enzyme displaying peroxidase activity has been extracted and purified 27-fold from the green alga *Enteromorpha linza*. The partially purified preparation was shown to contain two electrophoretically distinct components and their isoelectric points were calculated. The enzyme was estimated to have an unusually high MW of 220 000 as determined by gel filtration. It was strongly inhibited by cyanide and azide and these reagents acted competitively with respect to H_2O_2, suggesting that the enzyme is a haemoprotein. The enzyme preparation catalysed the formation of mono- and di-iodotyrosine in the presence of H_2O_2, as well as the oxidation of iodide.

INTRODUCTION

ALTHOUGH peroxidase (E.C. 1.11.1.7) has been isolated and characterised from many sources, the assignment of a precise physiological role to this enzyme has proved difficult and is only known with certainty in a limited number of organisms and tissues.

The iodide uptake mechanism in algae is still poorly understood, as is the mechanism of formation of iodine-containing organic compounds which occur in green,[1] as well as other marine algae.[2–6] Many workers have reported that radioactively labelled mono- and di-iodotyrosine are formed when marine algae are incubated with radioactive iodide.[3–5,7,8] Tong and Chaikoff[4] studied the mechanism by which radioactive iodide is incorporated into iodotyrosines in the brown alga *Nereocystis lutkeana* and demonstrated the ability of cell-free extracts to carry out the reaction. They concluded from inhibition studies, however, that peroxidase was not involved in the iodination reactions.

We have obtained, in partially purified form, a peroxidase from the green alga *Enteromorpha linza* and shown that it is capable of catalysing the oxidation of iodide and the formation of iodotyrosine in the presence of H_2O_2. A preliminary report of this work has been published.[9]

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RESULTS AND DISCUSSION

After a suitable method for the preparation of a cell-free extract was established, assays were carried out to detect and quantitate classical peroxidase activity as well as the ability to catalyse the iodination of tyrosine. A wide range of electron donors was found to be oxidized in the presence of the extract. These included *o*-dianisidine, guaiacol, *o*-tolidine and *p*-phenylenediamine. Cell-free extracts also catalysed the iodination of tyrosine. This reaction was carried out by incubating radioactive iodide with tyrosine and a peroxide generating system (glucose/glucose oxidase), together with an aliquot of the cell free extract and, after a suitable period, the reaction mixture was chromatographed; autoradiography revealed the formation of monoiodotyrosine. Radioactive diiodotyrosine was clearly seen to be formed when mono iodotyrosine was substituted for tyrosine in this reaction. A spectrophotometric assay based on the fact that the iodination reaction being catalysed must involve the prior oxidation of iodide was used to monitor the iodinating activity. A similar approach has been used in the purification of peroxidase from the thyroid.[10,11] Details of this and other assays are given in the Experimental.

Purification of the enzyme in cell free extract was achieved by an ammonium sulphate fractionation in the range 0·5–0·7 saturation. The precipitate was redissolved in 0·05 M glycine–NaOH, pH 9·0, dialysed, and chromatographed on Sephadex G200 equilibrated with the same buffer. The enzymatically active fractions of the eluate were pooled and used for further characterization studies. A summary of the purification procedure is shown in Table 1. As can be seen the iodide oxidizing activity closely parallels the classical peroxidase activity during the purification. A 27-fold purification was the best achieved overall. Studies on this enzyme preparation showed that it actively catalysed the iodination of tyrosine in the presence of H_2O_2 and iodide.

TABLE 1. SUMMARY OF ENZYME PURIFICATION

Fraction	Total protein (mg)	Specific activity as measured by iodide assay* (units/mg)	Purification factor (iodide assay)	Specific activity as measured by *o*-dianisidine assay* (units/mg)	Purification Factor (*o*-dianisidine assay)
Crude extract	874	14·5	(1)	3·26	(1)
0·5–0·7 saturated $(NH_4)_2SO_4$ precipitate	138	81	5·6	17·8	5·5
Eluate from Sephadex G200	25·2	392	27·0	83·3	25·6

* Assays were carried out as described in Experimental.

Starch gel electrophoresis of the enzyme preparation revealed, after staining for peroxidase activity, the presence of two bands which moved towards the anode, the fastest band being the major component. In order to investigate further the relationship between the iodide oxidizing activity of the algal preparation and classical peroxidase activity a staining

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procedure based on the oxidation of iodide was developed.[12] After electrophoresis, the same two bands were visualized by this technique, indicating that the same enzyme is responsible for both activities. Further confirmation of the presence of two components in the active enzyme preparation was obtained by isoelectric focusing. The elution pattern was identical regardless of whether *o*-dianisidine or iodide was used as substrate. The isoelectric point calculated for the major component was 4·45, and 4·7 for the minor. Possibly the two components are isoenzymes; peroxidase isoenzymes have been reported from many plant sources. Electrophoresis of the cell free extract did not show any additional peroxidase bands.

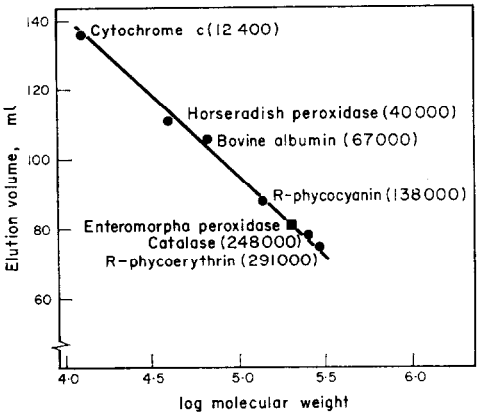

FIG. 1. MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION OF *E. linza* PEROXIDASE.

A column of Sephadex G200 was equilibrated with 0·1 M NaCl in 0·02 M sodium phosphate, pH 7·0. Elution of *E. linza* peroxidase was monitored by peroxidase assay, while proteins were followed by spectrophometry.

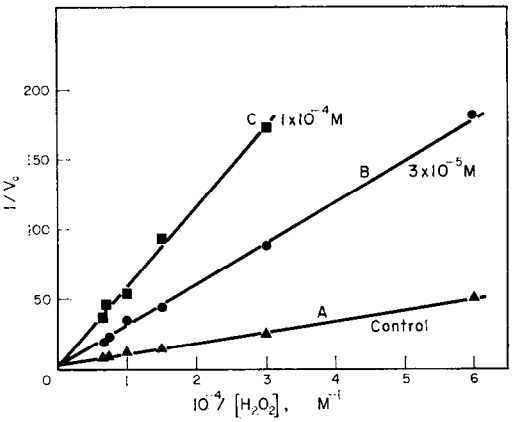

FIG. 2. LINEWEAVER–BURK PLOTS TO DETERMINE THE INHIBITION OF *E. linza* PEROXIDASE BY VARIOUS CONCENTRATIONS OF POTASSIUM CYANIDE.

Enzyme activity was measured using the standard *o*-dianisidine assay (see Experimental).

The MW of the enzyme was estimated at 220 000 by means of a calibrated Sephadex G200 column (Fig. 1). This is an unusually high value for a plant peroxidase but compares with MWs reported for some mammalian peroxidases.[13,14] The iodide assay has many short-comings when used for kinetic studies, the main one being that, as previously reported,[15] the absorbance at 350 nm disappears gradually at high H_2O_2 concentrations. Thus, in kinetic studies on the *Enteromorpha* enzyme *o*-dianisidine was used as substrate. Optimal H_2O_2 concentration was 0·8 mM; inhibition occurred at higher concentrations. An apparent K_m of 0·16 mM was calculated for H_2O_2 at a fixed concentration of *o*-dianisidine (0·5 mM). This algal enzyme is unusually thermostable.[16] When monitored with either iodine or *o*-dianisidine it retained all its activity when incubated at 50° for 20 min; at higher temperatures it was progressively inactivated, and boiling for 3 min caused complete inactivation. Based on the standard *o*-dianisidine assay the optimum temperature for

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peroxidase activity is 45–50°. The energy of activation for the enzyme-catalysed reaction was calculated from an Arrhenius plot as 11 700 cal/mol. The Q_{10} for this reaction, based on the energy of activation is 1·95. Similar Q_{10} values have been obtained for other peroxidase-catalysed reactions.[17,18]

A stability vs. pH plot showed that the enzyme is quite stable for long periods between pH 4 and 10 at 4°. Using a number of buffer systems a broad pH optimum around 6 was found for the enzyme when iodide was used as substrate.

The effect of bivalent cations and other potential inhibitors on the enzyme was studied by incubating with the peroxidase preparation for 20 min at 25°, before assay of a 0·1 ml sample with the *o*-dianisidine reaction. The enzyme activity was not altered significantly by Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+} or EDTA at 1 mol. Hg^{2+} at 1 mM inhibited the activity to 36% of the control (Table 2).

The enzyme was fully active in the presence of EDTA and other metal chelating reagents, *o*-phenanthroline and α,α-dipyridyl (inhibiting 0 and 12% resp.), indicating that it has no requirement for a non-haem metal cofactor.[5]

TABLE 2. EFFECT OF VARIOUS REAGENTS UPON THE PEROXIDASE ACTIVITY

Compound added*	Concn (mM)	Relative activity	Compound added*	Concn (mM)	Relative activity
None	—	(100)	Sodium fluoride	0·1	50
Hg^{2+}	1·0	36	Potassium cyanide	0·01	50
3-Amino-1,2,4-triazole	1·0	95	Sodium azide	0·04	50
Iodoacetic acid	1·0	97	Potassium thiocyanate	2·0	50
Sodium fluoride	1·0	8	Catalase	0·004	0

* The various compounds, at the final concentration indicated, were incubated with the enzyme in the standard *o*-dianisidine assay.

The lack of inhibition by acriflavine, a specific inhibitor of flavoproteins,[19] excludes the possibility that the enzyme has a flavine prosthetic group, as have some bacterial peroxidases.[20] 2,4-Dinitrophenol, an inhibitor of metal oxidases,[21] caused no inhibition of the algal peroxidase. The inhibition of thyroid peroxidase by 3-amino-1,2,4-triazole has been reported,[22] but no significant inhibition (5%) was caused to the green algal peroxidase. The absence of any peroxidatic action when catalase was added is to be expected. Low concentrations of some well known inhibitors of haematin enzymes caused inhibition, and these same reagents inhibited the algal enzyme-catalysed oxidation of iodide. However, no Soret peak was observable in absorption spectra of purified enzyme preparations.

Cyanide inhibits other haem-containing peroxidases by competing with H_2O_2 and therefore reciprocal plots of reaction rate in presence of algal enzyme versus H_2O_2 concentration were drawn at various concentrations of cyanide. Figure 2 shows that these plots are linear and extrapolate to a single point on the vertical axis, indicating that H_2O_2 and cyanide are competing for the same site on the enzyme. Similar results were obtained

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using azide. Inhibition of the tyrosine iodination reaction by low concentrations of cyanide was also observed.

These studies suggest that peroxidase from *Enteromorpha linza* is capable of iodinating organic compounds. In this property it contrasts with a peroxidase from red algae for which no role could be assigned in halogenation reactions.[16] It is also possible that the *E. linza* enzyme mediates the uptake of iodide by the alga. Shaw[7] proposed that iodide uptake by algae is catalysed by an oxidase; this, we suggest, may be composed of two enzymes, an iodide peroxidase, described here, and a peroxide generating sytem, so far unidentified.

EXPERIMENTAL

Preparation of cell free extract. Enteromorpha linza was collected during the Autumn on the shores of Galway Bay and stored at −30°. Cleaned algal tissue was frozen in liquid N_2 and powdered while still solid in a precooled mortar. To the powder, 3 vol. of ice-cold 9·1 M sodium phosphate, pH 7·0, was added and extraction continued overnight at 4°. The mixture was then centrifuged at 9000 *g* for 1 hr and the active supernatant decanted for further study.

Enzyme assays. A Unicam SP800 with a thermostat attachment to maintain the cuvette temperature at 25° was used in all assays. The standard *o*-dianisidine assay mixture contained in a final vol. of 3 ml: 1·5 μmol *o*-dianisidine, 2·4 μmol H_2O_2, 300 μmol sodium phosphate-citrate buffer, pH 6·0, and 0·1 ml of enzyme preparation. Under these conditions the absorbancy of oxidised *o*-dianisidine per mol H_2O_2 was $1{\cdot}08 \times 10^4\ cm^{-1}$ at 460 nm. Activity is expressed as μmol H_2O_2 consumed per min per 3 ml reaction mixture. The standard iodide assay system was based on methods developed by other workers.[22,23] The assay mixture contained in a final vol. of 3 ml was: 60 μmol potassium iodide, 0·5 μmol H_2O_2, 150 μmol sodium phosphate buffer, pH 6·8, and 0·1 ml of enzyme. A molar extinction coefficient of $22\,900\ cm^{-1}$ at 353 nm was used for tri-iodide.[22] Activity is expressed as μmol iodide oxidized per min per 3 ml reaction mixture.

Other methods. Electrophoresis was performed on horizontal 14% starch gels. Buffer systems used were Tris-citrate, pH 7·0, and Tris-maleate, pH 8·4. After electrophoresis the gels were sectioned longitudinally and stained using 3-amino-9-ethyl carbazole or iodide as electron donors. Isoelectric focusing experiments were carried out on a glass apparatus,[24] with the vertical column cooled to 8°. The synthetic ampholytes were in the range pH 3–10 (LKB-Ampholine 3141. Batch No. 21) and a sucrose density gradient was used.[25] Phosphoric acid (0·5%) and monoethanolamine (1·5%) were placed in the anode and cathode compartments, respectively. The isoelectric focusing usually lasted about 3 days. At the end of this period the column was drained, 1-ml fractions collected, and monitored for enzyme activity. The pH of each fraction was determined.

Preparation and equilibration of Sephadex columns, application of proteins and determination of MWs was carried out according to Andrews.[26]

Radioactive experiments were performed using I^{125} (Amersham/Searle). Tyrosine and the iodo-amino acids were separated by TLC.[27] Protein was estimated by the methods of Potty[28] and Lowry *et al.*[29]

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