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ISOLATION AND PROPERTIES OF CHLOROPEROXIDASE ISOZYMES

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Key Word Index—*Caldariomyces fumago*; fungi; chloroperoxidase; peroxidase; biological chlorination.

Abstract—A new purification method for chloroperoxidase from *Caldariomyces fumago* is described. This method involves dialysis, alumina gel adsorption, DEAE-cellulose chromatography at pH 6, and then at pH 3.85 and crystallization. Two isozymes have been isolated and one has been crystallized. The MWs estimated by gel filtration are 46000 for chloroperoxidase A and 40000 for chloroperoxidase B. The guaiacol peroxidation catalysed by these isozymes is proportional to their chlorination activity.

INTRODUCTION

The knowledge of biological mechanisms for the formation of a carbon-halogen bond is very limited despite the frequent occurrence of halogen-containing natural products [1–3]. In 1959 Shaw and Hager [4] discovered a fungal peroxidase which catalysed the formation of carbon-chlorine bonds. Subsequently this enzyme, chloroperoxidase, was purified in crystalline form [5]. A simpler method of preparation procedure was later published by Hager [6]. We would like to report our method of isolation of chloroperoxidase from *Caldariomyces fumago*, the discovery of an isozyme, and some properties of this enzyme.

RESULTS AND DISCUSSION

Preparation

Table 1 summarizes the process of purification of *C. fumago* chloroperoxidase. From 27 l. of culture medium, 12 mg of twice crystallized chloroperoxidase was obtained. An isozyme was also isolated but it did not crystallize under the same conditions. The crystalline chloroperoxidase is called chloroperoxidase A and the non-crystalline one is called chloroperoxidase B in this report.

Column isoelectric focusing showed that chloroperoxidases A and B had isoelectric pH values of 3.85 and 3.57, respectively. These two isozymes were eluted as a single protein-activity peak when chromatographed on a DEAE-cellulose column at pH 6. The combined chloroperoxidase fractions were then chromatographed on a pH 3.85 DEAE-cellulose column. Two chloroperoxidase peaks were eluted. The peak fractions showed constant A_{410}/A_{280} ratios for both isozymes ($A = 1.28 \pm 0.05$, $B = 1.30 \pm 0.05$). In order to show that these protein-activity peaks did not result from the changes of chromatographic conditions affecting one single protein, the combined fractions from each peak were rechromatographed separately on the same column. A single chloroperoxidase peak was obtained in each case. Samples taken from the two peak fractions were examined by disc electrophoresis on polyacrylamide gel. At pH 3.2, the peak A samples (chloroperoxidase A) showed one guaiacol staining band corresponding to the single Coomassie blue stained protein band with identical mobility. Peak B samples (chloroperoxidase B) showed two bands in both guaiacol and Coomassie blue stained gels. The minor band had a higher mobility identical to that of peak A. These chromatographic and electrophoretic results confirmed the presence of the two isozymes of chloroperoxidase.

Although isozymes among peroxidases are common, Morris and Hager [5] reported that the minor peak of activity in their preparation was due to artifacts caused by heat treatment of the growth medium, but no heat treatment was used in the present study.

Table 1. Summary of purification data on *C. fumago* chloroperoxidase

Step	Volume (ml)	Total activity (units × 10^3)	Total protein (mg)	Specific activity (units/mg)
Dialysed growth medium	27000	945	147	64
Alumina gel adsorption	3000	717	1100	640
DEAE-cellulose chromatography, pH 6.0	500	382	220	1740
DEAE-cellulose chromatography, pH 3.85				
Peak A	125	80.2	38.1	2100
Peak B	128	43.2	21.1	2050
1st crystallization of A		32.4	12.0	2700
2nd crystallization of A		—	—	2600

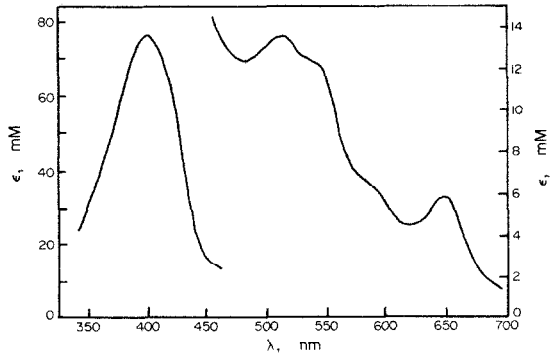

Fig. 1. Light absorption spectrum of chloroperoxidase A in deionized, distilled water.

Crystals of chloroperoxidase A were obtained in ammonium sulfate solution. These crystals appeared as fine red rosettes rather than monoclinic as reported by Morris and Hager [5]. It is not uncommon that protein crystals can be polymorphic. The A_{398}/A_{280} ratio of the twice crystallized chloroperoxidase A was 1.33 in deionized, distilled water. The same preparation when stained with 1% uranyl formate and examined in an electron microscope showed a highly homogeneous preparation of particles with a diameter of 4.75 nm. Fig. 1 shows the absorption spectrum of chloroperoxidase A having λ_{max} of 398, 513, 543 and 648 nm with millimolar absorptivity of 76.3, 13.5, 12.3 and 5.9, respectively. These absorption maxima are similar to those obtained in 0.1 M citrate phosphate buffer of pH 5.4 by Morris and Hager [5]. In addition, a well defined shoulder at *ca* 587 nm was observed.

The MW of chloroperoxidase A was estimated to be 46000 by gel filtration, and 42000 from electron microscopy [7]. The MW of chloroperoxidase B was estimated to be 40000 by gel filtration. Morris and Hager [5] reported a MW of *ca* 42000 for their crystalline enzyme.

Enzyme activity

The assaying method used throughout the isolation–purification procedure was based on the classical peroxidase–guaiacol method [8] in which the increase in A at 470 nm with time was measured as the guaiacol was being converted to an orange-brown product. However, the ability of both chloroperoxidases A and B to chlorinate needed to be shown.

To do so, these enzymes were mixed with monochlorodimedon, chloride and hydrogen peroxide. The reaction product was co-chromatographed on Si gel by TLC with authentic compounds. Both isozymes catalysed the conversion of monochlorodimedon to dichlorodimedon. Furthermore, the rate of conversion was proportional to the guaiacol peroxidation activity for both isozymes. The chlorination rates were the same for both isozymes under identical reaction conditions. Therefore, the guaiacol assay was shown to be a valid, useful and simple method for following chloroperoxidase activity in the purification procedures.

EXPERIMENTAL

Caldariomyces fumago strain Ag-92, was obtained from the London School of Hygiene and Tropical Medicine, University of London, U.K. Guaiacol (Matheson Chemical Company) was redistilled before use. Monochlorodimedon and dichlorodimedon were synthesized from dimedon according to the method of ref. [9]. Dichlorodimedon was purified by TLC on Si gel using EtOAc–Skellysolve B–HOAc–C_6H_6 (60:40:2:0.5) as solvent.

Growth of C. fumago. Stock cultures were carried on oat meal-supplemented agar slants with monthly transfer. In the prepn for large submerged cultures, mycelia taken from 500 ml bottle stock culture were blended with sterile H_2O at maximum speed for 30 sec using a Loudes Multimixer. The mycelial suspension from one 500 ml bottle stock culture was used to inoculate 10 l. of yeast extract supplemented, sterile Czapek-Dox medium [5] in a 50 l. carboy. The fungus was grown under submerged conditions in the carboy rolling at 76 rpm at 20–23°. A stream of moist air was directed into each carboy. The pH of the culture was maintained below 7. After the enzyme level in the medium had reached a plateau in 200–300 hr, the mycelia were filtered off and the filtrate was stored at 4° until further work-up. Little change in enzyme activity was observed after storage for 2–3 days.

The enzyme assay used for chloroperoxidase activity during routine purification work was based on the increase in A at 470 nm accompanying guaiacol peroxidation [8]. A standard 18 mm assay tube contained 4 ml 0.1 M KPi buffer, pH 6, 1 ml 0.1 M guaiacol, and 0.2 ml of peroxidase. H_2O_2 (1 ml of 6.3×10^{-4} M) was added to initiate the reaction. The assays were performed at room temp. One unit of enzyme activity is defined as 100 times the reciprocal of the time in sec required to reach an A of 0.2 under the stated conditions. A linear relationship between enzyme concn and reciprocal time was established.

Protein concn was determined by the method of ref. [10] using 1.8 cm matched test-tubes, with BSA as a standard.

Enzyme purification. The mycelia-free growth medium which contained the chloroperoxidase was dialysed against running tap H_2O (pH 7.5) for 3–4 hr. The pH of the dialysate was not higher than 6.5. To each 10 l. of dialysed growth medium, *ca* 2 l. of alumina gel suspension prepared according to the method described in ref. [11] (*ca* 30 mg/ml dry wt) was added. The mixture was stirred for 2 hr at 4°. The gel, after centrifugation, was eluted with 3–5 times of its vol. 50 mM KPi buffer of pH 6. The eluate (3 l.) was applied to a column of 21 × 6 cm DEAE-cellulose. This column was then washed with 60 mM KPi buffer of the same pH until the effluent had an A at 280 nm of less than 0.1. The flow cell had a 4 mm path. The chloroperoxidase was eluted with 90 mM KPi buffer of pH 6. One peroxidase peak was obtained. These fractions were combined and dialysed against 50 mM KOAc buffer of pH 3.85. The dialysate was applied to a DEAE-cellulose column (25 × 3 cm) equilibrated with the same buffer. Elution of the chloroperoxidase was achieved by a linear concentration gradient of pH 3.85 KOAc buffer of 50–300 mM. Two peaks with peroxidase activity were obtained. The major peak was designated chloroperoxidase A and the minor one B. The isozymes were concd by dialysing against saturated $(NH_4)_2SO_4$ soln at 4°. Crystallization was performed in the following way: the chloroperoxidase was precipitated by $(NH_4)_2SO_4$ and was redissolved at 0° with a slight excess of 65% saturated $(NH_4)_2SO_4$ soln. The soln was centrifuged at 20000 *g* at 0° to remove undissolved material. The supernatant liquid was then kept in a closed chamber of 18–20° with the top of the enzyme container uncovered for slow evapn. Crystals appeared after *ca* 1 week and crystallization was completed in *ca* another week. Recrystallization was performed by the same procedure. Crystals were collected by centrifugation. The enzyme can be dialysed against deionized H_2O and the protein can be lyophilized salt-free.

Isoelectric focusing. An aliquot of enzyme soln obtained from pH 6 DEAE-cellulose chromatography was dialysed against a 1% glycine soln. An aliquot of the dialysed enzyme soln containing 1.08 mg of protein was put into a 110 ml LKB electrofocusing column. A sucrose gradient with pH 3–6 Ampholine was used with the anode as the bottom electrode. The starting voltage of 300 was increased to 600 after 1 hr and was maintained for an additional 37 hr at 15–17°. Fractions (1 ml) were collected at the rate of 2 ml/min. *A* at 280 nm, peroxidase activity and pH of each fraction were measured.

Gel electrophoresis. Polyacrylamide gels (7%) were prepared in Pyrex tubing of 65 × 5 mm according to the method of ref. [12] using formate buffers (pH 3.2). To stain for protein, the gels were fixed in 20% sulfosalicylic acid for 20 min and then immersed in 0.25% aq. Coomassie brilliant blue soln for 1 hr. Background destaining was accomplished by rinsing for several hr with deionized H_2O and the gels were covered with 0.1 M guaiacol soln for 5 mins, rinsed once with distilled H_2O, and then immersed in 0.1 N H_2O_2 soln.

The MW of chloroperoxidase was estimated by gel filtration [18] through a Sephadex G100 column. MW standards (5 mg/ml) were BSA (70000), pepsin (35000) and horse heart myoglobin (16900).

Electron microscopy. The twice crystallized chloroperoxidase A was dialysed against deionized H_2O and the dialysate was adjusted to *ca* 1.2 mg protein per ml. The soln was put on a thin carbon membrane, supported by carbon-coated formvar-net on a 200-mesh copper screen, and stained with 1% uranyl formate at pH 3.5. The specimen was air-dried and examined at 75 kV. The specimen chamber was cooled by liquid N_2.

TLC of chlorination product. Chlorination was performed by mixing 12 μmol monochlorodimedon, 1.5 mmol KPi buffer of pH 2.75, 300 mol/KCl, 6 μmol H_2O_2 and 60–70 guaiacol units of peroxidase in a total vol. of 5 ml. The mixture was incubated for 15 min at room temp. and then extracted with 3 successive portions of C_6H_6. The combined extracts were evapd to dryness and the residue dissolved in 0.2 ml C_6H_6. Aliquots of this soln were spotted on Si gel TLC plates with fluorescence indicator and the plates developed in a Skellysolve B–EtOAc (9:1) as solvent. Zones were visualized under UV.

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REFERENCES

1. Petty, M. A. (1961) *Bacteriol. Rev.* **25**, 111.
2. Ashworth, R. A. and Cormier, M. J. (1967) *Science* **155**, 1558.
3. Webb, J. C. S. (1967) *Science* **158**, 522.
4. Shaw, P. D. and Hager, L. P. (1959) *J. Am. Chem. Soc.* **81**, 6527.
5. Morris, D. R. and Hager, L. P. (1966) *J. Biol. Chem.* **241**, 1763.
6. Hager, L. P. (1970) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.) Vol. XVIIA, p. 648. Academic Press, New York.
7. Slayter, E. M. (1969) in *Physical Principles and Techniques of Protein Chemistry, Part A* (Leach, S. J., ed.) p. 1. Academic Press, New York.
8. Maehly, A. C. and Chance, B. (1954) in *Methods of Biochemical Analysis* (Glick, D., ed.) Vol. 1, p. 357. Interscience, New York.
9. Hager, L. P., Morris, D. R., Brown, F. S. and Eberwein, H. (1966) *J. Biol. Chem.* **241**, 1769.
10. Miller, G. L. (1959) *Analyt. Chem.* **31**, 964.
11. Dixon, M. and Webb, E. C. (1964) *Enzymes*, 2nd edn, p. 42. Academic Press, New York.
12. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404.
13. Whitaker, J. R. (1963) *Analyt. Chem.* **35**, 1950.