Expression of the *Caldariomyces fumago* chloroperoxidase in *Aspergillus niger* and characterisation of the recombinant enzyme

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The abbreviations used are: CPO/nCPO, chloroperoxidase from *Caldariomyces fumago*; rCPO, recombinant CPO expressed in *Aspergillus niger*. MCD, monochlorodimedon; HRP, horseradish peroxidase. AMM: *Aspergillus* minimal growth medium.
Summary:

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 behaviour of recombinant chloroperoxidase (rCPO) was studied and compared with that of native CPO. The specific chlorination activity (47 U/nmol) of rCPO and its pH-optimum (pH 2.75) were very similar to those of native CPO. rCPO catalyses the oxidation of various substrates in comparable yields and selectivities to native CPO. Indole was oxidised to 2-oxindole with 99% selectivity and thioanisole to the corresponding R-sulfoxide (e.e. > 98%). Incorporation of $^{18}\text{O}$ from labelled $\text{H}_2^{18}\text{O}_2$ into the oxidised products was 100% in both cases.

INTRODUCTION

Chloroperoxidase (CPO; E.C. 1.11.1.10) is a heavily glycosylated monomeric hemoprotein, with a sugar content of 18% of its molecular weight 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 catalyses oxidative chlorination. In *vivo*, in the absence of Cl$^-$, CPO catalyses a variety of synthetically useful (enantioselective) oxygen transfer reactions (3, 4, 5) e.g. asymmetric epoxidation of olefins (6, 7, 8), allylic, benzylic, and propargylic hydroxylation (9, 10, 11), asymmetric sulfoxidation (12, 13, 14, 15) and oxidation of indoles to the corresponding 2-oxindoles (16, 17). In catalysing these oxygen transfer reactions CPO behaves more like the P-450 cytochromes than like classical peroxidases such as the peroxidases from horseradish roots, soy beans and the fungus *Coprinus cinereus*, which mostly catalyse one electron oxidations, e.g. polymerisation of phenol and anilinic compounds (18, 19). Moreover, the iron protoporphyrin in CPO is ligated to the active site through a cysteine residue (20, 21, 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, 25, 26, 27, 28) or to known microbial vanadium haloperoxidases (29, 30, 31) but is most similar to the *Aspergillus nidulans* stcC (32) a member of the sterigmatocystin biosynthetic gene cluster and shows also 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); especially horseradish peroxidase (HRP) has been studied in great detail by Morishima and co-
workers (33, 34, 35, 36) and by Smith and co-workers (37, 38, 39, 40). Furthermore, Ortiz de Montellano and co-
workers have used site-specific mutagenesis to engineer HRP with oxygen transfer catalytic properties, suitable for
enantioselective sulfoxidation and epoxidation reactions (41, 42, 43, 44). 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, disulphide-bridge formation, cleavage of
N-terminal and C-terminal sequences and prosthetic group incorporation (1), prokaryotic hosts appear not suitable for
synthesising the active protein. Indeed, Zong et al. (45), reporting the expression of cpo in E. 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 limited amounts of the active holoenzyme could 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 Caldariomyces 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 (Hager,
personal communication).

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 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
opposite 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, 50, 51, 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
Aspergillus niger. Fully active recombinant CPO was produced and purified. Its catalytic properties were compared with
those of the native CPO from *Caldariomyces fumago*.
EXPERIMENTAL PROCEDURES

Strains
Escherichia coli DH5α was used for construction and propagation of vector molecules. A. niger MGG029 (prtT, gla::floR, pyrG, (52)) was used as recipient strain in transformation experiments.

Reagentia
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 R₂ 1.23 (R₂ = purity standard = A₄₀₀/A₂₈₀ = 1.44 for pure enzyme) and an activity of 22.8 kU/ml (standard monochlorodimedon (MCD) assay as described by Morris and Hager (2)). O-anisidine was purchased from Fluka and hemin from Sigma. Gel filtration low molecular weight calibration kit was purchased from Pharmacia. Indole, 5-bromoindole, 5-chloroindole, 5-methoxyindole, thioanisole, ethyl phenyl sulphide, and methyl p-methoxyphenyl sulphide, were purchased from Aldrich Chemical Company. The corresponding sulfoxides were prepared by chemical oxidation according to Drabowicz et al. (53). ¹⁸O labelled hydrogen peroxide (H₂¹⁸O₂; 90% ¹⁸O) 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 H₂O₂. Enzyme purification was performed with a Waters Delta Prep 4000 HPLC system equipped with a Pharmacia 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 (Pharmacia, 10 x 300 mm) with a Waters 590 programmable HPLC pump with detection on a Waters 486 tunable absorbance detector at 280 nm or 400 nm with Waters Millennium32 software. Fractions were collected using a Waters fraction collector. Samples for analysing the enantioselective oxidation of sulphides were quenched with sodium sulphite, diluted with a hexane/isopropyl alcohol mixture of 75:25 (v:v) and dried over Na₂SO₄. After centrifugation, the samples were
analysed on chiral HPLC using a Chiralcel OD column (Daicel Chemical Industries, Ltd., 250 x 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 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 analysing indole oxidation were quenched with a saturated sodium sulphite solution and diluted with methanol. After centrifugation, the samples were analysed with reversed phase HPLC using a custom-packed Symmetry C₁₈ cartridge (Waters Radial-Pak, 8 x 100 mm, 7 μm) contained in a Waters RCM 8x10 compression unit, with simultaneous detection on a Waters 410 differential refractometer and a Waters 486 tunable absorbance detector at 254 nm with Waters Millennium 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 (25m x 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 one unit of chloroperoxidase activity as the amount enzyme that catalyses the formation of 1 μmole of dichlorodimedon in one 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 two minutes with CO. The reactivity of the recombinant enzyme to the Ellmann reagent for free SH-groups was carried out as described (54).

**Construction of cpo expression vector**

pCf6, a plasmid containing cpo genomic clone, was a gift from Dr. Hager (University of Illinois at Urbana-Champaign). Primers CLP15E/A (5’-GGAATTCACATGTTCTCCAAGGTCC-3’) and CLP1CTERM3 (5’-CGCGCGGATCCAAGCTTAAAGGTTGGGCGG-3’) were used to amplify the DNA sequence encoding the full-length CPO precursor (GeneBank accession number AJ300448) from pCf6 and introduce suitable cloning sites. The resulting PCR product was *EcoRI/BamHI* digested and cloned into pUC19 to render pCPO3. The amplified cpo fragment was checked by sequence analysis, excised from pCPO3 as an *AflIII/HindIII* fragment and cloned into the pAN52-10Not *Aspergillus* expression vector (55) at the *NcoI/HindIII* 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.
nidulands trpC terminator. Finally, the A. nidulands AmdS selection marker (56) was introduced in pCPO3.I at a unique NotI site to obtain the cpo expression vector pCPO3.I-AmdS (Fig. 1).
Transformation procedures
Fungal co-transformation was carried out as described (57), using pCPO3.1-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\textsubscript{2}O\textsubscript{2} in 0.1 M Na-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 RNAzo\textsuperscript{TM} kit from CINNA/BIOTECX. For Northern analysis experiments a 1 kb. Styl fragment from pCF6 containing most of the cpo coding region was used as a probe. SDS-PAGE was performed with a BioRad MiniprotII system using the Tris-glycine method and 10% polyacrylamide gels. N-terminus determination of rCPO was performed by Edman degradation after SDS-PAGE of the purified protein and blotting onto a PVDF membrane. For deglycosylation experiments, proteins were treated with EndoH endoglycosidase (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-0.3% HCl to remove the heme group (62) and both holo and apochloroperoxidase were used for rabbit immunisation. Immunisations were done in duplo using 100 µg protein in Freund’s Complete Adjuvant-H\textsubscript{2}O\textsubscript{2} (1:1). Boosters were done after two and sixteen weeks after immunisation using 100 µg protein in Freund’s Incomplete Adjuvant. Rabbits were bled one week after the last booster and optimal sera dilution was determined by ELISA.

Production and purification of rCPO
Fungal culturing was carried out in two-liter Erlenmeyer flasks containing 500 ml Aspergillus minimal growth medium (AMM, 63) with 5% maltodextrin and supplemented with 0.5% casein amino acids and 500 mg/L hemin.
Cultures were inoculated with 5·10^8 conidia and grown for 48 h at 30ºC or 22ºC 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; -20ºC) was slowly added at 4ºC, and after one hour incubation at -20ºC, precipitated impurities were removed by centrifugation (4400 min^-1; 20 min; 0ºC). Cold acetone was then slowly added to the supernatant (1000 ml; final concentration 60 % v:v; -20ºC) 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 (Pharmacia, 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 hour. The enzyme was eluted with a 20-200 mM phosphate buffer gradient (pH 5.8; 10 ml min^-1) during 4 hours. Fractions having peroxidase activity (MCD assay) above 0.25 U/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 (Pharmacia, 10 x 300 mm; phosphate buffer pH 5.2; 200 mM; 0.5 ml min^-1).

**Oxidation of sulphides**

For sulphide oxidation reactions, 50 µmol sulphide was dissolved at room temperature in 1.0 ml solvent (0.2 M phosphate buffer pH 5.2). 24 U 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./2h 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 homogenised by the addition of isopropyl alcohol (400 µl) and analysed by chiral HPLC.

Oxidation of thioanisole with H_2¹⁸O_2 was performed at 0.5 ml scale. Oxidation was started with the stepwise addition of H_2¹⁸O_2 (1.0 %; 5 µl each minute to a total of 95 µl). 5 minutes after the last addition the reaction mixture was extracted with dichloromethane and the reaction products were analysed with GC-MS.


Oxidation of substituted indoles

Oxidation of substituted indoles were performed at room temperature in 1.0 ml aliquots containing 10 µmol indole derivative dissolved in tert-butyl alcohol/0.2 M phosphate buffer pH 5.2 (50:50, v/v). 8 U chloroperoxidase were added to the reaction mixture, stirred for 5 min., and the reaction was started by the continuous addition of H₂O₂ (0.15 M) at a rate of 1 eq./h, to a total of 1.1 eq. of H₂O₂. The reactions were monitored by removing aliquots and analysing by HPLC.

The oxidation of indole with H₂¹⁸O₂ was performed at 0.5 ml scale. Oxidation was started with the stepwise addition of H₂¹⁸O₂ (0.4 %; 5 µl each minute to a total of 55 µl). 5 Minutes after the last addition the reaction mixture was extracted with dichloromethane and the reaction products were analysed with GC-MS.

RESULTS

Isolation of Aspergillus 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 utilising transformants were obtained and were transferred to both acrylamide and o-anisidine containing plates. Efficient growth and sporulation on acrylamide plates reflects multicopy integration of the transforming vector (59), and coloured 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 hours and analysed 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#5, was selected for production and purification recombinant CPO (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 a 10 fold upon hemin addition to the culture medium at a concentration of 500 mg/L. 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/L rCPO could be
produced from strain [MGG029]pCPO3#5.

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 1.

**Molecular characterisation of rCPO**

Figure 2 shows the UV-spectra of purified rCPO and native CPO (nCPO, commercial preparation with Rz 1.23). As it can be seen, the ratio between $A_{400}$ (indicating heme-containing protein) and $A_{280}$ (indicating total protein), or Rz-value, is lower for rCPO (0.54) in comparison to nCPO. Homogeneous CPO from *C. fumago* has a Rz 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 Cys29 (64).

To further characterise rCPO we compared the behaviour 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 5-10 kDa, respectively, higher than the native isozymes. As we suspected that this difference in size was due to overglycosylation of the recombinant enzyme, we treated both rCPO and nCPO with endoglycosidase H (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 disulphide bridge present in chloroperoxidase (65).
To analyse 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 analyse whether the recombinant CPO was fully active some of its catalytic properties were measured. The specific chlorination activity (MCD assay as described by Morris and Hager, (2)) was determined. The specific chlorination activity of purified rCPO was 47 U/nmol heme. The pH optimum for the chlorination of monochlorodimedon 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 2, 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 % e.e. Experiments with labelled 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 3 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 labelled 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 *Aspergillus 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 *Caldariomyces fumago* cpo gene has been efficiently expressed in *A. niger* and the recombinant enzyme was secreted into the culture medium as an active protein.

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 (MnP) 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 behaviour may be the different nature of heme attachment in the MnP (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 characterisation 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 (1), maintain the same specific activity. 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 *Aspergillus niger* production system for CPO, we have assessed whether the structural and catalytic properties of rCPO were comparable with those of native CPO. Experimental data showed the correct formation of the heme thiolate ligand as well as single disulphide bond in the recombinant CPO. The specific chlorination activity of rCPO (47 U/nmol heme) was in agreement with the activities reported by Morris and Hager (2): 70 U/nmol; Van Deurzen *et al.* (15): 53 U/nmol; Libby *et al.* (68): 59 U/nmol. Also 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 2) 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 3) 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 catalysed 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 labelled H$_2$H$_2$O$_2$ into thioanisole sulfoxide and 2-oxindole. This is in agreement with the results of labelling 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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REFERENCES


**FOOTNOTES**

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FIGURE LEGENDS

Scheme 1. Oxidation of sulphides. R=H or -OCH$_3$; R’=-CH$_3$ or -CH$_2$-CH$_3$.

Scheme 2. Oxidation of substituted indoles. R=-Br, -Cl, or -OCH$_3$.

Figure 1. *cpo* expression vector pCPO3.1-AmdS.

Figure 2. UV-spectra of rCPO (thick line) and nCPO (thin line).

Figure 3. Western blotting analysis of recombinant chloroperoxidase (rCPO) and native chloroperoxidase (nCPO). Proteins were detected with a "CPO polyclonal antiserum (see M&M). Proteins were partially deglycosylated by treatment with EndoH (+). The deglycosylation protein bands are indicated by an arrow.

TABLES

Table 1. Purification of rCPO.

<table>
<thead>
<tr>
<th></th>
<th>Volume (ml)</th>
<th>Activity (U/ml)</th>
<th>Enzyme (U)</th>
<th>Yield (%)</th>
<th>R$<em>Z$ (A$</em>{400}$/A$_{280}$)</th>
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<td>Crude</td>
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<td>234</td>
<td>11</td>
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**Table 2.** Oxidation of sulphides by native and recombinant CPO.

<table>
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<tr>
<th>Sulphide</th>
<th>rCPO Conversion (%)</th>
<th>rCPO e.e. (%)</th>
<th>Native CPO&lt;sup&gt;a&lt;/sup&gt; Conversion (%)</th>
<th>Native CPO&lt;sup&gt;a&lt;/sup&gt; e.e. (%)</th>
<th>Native CPO&lt;sup&gt;b&lt;/sup&gt; Conversion (%)</th>
<th>Native CPO&lt;sup&gt;b&lt;/sup&gt; e.e. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S─S</td>
<td>77</td>
<td>98</td>
<td>65</td>
<td>98</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>S─S</td>
<td>91</td>
<td>99</td>
<td>94</td>
<td>98</td>
<td>83</td>
<td>99</td>
</tr>
<tr>
<td>H₂C─O─S─S─O</td>
<td>58</td>
<td>94</td>
<td>62</td>
<td>97</td>
<td>53</td>
<td>99</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results obtained in a 1 ml scale experiment with native CPO from Chirazyme Labs.

<sup>b</sup> Results reported by van Deurzen et al. (15) for a 50 ml scale experiment.

**Table 3.** Oxidation of substituted indoles by native and recombinant CPO.

<table>
<thead>
<tr>
<th>Indole derivative</th>
<th>rCPO Conversion (15 min) (%)</th>
<th>rCPO Conversion (60 min) (%)</th>
<th>Native CPO&lt;sup&gt;a&lt;/sup&gt; Conversion (15 min) (%)</th>
<th>Native CPO&lt;sup&gt;a&lt;/sup&gt; Conversion (60 min) (%)</th>
<th>Native CPO&lt;sup&gt;b&lt;/sup&gt; Conversion (15 min) (%)</th>
<th>Native CPO&lt;sup&gt;b&lt;/sup&gt; Conversion (60 min) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>N─H─N─Br</td>
<td>24</td>
<td>78</td>
<td>24</td>
<td>83</td>
<td>25</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>n.d.</td>
<td>24</td>
<td>n.d.</td>
<td>37</td>
<td>9</td>
<td>19</td>
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<td>14</td>
<td>35</td>
<td>16</td>
<td>45</td>
<td>19</td>
<td>47</td>
</tr>
<tr>
<td>Cl─H─N─H</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>H₂C─O─N─H─N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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.
Fig. 1
Scheme 1

\[
\begin{align*}
\text{R-Sulfoxide} & \quad \text{R} \quad \text{S} \quad \text{R'} \\
& \quad \text{CPO} \quad \text{H}_2\text{O}_2
\end{align*}
\]
Scheme 2

\[ R \text{ benzene ring} \xrightarrow{\text{CPO, H}_2\text{O}_2} R \text{ indole ring} \]
Expression of the Caldariomyces fumago chloroperoxidase in Aspergillus niger and characterisation of the recombinant enzyme
Ana Conesa, Fred van de Velde, Fred van Rantwijk, Roger A. Sheldon, Cees A.M.J.J. van den Hondel and Peter J. Punt

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