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Expression of the *Caldariomyces fumago* Chloroperoxidase in *Aspergillus niger* and Characterization of the Recombinant Enzyme*

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The *Caldariomyces fumago* chloroperoxidase was successfully expressed in *Aspergillus niger*. The recombinant enzyme was produced in the culture medium as an active protein and could be purified by a three-step purification procedure. The catalytic behavior of recombinant chloroperoxidase (rCPO) was studied and compared with that of native CPO. The specific chlorination activity (47 units/nmol) of rCPO and its pH optimum (pH 2.75) were very similar to those of native CPO. rCPO catalyzes the oxidation of various substrates in comparable yields and selectivities to native CPO. Indole was oxidized to 2-oxindole with 99% selectivity and thioanisole to the corresponding *R*-sulfoxide (enantiomeric excess >98%). Incorporation of ^{18}O from labeled $H_2{}^{18}O_2$ into the oxidized products was 100% in both cases.

Chloroperoxidase (CPO[1]; EC 1.11.1.10) is a heavily glycosylated monomeric hemoprotein, with a sugar content of 18% of its molecular mass of 42 kDa (1). The chloroperoxidase is secreted by the filamentous fungus *Caldariomyces fumago* and was first purified and described in 1966 by Morris and Hager (2). *In vivo*, CPO catalyzes oxidative chlorination. *In vitro*, in the absence of Cl^-, CPO catalyzes a variety of synthetically useful (enantioselective) oxygen transfer reactions (3–5), *e.g.* asymmetric epoxidation of olefins (6–8); allylic, benzylic, and propargylic hydroxylation (9–11); asymmetric sulfoxidation (12–15); and oxidation of indoles to the corresponding 2-oxindoles (16, 17). In catalyzing these oxygen transfer reactions, CPO behaves more like the P-450 cytochromes than like classical peroxidases such as the peroxidases from horseradish roots, soybeans, and the fungus *Coprinus cinereus*, which mostly catalyze one-electron oxidations, *e.g.* polymerization of phenol and anilinic compounds (18, 19). Moreover, the iron protoporphyrin in CPO is ligated to the active site through a cysteine residue (20–22), as characteristic of P-450 cytochromes, whereas the axial ligand in peroxidases normally is a histidine residue (23). Interestingly, the *C. fumago* CPO shows no sequence similarity to other extracellular heme peroxidases (24–28) or to known microbial vanadium haloperoxidases (29–31) but is most similar to the *Aspergillus nidulans stcC* (32), a member of the sterigmatocystin biosynthetic gene cluster, and also shows significant sequence similarity to a *Agaricus bisporus* cellulolytic gene (accession number AJ293759).

Site-directed mutagenesis has proved to be a powerful tool in exploring structure-function relationships in classical peroxidases (23); in particular, horseradish peroxidase has been studied in great detail by Morishima and co-workers (33–36) and by Smith and co-workers (37–40). Furthermore, Ortiz de Montellano and co-workers (41–44) have used site-specific mutagenesis to engineer horseradish peroxidase with oxygen transfer catalytic properties, suitable for enantioselective sulfoxidation and epoxidation reactions. The use of such an approach for the *C. fumago* CPO could help in revealing the structural basis of the unique properties of this enzyme and to explore further possibilities.

For site-directed mutagenesis studies, an efficient expression system for the *cpo* gene is required. As CPO is a protein with several post-translational modifications, *i.e. N*- and *O*-glycosylation, disulfide bridge formation, cleavage of N-terminal and C-terminal sequences, and prosthetic group incorporation (1), prokaryotic hosts appear not suitable for synthesizing the active protein. Indeed, Zong *et al.* (45), reporting the expression of *cpo* in *Escherichia coli*, showed that the non-glycosylated enzyme was secreted into the periplasm in its apoform and only after a tedious high pressure-assisted reconstitution process could limited amounts of the active holoenzyme be recovered. Therefore, other, eukaryotic, expression systems have been considered. Expression of the *cpo* using the baculovirus system resulted in the production of extracellular inactive CPO, which could not be reconstituted to active protein (46). Similarly, attempts to produce CPO in *Saccharomyces cerevisiae* and *Pichia pastoris* have been unsuccessful (46, 47). Recently, the genetic transformation of *C. fumago* and the expression of mutant forms of CPO in the parental host have been reported (48). However, this system has the inconvenience of the presence of native CPO background, which hampers the screening for recombinant CPO producing strains, and has failed in providing specific CPO mutant proteins.[2]

We have explored the possibility of producing CPO in another filamentous fungal expression host, namely *Aspergillus niger*. Filamentous fungi are capable of secreting large amounts of proteins in the extracellular medium. Since versatile DNA transfer and gene expression systems are available

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[1] The abbreviations used are: CPO/nCPO, chloroperoxidase from *C. fumago*; rCPO, recombinant CPO expressed in *A. niger*; MCD, monochlorodimedone; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; GC, gas chromatography; MS, mass spectroscopy; EndoH, endoglycosidase H.

[2] L. P. Hager, personal communication.

for these organisms, the necessary tools are available for the production of recombinant proteins. Furthermore, *A. niger* has no detectable extracellular peroxidases, and therefore, in contrast to the *C. fumago* system, no interference of endogenous oxidising activities when screening for CPO producing transformants. To date several reports on the expression of fungal metalloproteins in filamentous fungi have been published (49–52). However, although production of active recombinant enzymes was found in most cases, yield levels were still far from those obtained for less complex fungal proteins, making the secretion of metalloproteins an intriguing subject of study. Here, we describe the expression of the *C. fumago cpo* gene in *A. niger*. Fully active recombinant CPO was produced and purified. Its catalytic properties were compared with those of the native CPO from *C. fumago*.

EXPERIMENTAL PROCEDURES

Strains—Escherichia coli DH5α was used for construction and propagation of vector molecules. *A. niger* MGG029 (*prtT*, *gla::fleo*^{r}, *pyrG*; Ref. 52) was used as recipient strain in transformation experiments.

Reagents—Native chloroperoxidase from *Caldariomyces fumago* was obtained from Chirazyme Labs (Urbana, IL) and used without further purification. The enzyme solution contained 11.4 mg/ml CPO with R_z 1.23 (R_z = purity standard = A_{400}/A_{280} = 1.44 for pure enzyme) and an activity of 22.8 kilounits/ml (standard monochlorodimedone (MCD) assay as described by Morris and Hager (Ref. 2)). *o*-Anisidine was purchased from Fluka and hemin from Sigma. Gel filtration low molecular weight calibration kit was purchased from Amersham Pharmacia Biotech. Indole, 5-bromoindole, 5-chloroindole, 5-methoxyindole, thioanisole, ethyl phenyl sulfide, and methyl *p*-methoxyphenyl sulfide, were purchased from Aldrich. The corresponding sulfoxides were prepared by chemical oxidation according to Drabowicz *et al.* (53). ^{18}O-Labeled hydrogen peroxide ($H_2{}^{18}O_2$; 90% ^{18}O) was obtained from Campro Scientific.

Analysis and Equipment—UV measurements were performed on a Cary 3 spectrophotometer from Varian. A Megafuge 2.0R from Heraeus Instruments was used for centrifugation. A Metrohm Dosimat 665 was used for continuous addition of H_2O_2.

Enzyme purification was performed with a Waters Delta Prep 4000 HPLC system equipped with an Amersham Pharmacia Biotech fast flow column (d = 5 cm; 750-ml DEAE-Sepharose) and a Waters fraction collector.

Gel filtration chromatography was done using a Superose 12 HPLC column (Amersham Pharmacia Biotech, 10 × 300 mm) with a Waters 590 programmable HPLC pump with detection on a Waters 486 tunable absorbance detector at 280 or 400 nm with Waters Millennium^{32} software. Fractions were collected using a Waters fraction collector.

Samples for analyzing the enantioselective oxidation of sulfides were quenched with sodium sulfite, diluted with a hexane/isopropyl alcohol mixture of 75:25 (v:v), and dried over Na_2SO_4. After centrifugation, the samples were analyzed on chiral HPLC using a Chiralcel OD column (Daicel Chemical Industries, Ltd., 250 × 4.6 mm), eluent flow 0.6 ml min^{-1}, and detected on a Waters 486 tunable absorbance detector at 220 nm with Waters Millennium^{32} software. A hexane/isopropyl alcohol mixture of 75:25 (v:v) was used as eluent. 1,2,3-Trimethoxybenzene was used as internal standard.

Samples for analyzing indole oxidation were quenched with a saturated sodium sulfite solution and diluted with methanol. After centrifugation, the samples were analyzed with reversed phase HPLC using a custom-packed Symmetry C_{18} cartridge (Waters Radial-Pak, 8 × 100 mm, 7 μm) contained in a Waters RCM 8 × 10 compression unit, with simultaneous detection on a Waters 410 differential refractometer and a Waters 486 tunable absorbance detector at 254 nm with Waters Millennium^{32} software. *tert*-Butyl alcohol was used as internal standard. A methanol/water mixture of 70:30 (v/v) at 1.0 ml min^{-1} was used as eluent for all indole derivatives.

GS-MS analysis was performed on a CP SIL5CB MS column (25 m × 0.25 mm) and a VG 70-SE mass spectrometer.

Chloroperoxidase activity was determined by the standard chlorination method as described by Morris and Hager (2), defining 1 unit of chloroperoxidase activity as the amount enzyme that catalyzes the formation of 1 μmol of dichlorodimedone in 1 min. Total protein content was determined by the method of Bradford using bovine serum albumin as a standard. To obtain the ferrous-CO complex of CPO (48), the Fe(III) was first reduced to Fe(II) with dithionite and then incubated for 2 min with CO. The reactivity of the recombinant enzyme to the Ellmann reagent for free SH-groups was carried out as described (54).

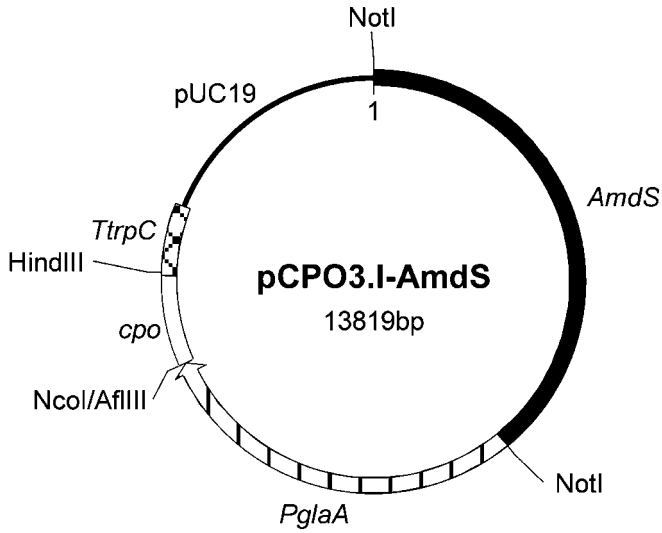


FIG. 1. **The *cpo* expression vector pCPO3.I-AmdS.**

Construction of cpo Expression Vector—pCf6, a plasmid containing *cpo* genomic clone, was a gift from Dr. Hager (University of Illinois, Urbana-Champaign, IL). Primers CLP15E/A (5′-GGAATTCACATGTT-CTCCAAGGTCC-3′) and CLP1CTERM3 (5′-CGCGCGGATCCAAGCT-TAAAGGTTGCGGG-3′) were used to amplify the DNA sequence encoding the full-length CPO precursor (GenBank™ accession no. AJ300448) from pCf6 and introduce suitable cloning sites. The resulting PCR product was *Eco*RI/*Bam*HI-digested and cloned into pUC19 to render pCPO3. The amplified *cpo* fragment was checked by sequence analysis, excised from pCPO3 as an *Afl*III/*Hin*dIII fragment and cloned into the pAN52–10Not *Aspergillus* expression vector (55) at the *Nco*I/*Hin*dIII cloning sites, which resulted in pCPO3.I. In vector pCPO3.I, the CPO coding sequence is placed under control of the *A. niger* glucoamylase promoter and *A. nidulans trpC* terminator. Finally, the *A. nidulans AmdS* selection marker (56) was introduced in pCPO3.I at a unique *Not*I site to obtain the *cpo* expression vector pCPO3.I-AmdS (Fig. 1).

Transformation Procedures—Fungal co-transformation was carried out as described (57), using pCPO3.I-AmdS and pAB4–1 (58) plasmids. Transformants were selected on fructose minimal medium plates without uridine and containing acetamide as sole nitrogen source. Transformants were selected for multicopy integration of the expression cassettes on acrylamide plates (59) and for extracellular peroxidase activity on *o*-anisidine plates as described (52) using 0.05% H_2O_2 in 0.1 M sodium phosphate buffer, pH 2.7, as developing buffer.

Molecular and Protein Methods—Molecular methods were carried out essentially as described (60). Total fungal RNA was isolated using the RNAzol™ kit from Cinna/Biotecx. For Northern analysis experiments, a 1-kilobase *Sty*I fragment from pCF6 containing most of the *cpo* coding region was used as a probe. SDS-PAGE was performed with a Bio-Rad MiniprotII system using the Tris-glycine method and 10% polyacrylamide gels. N-terminal determination of recombinant CPO (rCPO) was performed by Edman degradation after SDS-PAGE of the purified protein and blotting onto a polyvinylidene difluoride membrane. For deglycosylation experiments, proteins were treated with endoglycosidase H (EndoH; New England Biolabs) following manufacturer's instructions.

Polyclonal Antisera—For preparation of polyclonal antibodies, CPO from *C. fumago* IMI 089362 was purified according to van Deurzen *et al.* (61). A 3-mg aliquot of the purified CPO was treated with acetone plus 0.3% HCl to remove the heme group (62), and both holo- and apochloroperoxidase were used for rabbit immunization. Immunizations were performed in duplicate using 100 μg of protein in Freund's complete adjuvant-H_2O_2 (1:1). Boosters were administered after 2 and 16 weeks after immunization using 100 μg of protein in Freund's incomplete adjuvant. Rabbits were bled 1 week after the last booster, and optimal sera dilution was determined by enzyme-linked immunosorbent assay.

Production and Purification of rCPO—Fungal culturing was carried out in 2-liter Erlenmeyer flasks containing 500 ml of *Aspergillus* minimal growth medium (63) with 5% maltodextrin and supplemented with 0.5% casein amino acids and 500 mg/liter hemin. Cultures were inocu-

lated with 5×10^8 conidia and grown for 48 h at 30 °C or 22 °C in a rotary shaker revolving at 300 rpm. Medium samples were obtained by filtering the fungal cultures through a Miracloth.

To the filtered medium (1300 ml) cold acetone (1000 ml; 45% v/v; −20 °C) was slowly added at 4 °C, and after 1 h of incubation at −20 °C, precipitated impurities were removed by centrifugation (4400 min^{-1}; 20 min; 0 °C). Cold acetone was then slowly added to the supernatant (1000 ml; final concentration 60% v/v; −20 °C), and CPO precipitation occurred overnight at −20 °C. The supernatant was removed by decanting, and the precipitated protein was dried for 10 min. The protein pellet was dissolved in phosphate buffer (300 ml; 10 mM; pH 5.2), adjusted to pH 5.8 with 10 mM H_3PO_4, and brought onto a DEAE-Sepharose (Amersham Pharmacia Biotech, 750 ml) fast flow column in phosphate buffer (20 mM; pH 5.8; flow 10 ml min^{-1}). The column was washed with phosphate buffer (20 mM; pH 5.8; 10 ml min^{-1}) for 1 h. The enzyme was eluted with a 20–200 mM phosphate buffer gradient (pH 5.8; 10 ml min^{-1}) during 4 h. Fractions having peroxidase activity (MCD assay) above 0.25 units/ml were pooled, adjusted to pH 5.2, and concentrated over a 30-kDa membrane (Centriprep-30 concentrator, Amicon) at a speed of 1800 rpm. Further purification was done by gel filtration on a Superose 12 HPLC column (Amersham Pharmacia Biotech, 10 × 300 mm; phosphate buffer, pH 5.2; 200 mM; 0.5 ml min^{-1}).

Oxidation of Sulfides—For sulfide oxidation reactions, 50 μmol of sulfide was dissolved at room temperature in 1.0 ml solvent (0.2 M phosphate buffer, pH 5.2). 24 units of chloroperoxidase were added to the reaction mixture and stirred for 5 min. The reaction was started by the continuous addition of H_2O_2 (0.15 M) at a rate of 1eq/2 h to a total of 1.1 eq. H_2O_2. The reaction was quenched after 2.5 h by the addition of an excess of Na_2SO_3. The reaction mixture was homogenized by the addition of isopropyl alcohol (400 μl) and analyzed by chiral HPLC.

Oxidation of thioanisole with $H_2{}^{18}O_2$ was performed at 0.5-ml scale. Oxidation was started with the stepwise addition of $H_2{}^{18}O_2$ (1.0%; 5 μl/min to a total of 95 μl). 5 min after the last addition, the reaction mixture was extracted with dichloromethane and the reaction products were analyzed with GC-MS.

Oxidation of Substituted Indoles—Oxidation of substituted indoles were performed at room temperature in 1.0-ml aliquots containing 10 μmol of indole derivative dissolved in *tert*-butyl alcohol, 0.2 M phosphate buffer, pH 5.2 (50:50, v/v). 8 units of chloroperoxidase were added to the reaction mixture, stirred for 5 min, and the reaction was started by the continuous addition of H_2O_2 (0.15 M) at a rate of 1 eq/h, to a total of 1.1 eq of H_2O_2. The reactions were monitored by removing aliquots and analyzing by HPLC.

The oxidation of indole with $H_2{}^{18}O_2$ was performed at 0.5-ml scale. Oxidation was started with the stepwise addition of $H_2{}^{18}O_2$ (0.4%; 5 μl/min to a total of 55 μl). 5 minutes after the last addition, the reaction mixture was extracted with dichloromethane and the reaction products were analyzed with GC-MS.

RESULTS

Isolation of A. niger Transformants Producing rCPO—In a co-transformation experiment, *A. niger* strain MGG029 was transformed with a mixture of plasmids pCPO3.I-AmdS and pAB4–1. Several uridine prototrophic, acetamide utilizing transformants were obtained and were transferred to both acrylamide and *o*-anisidine containing plates. Efficient growth and esporulation on acrylamide plates reflects multicopy integration of the transforming vector (59), and colored halo formation on *o*-anisidine plates indicates extracellular peroxidase activity (52). Four transformants growing vigorously on acrylamide and developing an intense halo with the *o*-anisidine test were selected. These four strains were cultured on maltose minimal medium for 48 h and analyzed for *cpo* mRNA synthesis by Northern blotting and extracellular CPO production by Western analysis (data not shown). From this analysis, the best producing transformant, strain [MGG029]pCPO3.I#5, was selected for production and purification of rCPO.

Production and Purification of rCPO—Extracellular production of rCPO could be readily detected in shake-flask cultures of strain [MGG029]pCPO3.I#5 without the need of extra heme supplementation. However, rCPO production levels could be increased by 10-fold upon hemin addition to the culture medium at a concentration of 500 mg/liter. An additional 5-fold increase was achieved by switching the culturing temperature from 30 °C to 22 °C. Under these conditions up to 10 mg/liter rCPO could be produced from strain [MGG029]pCPO3.I#5.

TABLE I
Purification of rCPO

	Volume ml	Activity units/ml	Enzyme units	Yield %	R_z A_{400}/A_{280}
Crude	1300	1.6	2080	100	ND
Acetone precipitation	300	ND			<0.05
DEAE-Sepharose	9	74	666	32	0.31
GPC	6	39	234	11	0.54

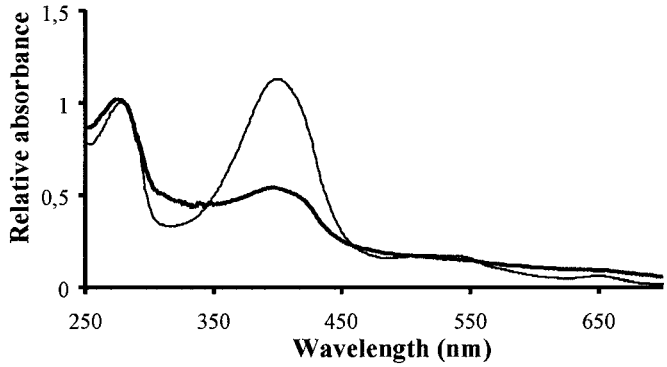


FIG. 2. **UV spectra of rCPO (*thick line*) and nCPO (*thin line*).**

rCPO was purified to electrophoretic homogeneity by acetone precipitation and column chromatography as reported for CPO from *C. fumago* (47, 61). The figures corresponding to the purification of rCPO are given in Table I.

Molecular Characterization of rCPO—Fig. 2 shows the UV spectra of purified rCPO and native CPO (nCPO, commercial preparation with R_z 1.23). As it can be seen, the ratio between A_{400} (indicating heme-containing protein) and A_{280} (indicating total protein), or R_z value, is lower for rCPO (0.54) in comparison to nCPO. Homogeneous CPO from *C. fumago* has a R_z of 1.44. This suggests that rCPO is only partly (~40%) occupied with heme. Similarly to nCPO, the absorption spectrum of the ferrous-CO complex of rCPO showed a Soret peak at 450 nm (data not shown), indicating the correct formation of the heme thiolate ligand with Cys^{29} (64).

To further characterize rCPO, we compared the behavior on SDS-PAGE of the native and recombinant proteins. Two major protein bands could be detected in the nCPO preparate, possibly corresponding to isozymes A and B. These two CPO forms have the same amino acid composition and specific activity, but they differ in the carbohydrate composition (1). rCPO migrated as a single band at a position that is 5–10 kDa, respectively, higher than the native isozymes. As we suspected that this difference in size was due to a overglycosylation of the recombinant enzyme, we treated both rCPO and nCPO with EndoH to remove *N*-linked glycans. As previously reported (1), EndoH digestion of nCPO produced two species of reduced molecular weight. Both deglycosylated rCPO and nCPO shifted to a similar position on SDS-PAGE (Fig. 3), indicating that the differences in size could indeed be attributed to overglycosylation of the recombinant enzyme. Furthermore, similarly to nCPO, the recombinant enzyme was not reactive to the Ellmann reagent (54), indicating a correct formation of the single disulfide bridge present in chloroperoxidase (65).

To analyze whether the CPO signal sequence was correctly processed in *A. niger*, the purified extracellular rCPO was submitted to sequencing of its N terminus. However, no amino acid sequence could be recovered from this analysis, suggesting that the recombinant enzyme was blocked at its N terminus.

Catalytic Properties—To analyze whether the recombinant

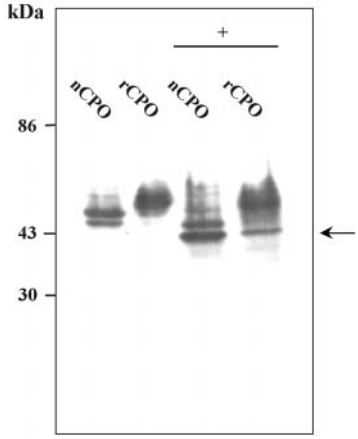
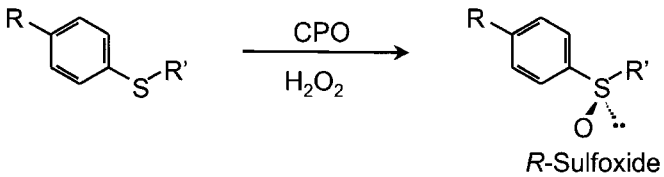


FIG. 3. **Western blotting analysis of rCPO and nCPO.** Proteins were detected with a CPO polyclonal antiserum (see "Experimental Procedures"). Proteins were partially deglycosylated by treatment with EndoH (+). The deglycosylation protein bands are indicated by an *arrow*.



SCHEME 1. **Oxidation of sulfides.** R = H or $-OCH_3$; R' = $-CH_3$ or $-CH_2$-CH_3.

CPO was fully active some of its catalytic properties were measured. The specific chlorination activity (MCD assay as described by Morris and Hager (Ref. 2)) was determined. The specific chlorination activity of purified rCPO was 47 units/nmol of heme. The pH optimum for the chlorination of monochlorodimedone was measured for rCPO and native CPO. rCPO and nCPO showed the same pH profile with a pH optimum at pH 2.75.

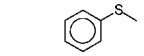
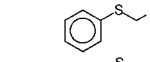
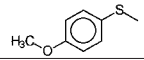
The enantioselective sulfoxidation of thioanisole and derivatives (see Scheme 1) was used to monitor the enantioselective properties of the enzyme. Although, as shown in Table II, results obtained in 1-ml scale experiments differed slightly from the results published for 50-ml scale experiments, similar to the native CPO, recombinant CPO produced predominantly the *R*-sulfoxide in up to 99% enantiomeric excess. Experiments with labeled $H_2{}^{18}O_2$ showed 100% incorporation of ^{18}O into thioanisole sulfoxide for both nCPO and rCPO (data not shown).

The regioselectivity of rCPO was studied by means of the oxidation of indole and derivatives (see Scheme 2). As shown in Table III, the conversions obtained with rCPO was slightly lower than those obtained with native CPO. However, both rCPO and native CPO yield the corresponding 2-oxindoles in virtually quantitative yield. Experiments with labeled $H_2{}^{18}O_2$ showed 100% incorporation of ^{18}O into 2-oxindole for both nCPO and rCPO (data not shown).

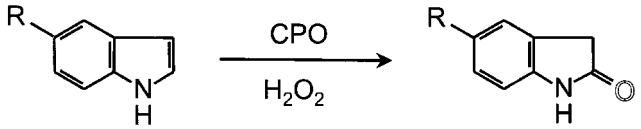
DISCUSSION

Chloroperoxidase from the filamentous fungus *Caldariomyces fumago* is an enzyme of unique versatility as a catalyst for synthetically useful oxygen transfer reactions. Structurally, the enzyme shares characteristics of the P450 cytochromes and the heme peroxidases. These features make CPO a very attractive example for function-structure relationship studies of oxidative enzymes. To make this possible, an efficient recombinant expression system for the *cpo* gene is required. Recently, we reported the expression of two fungal heme-containing peroxidases in the filamentous fungus *A. niger* (52). Production of the recombinant proteins was achieved by placing the peroxidase coding sequences under control of efficient *Aspergillus* expression signals. Using a similar approach, the *C. fumago cpo* gene has been efficiently expressed in *A. niger* and the recombinant enzyme was secreted into the culture medium as an active protein.

TABLE II
Oxidation of sulfides by native and recombinant CPO

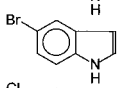
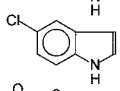
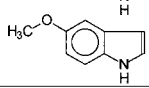
Sulfide	rCPO Conversion (%)	rCPO e.e. (%)	Native CPO[a] Conversion (%)	Native CPO[a] e.e. (%)	Native CPO[b] Conversion (%)	Native CPO[b] e.e. (%)
	77	98	65	98	100	99
	91	99	94	98	83	99
	58	94	62	97	53	99

[a] Results obtained in a 1-ml scale experiment with native CPO from Chirazyme Labs.
[b] Results reported by van Deurzen *et al.* (15) for a 50-ml scale experiment.



SCHEME 2. **Oxidation of substituted indoles.** R = $-$Br, $-$Cl, or $-OCH_3$.

TABLE III
Oxidation of substituted indoles by native and recombinant CPO

Indole derivative	rCPO Conversion (15 min) (%)	rCPO Conversion (60 min) (%)	Native CPO[a] Conversion (15 min) (%)	Native CPO[a] Conversion (60 min) (%)	Native CPO[b] Conversion (15 min) (%)	Native CPO[b] Conversion (60 min) (%)
	24	78	24	83	25	96
	n.d.	24	n.d.	37	9	19
	14	35	16	45	19	47
	5	5	5	5	9	10

[a] Results obtained in a 1-ml scale experiment with native CPO from Chirazyme Labs.
[b] Results reported by van Deurzen *et al.* (17) for a 50-ml scale experiment.

The production of rCPO could be increased by heme addition to the culture medium. Similar results have been obtained in previous studies by our and other groups on the expression of fungal peroxidases in *Aspergillus* species (50, 52, 66). However, our results show that despite heme supplementation, rCPO was only partially (40%) incorporated with heme. This is in contrast to our observations on the production of *Phanerochaete chrysosporium* manganese peroxidase in *A. niger* (52), where the recombinant enzyme could be produced with the same heme content as the native protein. A possible reason for this different behavior may be the different nature of heme attachment in the manganese peroxidase (axial ligand histidine) and CPO (axial ligand cysteine) protein.

EndoH treatment and SDS-PAGE analysis revealed a higher molecular weight of rCPO in comparison to nCPO as a result of overglycosylation of the recombinant enzyme. Overglycosylation has been reported for the expression of other heterologous proteins in *Aspergillus* spp. (52, 67). In these reports, it was shown

that the excess of glycosyl groups did not have a major effect on the properties of the recombinant enzymes. Our results on the characterization of the recombinant chloroperoxidase indicate that this is also the case for rCPO. This is in agreement with the observation that CPO isozymes, differing in glycosylation pattern, maintain the same specific activity (1). Furthermore, Zong *et al*. (45) in their studies on the expression of chloroperoxidase in *E. coli* showed that glycosylation is not an essential requirement for the activity of this enzyme.

The N terminus of rCPO appeared to be blocked. This was not completely surprising, since native CPO is known to posses a N-terminal glutamic acid residue, which is mostly cyclized into a pyrrolidone carboxylic acid (1). Such molecules, whose formation is induced in acidic environments, are unreactive to the Edman's reagent. As the culture medium of *A. niger* reaches a pH = 2, this may explain the N-terminal blockage of rCPO in case the *A. niger*-produced protein would have the native N terminus.

To further validate the *A. niger* production system for CPO, we have assessed whether the structural and catalytic properties of rCPO were comparable with these of native CPO. Experimental data showed the correct formation of the heme thiolate ligand as well as single disulfide bond in the recombinant CPO. The specific chlorination activity of rCPO (47 units/nmol of heme) was in agreement with the activities reported by Morris and Hager (Ref. 2; 70 units/nmol), Van Deurzen *et al*. (Ref. 15; 53 units/nmol), and Libby *et al*. (Ref. 68; 59 units/nmol). Additionally, the pH optimum for this chlorination reaction (pH 2.75 for rCPO) coincided with that of the native CPO and with the pH of the MCD assay as described by Morris and Hager (2). From these results we conclude that the natural chlorination activity of CPO is completely present in the recombinant enzyme.

Similarly, the oxygen transfer properties of CPO were not changed upon expression of the enzyme in *Aspergillus*. Recombinant CPO showed an enantioselectivity of 99% for the sulfoxidation of thioanisole derivatives (the *R*-sulfoxide being predominantly formed) and a regioselectivity of 99% for the oxidation of indole derivatives to the corresponding 2-oxindoles. In aqueous buffer solutions (sulfoxidation reaction, Table II), the yields obtained with rCPO were comparable with those obtained with native CPO. However, when a mixture of *tert*-butyl alcohol and aqueous buffer (50:50 (v/v)) was used (oxidation of indoles, Table III), rCPO resulted in a slightly lower yield than native CPO. Although the reasons for this result are not clear, it is possible that different glycosylation of rCPO has an influence on the stability of the enzyme in mixtures of *tert*-butyl alcohol and aqueous buffer.

Regio- and enantioselective oxidation reactions catalyzed by CPO are known to be oxygen transfer reactions in which the oxygen atom from CPO compound I is directly transferred to the substrate molecule. For rCPO we found 100% incorporation of ^{18}O from labeled $H_2{}^{18}O_2$ into thioanisole sulfoxide and 2-oxindole. This is in agreement with the results of labeling studies with native CPO, as reported for sulfoxidations (69) and oxidation of indole (17). Hence, we conclude that both the chlorination activity and the oxygen transfer properties of CPO are fully retained in the recombinant enzyme.

To our knowledge, this is the first report of the production of fully active chloroperoxidase in a heterologous expression system. We have shown that the catalytic properties of the enzyme remained basically unchanged, which makes of the *A. niger* expression system a suitable system for mechanistic and mutagenesis studies of this unique enzyme.

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Expression of the *Caldariomyces fumago*Chloroperoxidase in *Aspergillus niger* and Characterization of the Recombinant Enzyme

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