Chloroperoxidases from *Caldariomyces* (= *Leptoxyphium*) Cultures: Glycoproteins with Variable Carbohydrate Content and Isoenzymic Forms

By ATSUMI HASHIMOTO AND MICHAEL A. PICKARD*

Department of Microbiology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

(Received 23 February 1984)

Twenty-six fungal cultures, including ten *Caldariomyces* (= *Leptoxyphium*) cultures and five other capnodiaceous fungi, were examined for extracellular chloroperoxidase production on four growth media. Only the *Caldariomyces* cultures produced enzyme activity and enzyme production was highest on growth in a phytone medium. Chloroperoxidase was partly purified from the ten *Caldariomyces* cultures. All preparations were glycoproteins, with different carbohydrate content. The absorption spectra of the ten samples were indistinguishable and this was reflected in similar R_z (A_{403}/A_{280}) values. SDS-PAGE revealed two major bands of protein in all preparations but in different proportions: staining for carbohydrate confirmed these bands to be glycoproteins. PAGE at pH 3 showed each preparation to be composed of several isoenzymes and these could be grouped according to their migration patterns. Kinetic constants, where determined, and pH optima showed no differences among the ten preparations and all exhibited catalase and peroxidase activities in the same relative proportions to chlorinating activity.

INTRODUCTION

Chloroperoxidase (chloride peroxidase, EC 1.11.1.10) is an extracellular haem glycoprotein produced by the imperfect fungus *Caldariomyces fumago*, from which it has been crystallized and characterized (Morris & Hager, 1966). The enzyme catalyses the formation of a stable carbon-halogen bond with chloride, bromide and iodide, and under appropriate conditions can catalyse the formation of heterogeneous organic dihalides (Geigert *et al.*, 1983*a, b, c*; Neidleman & Geigert, 1983). It also has a potential commercial use in the production of alkene oxides, the precursors of various plastics (Eveleigh, 1981).

In the reduced (Fe^{2+}) form and in the presence of carbon monoxide, the absorption spectrum of chloroperoxidase reveals a striking resemblance to that of cytochrome P-450_{cam}. As such it has been used as a model system for the study of structural properties unique to cytochrome P-450 and the molecular mechanism of peroxidase enzymes (Hollenberg & Hager, 1973; Remba *et al.*, 1979; Palcic *et al.*, 1980; Araisio *et al.*, 1981; Lambeir *et al.*, 1983).

Most chloroperoxidase studies have been carried out on the enzyme produced by *C. fumago* ATCC 16373 (Hollenberg & Hager, 1978) while some were performed on the enzyme from *C. fumago* CMI 89362 (Shaw *et al.*, 1959; Morris & Hager, 1966; Sae & Cunningham, 1979). In most cases the fungi have been grown in the modified Czapek-Dox medium of Clutterbuck *et al.* (1940) supplemented with malt extract (Hollenberg & Hager, 1978) or with yeast extract (Morris & Hager, 1966; Sae & Cunningham, 1979). More recently a defined medium containing fructose as the carbon source has been shown to support good enzyme production by both strains (Pickard, 1981). A number of related *Caldariomyces* (= *Leptoxyphium*) cultures were also found to produce enzyme activity when grown on the glucose/malt extract medium, but at a much lower level (Pickard, 1981).

In an early study of chlorine metabolism by moulds (Clutterbuck *et al.*, 1940), five organisms

were described which metabolized more than 25% of the chloride in the medium: two strains of *C. fumago*, two strains of *Aspergillus terreus*, and *Absidia spinosa*. These organisms, where available, or related available strains and a number of capnodiaceous fungi (Hughes, 1976) related to *C. fumago* were examined for chloroperoxidase production. The enzyme has been partly purified from ten fungi and some properties of these preparations have been compared.

METHODS

Micro-organisms and culture conditions. *Caldariomyces fumago* ATCC 16373 and *C. fumago* ATCC 11925 were obtained from the American Type Culture Collection (Rockville, MD, USA). *Caldariomyces fumago* CMI 89362, *Aspergillus terreus* CMI 44339 and *Absidia spinosa* CMI 89279 were from the Commonwealth Mycological Institute (Kew, Surrey, UK). *Leptoxylum* sp. DAOM 145537, DAOM 138163, DAOM 119795, DAOM 137632, DAOM 167770, DAOM 166438 and DAOM 165359, *Scorias spongiosa* DAOM 167394, *Ciferrioxylum chaetomorphum* DAOM 153106, and *Polychaeton* sp. DAOM 155925, DAOM 166385 and DAOM 1816076 were kindly supplied by Dr S. J. Hughes of the Biosystematics Research Institute, Agriculture Canada, Ottawa, Ont., Canada. *Aspergillus terreus* UAMH 327, UAMH 1897, UAMH 2035 and UAMH 3627 were from the University of Alberta Mold Herbarium, Edmonton, Alta., Canada, courtesy of Ms L. Sigler; *A. terreus* PRL 1551, PRL 1552, PRL 1809 and PRL 2384 were from the Prairie Research Laboratory, National Research Council, Saskatoon, Sask., Canada; *A. terreus* NRRL 255 was kindly supplied by Dr A. Jones, Department of Chemistry, University of Calgary, Calgary, Alta., Canada.

The basal fructose/salts medium contained (per litre): fructose, 40 g; NaNO₃, 2 g; KCl, 2 g; KH₂PO₄, 2 g; MgSO₄ · 7H₂O, 1 g; FeSO₄ · 7H₂O, 20 mg. Yeast extract (Difco), phytone (BBL) or peptone (Difco) when present were added to the fructose/salts medium at 20 g l⁻¹. Conditions for growth and culture maintenance were otherwise as described previously (Pickard, 1981).

Cell breakage. Washed fungal mycelium (2–3 g wet weight) was suspended in 10 ml 0.1 M-KHPO₄ buffer pH 5 and homogenized for 20 s in a blender (Sorvall Omnimixer). Glucuronidase (22000 units) and aryl sulphatase (6300 units) (Glusulase, Endo Laboratories, NY, USA) were added and the dispersed mycelial fragments were incubated with shaking for 30 min at 30 °C in the presence of 2-mercaptoethanol (10 mM final concentration). The mycelial suspension was then sonicated for 6 × 20 s, with cooling using a Branson sonifier, which, as judged by microscopic examination, produced greater than 50% cell breakage.

Enzymes assays. Chlorinating activity was assayed by the chlorodimedone method of Morris & Hager (1966) as described previously (Pickard, 1981). Peroxidase activity was determined by the kinetic assay of *o*-dianisidine oxidation at 460 nm (Worthington Enzyme Manual, 1972), but in 50 mM-phosphate buffer pH 3. Catalase activity was assayed spectrophotometrically by following the loss of absorbance at 240 nm (Beers & Sizer, 1952), also at pH 3.

Protein determinations. During enzyme purification, protein content was monitored using the A_{280}/A_{260} ratio method of Warburg & Christian (1941). Purified samples were analysed by the Lowry method.

Enzyme purification. Chloroperoxidase was purified essentially as described by Hollenberg & Hager (1978) with modifications (Pickard, 1981). Column chromatography was carried out first on DEAE-cellulose (DE52, Whatman) using a phosphate gradient, followed by gel filtration on BioGel P-60 in 50 mM-phosphate buffer pH 5. Samples were stored at –20 °C in 50 mM-phosphate buffer pH 5 between stages and after purification.

Carbohydrate determinations. Free and bound carbohydrate was determined by the phenol/H₂SO₄ method of Dubois *et al.* (1956) using arabinose as the standard, since arabinose is the major carbohydrate component of the glycoprotein (Morris & Hager, 1966).

Gel electrophoresis. Gel electrophoresis under denaturing conditions (SDS-PAGE) was carried out as described by Laemmli (1970). Electrophoresis at pH 3 was performed in glycine/H₃PO₄ as described by Pickard & Hashimoto (1982). Periodate-Schiff staining of SDS gels was by the method of Fairbanks *et al.* (1971).

RESULTS AND DISCUSSION

Survey of fungal cultures for enzyme production

Of the twenty-six fungal cultures examined, only the ten *Caldariomyces* (= *Leptoxylum*) strains produced chloroperoxidase. These ten cultures produced different levels of extracellular activity depending on the growth medium employed (Table 1), the activity being generally highest in the fructose/phytone medium and lowest in the defined fructose/salts medium. The exception was *C. fumago* ATCC 16373 which is most productive when grown in fructose/salts or fructose/malt extract (Pickard, 1981). Five isolates of taxonomically related capnodiaceous fungi (Hughes, 1976), *Scorias spongiosa*, *Ciferrioxylum chaetomorphum*, and three isolates of *Polychaeton* sp., ten *Aspergillus terreus* strains, and *Absidia spinosa*, were examined for enzyme

Table 1. *Effect of growth medium on chloroperoxidase production by Caldariomyces (= Leptoxyphium) cultures*

Fungal strain	Chloroperoxidase activity of growth medium (enzyme units ml ⁻¹)*			
	Fructose/salts plus			
	Fructose/ salts	Yeast extract	Phytone	Peptone
<i>Leptoxyphium</i> sp. DAOM 138163	3.2	89	102	71
<i>Leptoxyphium</i> sp. DAOM 165359	0.9	48	85	26
<i>Leptoxyphium</i> sp. DAOM 166438	6.9	35	279	43
<i>Leptoxyphium</i> sp. DAOM 145537	4.8	67	139	43
<i>Leptoxyphium</i> sp. DAOM 137632	38	175	249	66
<i>Leptoxyphium</i> sp. DAOM 167770	19	89	121	34
<i>Leptoxyphium</i> sp. DAOM 119795	3.4	48	100	16
<i>C. fumago</i> ATCC 11925	62	109	157	78
<i>C. fumago</i> ATCC 16373	240	18	98	5
<i>C. fumago</i> CMI 89362	185	37	295	52

* Maximum activity, observed after 10–15 d growth.

Table 2. *Relative purity (Rz*) and carbohydrate content of chloroperoxidase preparations following ion-exchange and gel chromatography*

Enzyme source	DEAE-cellulose		BioGel P-60	
	Rz	Carbohydrate/ protein	Rz	Carbohydrate/ protein
<i>Leptoxyphium</i> sp. DAOM 138163	1.27	0.85	1.39	0.89
<i>Leptoxyphium</i> sp. DAOM 165359	1.10	1.00	1.30	0.83
<i>Leptoxyphium</i> sp. DAOM 166438	1.05	2.44	1.19	0.62
<i>Leptoxyphium</i> sp. DAOM 145537	1.00	2.81	1.23	0.55
<i>Leptoxyphium</i> sp. DAOM 137632	1.01	0.56	1.15	0.27
<i>Leptoxyphium</i> sp. DAOM 167770	1.22	0.41	1.29	0.22
<i>Leptoxyphium</i> sp. DAOM 119795	1.06	0.68	1.25	0.23
<i>C. fumago</i> ATCC 11925	1.21	1.22	1.38	0.28
<i>C. fumago</i> ATCC 16373	1.27	1.48	1.46	0.35
<i>C. fumago</i> CMI 89362	1.28	1.21	1.40	0.15

* Rz (Reinheitszahl), the ratio of absorbance at 403 nm (due to haem) to that at 280 nm (due to protein), is used as a measure of purity for haemoproteins. The Rz of pure chloroperoxidase is 1.44 (Hollenberg & Hager, 1978).

production. All these organisms grew well in the four media, producing from 5 to 50 g wet weight of mycelium from a litre of medium. However, no chloroperoxidase activity was detected in either the growth medium, broken cell suspensions, or the cell-free extracts. Thus the production of chloroperoxidase appears to be restricted to a group of closely related fungal strains of the genus *Caldariomyces*. None of the other capnodiaceous fungi produced the enzyme nor did the organisms used in the early survey of fungal chlorine metabolism by Raistrick (Clutterbuck *et al.*, 1940). The enzyme activities produced by the ten *Caldariomyces* cultures all fell into the range 100–300 units ml⁻¹ when the fungi were grown on the fructose/phytone medium, with seven cultures in the 100–150 range. These data indicate a relatively high and constant capacity for enzyme production by all producing cultures.

Preparation and purification of chloroperoxidase

The ten enzyme-producing cultures were grown in batches of from 4 to 10 l, depending on the efficiency of chloroperoxidase recovery from the culture medium. *Caldariomyces fumago* strains ATCC 16373 and CMI 89362 were grown in fructose/salts medium while the remaining eight cultures were grown in the fructose/phytone medium. After a single passage of the ethanol-precipitated culture medium concentrates through a DEAE-cellulose column, all preparations

Fig. 1. Absorption spectra of chloroperoxidase preparations ($2-4 \text{ mg protein ml}^{-1}$) in 0.1 M -phosphate buffer pH 5. Samples were scanned separately from 350 to 450 nm with full scale at 1.0 and from 450 to 700 nm with full scale at 0.2, using the recorder offset to separate the curves. 1, *Leptoxiphium* sp. DAOM 138163; 2, *C. fumago* CMI 89362; 3, *Leptoxiphium* sp. DAOM 145537; 4, *Leptoxiphium* sp. DAOM 165359; 5, *C. fumago* ATCC 16373; 6, *Leptoxiphium* sp. DAOM 119795; 7, 8, *C. fumago* ATCC 11925 and *Leptoxiphium* sp. DAOM 137632 (superimposed); 9, *Leptoxiphium* sp. DAOM 167770; 10, *Leptoxiphium* sp. DAOM 166438.

Fig. 2. SDS-PAGE of chloroperoxidase samples. (a) Protein stain. (b) Carbohydrate (periodic acid-Schiff) stain. Well 1, *Leptoxiphium* sp. DAOM 138163; well 2, *Leptoxiphium* sp. DAOM 165359; well 3, *Leptoxiphium* sp. DAOM 166438; well 4, *Leptoxiphium* sp. DAOM 145537; well 5, *Leptoxiphium* sp. DAOM 137632; well 6, *Leptoxiphium* sp. DAOM 167770; well 7, *Leptoxiphium* sp. DAOM 119795; well 8, *C. fumago* ATCC 11925; well 9, *C. fumago* ATCC 16373; well 10, *C. fumago* CMI 89362. Wells contained $10-20 \mu\text{g}$ protein.

Fig. 3. Electrophoresis of chloroperoxidase preparations under non-denaturing conditions at pH 3. (a) Protein stain. (b) Enzyme stain: oxidation of 3,3',5,5'-tetramethylbenzidine (Thomas *et al.*, 1976). The sources of the preparations in the different wells were as described in the legend to Fig. 2.

had a purity index (R_z) of 1.0 or greater (Table 2). However, when these preparations were analysed by PAGE and stained for protein, several of the samples smeared. These samples were also more viscous than those which migrated without smearing. The viscous samples were found to have carbohydrate contents considerably above the 30% reported for the crystalline glycoprotein (Morris & Hager, 1966). To remove this presumably non-covalently bound carbohydrate, small portions were submitted to gel exclusion chromatography. Initially Sephadex G-75 Superfine was used, to take advantage of the narrow elution zones, but the oxidative properties of this enzyme caused extensive breakdown of the gel matrix, requiring a change to the more stable acrylamide-based BioGel P-60. In almost all cases some polysaccharide material was eluted from the column in advance of the enzyme peak, resulting in a lower carbohydrate content of the enzyme preparations and an improvement (increase) in the R_z (Table 2). Reapplication of these pooled, enzymically active, fractions to a second BioGel P-60 column resulted in a symmetrical elution profile with the individual fractions exhibiting a constant carbohydrate/protein ratio. Unfortunately only small samples, 2–3% of the column volume, could be treated in this way because of the similar hydrodynamic properties of the enzyme and the contaminating polysaccharide. These partly purified preparations were found to contain between 13% and 47% carbohydrate (Table 2).

Spectral properties of the enzyme preparations

The absorption spectra of the ten preparations are shown in Fig. 1. Equivalent enzyme concentrations were prepared in 0.1 M-phosphate buffer pH 5 and the spectra recorded between

Table 3. *Relative activity of chloroperoxidase preparations for catalase and peroxidase reactions*

Values are averages of three determinations.

Enzyme source	Chlorination activity (units ml ⁻¹)	Activity relative to chlorination (= 100%)	
		Catalase	Peroxidase
<i>Leptoxylum</i> sp. DAOM 138163	2572	86	3
<i>Leptoxylum</i> sp. DAOM 165359	2555	50	4
<i>Leptoxylum</i> sp. DAOM 166438	1967	82	4
<i>Leptoxylum</i> sp. DAOM 145537	953	56	3
<i>Leptoxylum</i> sp. DAOM 137632	2818	73	3
<i>Leptoxylum</i> sp. DAOM 167770	2223	93	4
<i>Leptoxylum</i> sp. DAOM 119795	2131	80	5
<i>C. fumago</i> ATCC 11925	3362	55	2
<i>C. fumago</i> ATCC 16373	2736	82	4
<i>C. fumago</i> CMI 89362	2674	90	4

350 and 700 nm, using the offset to separate the curves. No detectable differences could be observed between any of the preparations: absorption maxima typical of chloroperoxidase were recorded at 403, 515, 540 and 650 nm (Morris & Hager, 1966).

Analysis of chloroperoxidase preparations by PAGE

Under denaturing conditions, SDS-PAGE revealed only minor differences between the ten preparations (Fig. 2). Two major bands were visible in eight of the ten samples, when stained for protein (Fig. 2a). The samples from *Leptoxylum* sp. DAOM 138163 (well 1), and *Leptoxylum* sp. DAOM 165359 (well 2) did not resolve clearly into two bands, but with oblique lighting under the gel two areas of staining could be seen. A duplicate gel stained for carbohydrate by the periodic acid-Schiff reagent (Fig. 2b) produced an almost identical staining pattern to the protein-stained gel, indicating that the two major enzyme forms produced by each organism are glycoproteins. Minor bands migrating ahead of the doublet in wells 5 and 6 appeared to be contaminating protein as they were absent from the periodic acid-Schiff-stained gel. These two bands are believed to correspond to the chloroperoxidase isoenzymes A and B described by Sae & Cunningham (1979) and Pickard & Hashimoto (1982). Sae & Cunningham (1979) estimated the molecular weight of the isoenzymes by gel filtration to be 46000 (A) and 40000 (B). Our estimate of apparent molecular weights on gels using marker proteins gave values that were 30% higher than expected; however, SDS-PAGE is known to be unreliable for determining the molecular weights of glycoproteins (Segrest & Jackson, 1972; Sharon & Lis, 1982).

Under non-denaturing conditions, at pH 3, a significantly different pattern of protein bands was observed (Fig. 3a). Almost all of these bands were demonstrated to be catalytically active when a duplicate gel was stained for peroxidase activity by the tetramethylbenzidine method (Thomas *et al.*, 1976) (Fig. 3b). The preparations in wells 5, 6 and 7 contained a minor, more mobile species with no enzyme activity. On the basis of these gel patterns there appeared to be at least two groups of enzymes: the multiple (four to six) bands of the *Leptoxylum* sp. DAOM 166438 (well 3) and *C. fumago* ATCC 11925 (well 8) constituted one group and the other eight preparations, with two major and one or two minor bands, constituted the other.

Substrate specificity and effect of pH

Chloroperoxidase is known to exhibit a broad specificity towards its non-halogen substrates and can catalyse peroxidase and catalase reactions as well as halogenations (Thomas *et al.*, 1970; Hollenberg & Hager, 1978; Araiso *et al.*, 1981). To compare the similarity of the ten preparations, peroxidase and catalase activities were determined, and these are presented in Table 3 as percentages of the chlorinating activity. All the preparations catalysed all three reactions and to similar levels. Catalase activity was always lower than chlorinating activity: in

most cases in the range 80–90% but as low as 50–55% in three samples. Peroxidase activity was much lower, ranging from 2% to 5% of the chlorination activity.

The pH optimum for the peroxidase assay has been reported to range from 4 to 7 depending on the substrate (Hollenberg & Hager, 1978) but in our hands peroxidase activity dropped by 77% when the pH was raised from 3 to 4. Thus, for all three catalytic assays, reaction mixtures were maintained at pH 3. The pH-activity profile was recorded over the pH range 2.6 to 3.6 for all preparations before and after the gel filtration stage of purification. All preparations showed minor differences in their pH-activity profiles and while removal of extraneous polysaccharide changed the shape of some of these profiles, this effect was not consistent among samples. At the substrate concentrations used the pH optimum was 3.0.

Apart from minor variations in the pH-activity curves, we detected no differences in catalytic properties among the enzyme preparations. While kinetic parameters for substrates have not been determined for all the preparations, those which have been studied fall within the ranges described for the enzyme from *C. fumago* Ag92 (= CMI 89362) (Hager *et al.*, 1966). From these data it appears that the enzymes formed by the ten fungi studied are catalytically indistinguishable but contain variable amounts of polysaccharide, resulting in the formation of different patterns of isoenzymes: examples of glycoprotein 'microheterogeneity' (Cunningham, 1971).

The technical assistance of Sherry McLaren and Tom Hantos, and the mycological expertise of Ms L. Siger and Dr S. J. Hughes, are gratefully acknowledged. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (A6482) to M. A. Pickard.

REFERENCES

- ARAI, T., RUTTER, R., PALCIC, M. M., HAGER, L. P. & DUNFORD, H. B. (1981). Kinetic analysis of compound I formation and the catalytic activity of chloroperoxidase. *Canadian Journal of Biochemistry* **59**, 233–236.
- BEERS, R. F. & SIZER, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry* **195**, 133–140.
- CLUTTERBUCK, P. W., MUKHOPADHYA, S. L., OXFORD, A. E. & RAISTRICK, H. (1940). Studies in the biochemistry of micro-organisms. 65(a) A survey of chlorine metabolism by moulds. (b) *Caldariomycin*, $C_5H_8O_2Cl_2$, a metabolic product of *Caldariomyces fumago* Woronichin. *Biochemical Journal* **34**, 664–677.
- CUNNINGHAM, L. W. (1971). Microheterogeneity and function of glycoproteins. In *Glycoproteins of Blood Cells and Plasma*, pp. 16–67. Edited by G. A. Jamieson & T. J. Greenwalt. Philadelphia: Lippincott.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for the determination of sugars and related substances. *Analytical Chemistry* **28**, 350–355.
- EVELEIGH, D. E. (1981). The microbiological production of industrial chemicals. *Scientific American* **245**, 154–178.
- FAIRBANKS, G., STECK, T. L. & WALLACH, D. H. F. (1971). Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**, 2606–2617.
- GEIGERT, J., NEIDLEMAN, S. L., DALIETOS, D. J. & DEWITT, S. K. (1983a). Haloperoxidases: enzymatic synthesis of $\alpha\beta$ -halohydrins from gaseous alkenes. *Applied and Environmental Microbiology* **45**, 366–374.
- GEIGERT, J., NEIDLEMAN, S. L. & DALIETOS, D. J. (1983b). Novel haloperoxidase substrates alkynes and cyclopropanes. *Journal of Biological Chemistry* **258**, 2273–2277.
- GEIGERT, J., NEIDLEMAN, S. L., DALIETOS, D. J. & DEWITT, S. K. (1983c). Novel haloperoxidase reaction: synthesis of dihalogenated products. *Applied and Environmental Microbiology* **45**, 1575–1581.
- HAGER, L. P., MORRIS, D. R., BROWN, F. S. & EBERWEIN, H. (1966). Chloroperoxidase. II. Utilization of halogen ions. *Journal of Biological Chemistry* **241**, 1769–1777.
- HOLLENBERG, P. F. & HAGER, L. P. (1973). The P-450 nature of the carbon monoxide complex of ferrous chloroperoxidase. *Journal of Biological Chemistry* **248**, 2630–2633.
- HOLLENBERG, P. F. & HAGER, L. P. (1978). Purification of chloroperoxidase from *Caldariomyces fumago*. *Methods in Enzymology* **52**, 521–529.
- HUGHES, S. J. (1976). Sooty moulds. *Mycologia* **68**, 693–820.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- LAMBEIR, A.-M., DUNFORD, H. B. & PICKARD, M. A. (1983). Kinetics of cyanide binding to chloroperoxidase in the presence of nitrate: detection of the influence of a heme-linked acid group by shift of the apparent pK_a value. *Journal of Inorganic Biochemistry* **19**, 291–300.
- MORRIS, D. R. & HAGER, L. P. (1966). Chloroperoxidase. I. Isolation and properties of the crystalline glycoprotein. *Journal of Biological Chemistry* **241**, 1763–1768.
- NEIDLEMAN, S. L. & GEIGERT, J. (1983). The enzymatic synthesis of heterogeneous dihalide derivatives: a unique biocatalytic discovery. *Trends in Biotechnology* **1**, 21–25.

- PALCIC, M. M., RUTTER, R., ARAISO, T., HAGER, P. P. & DUNFORD, H. B. (1980). Spectrum of chloroperoxidase compound I. *Biochemical and Biophysical Research Communications* **94**, 1123–1127.
- PICKARD, M. A. (1981). A defined growth medium for the production of chloroperoxidase by *Caldariomyces fumago*. *Canadian Journal of Microbiology* **27**, 1298–1305.
- PICKARD, M. A. & HASHIMOTO, A. (1982). Isoenzymes of chloroperoxidase from *Caldariomyces fumago*. *Canadian Journal of Microbiology* **28**, 1382–1388.
- REMBE, R. D., CHAMPION, P. M., FITCHEN, D. B., CHIANG, R. & HAGER, L. P. (1979). Resonance Raman investigations of chloroperoxidase, horseradish peroxidase and cytochrome *c* using Soret band laser excitation. *Biochemistry* **18**, 2280–2290.
- SAE, A. S. W. & CUNNINGHAM, B. A. (1979). Isolation and properties of chloroperoxidase isoenzymes. *Phytochemistry* **18**, 1785–1787.
- SEGREST, J. P. & JACKSON, R. L. (1972). Molecular weight determination of glycoproteins by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. *Methods in Enzymology* **28**, 54–63.
- SHARON, N. & LIS, H. (1982). Glycoproteins. In *The Proteins*, vol. 5, 3rd edn, pp. 1–144. Edited by H. Neurath & R. L. Hill. New York: Academic Press.
- SHAW, P. D., BECKWITH, J. R. & HAGER, L. P. (1959). Biological chlorination. II. The biosynthesis of δ -chlorolevulinic acid. *Journal of Biological Chemistry* **234**, 2560–2564.
- THOMAS, J. A., MORRIS, D. R. & HAGER, L. P. (1970). Chloroperoxidase. VIII. Formation of peroxide and halide complexes and their relation to the mechanism of the halogenation reaction. *Journal of Biological Chemistry* **245**, 3135–3142.
- THOMAS, P. E., RYAN, D. & LEVIN, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Analytical Biochemistry* **75**, 168–176.
- WARBURG, O. & CHRISTIAN, W. (1941). Isolierung und Krystallisation des Gärungsfermens Enolase. *Biochemische Zeitschrift* **310**, 384–421.
- WORTHINGTON ENZYME MANUAL (1972). Peroxidase (horseradish), p. 43. Freehold, New Jersey: Worthington Biochemical Corporation.