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ISOLATION AND CHARACTERIZATION OF A NOVEL NONHEME CHLOROPEROXIDASE

Te-Ning Ernie Liu**, Thabiso M'Timkulu, John Geigert*, Beverly Wolf***, Saul L. Neidleman, Deborah Silva***, and Jennie C. Hunter-Cevera

Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608

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Chloroperoxidase, purified from the fermentation of Curvularia inaequalis, had a molecular weight of approximately 240,000 and was composed of 4 subunits of identical molecular weight (M_r 66,000). The enzyme was specific for I^-, Br^- and Cl^-, and inactive toward F^-. The optimum pH of the enzyme was centered around 5.0. X-ray fluorescence revealed that the enzyme contained 2.2 atoms of zinc and 0.7 atom of Fe per molecule of protein. The enzyme had no heme-like compound as a prosthetic group, making it the first nonheme chloroperoxidase to be reported. Under oxidative conditions that rapidly inactivated other haloperoxidases, this enzyme was remarkably stable.

A diversity of peroxidases, which catalyze the peroxidation of many compounds, are widely distributed throughout nature. Among them are the haloperoxidases that introduce halogen-carbon bonds in many organic chemicals according to the following general equation:

$$A\text{-}H + H_2O_2 + X^- + H^+ \longrightarrow A\text{-}X + 2H_2O$$

(A-H is the substrate, X^- is halide ion)

Haloperoxidases are divided into three groups according to their specificity for halide ion (1). Iodoperoxidases catalyze the oxidation of only I^-. Thyroid peroxidase and horseradish peroxidase are representatives of this group. Bromoperoxidases are specific for I^- and Br^-, but they cannot activate Cl^-. Lactoperoxidase and the many haloperoxidases isolated from the red and green algae are representatives of this group. Chloroperoxidases catalyze the oxidation of all halide ions except F^-. Myeloperoxidase and the chloroperoxidase from Caldariomyces fumago are representatives of this group.

* To whom correspondence should be addressed.
** Present Address: Biotech Connections, Martinez, CA 94553.
*** Presently in private practice.

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The above haloperoxidases are heme-containing enzymes. The heme prosthetic group plays an important function in the catalytic activity, with both the iron and the porphyrin being involved in the storing of oxidizing equivalents and the regulating of oxidation-reduction potential (2). However, among the bromoperoxidases, several nonheme enzymes have now been reported: a nonheme lactoperoxidase from bovine milk (3), a nonheme vanadium-containing bromoperoxidase from the marine alga Ascophyllum nodosum (4) and a nonheme iron-containing bromoperoxidase from the seaweed Corallina pilulifera (5).

During the course of our screening for novel enzymes, a haloperoxidase was discovered in a dematiaceous hyphomycete, Curvularia inaequalis. We undertook the isolation and characterization of this enzyme, and found it to be a nonheme, zinc-containing chloroperoxidase showing high stability under oxidative conditions.

MATERIALS AND METHODS

Fungal Organism and Culture Conditions - Curvularia inaequalis CMCC 755 was grown on agar seed plates at 25°C for 7 days. The spore mass was used to inoculate sterile FA-1 germination media in flasks that were then incubated at 25°C with agitation for 3 days. The growth material was used to inoculate sterile FP fermentation media in flasks that were then incubated at 25°C with agitation for 12 days, as previously described (6).

Isolation and Purification - The fungal growth material was disrupted by sonication and then filtered. $(NH_4)_2SO_4$ was added to the filtrate, under stirring, to 40% of saturation. After 1 hr, the precipitate was collected and discarded. $(NH_4)_2SO_4$ was then added to 55% of saturation. After 1 hr, the precipitate was collected by centrifugation and dissolved in a minimal volume of 0.1M phosphate buffer pH 6.0. The solution was applied to a DEAE-Sepharose CL-6B column. Protein was eluted from the column with a linear gradient of 50 mM to 500 mM sodium phosphate buffer pH 6.0. The haloperoxidase eluted at about 400 mM.

Activity Assays - The chlorinating activity of the purified enzyme was determined by monitoring the decrease in A_{292nm} due to the chlorination of monochlorodimedone (MCD) at 25°C. The reaction mixture consisted of 2 ml of potassium phosphate buffer (100 mM, pH 5.0) KCl (1 mM), MCD (0.005 mM) and H_2O_2 (0.1 mM). To initiate the reaction, 0.01 ml of sample was added.

Substrate Reactivity - In addition to the chlorination of the β-diketone monochlorodimedone (MCD), the chloroperoxidase from C. inaequalis also catalyzed the chlorination of the alkene allyl chloride (Fig. 1). The reaction protocol previously described for the chloroperoxidase from Caldariomyces fumago was used (1), but the reaction pH was 6.0 rather than 3.0.

Unlike the chloroperoxidase from C. fumago, the chloroperoxidase from C. inaequalis did not catalyze the peroxidation of primary alcohols to aldehydes nor the epoxidation of alkenes. The reaction protocols previously described for allyl alcohol and styrene were used (7,8).

Kinetic Properties - Using as a spectrophotometric assay the chlorination of monochlorodimedone (MCD), we examined the activity of the chloroperoxidase in 50 mM potassium phosphate buffer at 25°C as a function of H_2O_2

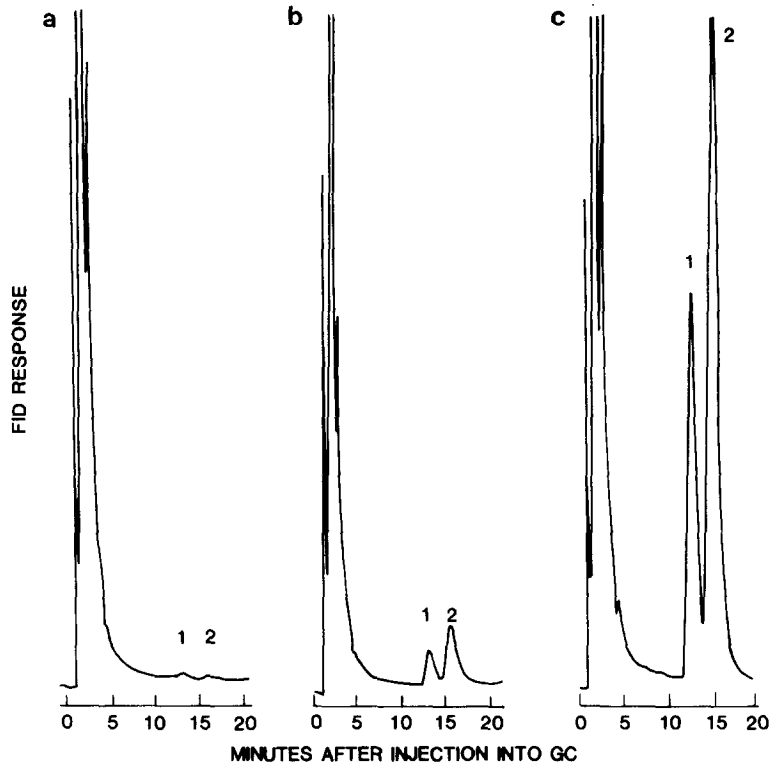

Figure 1. Gas chromatographic analysis of the chloroperoxidase-catalyzed chlorination of allyl chloride. Time after initiation of enzymatic reaction: (a) 15 minutes, (b) 2 hours and (c) 24 hours. Peak identification: (1) 1,3-dichloro-2-propanol and (2) 1,2-dichloro-3-propanol.

concentration, Cl^- concentration, and pH. As has been shown for the other haloperoxidases (9), there is an interrelationship between the H_2O_2 and Cl^- concentrations that affects the optimum pH and K_m values. At the concentrations normally run in the MCD assay (0.1 mM H_2O_2 and 1 mM Cl^-), the chloroperoxidase had a pH optimum of 5.0 and a turnover number of 240 mol of MCD per mol of enzyme per s. For comparison, the chloroperoxidase from Caldariomyces fumago (Sigma Chemical Co., St. Louis, MO) had a pH optimum of 3.0 and a turnover number of 1500 mol of MCD per mol of enzyme per s.

Protein Measurement - Protein concentrations were determined by use of the Bio-Rad protein reagent and bovine serum albumin as standard.

Molecular Weight and Subunit Composition - The molecular weight of the purified chloroperoxidase was determined by gel filtration chromatography with a Sephacryl S-300 column. The column was calibrated with a gel filtration standard containing (molecular weight) catalase (232,000), aldolase (158,000), bovine serum albumin (67,000) and ovalbumin (44,000). The apparent molecular weight of the chloroperoxidase was estimated to be 240,000.

The chloroperoxidase was dissociated into component subunits and their molecular weights were estimated by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). A protein band of molecular weight 66,000 was observed whether or not 2-mercaptoethanol was present. The chloroperoxidase appears to be composed of four subunits of equal size and these are associated entirely by noncovalent forces.

Optical Spectra - The chloroperoxidase exhibits a single absorption band in the ultraviolet range, centered at 280 nm. The molar extinction

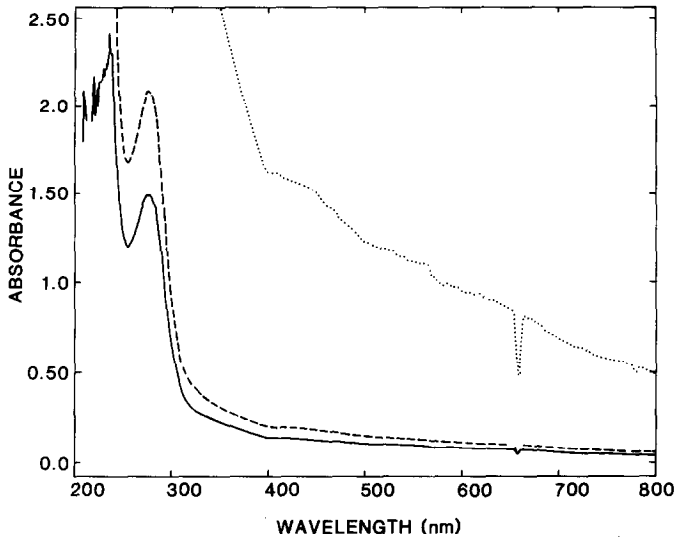

Figure 2. Absorbance spectrum of chloroperoxidase from C. inaequalis. Spectrum was recorded with a Hewlett-Packard 8450 spectrophotometer and printed out at three different amplifications. Enzyme at 0.30 mg/ml in 50 mM potassium phosphate buffer pH 5.0 was examined.

coefficient, calculated on the basis of a molecular weight of 240,000, was 1.2×10^6 $M^{-1}cm^{-1}$ at this wavelength. In the visible region there was no measurable absorbance in the region of 400-420 nm (Fig. 2). Since the characteristic of heme-containing proteins is the Soret band absorbance in this visible region, the chloroperoxidase is devoid of heme.

Metal Content - The pure chloroperoxidase was analyzed by X-ray fluorescence. Zinc was present at 2.2 atom per molecule, whereas Fe was present at 0.7 atom per molecule. As a control, lactoperoxidase (Sigma Chemical Co., St. Louis, MO) was measured at 1.0 atom of Fe per molecule, with no detectable Zn.

Carbohydrate Content - The carbohydrate content of the purified chloroperoxidase was estimated according to the method described by Dubois, et al. (10). This enzyme had a 9% (w/w) carbohydrate content. As a control, glucose oxidase (Sigma Chemical Co.) was measured at 13% (w/w).

RESULTS AND DISCUSSION

The chloroperoxidase from Curvularia inaequalis is the only nonheme chloroperoxidase reported to date. The other known chloroperoxidases are all heme-containing. The catalytic cycle of these heme-containing haloperoxidases have been well-studied (1). The cycle involves alternate divalent oxidation and reduction of the heme, with an Fe (IV) π-cation radical of the heme representing the oxidized state referred to as Compound I. For the chloroperoxidase from C. inaequalis, which has no heme, hence no Compound I, the mechanism for catalysis is unknown.

One of the serious limitations for chloroperoxidase-catalyzed chlorination is the destructive nature of the reactive intermediate, HOCl, on the enzyme itself. Chlorination of heme leads to the inactivation of the

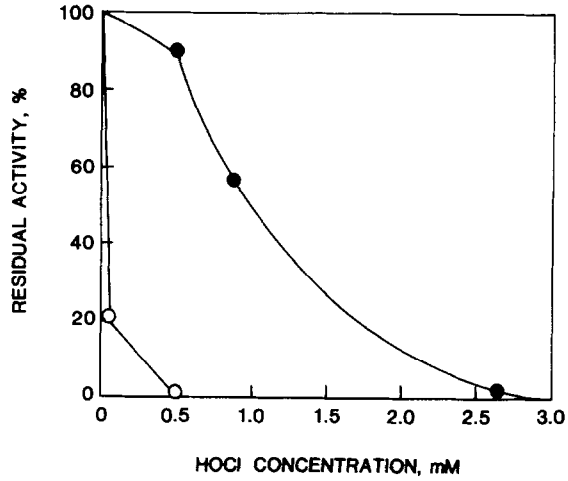

Figure 3. Oxidative stability comparison of heme-containing chloroperoxidase (○, from C. fumago) and heme-minus chloroperoxidase (●, from C. inaequalis) upon exposure to various concentrations of HOCl for 2 minutes.

enzyme. Since the chloroperoxidase from C. inaequalis lacks heme, it should be more stable in the presence of HOCl. It should also be more stable to the oxidative reagent, H_2O_2, which is utilized in the haloperoxidase reaction. This was demonstrated by incubating at room temperature both the heme-containing chloroperoxidase from C. fumago and the heme-minus chloroperoxidase from C. inaequalis in the presence of added HOCl. At a HOCl concentration of 0.5 mM, the heme-containing chloroperoxidase was completely inactivated within 2 minutes, while the heme-minus chloroperoxidase still retained 90% of its initial activity (Fig. 3). In addition, when the chloroperoxidases were incubated at a constant H_2O_2 concentration (200 mM), the heme-containing chloroperoxidase was again completely inactivated within 2 minutes, while the heme-minus chloroperoxidase showed no significant inactivation even after 25 hours of incubation. It has not been reported if the heme-minus bromoperoxidases demonstrate this stability.

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