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PEROXIDASE FROM THE RED ALGA *CYSTOCLONIUM PURPUREUM*

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Abstract—A particulate peroxidase has been extracted from the marine red alga *Cystoclonium purpureum*. Solubilisation was achieved by the use of either digitonin or a brief tryptic digestion and the enzyme was purified over 80-fold. The peroxidase was found to have a MW of 40 000–50 000 and to exhibit multiple molecular components after electrophoresis. The enzyme has a restricted substrate specificity and does not appear to catalyse the formation of halogenated organic compounds. It is strongly inhibited by cyanide and azide.

INTRODUCTION

PEROXIDASE (E.C. 1.11.1.7) of marine red algae has attracted little attention since the mid-forties when it was reported that earlier observations of its occurrence were based on assays of artifacts.[1] Halogenated compounds have been reported in numerous red algae[2–6] but little is known about their biosynthesis. The role of peroxidases in the formation of halogenated compounds in the thyroid[7,8] and the fungus *Caldariomyces fumago*[9] suggested to us that peroxidase may have a similar function in algae. We now report a partial purification and characterisation of peroxidase from the red alga *Cystoclonium purpureum*. A preliminary report was published earlier.[10]

RESULTS AND DISCUSSION

Solubilization and Partial Purification

After an extensive search for peroxidase activity in marine red algae, *Cystoclonium purpureum* was chosen for special attention because of the ease with which the enzymic activity could be extracted, combined with the relatively high specific activity of the enzyme from this source. EDTA in the extracting buffer at mM concentration increased the peroxidase activity 30–40%; the optimal extraction time was 2–3 hr.

[1] S. RONNERSTRAND, *K. Fysiogr. Sallsk., Lund Forh.* **16,** 117 (1946).
[2] A LEMAN, *Bull. Soc. Chim., France, Memoires* **11,** 64 (1944).
[3] C. Ó hEOCHA, *Cooloq. Int. Cent. Nat. Rech. Sci., Paris.* No. 103, 121 (1961).
[4] J. J. SIMS, W. FENICAL, R. M. WING and P. RADLICK. *Tetrahedron Letters* 195 (1972).
[5] J. S. CRAIGIE and D. E. GRUENIG, *Science* **157,** 1058 (1967).
[6] T. IRIE, M. IZAWA and E. KUROSAWA, *Tetrahedron* **26,** 851 (1970); T. IRIE, M. SUZUKI, E. KUROSAWA and T. MATSUME, *Tetrahedron* **26,** 3271 (1970); M. SUZUKI, E. KUROSAWA and T. IRIE, *Tetrahedron Letters,* 4995 (1970); A. FUKUZAWA, E. KUROSAWA and T. IRIE, *Tetrahedron Letters* 3 (1972).
[7] T. HOSOYA and M. MORRISON, *J. Biol. Chem.* **242,** 2828 (1967).
[8] M. L. COVAL and A. TAUROG, *J. Biol. Chem.* **242,** 5510 (1967).
[9] D. R. MORRIS and L. P. HAGER, *J. Biol. Chem.* **241,** 1763 (1966).
[10] M. J. MURPHY and C. Ó hEOCHA, *Biochem. J.* **115,** 12P (1969).

The distribution of the enzyme in the algal extract was studied by differential centrifugation (Table 1). While the bulk of the protein was in the supernatant, most peroxidase activity appeared to be particle-bound as it sedimented during high-speed centrifugation.

TABLE 1. SUBCELLULAR DISTRIBUTION OF PEROXIDASE ACTIVITY IN *C. purpureum*

Fraction	Protein		*o*-Dianisidine assay*	
	mg Protein/g wet tissue	mg Protein (ml)	Units of activity/g wet tissue	Specific activity (units/mg protein)
Extract	2·55	1·7	0·667	0·392
Residue after 1500 *g* for 10 min	0·34	0·23	0·063	0·274
Residue after 35 000 *g* for 2 hr	0·52	0·35	0·459	1·31
Supernatant after 35 000 *g* for 2 hr	1·60	1·10	0·125	0·114

* Measured using standard enzyme assay as described in Experimental.

This was confirmed using gel filtration; the pellet obtained by differential centrifugation was resuspended in 0·1 M phosphate buffer, pH 7·0, dialysed and applied to a Sephadex G200 column; the peroxidase activity was completely excluded from the gel.

TABLE 2. EXTRACTION OF PEROXIDASE FROM PARTICULATE FRACTION OF *C. purpureum* BY VARIOUS SURFACE ACTIVE AGENTS

Type of extracting reagent	Solubilization* %	Type of extracting reagent	Solubilization* %
Cholate (anionic)	12	Nonidet P40 (non ionic)	0
Deoxycholate (anionic)	34	Triton X100 (non ionic)	18
Digitonin (non ionic)	84	Tween 80 (non ionic)	7
Emulphogene (non ionic)	7	5% *n*-Butanol (non ionic)	0
Hexadecyltrimethylammonium bromide (cationic)	0		

* The degree of solubilization is expressed as a percentage of the residual activity in the supernatant after centrifugation at 35 000 *g* for 2 hr.

It has been suggested that many of the so-called particle-bound enzymes found in plant extracts are artifacts which arise from the formation of insoluble protein-tannin complexes.[11–14] However, the peroxidase from *C. purpureum* retained its particulate nature when reagents which inhibit complex formation, such as polyvinylpyrrolidone, diethyldithiocarbamate or thiol reducing reagents were present during extraction. Horseradish peroxidase added to an algal extract which was subsequently subjected to high-speed centrifugation remained in the supernatant. These findings indicate that the *C. purpureum* enzyme is particle-bound *in vivo*.

[11] E. H. NEWCOMB, *A. Rev. Pl. Physiol.* **14**, 43 (1963).
[12] A. C. HULME, J. D. JONES and L. S. C. WOOLTORTON, *Phytochem.* **3**, 173 (1964).
[13] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **4**, 185 (1965).
[14] J. S. HAWKER, *Phytochem.* **8**, 377 (1969).

Numerous methods and reagents were employed in an effort to solubilize the enzyme (Table 2). Digitonin was the most effective reagent used; indeed the effect of digitonin was even more significant than is suggested by the figures in Table 2 because digitonin caused a 1·5- to 2·0-fold increase in perodiase activity, as compared to controls containing particle-bound enzyme. This indicates that digitonin unmasks latent peroxidase activity, a phenomenon not duplicated with any of the other detergents, with the exception of sodium deoxycholate which caused a 10% increase in overall activity. The peroxidase was solubilized to the extent of 80% by a brief exposure of the extract to trypsin. This treatment caused no significant increase in overall activity relative to controls and thus digitonin treatment became the method of choice.

TABLE 3. SUMMARY OF ENZYME PURIFICATION

Fraction	Total protein (mg)	Total activity* units	Specific activity (units/mg)	Yield (%)	Purification factor
1 Crude extract	2040	534	0·261	(100)	(1·0)
2 Particulate suspension	420	345	0·828	64	3·2
3 Solubilised supernatant	280	411	1·47	77	5·6
4 Acetone	84	329	3·92	61	15·0
5 Sephadex	11·5	261	22·6	49	86·5

The enzyme was further purified in good yield by fractionation with ice-cold acetone followed by gel filtration on a column of Sephadex G75 equilibrated with 0·02 M glycine–NaOH buffer, pH 9·0. Active fractions were pooled and used for further study of the enzyme. The purification procedure is summarised in Table 3. An overall 86-fold purification was the best achieved.

Characterization of the Peroxidase

Based on a standard peroxidase assay, the initial rate of reaction catalysed by solubilized, partially purified, enzyme preparation was directly proportional to the quantity used over a 7-fold range of concentration. The enzyme displayed a broad pH optimum between 5 and 6; for routine assays, pH 5·4 was employed. A pH stability experiment showed that the enzyme is moderately stable at alkaline pH values but very labile under acid conditions. Similar pH optimum and stability characteristics were found for the particle-bound peroxidase.

Horseradish peroxidase lost no activity on heating for 90 min at 40°. At this temperature the algal enzyme had lost 90% activity in 30 min. At 30° the loss was 40% and at 25° the loss was about 10% in 30 min. The algal enzyme is less stable at high temperatures than is horseradish peroxidase and, in this property, it differs from peroxidases from most plant sources, which are relatively insensitive to high temperatures.[15] Thyroid peroxidase on the other hand is rather labile at room temperature.[16]

[15] B. C. SAUNDERS, A. G. HOLMES-SEIDLE and B. P. STARK, *Peroxidase*, p. 135, Butterworths, London (1964).
[16] T. HOSOYA, Y. KONDO and N. UI, *J. Biochem. Tokyo* **52**, 180 (1962).

The solubilized enzyme preparation gave rise to multiple components on starch gel electrophoresis, followed by staining for peroxidase activity. Four bands migrated to the anode, the leading band being the weakest. The remaining three bands were spaced evenly. The two bands nearest the origin were more intense than the second fastest band. The band remaining at the origin, which was immobile at all pH values tested, is not a true multiple form of the enzyme. A band also migrated to the cathode having the same speed (in the opposite direction from the origin) as the slowest anodic band. The enzyme solubilized by the trypsin treatment was concentrated and electrophoresed without subjecting it to the acetone fractionation step, lest acetone might be responsible for artifacts. An identical pattern was obtained with this preparation, however. Fractionation with acetone was unavoidable when digitonin was used to solubilize the enzyme because the location of peroxidase activity after electrophoresis was not possible due to discolouration of the gel by chlorophyll.

The MW of the enzyme was estimated by gel filtration on Sephadex G75 (Fig. 1); the deviation of horseradish peroxidase from the general linear relationship has been explained by Andrews).[17] A MW of 40 000–50 000 was estimated for the *C. purpureum* enzyme. This accords with generally accepted values for plant peroxidases. The elution volume of the peroxidase was independent of the method used to solubilize it (digitonin or trypsin).

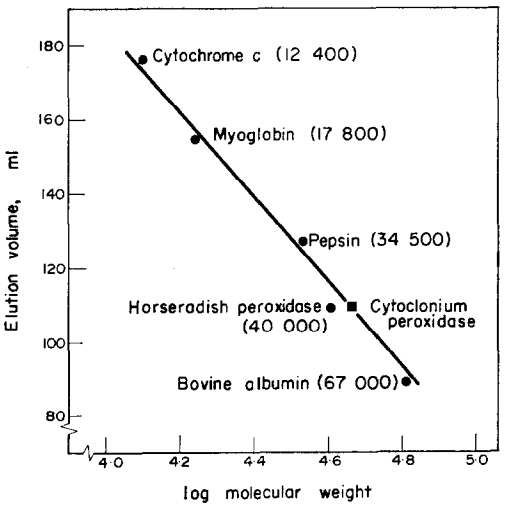

FIG. 1. MOLECULAR WEIGHT ESTIMATION OF *C. purpureum* PEROXIDASE BY GEL FILTRATION THROUGH SEPHADEX G75.

The gel was equilibrated and subsequently eluted with 0·1 M glycine–NaOH, pH 9·0. Elution of *C. purpureum* peroxidase was monitored by the standard assay method (see Experimental). Elution of other proteins was followed spectrophotometrically: myoglobin and cytochrome *c* at 408 nm and others at 280 nm.

The purest samples of algal peroxidase did not display a Soret band, but other properties suggest that it is a haemoprotein. Thus it is sensitive to cyanide (50% inhibition at 5×10^{-6} M) and azide (50% inhibition at 10^{-3} M) but not to 8-hydroxyquinoline, *o*-phenanthroline or acriflavine. These findings rule out a flavin prosthetic group as has been

[17] P. ANDREWS, *Biochem. J.* **91**, 222 (1964).

reported in some bacteria.[18] *o*-Dianisidine, *o*-tolidine, leuco-2,3,6-trichloroindophenol, 3-amino-9-ethyl carbazole, pyrogallol and *p*-phenylenediamine served as effective electron donors in *C. purpureum* peroxidase-catalysed reactions. However, benzidine, iodide and guaiacol, compounds often used in peroxidase assays, did not serve as electron donors at concentrations ranging from 0·5 to 5 mM. Siegel and Siegel[19] have reported similar results based on their survey of red and brown algae. *C. purpureum* peroxidase did not catalyse the formation of mono- or di-iodotyrosine from tyrosine and radioactive iodide. The optimum hydrogen peroxide concentration for most of the effective donors at fixed concentration (0·5 mM) was 1 mM. The apparent K_m for the enzyme, at a fixed concentration of *o*-dianisidine (0·5 mM), was 0·11 mM. The inability of the peroxidase to catalyse the oxidation of iodide, whose redox potential for the couple ($2I^- — I^2$) is the lowest of any of the halides, is surprising and suggests that it may not participate in halogenation reactions in *C. purpureum*.

EXPERIMENTAL

Preparation and purification. Algae were collected on the shores of Galway Bay and stored at −20°. The plants were disrupted with a triple-roll mill (Pascall, Crawley, England) and extracted with 0·1 M phosphate buffer (1 mM EDTA), pH 7·0. The pellet obtained after centrifugation was resuspended using a Potter–Elvehjem homogenizer. Solubilization with detergent was achieved by adding an equal vol. of a 2% digitonin solution adjusted to pH 7·0.[20] To achieve solubilisation with trypsin the pellet was suspended in 0·1 M phosphate buffer, pH 7·0, and trypsin (Sigma Type 11) added to a final concentration of 0·5 mg/ml. The suspension was stirred at room temp. for 2 hr. Soybean trypsin inhibitor (Sigma Type 11S) was then added to a final concentration of 1 mg/ml and the suspension centrifuged. Acetone fractionation was achieved by adding acetone, cooled to −30°, to the solubilised enzyme at 0° to a final concentration of 50%. After centrifugation the residue was discarded and the supernatant brought to a final concentration of 72% acetone. After further centrifugation the precipitate, containing the bulk of the peroxidase activity, was dialysed against 0·02 M glycine–NaOH buffer, pH 9·0.

Standard enzyme assay. Assay was by the oxidation of *o*-dianisidine (Worthington Biochemical Corporation Descriptive Manual). The assay mixture (final vol. 3 ml) contained 1·5 μmol *o*-dianisidine; 3 μmol H_2O_2; 300 μmol sodium phosphate–citrate buffer, pH 5·4 and 0·1 ml of enzyme preparation. Spectral measurements (460 nm) were made at 25°; the absorbancy of oxidized *o*-dianisidine per mol of 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.

Other methods. Electrophoresis was performed on vertical starch gels (Buchler Instruments, Fort Lee, New Jersey). Buffer systems used were phosphate, pH 7·4; Tris-EDTA-borate, pH 8·0 and Tris-maleate, pH 8·4. After electrophoresis the gels were stained with either *o*-dianisidine diHCl or 3-amino-9-ethyl carbazole, using an agar overlay.[21] Preparation and equilibration of Sephadex columns, application of proteins and estimation of MWs was carried out according to Andrews.[17] Protein was determined at the early stages of purification by the method of Potty[22] and at later stages by the method of Lowry *et al.*[23] Radioactive experiments were carried out according to Tong and Chaikoff[24] and products identified by TLC and autoradiography.

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[18] M. I. Dolin, *J. Biol. Chem.* **225**, 557 (1957).
[19] B. Z. Siegel and S. M. Siegel, *Am. J. Bot.* **57**, 285 (1970).
[20] N. O. Kaplan, S. P. Colowick and E. F. Neufeld, *J. Biol. Chem.* **205**, 1 (1953).
[21] W. H. P. Lewis and H. Harris, *Nature, Lond.* **215**, 351 (1967).
[22] V. H. Potty, *Anal. Biochem.* **29**, 535 (1969).
[23] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
[24] W. Tong and I. L. Chaikoff, *J. Biol. Chem.* **215**, 473 (1955).