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Chloroperoxidase, a peroxidase with potential

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SUMMARY

Chloroperoxidase is an extracellular heme glycoprotein produced by the imperfect fungus *Caldariomyces fumago*. The enzyme can catalyse chlorination reactions as well as act as a catalase or a peroxidase. As a peroxidase, it has a wide substrate specificity and we are interested in some applied aspects of this activity, requiring the production and purification of moderate quantities of the enzyme. High levels of chloroperoxidase are produced in a fructose synthetic medium, and highest enzyme production occurs in a low-shear environment. Fungal pellets produce enzyme continuously at low medium replacement rates and at up to 0.6 g enzyme per l: chloroperoxidase is essentially the only extracellular enzyme produced. Enzyme purification is uncomplicated and gives good yields of high purity. Pure enzyme is stable for weeks at room temperature and under pH control. Chloroperoxidase can be ionically bound to aminopropyl glass, then covalently immobilized by glutaraldehyde crosslinking. Immobilized preparations have been washed and re-used five times, and are most stable at pH 5.5–6. Like many peroxidases, chloroperoxidase will oxidize phenols and phenolics, often causing a precipitate, and can totally remove phenols at low aqueous concentrations. Chloroperoxidase incubation with the petroporphyrin component of crude oil asphaltene (fraction 5) causes a reduction or removal of the Soret band (410 nm) and the α-peak (573 nm). This petroporphyrin fraction is enriched with vanadium which poisons the chemical catalyst used in cracking crude oil.

INTRODUCTION

Chloroperoxidase (CPO) was first purified, crystallized and biochemically characterized in the mid-1960s [20], and most recently, the structural gene has been isolated, cloned and sequenced [6,22]. In between these times, the enzyme has been studied extensively because of its remarkable catalytic activities. The reduced, carbon monoxide treated enzyme has a spectrum similar to cytochrome P_{450} (cam), and CPO has been proposed as a model system to study the molecular mechanism of peroxidase action. A number of potential commercial uses for CPO have been proposed based on its halogenation activity [21] and several other uses based on its peroxidative activity. The potential uses of peroxidases extend to fields such as analytical diagnosis [18], pharmaceuticals, and in pollution control [21]. Despite these potential uses and glowing predictions [19], the industrial application of peroxidase has been limited, mainly due to their high isolation and purification costs, relative instability, and the difficulty in recovering active enzyme after completion of the catalytic process [14,15]. We have studied several aspects related to theses limitations of peroxidase application, using CPO as our model.

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MEDIUM DEVELOPMENT

Our interest in CPO began about 10 years ago as a collaboration with a colleague, Brian Dunford in our Chemistry Department, who had begun to work with CPO. At that time, the enzyme was available commercially at $26/mg: current catalog prices are $70/mg of enzyme with an R_z (absorbance at 403 nm/280 nm) = 1. Pure CPO has an R_z = 1.4.

Early attempts at CPO production were carried out using a glucose–malt extract medium [10]. In our hands, enzyme production in this medium was inconsistent: the fungus produced a black gelatinous pigment, making the spent medium difficult to handle, and as maximum CPO production approached, the medium turned alkaline, causing total loss of enzyme activity (Fig. 1). Substitution of fructose for glucose as the carbon source and removal of the malt extract from the medium of cultures grown under similar physical conditions produced the following changes [25]: (i) improved yield of enzyme; (ii) earlier maximum enzyme production (6–8 days rather than 10–12); (iii) pigment and viscosity reduced by 90%; and (iv) stabilization of medium pH, and therefore enzyme stability. This change in medium design has provided a more consistent fungal fermentation.

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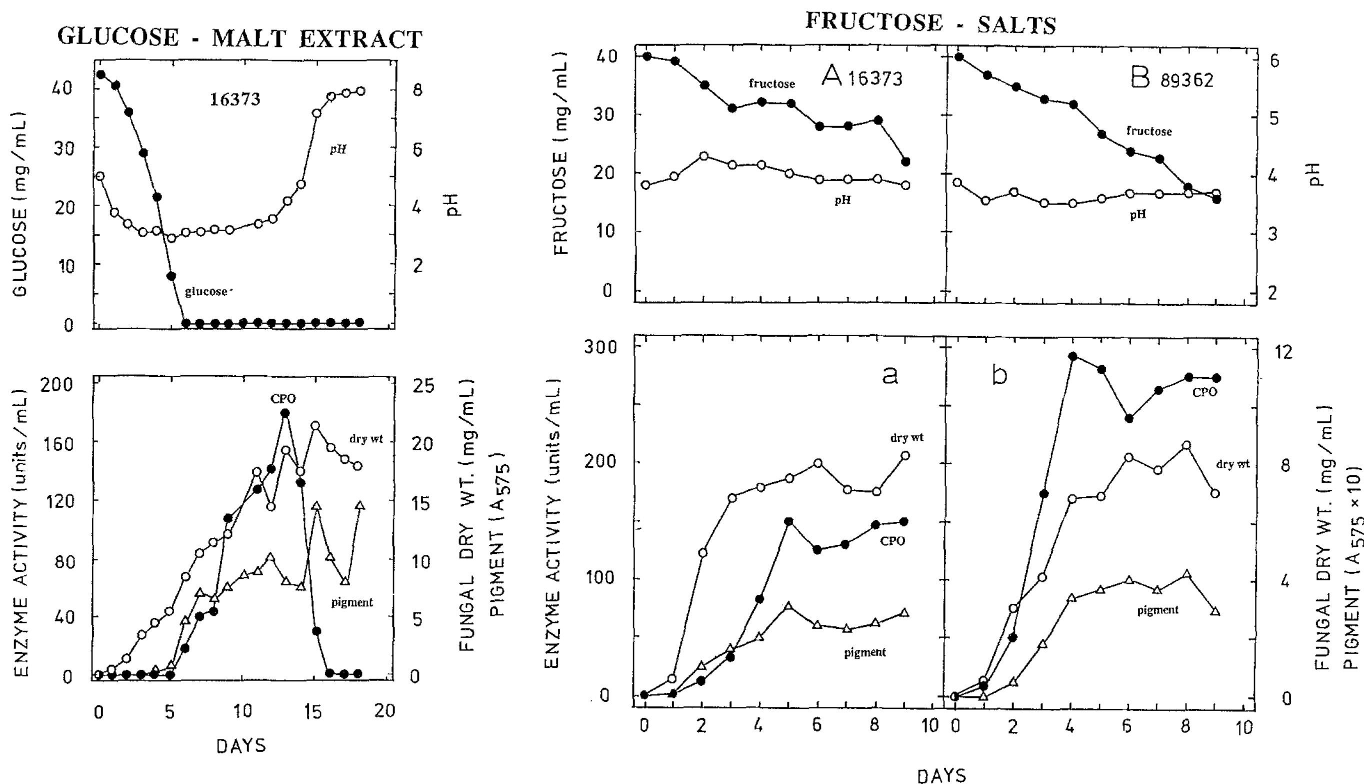

Fig. 1. Growth of *C. fumago* on glucose-malt extract and fructose-salts media. ([25]; with permission).

GROWTH AND PURIFICATION

Improving purification methodology is an ongoing process, and depends to an extent on the scale of the operation. On a small scale the published methodology [10] gave good recovery and purification but variable losses occurred when ethanol precipitation was used to remove pigment prior to ion exchange chromatography. Currently pigment removal is achieved with 10% polyethylene glycol 3500 [8]. *C. fumago* CMI 89362, a non-sporulating high CPO producing strain, is used in this laboratory and is maintained both on PDA plates at 4 °C and in 20% glycerol at − 80 °C. Typically, a 50-l fermentation is processed in three batches:

(i) A 2-l pelletized *C. fumago* CMI 89362 culture [3] grown for 10 days is used to inoculate 48 l of fructose-salts medium [25]. The stirred tank fermenter is operated as an air-lift, using 50 rpm agitation. After 10 days at 27 °C the medium contains > 100 mg CPO/l based on the specific activity of 1660 enzyme units/mg protein [20]. CPO is essentially the only extracellular protein produced.

(ii) The mycelium is filtered through nylon mesh and the spent medium is frozen and thawed twice in 25-l plastic buckets. Precipitated gel is removed by filtration and centrifugation, prior to concentration to 10% of the original volume using hollow fibre ultrafiltration (10K cutoff).

(iii) Remaining pigment is precipitated with 10% polyethylene glycol (PEG), centrifuged, diluted to 5% PEG, reconcentrated by ultrafiltration, and dialysed against 20 mM phosphate buffer pH 5.8.

(iv) Further purification is accomplished by ion exchange chromatography through DEAE cellulose (DE 52) using a gradient of 20–200 mM phosphate pH 5.8. Fractions with an R_z of > 1.25 are pooled, representing > 80% recovery. Small amounts of contaminating carbohydrate can be removed by gel exclusion chromatography [9] or ammonium sulfate precipitation [10].

SEMICONTINUOUS ENZYME PRODUCTION

The concept of using immobilized microbial cells for the production of fine chemicals and biochemicals is well established, but the use of fungal material for the continuous production of enzymes is less well documented [7,24]. Development of the defined fructose–salts medium resulted in a consistent level of CPO production, and

produced a spent medium in which the enzyme was stable and readily purified. Growth periods longer than about 10 days gave rise to increased pigment and viscosity with only small increases in CPO levels: the most productive part of the culture growth was in the early stages. However, inoculum preparation and growth of the fungus to a stage where it becomes productive are time consuming. The involvement of a preculture, found to produce the most consistent results, plus the production phase made the overall production cycle about 3 weeks. If the spent mycelium could be re-used once the CPO-containing supernatant had been removed, much economy of time and material could be achieved. Initially, fragments of mycelium were immobilized in carrageenan beads [11], for ease of handling, and grown in shake flasks. At the end of a growth period of about 10 days, the immobilized fungal beads were separated from the CPO-containing medium by filtration, washed in distilled water, and inoculated into fresh fructose–salts medium. Two of the

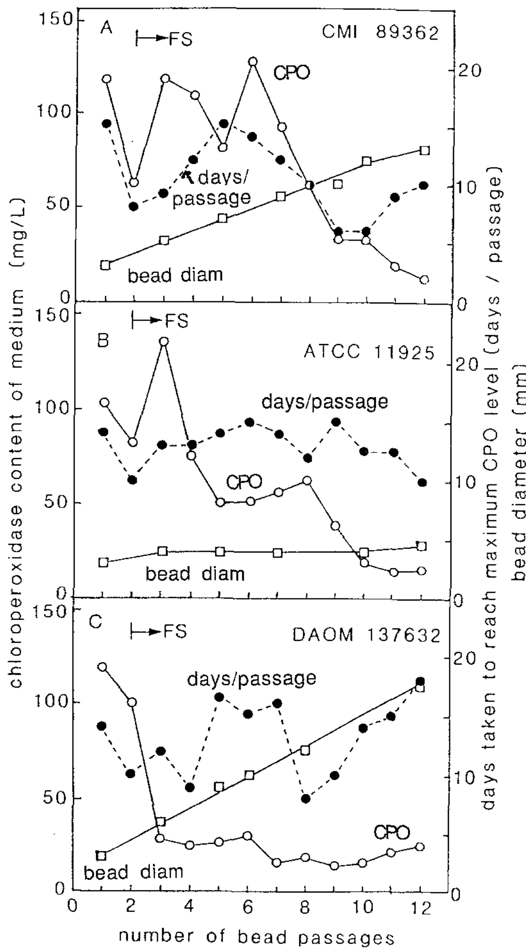

Fig. 2. Semicontinuous production of CPO by three strains of *C. fumago*. ([5]; with permission).

C. fumago strains produced between 70 and 100 mg CPO/l during 8 growth periods of 10 days, as is shown in Fig. 2. Enzyme production required a complete medium and fungal growth occurred at the bead surface causing mycelial fragments to be shed into the medium: these were removed during filtration between transfers. Despite this abrasion, the bead diameter grew about 1 mm in each passage, from 0.2 to 1.2 cm, until eventually the beads began to fragment due to crowding in the flask. The useful phase of CPO production under these conditions was about 3 months.

CONTINUOUS CPO PRODUCTION

We continued to investigate the long-term use of *C. fumago* mycelium for the continuous production of CPO using fungal pellets and an external-loop airlift fermenter. Fungal pellets could be size-selected by decantation of the inoculum: they behaved similarly to carrageenan-entrapped mycelium, and were easier to prepare. The external-loop airlift fermenter provided a non-abrasive, well-mixed system that permitted growth on the surface of the fungal pellets, but minimized fragmentation. Initially the fermenter was grown under batch conditions until the CPO content of the medium was 100 mg/l (on occasions the level rose to 450 mg CPO/l). At this point, fructose salts medium was simultaneously added and removed. Over a range of dilution rates, the CPO content of the effluent followed the theoretical washout rate for 48 h, then rose to a stable production level, determined by

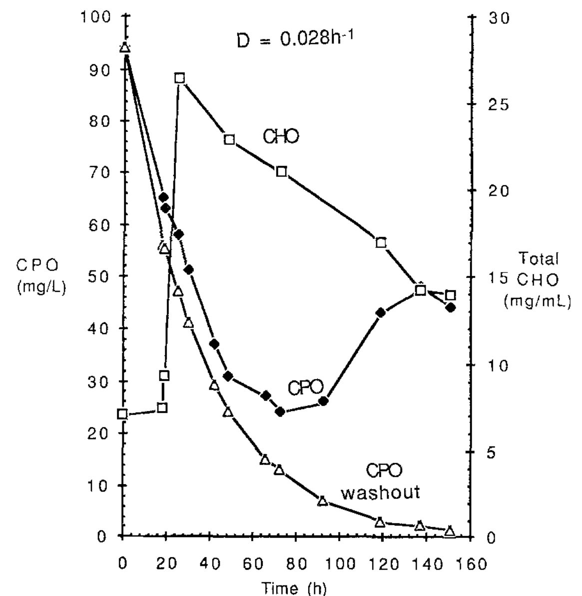

Fig. 3. Continuous production of CPO by mycelial pellets. ([3]; with permission).

the dilution rate. Fig. 3 shows the fermenter profile at a dilution rate of 0.028 h^{-1}. Over the dilution range 0.008 to 0.070 h^{-1}, the CPO content of the effluent medium was inversely proportional to the dilution rate so that the CPO production rate was constant at 1.2 mg CPO h^{-1} as shown in Fig. 4. Later studies showed that it was unnecessary to condition the fermenter by running in the batch mode before continuous operation [3]. Recently, Hager's group [1] have described a novel continuous CPO production system using a rotating plastic carboy with *C. fumago* growing on the scarified internal surface. This system can produce up to 600 mg CPO/l for 80 days and at a maximum rate of 117 mg CPO/day.

Thus there appear to be a number of systems that may lend themselves to the large scale and continuous production of CPO in a crude but readily purifiable form. This has been shown for several strains of *C. fumago*, so research on the methodology for CPO production and scale-up exists, should the demand occur.

IMMOBILIZATION

To test CPO as a recoverable and reusable catalyst, it was decided to immobilize the enzyme on glass beads, using the method developed by Weetall [29]. Acid-washed glass was derivatized with aminopropyl triethoxysilane, treated with glutaraldehyde and finally reacted with the enzyme. In several attempts a coupling efficiency of 6 mg CPO/mg glass was obtained, low compared to the 25 to 40 mg of alcohol dehydrogenase and lactic dehydrogenase reported by Brotherton et al. [2]. This low efficiency is believed to be due to the anionic nature of CPO which has a p*I* in the range 3.5–3.9 [9,20] and a corresponding large excess of acidic over basic amino acids [22].

During analysis of the methodology, it was observed that in low ionic strength buffer, pH 6, CPO bound ionically to aminopropyl glass and could be displaced by high ionic strength buffers. Treating the ionically bound enzyme with glutaraldehyde immobilized the CPO in an active form that was not displaced by high salt concentrations. This variation from the standard method is shown in Fig. 5. The loading of enzyme protein to the glass beads was highest, 100 mg protein/g glass, at high reaction ratios of CPO to glass. To determine enzyme activity for immobilized CPO, a colorimetric assay was devised using the oxidation of N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD; [12]) and the highest specific activity preparations were achieved at low enzyme-to-carrier ratios in the crosslinking mixtures [13].

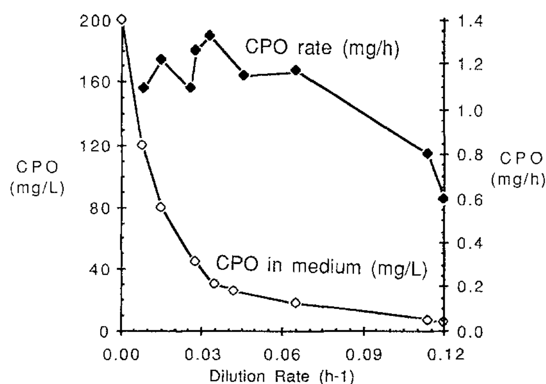

Fig. 4. Effect of dilution rate on the level and rate of CPO synthesis. ([3]; with permission).

CHARACTERISTICS OF SOLUBLE AND IMMOBILIZED CPO

Since immobilization of an enzyme may affect its stability characteristics and kinetic properties, we compared the properties of the soluble [26] and immobilized enzyme preparations. Virtually no differences were observed. In the presence of chloride, both preparations showed a biphasic activity response to pH, with maxima at pH 2.7 and pH 6 [28]. No differences could be detected in their stability to room temperature storage in different pH buffers, although the immobilized enzyme appeared to be more stable at pH 7, and no differences were detected in thermal stability. Thus CPO, like horseradish peroxidase [27], is no more stable to inactivation in the immobilized form than as a soluble enzyme.

Since the optimum conditions for enzyme assay are often unrelated to enzyme stability, and an essential property of an immobilized enzyme is that it be stable to re-use, the retention of activity with re-use was studied at pH values from 3.1 to 6.7. After five uses, the immobilized CPO retained full activity for TMPD oxidation between pH 6.0 to 6.7, but activity retention declined at more acidic pH values (Fig. 6). Thus for repeated oxidations at pH 6–6.5, immobilized CPO appears to have promise.

USES FOR CPO

CPO can carry out a wide range of reactions, based on halogenation and peroxidase activities, and a considerable number of potential uses for the enzyme have been proposed [21]. Our interest has centred around the peroxidase activity of CPO since, in laboratories, peroxidases have been shown to remove carcinogenic aromatic amines for industrial effluents [16], phenols from coal conversion waste water [17], coloured material from pulp mill effluents [23] and flavoured aromatics from drinking water [6a]. In a preliminary study [4], we have shown that CPO can carry out oxidation of a variety of substituted

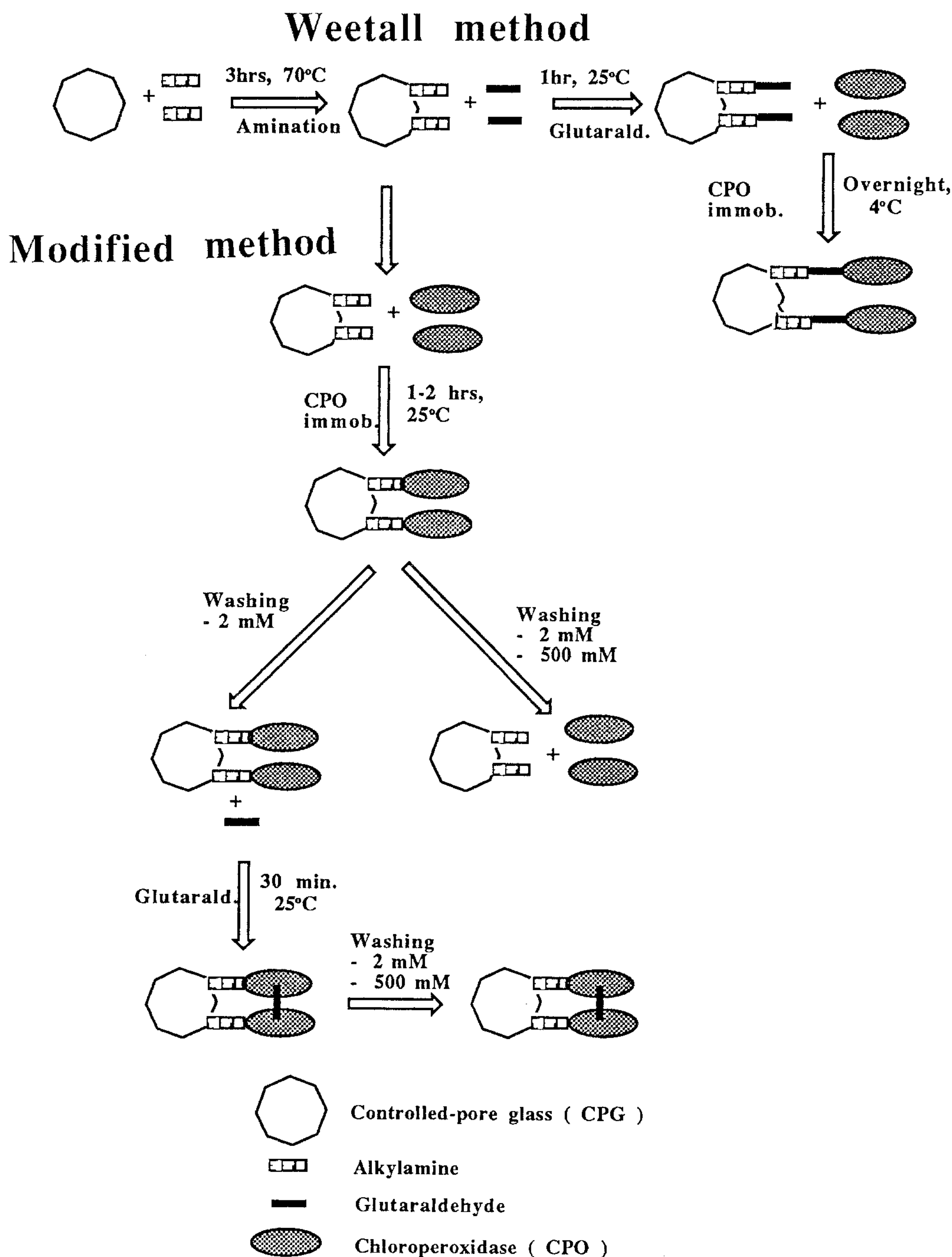

Fig. 5. Schemes for immobilizing CPO on glass beads.

phenolics at concentrations of up to 1 g phenolic/l. Under non-optimized conditions, 50–90% of individual phenolics was oxidized and in half the reactions insoluble products were formed, effectively removing the product from solution. Thus CPO may provide an alternative to the proposed use of horseradish peroxidase for phenolic removal from aqueous solutions. Another potential use for CPO involves a component of crude oil. In our department, a group (D.W.S. Westlake, P.M. Fedorak et al.) are studying the microbial metabolism of crude oil and its components. There is a pentane precipitable fraction of crude oil called asphaltene that contains many polycyclic aromatic hydrocarbons. This material can be further subdivided into size-classes by gel-permeation chromato-

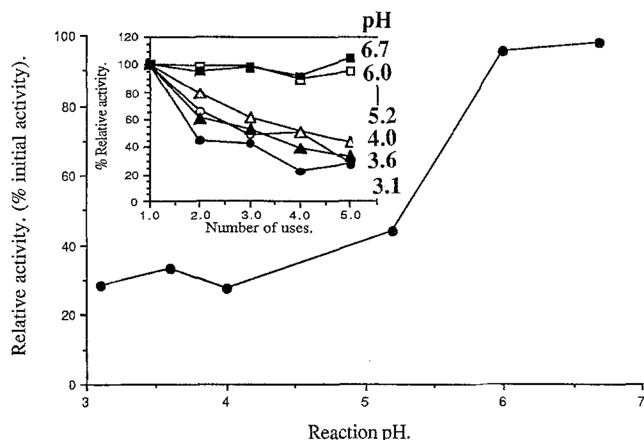

Fig. 6. Stability of immobilized CPO after repeated use.

graphy and the lowest molecular weight fraction, fraction 5, is enriched with petroporphyrins: tetrapyrroles containing coordinated metal ions such as nickel or vanadium, and exhibiting a characteristic porphyrin spectrum. Incubation of CPO with protoporphyrin IX or fraction 5 causes the loss of the Soret peak (410 nm) (Westlake and Fedorak, unpublished results). Horseradish peroxidase does not carry out this conversion. This reaction may therefore form the basis for an analytical probe for porphyrins in crude oil fractions. Furthermore, if the reaction causes the release of heavy metals, enzyme treatment may be a means of enhancing metal removal and protecting heavy-metal-sensitive catalysts involved in crude oil fractionation.

REFERENCES

1 Blanke, S.R., S. Yi, and L.P. Hager. 1989. Development of semi-continuous and continuous flow bioreactors for the high level production of chloroperoxidase. Biotechnol. Lett. 11: 769–774.

2 Brotherton, J.E., A. Emery and V.W. Rodwell. 1976. Characterization of sand as a support for immobilized enzymes. Biotechnol. Bioeng. 18: 527–543.

3 Carmichael, R.D. and M.A. Pickard. 1989. Continuous and batch production of chloroperoxidase by mycelial pellets of *Caldariomyces fumago* in an airlift fermentor. Appl. Environ. Microbiol. 55: 17–20.

4 Carmichael, R.D., P.M. Fedorak and M.A. Pickard. 1985. Oxidation of phenols by chloroperoxidase. Biotechnol. Lett. 7: 289–294.

5 Carmichael, R.D., A. Jones and M.A. Pickard. 1986. Semi-continous and continuous production of chloroperoxidase by *Caldariomyces fumago* immobilized in k-carrageenan. Appl. Environ. Microbiol. 51: 276–280.

6 Fang, G.H., P. Kenigsberg, M.J. Axley, M. Nuell and L.P. Hager. 1986. Cloning and sequencing of chloroperoxidase cDNA. Nucl. Acids Res. 14: 8061–8071.

6a Feissinger, F., S.W. Malony, J. Manem and J. Mallevialle. 1984. Potential use of enzymes as catalysts in drinking water for the oxidation of taste-causing substances. Aqua 2: 116–118.

7 Frein, E.M., B.S. Montenecourt and D.E. Eveleigh. 1982. Cellulase production by *Trichoderma viride* immobilized on k-carrageenan. Biotechnol. Lett. 4: 287–292.

8 Gonzalez-Vergara, E., D.C. Ales and H.M. Goff. 1985. A simple, rapid, high yield isolation and purification procedure for chloroperoxidase isoenzymes. Prep. Biochem. 15: 335–348.

9 Hashimoto, A. and M.A. Pickard. 1984. Chloroperoxidase from *Caldariomyces* (= *Leptoxyphium*) cultures: glycoproteins with variable carbohydrate content and isoenzymic forms. J. Gen. Microbiol. 130: 2051–2058.

10 Hollenberg, P.E. and L.P. Hager. 1970. Purification of chloroperoxidase from *Caldariomyces fumago*. Methods Enzymol. 52: 521–529.

11 Jones, A., D. Berk, B.H. Lesser, L.A. Behie and G.M. Gaucher 1983. Continuous production of patulin by immobilized cells of *Penicillium urticae* in a stirred-tank reactor. Biotechnol. Lett. 5: 785–790.

12 Kadima, T.A. and M.A. Pickard. 1990. A colorimetric assay for chloroperoxidase. Can. J. Microbiol. 36: 302–304.

13 Kadima, T.A. and M.A. Pickard. 1990. Immobilization of chloroperoxidase on aminopropyl-glass. Appl. Environ. Microbiol. 56: 3473–3477.

14 Kennedy, J.F. and J.M.S. Cabral. 1983. Immobilized enzymes. In: Solid Phase Biochemistry (W.H. Scouten, ed.), John Wiley and Sons, New York, NY, pp. 253–339.

15 Kennedy, J.F. and J.M.S. Cabral. 1987. Enzyme immobilization. In: Enzyme Technology (J.F. Kennedy, ed.), VCH Publishers, New York, NY, pp. 347–404.

16 Klibanov, A.M. and E.D. Morris. 1981. Horseradish peroxidase for the removal of carcinogenic aromatic amines from water. Enzyme Microb. Technol. 3: 119–122.

17 Klibanov, A.M., T.M. Tu and K.P. Scott. 1983. Peroxidase-catalyzed removal of phenols from coal-conversion waste-waters. Science 221: 259–261.

18 Manthey, J.A., L.P. Hager and K.D. McElvany. 1984. Protein bromination by bromoperoxidase from *Penicillus capitatus*. Methods Enzymol. 107: 439–445.

19 Martyn, R.P., S.C. Branzei and G.T. Sperl. 1981. Antimicrobial chlorinated b-diketones. Bios. 52: 8–12.

20 Morris, D.R. and L.P. Hager. 1966. Chloroperoxidase I. Isolation and properties of the crystalline glycoprotein. J. Biol. Chem. 241: 1763–1768.

21 Neidleman, S.L. and J. Geigert. 1986. Biohalogenation: principles, basic roles and applications. Ellis Horwood Limited, John Wiley and Sons, New York, NY.

22 Nuell, M.J., G.-H. Fang, M.J. Axley, P. Kenigsberg and L.P. Hager. 1988. Isolation and nucleotide sequence of the chloroperoxidase gene from *Caldariomyes fumago*. J. Bacteriol. 170: 1007–1011.

23 Paice, M.G. and L. Jurasek. 1984. Peroxidase-catalyzed color removal from bleach plant effluent. Biotechnol. Bioeng. 26: 477–480.

24 Peitersen, N. 1977. Continuous cultivation of *Trichoderma viride* on cellulose. Biotechnol. Bioeng. 19: 337–348.
25 Pickard, M.A. 1981. A defined medium for the production of chloroperoxidase by *Caldariomyes fumago*. Can. J. Microbiol. 27: 1298–1305.
26 Pickard, M.A. and A. Hashimoto. 1988. Stability and carbohydrate composition of chloroperoxidase from *Caldariomyces fumago* grown in a fructose-salt medium. Can. J. Microbiol. 34: 998–1002.
27 Small, N., J.L. Woodhead and A.D.B. Malcolm. 1984. The properties of immobilized horseradish peroxidase. Biochem. Soc. Trans. 12: 280.
28 Thomas, J.A., D.R. Morris and L.P. Hager. 1970. Chloroperoxidase VII. Classical peroxidatic, catalatic, and halogenating forms of the enzyme. J. Biol. Chem. 245: 3129–3134.
29 Weetall, H.H. 1976. Covalent coupling methods for inorganic support materials. Methods Enzymol. 44: 134–148.