The application of peroxidases in the synthesis of fine chemicals

Improvements in the operational stability

Proefschrift

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Er zijn geheimen die de Here, uw God, u niet heeft geopenbaard.
(Deuteronomium 29:29)

The secret things belong onto the Lord our God.
(Deuternomy 29:29)
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List of abbreviations

ABTS  2,2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
ACN  acetonitrile
AOT  Aerosol OT: sodium bis(2-ethylhexyl)sulfosuccinate
aw  water activity
C400  concentration CPO based on the absorbance at 400 nm
CcP  cytochrome c peroxidase
Cip  Coprinus cinereus peroxidase
CPO  chloroperoxidase from Caldariomyces fumago
CPO-EC  CPO-ethyl cellulose complex
CPO/GOX-PUR  CPO coimmobilized with GOX into polyurethane foam
CPO-PUR  CPO immobilized into polyurethane foam
CPO-SOS  CPO-sodium octadecylsulfate complex
DTPA  diethyltriaminepentaacetic acid
DMC  dimethoxyethane
DHF  dihydroxyfumaric acid
[E]  concentration metal-free phytase
EDTA  ethylenediaminetetraacetic acid
ee  enantiomeric excess
Emmob  immobilization efficiency: Ufound in foam/Ubound into foam
fM  fraction metal-free phytase
fv  fraction vanadate-incorporated phytase
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOX</td>
<td>glucose oxidase from <em>Aspergillus niger</em></td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IPA</td>
<td>isopropyl alcohol</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>$K_{d_{app}}$</td>
<td>apparent dissociation constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis Menten constant</td>
</tr>
<tr>
<td>LiP</td>
<td>lignin peroxidase</td>
</tr>
<tr>
<td>LPO</td>
<td>lactoperoxidase</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MCD</td>
<td>monochlorodimedone</td>
</tr>
<tr>
<td>MP-11</td>
<td>microperoxidase 11</td>
</tr>
<tr>
<td>MTBE</td>
<td>methyl tert-butyl ether</td>
</tr>
<tr>
<td>Phytase/GOX-PUR</td>
<td>phytase (from <em>Aspergillus niger</em>) coimmobilized with GOX into polyurethane foam</td>
</tr>
<tr>
<td>$R_z$</td>
<td>purity standard = $A_{400}/A_{280} = 1.44$ for pure CPO</td>
</tr>
<tr>
<td>SBP</td>
<td>soybean peroxidase</td>
</tr>
<tr>
<td>SBP/GOX-PUR</td>
<td>SBP coimmobilized with GOX into polyurethane foam</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SOS</td>
<td>sodium octadecylsulfate</td>
</tr>
<tr>
<td>STY</td>
<td>space time yield</td>
</tr>
<tr>
<td>TBHP</td>
<td>tert-butyl hydroperoxide</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TOP</td>
<td>turnover frequency</td>
</tr>
<tr>
<td>TON</td>
<td>turnover number</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Trolox\textsuperscript{TM}-C</td>
<td>6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid</td>
</tr>
<tr>
<td>TTN</td>
<td>total turnover number</td>
</tr>
<tr>
<td>VBPO</td>
<td>vanadium bromoperoxidase</td>
</tr>
<tr>
<td>VCPO</td>
<td>vanadium chloroperoxidase</td>
</tr>
<tr>
<td>$v$</td>
<td>rate of ration</td>
</tr>
<tr>
<td>$v_0$</td>
<td>rate of the metal-free phytase catalyzed oxidation</td>
</tr>
<tr>
<td>$[V_i]$</td>
<td>concentration vanadate</td>
</tr>
<tr>
<td>$[V_i]E$</td>
<td>concentration vanadate-incorporated phytase</td>
</tr>
<tr>
<td>$v_{max}$</td>
<td>rate of the vanadium-incorporated phytase catalyzed oxidation</td>
</tr>
<tr>
<td>$v_{total}$</td>
<td>overall rate of oxidation</td>
</tr>
<tr>
<td>Yield</td>
<td>mass protein bound to foam/mass protein in</td>
</tr>
</tbody>
</table>
1 General Introduction

1.1 Catalytic oxidation and fine chemicals

Selective catalytic oxidation with molecular oxygen is an important industrial technology (Sheldon, 1995) for the conversion of petroleum hydrocarbons into bulk chemicals, such as propylene oxide (Landau, 1979; Sheldon, 1980). In contrast, fine chemicals manufacture often involves the use of stoichiometric amounts of metal oxidants, such as chromium(VI) and manganese(VII) salts. However, the industry is under considerable pressure to replace these processes with catalytic alternatives, thereby reducing the amount of inorganic waste streams (Sheldon, 1994, 1996).
Catalytic oxygen transfer reactions can, in principle, be supported by a variety of oxygen donors (table 1, Sheldon, 1985). In addition to the costs, the practical utility is determined by the percentage of active oxygen and the nature of the coproduct. The former has a direct bearing on the productivity of the process (yield per unit of reactor volume per unit time) and the latter on the environmental acceptability. On the basis of these criteria it is readily apparent that the most commercially attractive oxidants are dioxygen and hydrogen peroxide since they produce water as the sole byproduct and their active oxygen content is high.

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>Active oxygen cont. (% weight)</th>
<th>Waste product</th>
</tr>
</thead>
<tbody>
<tr>
<td>O₂/reductant</td>
<td>50.0</td>
<td>H₂O</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>47.0</td>
<td>H₂O</td>
</tr>
<tr>
<td>O₃</td>
<td>33.3</td>
<td>O₂</td>
</tr>
<tr>
<td>NaOCl</td>
<td>21.6</td>
<td>NaCl</td>
</tr>
<tr>
<td>CH₃CO₂H</td>
<td>21.1</td>
<td>CH₃COOH</td>
</tr>
<tr>
<td>t-BuOOH</td>
<td>17.8</td>
<td>t-BuOH</td>
</tr>
<tr>
<td>C₆H₅NO₂⁻</td>
<td>13.6</td>
<td>C₆H₅NO</td>
</tr>
<tr>
<td>NaOBr</td>
<td>13.4</td>
<td>NaBr</td>
</tr>
<tr>
<td>KHSO₄</td>
<td>10.5</td>
<td>KHSO₄</td>
</tr>
<tr>
<td>NaIO₄</td>
<td>7.5</td>
<td>NaIO₃ (NaI)</td>
</tr>
<tr>
<td>PhIO</td>
<td>7.3</td>
<td>PhI</td>
</tr>
</tbody>
</table>

a) N-methylmorpholine-N-oxide (NMO).

In contrast to the continuous processes in dedicated plants characteristic of bulk chemicals manufacture, fine chemicals production is generally batch-wise and in multipurpose equipment. Furthermore, owing to the structural complexity and low volatility of fine chemicals, reactions are generally performed in the liquid phase. Enzymes are of growing importance for the synthesis of fine chemicals, as they act in aqueous solutions at moderate temperature. Moreover, they often exhibit high selectivities. In nature oxidations are catalyzed by redox-enzymes, viz. monoxygenases (Fang, 1995) and peroxidases (Van Deurzen, 1997a). Monoxygenases use molecular oxygen and a cofactor (reductant) to catalyze oxidation reactions, whereas peroxidases use hydrogen peroxide as the oxidant. In view of table 1, monoxygenases and peroxidases are attractive candidates to replace stoichiometric oxidations in fine chemicals manufacture by catalytic alternatives. For industrial application peroxidases are preferred over monoxygenases, as they do not require an expensive cosubstrate, such as NADH, to supply reducing equivalents.

1.2 Mechanism of catalytic oxidation

Metal catalyzed oxygen transfer processes can be divided into two major mechanistic categories, involving peroxometal and oxometal species as the active oxidant, respectively (Sheldon, 1985, 1993). The peroxometal mechanism is generally observed with early transition metals whereby high-valent peroxometal complexes of, e.g. molybdenum(VI), tungsten(VI), vanadium(V), titanium(IV), are the active oxidant (pathway a in scheme 1).
Catalysis by late and/or many first-row transition metals, e.g. chromium, manganese, iron, on the other hand, involves high-valent oxometal species as the active intermediate (pathway b). A characteristic feature of the latter category is that the presence of organic ligands is necessary to modulate the activity of the oxometal intermediate. In peroxidases and cytochrome P450 monooxygenases, a porphyrin ligand stabilizes the formally iron(V)oxo intermediate (*vide infra*).

### 1.3 Catalyst performance: key parameters

The commercial viability of a catalytic reaction is dependent on a number of key parameters (table 2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>term</th>
<th>dimension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalyst activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>turnover frequency</td>
<td>TOF</td>
<td>min⁻¹</td>
</tr>
<tr>
<td>Catalyst stability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>catalyst turnover number</td>
<td>TON</td>
<td>mol_{product} mol⁻¹_{catalyst}</td>
</tr>
<tr>
<td>catalyst total turnover number</td>
<td>TTN</td>
<td>mol_{product} mol⁻¹_{catalyst}</td>
</tr>
<tr>
<td>Productivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>catalyst productivity</td>
<td>g_{product} g⁻¹_{catalyst} d⁻¹</td>
<td></td>
</tr>
<tr>
<td>volumetric productivity</td>
<td>STY</td>
<td>g_{product} L_{reactor}⁻¹ d⁻¹</td>
</tr>
<tr>
<td>space-time yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td>final product concentration</td>
<td>g L⁻¹</td>
<td></td>
</tr>
<tr>
<td>Catalyst costs</td>
<td>$ kg_{product}⁻¹</td>
<td></td>
</tr>
</tbody>
</table>

a) measured in one reaction;  
b) measured over the catalyst lifetime (more reaction cycles are possible).

The first important parameter is the *activity* of the catalyst, defined as the amount of product obtained per unit weight of catalyst in a certain time. In chemocatalysis the activity of a catalyst is most frequently expressed in terms of *turnover frequency* (TOF: mol product formed per mol catalyst per unit time). In contrast, the activity of a biocatalyst is generally given by its specific activity (U mg⁻¹ = μmol of product formed or substrate converted per minute per mg biocatalyst). Only when the purity of the enzyme sample and the molecular mass of the protein are known, activities can be converted into turnover frequencies. The
Chapter 1

TOF of the biocatalyst is equal to its $k_{\text{cat}}$ in the Michaelis-Menten equation (2) as all the enzyme is saturated with substrate ([S] $\gg K_m$).

The Michaelis-Menten theory for enzyme kinetics is based on the assumption that the enzyme (E) and its substrate (S) associate reversibly to form an enzyme-substrate complex (ES). This association/dissociation is assumed to be in rapid equilibrium. The product (P) is formed in a second step when ES breaks down to yield E and P (equation 1).

$$E + S \rightleftharpoons ES \longrightarrow E + P$$  \hspace{1cm} (1)

The most important parameter in enzyme kinetics is the rate of product formation ($v$). Substitution of the Michaelis-Menten theory with the rate equation gives the Michaelis-Menten equation, which describes the relation between substrate concentration and the rate of reaction (equation 2; Garrett, 1995; Engel, 1996).

$$v = \frac{v_{\text{max}} [S]}{K_m + [S]} \quad \text{or} \quad \text{TOF} = \frac{v}{[E]} = \frac{k_{\text{cat}} [S]}{K_m + [S]}$$  \hspace{1cm} (2)

Besides the activity of the catalyst, its stability is also an important parameter. The (total) turnover number (TTN or TON: mol of product formed per mol of catalyst) is directly related to the catalyst cost for a process, and, therefore low turnover numbers are only acceptable for cheap catalysts. For enzymatic processes the catalyst’s price is high, and, therefore either the TTN has to be very high or the desired product must be very expensive before it becomes economically feasible.

The space-time yield (STY: g L$^{-1}$d$^{-1}$) describes the amount of product produced in a certain reactor volume per unit of time. The final product concentration is directly related to the costs for down-stream processing necessary for the isolation of the product. A rule of thumb is a STY of 100 g L$^{-1}$d$^{-1}$ for a product with a market price of $100 per kg and a final product concentration of 100 g L$^{-1}$ to make a process economically applicable. However all values given for these key parameters are strongly dependent on the price of the product.

2 Introduction to peroxidases

2.1 Sources and biological functions of peroxidases

Oxidation and reduction reactions in nature are catalyzed by redox enzymes, which are classified as oxidoreductases. This class is divided into four categories according to the oxidant they use and the reaction they catalyze:

1. Dehydrogenases (reductases)
   $$\text{RH}_2 + D \longrightarrow \text{R} + \text{DH}_2$$

2. Oxidases
   $$\text{RH}_2 + O_2 \longrightarrow \text{R} + 2\text{H}_2\text{O}_2$$

3a. Monooxygenases
   $$\text{RH} + O_2 + \text{DH}_2 \longrightarrow \text{ROH} + D + \text{H}_2\text{O}$$
3b Dioxygenases
\[ RH + O_2 \rightarrow ROOH \]

4 Peroxidases
\[ 2 AH + H_2O_2 \rightarrow 2 A^* + 2 H_2O \]
\[ R + H_2O_2 \rightarrow RO + H_2O \]

Peroxidases catalyze oxidative transformations of organic substrates with a peroxide as oxidant. Usually the oxidant is hydrogen peroxide, but also alkyl hydroperoxides, including tert-butyl hydroperoxide, are accepted by some peroxidases. In contrast to monooxygenases and dehydrogenases, peroxidases do not require expensive cosubstrates, such as NADH. Peroxidases are classified as EC 1.11.1.x, where x denotes a specific peroxidase, e.g. for chloroperoxidase x=11.

Peroxidases are ubiquitous in nature and are found in animals, plants, and microorganisms. Most of them are relatively stable, extracellular enzymes. Their names are derived from their sources, for example horseradish, soybean, peanut and tea-leaf peroxidase and lactoperoxidase, or from their substrates, e.g. bromo-, and chloroperoxidase or lignin, and cytochrome c and manganese peroxidase. Peroxidases exhibit a variety of biological functions, e.g. the synthesis of biomolecules and the detoxification of hydrogen peroxide. They can accommodate a broad range of substrates in a diversity of reactions.

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Source</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>Bovine liver</td>
<td>Detoxification of ( H_2O_2 )</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td>Mold Caldaromyces fumago</td>
<td>Biosynthesis of caldariomycin</td>
</tr>
<tr>
<td>Cytochrome c peroxidase</td>
<td>Yeast Saccharomyces cerevisiae</td>
<td>Reduction of ( H_2O_2 ) and oxidation cytochrome c</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>Plant roots of Armoracia rusticana</td>
<td>Biosynthesis of plant hormones</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>Bovine milk</td>
<td>Antimicrobial</td>
</tr>
<tr>
<td>Lignin peroxidase</td>
<td>Mold Phanerochaete chrysosporium</td>
<td>Lignin degradation</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>Human leukocytes</td>
<td>Antimicrobial</td>
</tr>
</tbody>
</table>

Based on their specific active center, peroxidases can be classified into three groups: heme peroxidases, vanadium peroxidases, and non-metal peroxidases. Best known are the heme peroxidases that bear an iron protoporphyrin IX molecule in their active site. Sources and functions of a selection of heme peroxidases are represented in table 3 (Adam, 1999). Vanadium haloperoxidases have a vanadate ion as their prosthetic group and they are mainly found in marine environments (Butler, 1993). The group of the bacterial metal-free haloperoxidases catalyzes the \textit{in situ} formation of peroxycarboxylic acids followed by non-enzymatic oxidation of their substrate (Picard, 1997). This catalytic mechanism is more comparable with lipases (Björkling, 1992; De Zee et al., 1993) than with the classical peroxidases.

2.2 Catalytic cycle of peroxidases

The generally accepted catalytic pathway for the heme-containing peroxidases is shown in scheme 2. Oxidation of the native peroxidase with hydrogen peroxide gives the active enzyme intermediate. This so-called compound I is formally an iron(V)oxo species, but in
most of the peroxidases it is present as an iron(IV)oxo porphyrin radical cation species (Dawson, 1988). In some cases the radical cation is not located at the porphyrin ring but at the protein, e.g. a tryptophan residue in the case of cytochrome c peroxidase (Sivaraja, 1989), yielding an iron(IV)oxo protein radical cation species. Compound I is comparable with the active intermediate in the monooxygenase catalytic cycle. It was generally believed that the formation of compound I was irreversible, but recently evidence has been presented that is consistent with compound I formation in peroxidases being reversible (Van Haandel, 1998).

Scheme 2. Reaction scheme for peroxidases [P= protoporphyrin IX].

Compound I can be reduced to the native enzyme via several pathways, depending on the type of reaction. In an oxygen transfer reaction (pathway 2 in scheme 2) compound I is reduced in one step to the native enzyme. In contrast, a classical peroxidase reaction (pathway 4) involves two separate electron transfer reactions from two substrate molecules. The first substrate molecule donates its electron to compound I resulting in the formation of an iron(IV)oxo intermediate, the so-called compound II (pathway 4a). The second substrate molecule reduces compound II back to the native enzyme (pathway 4b). In the catalase reaction (pathway 3) compound I is reduced by a second molecule of H$_2$O$_2$, resulting in the formation of singlet oxygen.

The catalytic cycle of the vanadium haloperoxidases involves a peroxometal mechanism in which the vanadium atom does not change its oxidation state. Steady-state kinetic studies on VCPO have shown that, in common with the heme peroxidases, the vanadium peroxidases exhibit a Ping-Pong type mechanism (Van Schijndel, 1994). Ping-Pong or double-displacement kinetics are characterized by the product of the enzyme's reaction with the first substrate (in the case of peroxidases this is the reagent H$_2$O$_2$) being released prior to reaction of the enzyme with the second substrate (Garrett, 1995). The hydrogen peroxide binds in the first step of the Ping-Pong mechanism resulting in the formation of the oxidized form of VCPO, followed by the halide ion. The VBPO catalyzed bromination reaction is electrophilic and not a radical process (Soedjak, 1995). Recently, the crystal structure of the peroxy form of VCPO from C. inaequalis was published (Messerschmidt, 1997), which revealed that the peroxide is bound in a η-peroxovanadium(V) complex (structure 4 in scheme 3).

The proposed catalytic cycle of vanadium chloroperoxidase is shown in scheme 3. In the native enzyme (structure 1) the apical oxygen is hydrogen-bonded to His404. The H$_2$O$_2$
approaches (structure 2) and displaces a water molecule from the vanadium-coordination sphere (structure 3). The OH ligand is then displaced by the peroxide oxygen (iii) giving a η-peroxovanadium(V) intermediate (structure 4). At this stage the chloride ion binds (iv) to the empty vanadium coordination site (structure 5). The peroxide moiety accepts two electrons from the chloride resulting in cleavage of the O-O bond with concomitant formation of ClO⁻. After uptake of a proton from the surrounding water, the OCl⁻ leaves the active site as HOCl (v). The formed OH⁻ binds to the vanadium site and rebuilds the native state.

Scheme 3. Proposed catalytic cycle of vanadium CPO from C. inaequalis (Messerschmidt, 1997).

2.3 Catalytic reactions of peroxidases

The application of peroxides as biocatalysts for selective oxidations has been extensively reviewed in general (Adam, 1999; Colonna, 1999; Van Deurzen, 1997c) and for CPO (Hager, 1998) and haloperoxidases (Littlechild, 1999) in particular. Therefore, only the highlights in peroxidase chemistry will be mentioned in this section. The peroxidase catalyzed reactions can be divided into four categories:

1. Oxygen transfer reactions
   (pathway 2 in scheme 2)
   \[ R + H_2O_2 \longrightarrow RO + H_2O \]

2. Oxidative dehydrogenation
   (classical peroxidase reaction, pathway 4a and 4b in scheme 2)
   \[ 2 AH + H_2O_2 \longrightarrow 2 A^+ + 2 H_2O \]

3. Oxidative halogenation
   (pathway 2 in scheme 2)
   \[ SH + H_2O_2 + H^+ + X^- \longrightarrow SX + 2 H_2O \]

4. Hydrogen peroxide disproportionation
   (catalase reaction, pathway 3 in scheme 2)
   \[ 2 H_2O_2 \longrightarrow 2 H_2O + O_2 \]
**Oxygen transfer reactions** are catalyzed by peroxidases, notably, CPO from *C. fumago*, which catalyzes a wide variety of synthetically useful oxygen transfer reactions, e.g. olefin epoxidation, benzylic, allylic, and propargylic hydroxylation, oxidation of sulfides, oxidation of indoles to the corresponding 2-oxindoles, oxidation of primary alcohols, and oxidation of arylamines to the corresponding nitroso derivatives (table 4).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Main product</th>
<th>TTN³ (-)</th>
<th>ee (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Substrate 1" /></td>
<td><img src="image2.png" alt="Main product 1" /></td>
<td>900</td>
<td>49</td>
<td>Dexter, 1995a</td>
</tr>
<tr>
<td><img src="image3.png" alt="Substrate 2" /></td>
<td><img src="image4.png" alt="Main product 2" /></td>
<td>1450</td>
<td>96</td>
<td>Allain, 1993</td>
</tr>
<tr>
<td><img src="image5.png" alt="Substrate 3" /></td>
<td><img src="image6.png" alt="Main product 3" /></td>
<td>4200</td>
<td>93</td>
<td>Lakner, 1996</td>
</tr>
<tr>
<td><img src="image7.png" alt="Substrate 4" /></td>
<td><img src="image8.png" alt="Main product 4" /></td>
<td>1700</td>
<td>96</td>
<td>Allain, 1993</td>
</tr>
<tr>
<td><img src="image9.png" alt="Substrate 5" /></td>
<td><img src="image10.png" alt="Main product 5" /></td>
<td>460</td>
<td>97</td>
<td>Zaks, 1995</td>
</tr>
<tr>
<td><img src="image11.png" alt="Substrate 6" /></td>
<td><img src="image12.png" alt="Main product 6" /></td>
<td>460</td>
<td>88</td>
<td>Zaks, 1995</td>
</tr>
<tr>
<td><img src="image13.png" alt="Substrate 7" /></td>
<td><img src="image14.png" alt="Main product 7" /></td>
<td>140</td>
<td>n.d.</td>
<td>Zaks, 1995</td>
</tr>
<tr>
<td><img src="image15.png" alt="Substrate 8" /></td>
<td><img src="image16.png" alt="Main product 8" /></td>
<td>590</td>
<td>94</td>
<td>Hu, 1999</td>
</tr>
<tr>
<td><img src="image17.png" alt="Substrate 9" /></td>
<td><img src="image18.png" alt="Main product 9" /></td>
<td>148 (\times 10^3)</td>
<td>99</td>
<td>Seelbach, 1997a</td>
</tr>
<tr>
<td><img src="image19.png" alt="Substrate 10" /></td>
<td><img src="image20.png" alt="Main product 10" /></td>
<td>90 (\times 10^3)</td>
<td>99</td>
<td>Van Deurzen, 1997b</td>
</tr>
<tr>
<td><img src="image21.png" alt="Substrate 11" /></td>
<td><img src="image22.png" alt="Main product 11" /></td>
<td>84 (\times 10^3)</td>
<td>99</td>
<td>Van Deurzen, 1997b</td>
</tr>
<tr>
<td><img src="image23.png" alt="Substrate 12" /></td>
<td><img src="image24.png" alt="Main product 12" /></td>
<td>41 (\times 10^3)</td>
<td>99</td>
<td>Allenmark, 1996</td>
</tr>
<tr>
<td><img src="image25.png" alt="Substrate 13" /></td>
<td><img src="image26.png" alt="Main product 13" /></td>
<td>850 (\times 10^3)</td>
<td>-</td>
<td>Van Deurzen, 1997c</td>
</tr>
<tr>
<td><img src="image27.png" alt="Substrate 14" /></td>
<td><img src="image28.png" alt="Main product 14" /></td>
<td>32 (\times 10^3)</td>
<td>-</td>
<td>Van Deurzen, 1997d</td>
</tr>
<tr>
<td><img src="image29.png" alt="Substrate 15" /></td>
<td><img src="image30.png" alt="Main product 15" /></td>
<td>312</td>
<td>-</td>
<td>Corbett, 1978</td>
</tr>
</tbody>
</table>

a) TTN: total turnover number calculated from experimental details.

Other peroxidases, such as HRP, LPO, and GiP, also catalyze oxygen transfer reactions, such as sulfoxidation (Colonna, 1995; Tuynman, 1998) albeit with lower selectivities and much lower TTN, compared to CPO. Their catalytic properties could be improved via site-directed mutagenesis (section 4.3).

When the oxidation of aniline derivatives was catalyzed by HRP a complex mixture of colored high molecular weight products was obtained (Corbett, 1978; Holland, 1968), pointing to a radical mechanism. However, selective oxygen transfer to N-atoms catalyzed by HRP
has also been reported by Zaks and coworkers (Kalliney, 1995). They studied the HRP-catalyzed gram-scale oxidation of the hydroxylamino and nitroso derivative of everninomicin (an antibiotic) to the nitro derivative.

**Oxidative halogenation** reactions are catalyzed by the so-called haloperoxidases. Both heme-dependent and vanadium-dependent haloperoxidases are known (Butler, 1993; Littlechild, 1999). One of the most versatile haloperoxidases is the chloroperoxidase from *C. fumago*, which is involved in the biosynthesis of the fungal metabolite caldariomycin (1,1-dichloro-2,5-dihydroxycyclopentane). The chlorination or bromination of monochlorodimedone (MCD, scheme 4) is widely used as an assay for activity determination of haloperoxidase.

\[
\begin{align*}
\text{H}_2\text{C} & \quad \text{CH}_3 \\
\text{O} & \quad \text{Cl} \\
\text{H}^+ & \quad X^\cdot & \quad \text{H}_2\text{O}_2 & \quad 2\text{H}_2\text{O} \\
\text{H}_2\text{C} & \quad \text{CH}_3 \\
\text{O} & \quad \text{Cl} & \quad X
\end{align*}
\]

**Scheme 4. Halogenation of monochlorodimedone by haloperoxidases (MCD-assay).**

Oxidative halogenation is believed to proceed via an active halide species. In the case of vanadium and heme peroxidases this active species is probably hypohalous acid, which is formed by oxidation of the corresponding halide ion (pathway 2 in scheme 2). The lack of stereospecificity observed for peroxidase-catalyzed oxidative halogenations is consistent with the participation of an active halogenation species that acts outside the catalytic center.

**Hydrogen peroxide disproportionation**, the so-called catalase activity, is catalyzed by many peroxidases, either directly (pathway 3 in scheme 2) or by the production of hypohalous acid, which subsequently reacts with $\text{H}_2\text{O}_2$. Especially CPO exhibits substantial catalase activity in the absence of oxidizable substrates (Sun, 1994). In order to obtain high oxidant selectivities in peroxidase-catalyzed reactions this catalase activity must be minimized.

**Oxidative dehydrogenation** is mainly restricted to heme peroxidases and is referred to as the classical peroxidase reaction. The latter involves single electron transfer processes and radical cations and radicals as reaction intermediates (pathway 4 in scheme 2). The iron(IV)oxo radical cation species (compound I), for example, is reduced to the native enzyme via two one-electron transfers, that are believed to proceed at the heme edge rather than at the iron oxo moiety. The intermediate iron(IV)oxo species, which is one oxidation state above the native enzyme, is called compound II.

Among the classical peroxidases, e.g. HRP, SBP, CcP, CiP, and LPO, horseradish peroxidase (HRP) is the one with the broadest substrate specificity for one-electron oxidations. Peroxidases catalyze a wide variety of one-electron oxidations of electron-rich aromatics (table 5), which include assays for activity determination based on guaiacol (entry 1) and ABTS (entry 2).
In addition to phenol derivatives anilines are also oxidized, leading to polymeric products (entry 3). The polymeric products formed upon oxidation of aromatics form water-insoluble aggregates, which are readily separated from solution by sedimentation or filtration. HRP is frequently studied for the removal of aromatic pollutants from waste waters produced by, for example, the paper and textile industries (Karam, 1997; Nicell, 1995; Peralta-Zamora, 1998). Pulp and paper bleaching is also possible with lignin peroxidases from white rot fungi, which catalyze the depolymerization of lignin (entry 4).

Dealkylation reactions are difficult to perform in organic chemistry and often harsh conditions or stoichiometric amounts of reagents are required. Heme proteins, peroxidases included, catalyze the oxidative dealkylation of heteroatoms under mild conditions, for example the O-demethylation of 9-methoxyellipticine (entry 5).

Table 4. Summary of peroxidase-catalyzed one-electron oxidations.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Product</th>
<th>Peroxidase</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1     | \[
\begin{array}{c}
\text{OCH}_3 \\
\text{OH}
\end{array}
\] | \[
\begin{array}{c}
\text{OCH}_3 \\
\text{O}
\end{array}
\] | all | Gallagher, 1923 |
| 2     | \[
\begin{array}{c}
\text{NH}_4 \\
\text{O}_3\text{S}
\end{array}
\] \[
\begin{array}{c}
\text{S} \\
\text{N}
\end{array}
\] \[
\begin{array}{c}
\text{S} \\
\text{N}
\end{array}
\] \[
\begin{array}{c}
\text{NH}_4 \\
\text{O}_3\text{S}
\end{array}
\] | all | Childs, 1975 |
| 3     | \[
\begin{array}{c}
\text{NH}_2 \\
\text{NH}_2
\end{array}
\] | \[
\begin{array}{c}
\text{N}-
\end{array}
\] | HRP | Kobayashi, 1995 |
| 4     | Wood (Lignine + cellulose) | Cellulose (+ depolymerized lignin) | LiP | Schoemaker, 1990 |
| 5     | \[
\begin{array}{c}
\text{H}_3\text{CO} \\
\text{H}_3
\end{array}
\] \[
\begin{array}{c}
\text{H}_3\text{CO} \\
\text{H}_3
\end{array}
\] | \[
\begin{array}{c}
\text{O} \\
\text{N}
\end{array}
\] \[
\begin{array}{c}
\text{N} \\
\text{N}
\end{array}
\] | HRP | Meunier, 1985 |

a) Lignin is a highly irregular three-dimensional biopolymer composed of oxygenated phenylpropane units, e.g. p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol.

3. Instability of peroxidases

Although peroxidases have enormous potential as biocatalysts, commercial applications are limited. For example, a combination of amylglucosidase, glucose oxidase, and lactoperoxidase is applied in toothpaste (Zendium™) to oxidize thiocyanate as an antimicrobial additive. Quest International (Naarden, The Netherlands) commercialized soybean peroxidase as an additive in dough preparation where it catalyzes the oxidative cross-linking of peptide chains.

The most developed field for commercial applications of peroxidases is in analytical diagnostics, for example in biosensors and immunoassays (Black, 1994; Tamaki, 1995; Yamazaki, 1995). In most diagnostic applications HRP is used to oxidize a chromogen with the
$\text{H}_2\text{O}_2$ produced by a substrate specific oxidase. For example glucose oxidase in the case of colorimetric diagnostic kits for the determination of glucose in blood serum or plasma (Wilson,1992).

To our knowledge there are no examples of the use of peroxidases as catalysts in industrial organic synthesis. The relatively low volumetric productivities (space-time yields) and the limited commercial availability and low stabilities seriously hamper their commercial application. In particular, heme peroxidases are readily deactivated by the oxidant, resulting in a low operational stability.

Heme enzymes are susceptible to oxidative destruction of the porphyrin ring, which may involve various activated oxygen species, e.g. hydrogen peroxide, superoxide anion, hydroxyl radicals or singlet oxygen. All these activated oxygen species may be produced during the catalytic cycle of peroxidases and may damage the peroxidase irreversibly. Heme oxidation in the presence of $\text{H}_2\text{O}_2$ has been reported for cytochrome P450 monoxygenases (Ortiz de Montellano,1986) as well as for HRP (Arnao,1995; Bagger,1971).

Alternatively, heme deactivation can involve irreversible reaction with so-called suicide inhibitors. An example of suicide deactivation is the heme alkylation observed during CPO-catalyzed epoxidation of 1-alkenes (Dexter,1995b). Suicide inhibitors are frequently used to study the active site structure and mechanistic details of peroxidases (DePillis,1990; Ortiz de Montellano,1988; Samokyszyn,1991). For example, phenylhydrazine, alkylhydrazines, and sodium azide, when oxidized by peroxidases yield radicals that react with the peroxidase active site.

\[
\begin{align*}
\text{P Fe}^{\text{II}} + \text{O}_2 & \rightarrow \text{P Fe}^{\text{III}} \text{O}_2^-
\text{P Fe}^{\text{III}} + \text{O}_2 & \rightarrow \text{P Fe}^{\text{IV}}\text{O}_2^-
\text{P Fe}^{\text{IV}} = \text{O} + \text{H}_2\text{O} & \rightarrow \text{P Fe}^{\text{IV}}\text{O}_2^- + \text{H}_2\text{O}
\end{align*}
\]

\textbf{Scheme 5.} Formation and mesomeric structures of peroxidase compound III $[\text{P}= \text{porphoporphyrin IX}].$

A third deactivation pathway is the formation of the so-called compound III, often referred to as oxyperoxidase (Scheme 5). There are three ways to obtain peroxidase compound III: addition of molecular oxygen to the ferrous form of peroxidases, addition of superoxide anion to the native enzyme, and addition of an excess of $\text{H}_2\text{O}_2$ to either the native peroxidase or its compound II. The first route to compound III is comparable to the cytochrome P450 catalytic cycle. However, in the P450 cytochromes reducing equivalents are available from NADH that reduce the iron(III)peroxide radical to the iron(III)-hydroperoxo intermediate that yields the active iron(V)oxo intermediate upon cleavage of the O-O bond. In peroxidases no reducing equivalents are available and the accumulation of compound III during catalysis leads to a decreasing amount of available enzyme. When compound III is not involved in the catalytic cycle, apparent deactivation takes place. An overview of the stability and structure of compound III from different peroxidases is given by Van Deurzen (1997a).

In contrast to heme peroxidases, the vanadium-dependent peroxidases are far more stable towards oxidative deactivation, since they do not bear a porphyrin ring (Tromp,1989).
However, deactivation was reported (Soedjaj, 1995) to occur at low pH (4 to 5) and very high hydrogen peroxide concentrations (100 mM). This oxidative deactivation resulted from the formation of 2-oxohistidine (Meister Winter, 1996). The formation of 2-oxohistidine is only observed under catalytic turnover when all the required components of turnover, bromide ion, $\text{H}_2\text{O}_2$, and VBPO, are available. It is tempting to speculate that active site histidine residues were involved in oxidative deactivation of vanadium peroxidases, however no results proving this hypothesis are available. The oxidative deactivation by 2-oxohistidine formation is prevented by the addition of organic substrates.

4. Stabilization of peroxidases

As outlined in the preceding sections, the application of peroxidases is limited by their low operational stability caused by oxidative deactivation by hydrogen peroxide. Several approaches have been used to increase the stability of peroxidases. One approach involves regulation of the hydrogen peroxide concentration using a feed-on-demand system or in situ generation of $\text{H}_2\text{O}_2$. Alternative approaches involve the improvement of the stability of the enzyme via protein engineering or via the design of synthetic peroxidases.

4.1 Feed-on-demand system

The mode of addition of $\text{H}_2\text{O}_2$ is crucial for obtaining high yields in sulfoxidation reactions catalyzed by CPO (Van Deurzen, 1994). Nowadays the use of syringes and pumps is quite common for CPO-catalyzed oxidations (Hu, 1999; Lakner, 1996). However continuous slow addition has two major drawbacks: a) the addition of $\text{H}_2\text{O}_2$ has to be stopped before or immediately after the reaction is completed, otherwise an excess of oxidant will cause a rapid deactivation of the enzyme, b) the rate of $\text{H}_2\text{O}_2$ addition needs to be adjusted to the rate of reaction. This complicates the procedure as the rate is different for every type of reaction and substrate. The reaction rate declines when the substrate is present in non-saturating concentrations and therefore the reaction always proceeds under non-optimal conditions. A peroxide-stat, which controls the $\text{H}_2\text{O}_2$ concentration at a constant low level by adjusting the delivery rate according to the progress of the reaction (feed-on-demand), would facilitate the oxidation procedure. Van Deurzen (1997c) and Seelbach (1997b) studied the use of a hydrogen peroxide-stat for the CPO-catalyzed oxidation of indole to 2-oxindole. The TTN for this reaction was increased 20-fold from $40 \cdot 10^3$ (Corbett, 1979) to $850 \cdot 10^3$ when a hydrogen peroxide-stat for the controlled addition of the oxidant was used. The highest STY (120 g L$^{-1}$d$^{-1}$) was obtained in a continuous system based on an enzyme membrane reactor.

![Scheme 6. Schematic representation of the sensor-controlled fed-batch apparatus.](image-url)
Introduction

As a spin-off of the study on the use of the sensor-controlled addition of H$_2$O$_2$, the deactivation of CPO during indole oxidation was studied (Van Deurzen, 1997c). This revealed that tert-butyl alcohol (used as a cosolvent) protects the enzyme against oxidative deactivation during catalytic oxidation. When the oxidation of indole was performed in a sensor-controlled procedure (30 μM H$_2$O$_2$) in aqueous buffer, CPO was deactivated. In contrast, no deactivation was observed in mixtures of tert-butyl alcohol and aqueous buffer (30:70 or 50:50; v:v). Probably, tert-butyl alcohol acts as a radical scavenger (chapter 3) and protects CPO against deactivation by reactive radicals.

Also sensor-controlled sulfoxidation and epoxidation reactions catalyzed by CPO were studied by Seelbach (1997a) The turnover numbers for these two reactions were also increased by the use of the H$_2$O$_2$-stat, however, the improvement was not as impressive as for the oxidation of indole. The TTN for the oxidation of thioanisole was increased from 41·10$^3$ (Fu, 1992) to 148·10$^3$ in a sensor-controlled batch-wise oxidation. For the epoxidation of cis-2-heptene the TTN was improved with a factor 4.3 from 1700 (Allain, 1993) to 7400 in a sensor-controlled batch-wise oxidation.

4.2 In situ formation of hydrogen peroxide

The feed-on-demand system afforded an improvement of the TTN of CPO-catalyzed oxidations. However, the concentrated hydrogen peroxide solution is introduced at a single point into the reaction vessel, which can result in hot spots around the entry point, resulting in deactivation of the peroxidase.

These 'hot spots' can be avoided by in situ generation of hydrogen peroxide from oxygen and a cosubstrate. For example, glucose oxidase catalyzes the formation of gluconate and hydrogen peroxide from glucose and oxygen. This approach was used in the CPO catalyzed production of epoxides and glycols (Neidlemann, 1981). Other oxidases in combination with other cosubstrates can, in principle, be used in this approach. The wide variety of oxidases available makes it possible to fine-tune the combination of oxidase/peroxidase system in terms of pH-optimum, (co)solvent, and cosubstrate.

Glucose oxidase is the enzyme of choice since it has been extensively studied and it is stable (Hecht, 1993a,b; Wilson, 1992), readily available and applied commercially in glucose sensors and diagnostic kits. The use of glucose oxidase for the in situ generation of H$_2$O$_2$ in peroxidase-catalyzed oxidation reactions is described in chapter 2.

Improvements made in the stabilization of CPO are summarized in table 6 for the sulfoxidation of thioanisole (the ee of the sulfoxide exceeded 99% for all the methods). Starting at a TTN of 41·10$^3$ a two-fold increase was made when the oxidant was added continuously instead of step-wise. Further improvement was made by a sensor-controlled addition of H$_2$O$_2$. And finally the in situ formation of H$_2$O$_2$ resulted in the highest TTN ever obtained. The high STY obtained in the continuous and feed-on-demand addition resulted from the use of a cosolvent that allowed higher substrate concentrations. The very low TOF and STY for the step-wise addition resulted from the long reaction time applied. The lower TOF observed with continuous addition versus feed-on-demand is consistent with the notion that the former leads to sub-optimum conditions. The lower TOF for the in situ generation of H$_2$O$_2$ (500 min$^{-1}$) suggests that the oxidation of glucose is the rate limiting step and no H$_2$O$_2$ build-up takes place.
Table 6. Improvements made in the CPO-catalyzed oxidation of thioanisole.

<table>
<thead>
<tr>
<th>Mode of H₂O₂ addition</th>
<th>TTN (10³)</th>
<th>TOF (min⁻¹)</th>
<th>STY (g L⁻¹d⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step-wise a</td>
<td>41</td>
<td>11</td>
<td>0.7</td>
<td>Fu,1992</td>
</tr>
<tr>
<td>Continuous b</td>
<td>108</td>
<td>900</td>
<td>84</td>
<td>Van Deurzen,1997b</td>
</tr>
<tr>
<td>Feed-on-demand c</td>
<td>148</td>
<td>1375*</td>
<td>57</td>
<td>Seelbach,1997a</td>
</tr>
<tr>
<td>In situ generation d</td>
<td>250</td>
<td>500*</td>
<td>23</td>
<td>Chapter 2</td>
</tr>
</tbody>
</table>

a) 10 mM in aqueous solution; 1.2 eq of H₂O₂ added in 40 aliquots over 60 h;
b) 50 mM in 50% t-BuOH; 1.1 eq of H₂O₂ added in 2 h;
c) 20 mM in 30% t-BuOH; H₂O₂ concentration controlled at 50 μM;
d) 15 mM in aqueous solution; 1 eq of glucose added in one step;
e) initial TOF.

4.3 Improvement of the catalytic properties by protein engineering

Site-directed mutagenesis has proved to be a powerful tool in exploring the structure-function relationships in classical peroxidases (Smith,1998). Especially horseradish peroxidase isoenzyme C, the crystal structure of which was solved in 1997 (Gaihede), has been studied in great detail. For example, Morishima and co-workers (Nagano,1996; Tanaka,1997) used site-directed mutagenesis to prove the catalytic role of the amino acid residues His42, Asn70, and Glu64. These amino acid residues form a long distance hydrogen-bonding network on the distal side of HRP that is essential for efficient catalysis.

Insights obtained with site-directed mutagenesis serve as a template for the design of peroxidases with enhanced catalytic properties. The almost negligible two-electron oxidation activity of HRP may result from steric restrictions hindering the access of substrate molecules to the ferryl species. Restricted access to the ferryl species does not limit the one-electron oxidations because these occur at the heme edge rather than by a direct transfer to the ferryl species (Savenkova,1998; and references therein). The crystal structure of HRP indicates that the key residues in HRP that limit the access to the ferryl species are Phe41, His42, and Arg38. Especially His42 is critical as it is positioned in the center of the access channel. Reduction of the size of these residues is not straightforward, as His42 and Arg38 are critical catalytic residues.

Table 7 summarizes the results obtained with different HRP mutants that were designed by Ortiz de Montellano and coworkers to be better oxygen transfer catalysts. Substitution of the bulky Phe41 for an alanine (F41A: Newmyer,1995) or a leucine (F41L: Ozaki,1994,1995) resulted in an improved sulfoxidation and epoxidation catalyst. Comparable results were obtained with mutants in which His42 was replaced with alanine or valine (H42A and H42V: Newmyer,1995). However the rate of compound I formation for these last two mutants was decreased by a factor 10⁶. The catalytic activity could be restored by the addition of exogenous imidazoles (Newmyer,1996) or by the introduction of a histidine on the position of the Phe41 (F41H/H42A: Savenkova,1996). In the latter mutant the catalytic activity was restored, but there is still a bulky residue at position 41 that restricts access to the iron center. In a recent study (Savenkova,1998) the possibilities for replacement of Arg38 were studied. Promising results were obtained with the R38H/H42V double mutant in which the distal histidine is moved deep into the active site. For example, the oxidation of thioanisole was catalyzed 1400 fold more efficiently compared with the wild type.
Introduction

Table 7. Oxidation of thioanisole and styrene catalyzed by HRP and its mutants\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (s\textsuperscript{-1})</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (mM\textsuperscript{-1}s\textsuperscript{-1})</th>
<th>ee (%)</th>
<th>$k_{\text{cat}}$ (s\textsuperscript{-1})</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (mM\textsuperscript{-1}s\textsuperscript{-1})</th>
<th>SO\textsuperscript{b} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native HRP</td>
<td>0.05</td>
<td>0.6</td>
<td>0.08</td>
<td>58 (S)</td>
<td>1.0 $\times$ 10\textsuperscript{4}</td>
<td>0.3</td>
<td>3.3 $\times$ 10\textsuperscript{6}</td>
<td>42</td>
</tr>
<tr>
<td>F41A</td>
<td>0.13</td>
<td>30 (S)</td>
<td></td>
<td></td>
<td>0.1 $\times$ 10\textsuperscript{3}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F41L</td>
<td>0.32</td>
<td>4.5</td>
<td>0.07</td>
<td>97 (S)</td>
<td>3.3 $\times$ 10\textsuperscript{4}</td>
<td>1.4</td>
<td>2.4 $\times$ 10\textsuperscript{5}</td>
<td>62</td>
</tr>
<tr>
<td>H42A</td>
<td>0.03</td>
<td>0.3</td>
<td>0.1</td>
<td>84 (S)</td>
<td>7.0 $\times$ 10\textsuperscript{3}</td>
<td>2.9</td>
<td>2.4 $\times$ 10\textsuperscript{3}</td>
<td>62</td>
</tr>
<tr>
<td>H42V</td>
<td>0.01</td>
<td>86 (S)</td>
<td></td>
<td></td>
<td>3.5 $\times$ 10\textsuperscript{4}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F41H/H42A</td>
<td>5.3</td>
<td>8.4</td>
<td>0.63</td>
<td>&gt;99 (S)</td>
<td>2.4 $\times$ 10\textsuperscript{2}</td>
<td>2.5</td>
<td>9.6 $\times$ 10\textsuperscript{3}</td>
<td>63</td>
</tr>
<tr>
<td>R38A</td>
<td>9.1</td>
<td>0.6</td>
<td>15</td>
<td>28 (R)</td>
<td>2.3 $\times$ 10\textsuperscript{2}</td>
<td>3.1</td>
<td>7.4 $\times$ 10\textsuperscript{3}</td>
<td>36</td>
</tr>
<tr>
<td>R38H</td>
<td>0.05</td>
<td>0.5</td>
<td>0.1</td>
<td>32 (S)</td>
<td>3.7 $\times$ 10\textsuperscript{3}</td>
<td>1.9</td>
<td>1.9 $\times$ 10\textsuperscript{3}</td>
<td>38</td>
</tr>
<tr>
<td>R38H/H42V</td>
<td>33</td>
<td>0.3</td>
<td>110</td>
<td>40 (S)</td>
<td>3.8 $\times$ 10\textsuperscript{3}</td>
<td>0.4</td>
<td>7.8 $\times$ 10\textsuperscript{3}</td>
<td>29</td>
</tr>
</tbody>
</table>


\textsuperscript{b} selectivity for the formation of styrene oxide.

Recently, the first report about mutagenesis on CPO was published by Hager and coworkers (Rai, 1999). Random mutagenesis was used to obtain mutants that were completely resistant to the suicide deactivation that occurs with the native CPO during the epoxidation of 1-alkenes (Dexter, 1995a,b). The best result was obtained with a fourth generation mutant that contained 7 mutated amino acid residues. All these mutated residues lie at the periphery of the enzyme, far away from the heme prosthetic group and the active site of CPO. The authors explained this contradiction by the suggestion that the mutations could promote the release of product from the heme-alkylated enzyme, but provided no evidence to support this. It was already shown for the wild type CPO that the active enzyme will spontaneously regenerate from inactive CPO (Dexter, 1995b). However, regeneration took place on a time scale of hours, whereas the deactivation tooks place in seconds. The authors suggested that the mutated amino acid residues catalyze the release of the alkyl group from the alkylated porphyrin group at a higher rate than the wild type residues.

In common with peroxidases, myoglobin (Mb) bears a protoporphyrin IX as a prosthetic group. Its major physiological role is the storage and transfer of molecular oxygen. However, in the presence of peroxide Mb catalyzes the one- and two-electron oxidation of a variety of substrates at very low rates (Levinger, 1995).

Appropriate site-directed mutagenesis in the active site (F43H/H64L, H64S, H64A, and H64L Mb) turned Mb into a semi-synthetic peroxidase showing the characteristic ferryl porphyrin radical cation species as a reactive intermediate in two-electron oxidations (Matsui, 1996; Adachi, 1993). Changes in the position of the distal histidine (L29H/H64L and F43H/H64L) turned Mb into a peroxidase that exhibited catalytic turnover with high stereospecificity (Ozaki, 1996, 1997, 1999). The L29H/H64L mutant yielded the highest ee in the oxidation of sulfides (97%; the $R$-enantiomer is predominantly formed) and in the oxidation of cis-$\beta$-methylstyrene (99%). The F43H/H64L mutant yielded the highest rates of oxidation, for example 47 min$^{-1}$ for thioanisole, which is still much lower than rates obtained with CPO (1375 min$^{-1}$; Seelbach, 1997a).
Table 8. Enantioselective oxidations catalyzed by myoglobin mutants (Ozaki, 1999).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>wild type</th>
<th>L29H/H64L</th>
<th>F43H/H64L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TOF (min⁻¹)</td>
<td>ee (%)</td>
<td>TOF (min⁻¹)</td>
</tr>
<tr>
<td>[Structure]</td>
<td>0.25</td>
<td>25 (R)</td>
<td>5.5</td>
</tr>
<tr>
<td>[Structure]</td>
<td>0.46</td>
<td>8 (R)</td>
<td>6.5</td>
</tr>
<tr>
<td>[Structure]</td>
<td>0.65</td>
<td>27 (R)</td>
<td>1.6</td>
</tr>
<tr>
<td>[Structure]</td>
<td>2.2</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>[Structure]</td>
<td>0.8</td>
<td>5 (R)</td>
<td>3.2</td>
</tr>
<tr>
<td>[Structure]</td>
<td>0.4</td>
<td>4 (R)</td>
<td>2.4</td>
</tr>
<tr>
<td>[Structure]</td>
<td>0.015</td>
<td>9 (R)</td>
<td>0.14</td>
</tr>
<tr>
<td>[Structure]</td>
<td>0.076</td>
<td>39 (1R,2R)</td>
<td>0.29</td>
</tr>
<tr>
<td>[Structure]</td>
<td>0.003</td>
<td>3 (1R,2S)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

4.4 Design of a semi-synthetic peroxidase

Stability improvements based on modification of known peroxidases have their limitations. For example, heme-dependent peroxidases will always contain the heme moiety that is intrinsically unstable. The design of semi-synthetic peroxidases that do not bear a porphyrin moiety, will circumvent this instability. Two examples of semi-synthetic peroxidases will be mentioned in this section: based on selenium and on vanadium.

Seleno-subtilisin is a glutation peroxidase mimic which was originally designed and synthesized by Wu and Hilvert (1989, 1990). The active site serine (Ser221) of subtilisin is converted into a selenic acid residue. Subtilisin is one of the most important commercially produced enzymes (world market of 500 tons year⁻¹) and is mainly applied as a detergent additive. Hence, it is a readily available starting material for the synthesis of semi-synthetic peroxidases.

The synthesis of seleno-subtilisin was reported on 10 g ram-scale with an overall yield of 20 to 25% (Häring, 1998a) using a three-step protocol (scheme 7). In the first step the Ser221 was activated by reaction with phenylmethanesulfonyl fluoride. Selenium was subsequently introduced into the active site by reaction of the activated Ser221 with sodium hydrogen selenide. Finally, the seleno-subtilisin was oxidized with H₂O₂ to its selenic acid form.

Seleno-subtilisin catalyzes the reduction of hydroperoxides with thiols via the catalytic cycle of glutathion peroxidase (scheme 8). This reaction can be used for the kinetic resolution of racemic hydroperoxides (Häring, 1997, 1998b). As shown in table 9 moderate to excellent ees were reported for both the remaining hydroperoxide and for the formed alcohol. Also the kinetic parameters (kcat up to 2443 min⁻¹) reached normal enzymatic proportions. A stable heterogeneous semi-synthetic peroxidase was obtained when first
cross-linked enzyme crystals (CLC) from subtilisin were prepared followed by the introduction of the seleno moiety (Haring, 1998c, 1999). CLC seleno-subtilisin is an immobilized biocatalyst that can be recycled ten times without loss of activity and selectivity. However there is one major drawback of this method: it consumes 2 eq of 5-thio-2-nitrobenzoic acid per equivalent of alkyl hydroperoxide.

![Scheme 7](image)

Scheme 7. Schematic three-step synthesis of seleno-subtilisin. Ser221 of subtilisin (Enz-OH) is transferred into selenic acid.

![Scheme 8](image)


### Table 9. Enantioselectivities of the seleno-subtilisin-catalyzed kinetic resolution of hydroperoxides

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Hydroperoxide ee (%)</th>
<th>Alcohol ee (%)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/K_m$ (mM⁻¹ min⁻¹)</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 (R)</td>
<td>52 (R)</td>
<td>60 (S)</td>
<td>2125</td>
<td>135</td>
<td>6.6</td>
</tr>
<tr>
<td>48 (R)</td>
<td>48 (R)</td>
<td>56 (S)</td>
<td>1723</td>
<td>287</td>
<td>5.6</td>
</tr>
<tr>
<td>98 (S)</td>
<td>98 (R)</td>
<td>98 (R)</td>
<td>2243</td>
<td>1150</td>
<td>&gt;200</td>
</tr>
<tr>
<td>40 (S)</td>
<td>40 (S)</td>
<td>42 (R)</td>
<td>1745</td>
<td>97</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Vanadium-incorporated phytase is a semi-synthetic peroxidase that mimics the vanadium-dependent haloperoxidases. In common with seleno-subtilisin, the vanadate-phytase is based on a cheap and commercially available enzyme (phytase is used on a large scale in animal feed). Sequence alignment of the vanadium chloroperoxidase from *C. officinalis* revealed that this enzyme is structurally closely related to the (membrane-bound) acid phosphatases (Hemrika, 1997; Neuwald, 1997). Moreover, the apo-vanadium CPO was shown to exhibit phosphatase-like activity. Incorporation of vanadate into the active site of acid phosphatases and the related phytases and sulfatase led to the formation of stable semi-synthetic peroxidases that catalyze enantioselective sulfoxidation (chapter 8).
5 Scope of this thesis

This thesis, entitled The application of peroxidases in the synthesis of fine chemicals, is based on the PhD-project that had the same title and was financed by the Dutch Innovation Oriented Program on Catalysis (IOP-catalysis; project IKA94013). A characteristic feature of IOP catalysis projects is their relevance to industrial problems, which is essential for justification of the project. The objective and justification of this IOP catalysis project, as formulated in its fact sheet are:

**Objective:** The development of catalysis by peroxidases in the manufacture of fine chemicals and chiral intermediates.

**Justification:** Oxidation procedures that use high-valency metal compounds in stoichiometric amounts are environmentally unacceptable and economically unattractive. Peroxidases are potentially attractive catalysts that use hydrogen peroxide as a clean and inexpensive oxidant.

Unlike monooxygenases, peroxidases do not require a stoichiometric amount of a cosubstrate such as NADH to supply reducing equivalents. Furthermore, some peroxidases, notably haloperoxidases, are able to catalyze synthetically useful oxygen transfer reactions with high selectivities. However, the application of peroxidases is seriously hampered by their low operational stability. Hence, a major theme of this thesis is the improvement of the operational stability of peroxidases. Commercial viability is, in the final analysis, determined by the enzyme costs per kg of product which are largely dependent on the price of the enzyme and its operational stability.

Throughout this thesis two model reactions were used to study the stability of the peroxidases: the regioselective oxidation of indole to oxindole (scheme 9) and the enantioselective oxidation of thioanisole (methyl phenyl sulhide a prochiral substrate; scheme 10).

![Scheme 9. Regioselective oxidation of indole to 2-oxindole.](image)

![Scheme 10. Enantioselective oxidation of thioanisole.](image)

Our initial studies were concerned with the stabilization of native peroxidases. The feed-on-demand and continuous addition of H₂O₂ still have the disadvantage that a concentrated oxidant solution is added that can result in local high concentrations. The use of glucose oxidase to produce H₂O₂ in situ from glucose and oxygen to avoid these 'hot spots' is described in chapter 2. An attempt to use dioxygen as primary oxidant in the presence of a sacrificial reductant is discussed in chapter 3.

The immobilization of peroxidases and their use as stable catalysts for enantioselective oxidations in predominantly organic media is described in chapters 4 and 5, respectively.
Since modification of external factors did not prove sufficient to render peroxidases stable enough for industrial viability the peroxidase itself has to be modified. Chapter 6 describes attempts to modify the iron-center of CPO, as well as the genetic modification of CPO in its natural organism: the mold Caldariomyces fumago. The expression of recombinant CPO in another host, the fungus Aspergillus niger, which offers the possibility of homologous expression of mutant CPO, is discussed in chapter 7.

However, stability improvements based on modification of heme-dependent peroxidases appear to be limited by the intrinsically low stability of the heme moiety to oxidative degradation. Vanadium-dependent peroxidases, on the other hand, exhibit higher stabilities but are hampered by narrow scope and/or low activities. Hence, attention was turned to the design of semi-synthetic vanadium-dependent peroxidases (chapter 8).
Abstract

The stability of peroxidases was considerably enhanced by generating hydrogen peroxide in situ from glucose and oxygen. For example, the total turnover number of microperoxidase-11 for the oxidation of thioanisole was increased 7-fold compared to that obtained with continuous addition of \( \text{H}_2\text{O}_2 \). Coimmobilization of peroxidases with glucose oxidase into polyurethane foams afforded heterogeneous biocatalysts in which the hydrogen peroxide is formed inside the polymeric matrix from glucose and oxygen. The total turnover number of chloroperoxidase for the oxidation of thioanisole and cis-2-heptene was increased to new maxima of \( 250 \times 10^3 \) and \( 10 \times 10^3 \), respectively, upon coimmobilization with glucose oxidase. Soybean peroxidase, which normally shows only classical peroxidase activity, was transformed into an oxygen transfer catalyst when coimmobilized with glucose oxidase. The combination catalyst mediated the enantioselective oxidation of thioanisole (50% ee the \( S \)-enantiomer being predominantly formed) with 210 catalyst turnovers.
Chapter 2

1 Introduction

The continuous addition of hydrogen peroxide, using a syringe or pump is quite common in peroxidase-catalyzed oxidations, because it dramatically reduces the deactivation of the enzyme (Hu, 1999; Lakner, 1996; Van Deurzen, 1994). However, the rate of addition of the oxidant has to be carefully tuned to the known rate of the reaction to keep the hydrogen peroxide concentration as low as possible. The use of a hydrogen peroxide-stat, which controls the $\text{H}_2\text{O}_2$ concentration at a constant low level by adjusting the delivery rate according to the progress of the reaction, has proved to be quite effective for CPO-catalyzed oxidations (Van Deurzen, 1997c; Seelbach, 1997b). The TTN for the oxidation of indole (3a) was increased from $40 \cdot 10^3$ (Corbett, 1979) to $850 \cdot 10^3$ in a sensor-controlled fed-batch reaction. Sensor-controlled sulfoxidation and epoxidation reactions catalyzed by CPO were studied by Seelbach (1997a), but the improvement was not as impressive as for the oxidation of indole. The TTN for the oxidation of thioanisole (2a) was increased from $41 \cdot 10^3$ (Fu, 1992) to $148 \cdot 10^3$ by sensor-control. For the epoxidation of cis-2-heptene (4a) the TTN was improved by a factor of 4.3 from 1700 (Allain, 1993) to 7400 in a sensor-controlled batch-wise reaction.

![Scheme 1. Reactions of glucose oxidase and peroxidases.](image)

The continuous or sensor-controlled addition of hydrogen peroxide still has the disadvantage that local high concentrations may occur around the entry point, resulting in deactivation of the peroxidase. These ‘hot spots’ can be avoided by in situ generation of hydrogen peroxide from oxygen and a cosubstrate. For example, glucose oxidase uses oxygen to oxidize D-glucose (1a) to $\text{H}_2\text{O}_2$ and D-gluconolactone (1b); the latter hydrolyzes rapidly to D-gluconic acid (1c). This approach was used for the CPO-catalyzed production of epoxides and glycols (Neidleman, 1981) and for the polymerization of phenolic compounds catalyzed by HRP (Uyama, 1997). Other oxidases in combination with other cosubstrates can, in principle, be used in this approach. The wide variety of oxidases makes it possible to fine-tune the combination of oxidase/peroxidase in terms of pH-optimum, (co)solvent, and cosubstrate. Glucose oxidase is the enzyme of choice since it has been extensively studied and it is stable (Hecht, 1993a, b; Wilson, 1992), readily available and applied commercially in glucose sensors. This prompted us to study the combination of peroxidases with glucose oxidase to afford a stable oxidation catalyst.
2 Results and discussion

2.1 Native enzymes in solution

Initially we studied the effect of *in situ* formation of H$_2$O$_2$ with three native peroxidases in solution by comparing continuous addition of H$_2$O$_2$ with continuous addition of glucose in the same concentration. In the latter case a 25-fold excess of glucose oxidase (enzyme activity with respect to the rate of glucose addition) was added to the reaction mixture to obtain instantaneous oxidation of glucose to gluconic acid and H$_2$O$_2$.

The procedure was carried out with microperoxidase-11 (MP-11, a cytochrome c digest that contains 11 amino acid residues covalently bound to a heme system) and soybean peroxidase (SBP). Total catalyst turnover numbers (TTN) were determined for the oxidation of thioanisole (2a). The effect on CPO was measured in the oxidation of 5-methoxyindole (3a) and cis-2-heptene (4a), which react with moderate TTN in the normal procedure. Measured TTNs are given in table 1.

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Substrate</th>
<th>H$_2$O$_2$ TTN$^a$ (-)</th>
<th>Glucose / O$_2$ TTN$^a$ (-)</th>
<th>ee (%)</th>
<th>TTN$^a$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP-11$^b$</td>
<td>thioanisole</td>
<td>340</td>
<td>2500</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SBP$^c$</td>
<td>thioanisole</td>
<td>1.6</td>
<td>24 (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPO$^d$</td>
<td>5-methoxyindole</td>
<td>7150</td>
<td>12500</td>
<td>&gt;95</td>
<td>&gt;95</td>
</tr>
<tr>
<td>CPO$^e$</td>
<td>cis-2-heptene</td>
<td>6300</td>
<td>11500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) TTN: total turnover number (μmol product/μmol peroxidase);
b) 5 ml scale (50% MeOH); 25 mM thioanisole; oxidant: 1 eq / 10h;
c) 20 ml scale; 1 mM thioanisole; oxidant: 1 eq / 50h;
d) 50 ml scale (50% t-BuOH); 10 mM 5-methoxyindole; oxidant: 1 eq / h;
e) 25 ml scale (30% t-BuOH); 26 mM cis-2-heptene; oxidant: 1 eq / 4h;
f) no detectable amount of sulfoxide formed.

Microperoxidase is known to be operationally unstable. *In situ* generation of H$_2$O$_2$ resulted in a 7-fold improvement of the TTN. SBP did not exhibit oxygen transfer activity, even when H$_2$O$_2$ was added continuously and at a very low rate to the reaction mixture. However, with *in situ* formation of H$_2$O$_2$ a very low conversion (1.6 turnovers) to thioanisole sulfoxide (24% ee; the S-enantiomer being predominantly formed) was obtained. The effect on CPO was much less, but still a nearly two-fold increase of the TTN could be obtained.

2.2 Coimmobilization of peroxidases with glucose oxidase

The enzyme-catalyzed oxidation of sulfides by hydrogen peroxide is always in competition with non-enzymatic oxidation. A highly active biocatalyst, e.g. the chloroperoxidase from *C. fumago*, gave enantiomerically pure sulfoxides, with no detectable background reaction (Van Deurzen,1997c). However when a less active catalyst, e.g. phytase (chapter 8) was used at relatively high oxidant concentrations, considerable amounts of racemic sulfoxide were formed. The oxidant concentration in solution and, hence, the blank reaction can be reduced by continuously generating the H$_2$O$_2$ inside a matrix in which the peroxidase is entrapped. The H$_2$O$_2$ is then consumed before it diffuses into the bulk solution.
This was achieved by coimmobilizing peroxidases and glucose oxidase into polyurethane (PUR) foams. Immobilization into PUR-foams has proved to be an efficient method for the immobilization of extracellular glycosylated enzymes, such as peroxidases (chapter 4). The glucose oxidase from *Aspergillus niger* is also a glycoprotein, with a carbohydrate content of 16% (Hecht, 1993a,b; Wilson, 1992) and, therefore, suitable for immobilization into PUR-foam. When GOX was covalently by coimmobilized with CPO into PUR-foam, no leaching was detected under washing conditions, indicating that all the protein was covalently bound into the polymeric matrix.

### Table 2. Application of coimmobilized peroxidases with glucose oxidase in PUR-foams.

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Reactant</th>
<th>H$_2$O$_2$</th>
<th>Glucose / O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial rate$^a$</td>
<td>TTN$^b$</td>
<td>ee</td>
</tr>
<tr>
<td>CPO$^c$</td>
<td>indole</td>
<td>2.0</td>
<td>7885</td>
</tr>
<tr>
<td>Phytase$^d$</td>
<td>thioanisole</td>
<td>1.3</td>
<td>24</td>
</tr>
<tr>
<td>SBP$^e$</td>
<td>thioanisole</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- a) initial rate in μmol product per min per gram foam;
- b) total turnover number (μmol product/μmol peroxidase);
- c) 50 ml scale (40% t-BuOH); 50 mM indole; H$_2$O$_2$ added with H$_2$O$_2$-stat (set point: 30 μM); 1 eq of glucose was added at once;
- d) 20 ml scale (10% t-BuOH); 10 mM thioanisole; 1.1 eq of oxidant was added at once;
- e) 25 ml scale; 5 mM thioanisole; 0.5 eq of glucose was added at once;
- f) no detectable amounts of product formed.

Table 2 summarizes the results obtained with peroxidases coimmobilized with glucose oxidase into PUR-foams. The results obtained with phytase and SBP confirmed the notion that enantioselectivities could be increased by the formation of the oxidant inside a peroxidase-containing matrix. For phytase the ee of the formed sulfoxide increased from 21% to 69%, the maximum ee of phytase for this reaction (chapter 8), confirming that no background reaction took place when the H$_2$O$_2$ was generated inside the matrix. Furthermore, the rate of reaction was increased in the system in which H$_2$O$_2$ was formed inside the foam compared to external H$_2$O$_2$ addition. The results obtained with soybean peroxidase coimmobilized with GOX showed an increase of the TTN from 1.6 to 210 while the ee increased from 20% to 50%, compared to free enzymes in solution.

### 2.3 Detailed analysis of CPO/GOX-PUR

As mentioned in the introduction of this thesis, CPO from *C. fumago* is the most attractive peroxidase for synthetic applications and the CPO/GOX-PUR was studied in more detail using thioanisole oxidation as the test reaction. Figure 1 shows the concentration versus time curves of both glucose and thioanisole sulfoxide in the experiment in which 40 mg CPO/GOX-PUR was used to oxidize 15 mM thioanisole in the presence of 19 mM glucose. The rate of the oxidation of glucose (7.8 mM h$^{-1}$) is equal to the rate of the sulfoxidation of thioanisole (7.9 mM h$^{-1}$) and, therefore, we concluded that the oxidation of glucose is the rate limiting step. The ee of the formed sulfoxide exceeded 99%, which is equal to the enantioselectivity of native CPO (Van Deurzen, 1997b) and, hence, we conclude that no H$_2$O$_2$ diffused into the bulk solution.
**Figure 1.** Oxidation of thioanisole catalyzed by CPO/GOX-PUR: concentration glucose (●); concentration thioanisole sulfoxide (◆).

The effect of the glucose concentration on the rate of thioanisole oxidation was studied with 40 mg CPO/GOX-PUR and 15 mM thioanisole at glucose concentrations varying from 5 to 25 mM. In figure 2 the formation of thioanisole sulfoxide is plotted versus time and in figure 3 the initial rate is plotted versus the glucose concentration. The final product concentration was equal to the initial glucose concentration when an excess of thioanisole was used, whereas 100% conversion was achieved in experiments with an excess of glucose. In all experiments an ee of the formed sulfoxide of above 99% was obtained. The initial rate of thioanisole oxidation showed saturation kinetics with respect to the glucose concentration. From the data shown in figure 3 a $K_m$ for glucose of around 50 mM was estimated, which is higher than the $K_m$ of 20 mM reported for native glucose oxidase (Wilson, 1992). This increased $K_m$ for glucose suggests that immobilization of glucose oxidase into PUR-foam leads to diffusion limitations for glucose.

**Figure 2.** Plot of the sulfoxide concentration versus time for the oxidation of thioanisole (15 mM) at different amounts of glucose: 4.9 mM (●); 9.6 mM (◆); 14.2 mM (■); 22.7 mM (▲).

**Figure 3.** Plot of the initial rate versus the initial glucose concentration for the oxidation of thioanisole.
Chapter 2

The practical application of peroxidases is limited by the low solubility of most substrates in aqueous buffer solutions. This problem is usually solved by means of a mixture of aqueous buffer solution with a miscible organic solvent, such as tert-butyl alcohol (Van Deurzen, 1997b) or acetone (Allain, 1993). The addition of tert-butyl alcohol to the reaction mixture has a two-fold effect on the catalyst. The chloroperoxidase is stabilized by tert-butyl alcohol towards deactivation by $H_2O_2$ (Van Deurzen, 1997c), but, at the same time, its activity is decreased at increasing tert-butyl alcohol concentrations resulting in a lower TTN (Van Deurzen, 1994). The influence of the tert-butyl alcohol concentration on the activity and stability of CPO/GOX-PUR was studied at 15 mM thioanisole and 14 mM glucose.

![Graph showing sulfoxide formed versus time for the oxidation of thioanisole at different tert-butyl alcohol concentrations.](image1)

![Graph showing relative rate versus concentration of tert-butyl alcohol.](image2)

Figures 4 and 5 show the results of thioanisole oxidation catalyzed by CPO/GOX-PUR (40 mg) in aqueous buffer solution containing different amounts of tert-butyl alcohol. The relative rate of oxidation decreased to a stable value of 0.8 upon addition of tert-butyl alcohol. At tert-butyl alcohol concentrations above 70% the relative rate dropped dramatically to 0.1 at 90%. Similarly, the ee of the formed sulfoxide was maintained at 99% up to a tert-butyl alcohol concentration of 75% but dropped to 88% at 90% tert-butyl alcohol. The influence of the tert-butyl alcohol concentration on the TTN of the reaction was studied at a different substrate/enzyme-ratio (5 mg CPO/GOX-PUR and 34 mM thioanisole). When the tert-butyl alcohol concentration was increased from 0 to 50%, the TTN of the CPO/GOX-PUR catalyzed oxidation of thioanisole stayed at a constant value of around 200·10³. Above 50% tert-butyl alcohol the TTN decreased slightly to 120·10³ at 75% tert-butyl alcohol, followed by a significant drop to only 13·10³ at 90% tert-butyl alcohol. Compared to free CPO (Van Deurzen, 1994), the CPO/GOX-PUR catalyst is much more stable in the presence of organic cosolvent, as only a slight decrease in TTN was observed at 70% tert-butyl alcohol.

The operational stability of CPO/GOX-PUR was studied by decreasing the amount of enzyme used in the oxidation of thioanisole (15 mM). In an experiment in which 6.4 mg of foam was used a TTN for CPO of 250·10³ was reached after 24 hours at 100% conversion of thioanisole. The formed thioanisole sulfoxide had an enantiomeric purity of over 99%. This
In situ generation of hydrogen peroxide

TTN of 250·10^3 for CPO/GOX-PUR is much higher than the values reported in literature: 41·10^3 for the oxidation in aqueous buffer solution (Fu, 1992) and 148·10^3 for the sensor-controlled oxidation in aqueous buffer solution containing 30% (v:v) tert-butyl alcohol (Seelbach, 1997a). Compared to the best reactor design known for CPO today (sensor-control) the operational stability of CPO has been increased nearly two-fold upon coimmobilization with glucose oxidase.

The TOF of the CPO/GOX-PUR catalyst at this TTN is 500 min^{-1} based on the number of CPO molecules that is present in the foam. This TOF is lower than that of free CPO under the optimum conditions in H_2O_2 feed-on-demand oxidation of thioanisole (1375 min^{-1}; Seelbach, 1997a). This is consistent with the oxidation of glucose by glucose oxidase being the rate limiting step in CPO/GOX-PUR-catalyzed oxidation. Consequently, no build up of H_2O_2 should take place in the foam.

The epoxidation of cis-2-heptene (19 mM) catalyzed by CPO/GOX-PUR (10 mg) was performed in a mixture of aqueous buffer solution and tert-butyl alcohol (30% v:v) by the addition of 2.5 mM glucose every 1.5 hour. Under these conditions a TTN of 10·10^3 was obtained, which is in the same order as that obtained with free enzymes (table 1). These TTNs exceed the TTN obtained in a feed-on-demand procedure, which was the most successful method until now (Seelbach, 1997a). Initial TOF of the CPO/GOX-PUR catalyst for the epoxidation of cis-2-heptene is 50 min^{-1}. This TOF is lower than that of free CPO in a H_2O_2 feed-on-demand oxidation of cis-2-heptene (180 min^{-1}; Seelbach, 1997a). The operational stability of CPO in the epoxidation reaction probably could be increased by lowering the rate of H_2O_2 generation, either by decreasing the glucose concentration or by changing the CPO to GOX ratio in the PUR-foam.

3 Concluding remarks

In this chapter the approach to improve the stability of peroxidases via the in situ generation of hydrogen peroxide from glucose and oxygen has been discussed. Improvements varying from a two-fold to a 7-fold increase of the TTN for respectively CPO and the much more unstable microperoxidase-11 were obtained when continuous addition of H_2O_2 was replaced by in situ generation.

Coimmobilization of chloroperoxidase and glucose oxidase into polyurethane foams produces a biocatalyst that is extremely efficient in enantioselective sulfoxidation via in situ hydrogen peroxide formation from glucose and oxygen. The operational stability was enhanced, as the total turnover number for the oxidation of thioanisole was increased to a new maximum of 250·10^3. At 100% conversion thioanisole sulfoxide with 99% ee was formed. Moreover, the stability in mixtures of aqueous buffer solution and organic cosolvent was increased.

Enantioselectivities of peroxidase-catalyzed sulfoxidation reactions were increased by the formation of the oxidant inside the peroxidase-containing matrix. The ee of thioanisole sulfoxide produced by phytase and soybean peroxidase, increased from 24 to 69% and from 0 to 50%, respectively, when H_2O_2 was continuously generated inside the foam instead of being added to the bulk solution.
Experimental part

Enzyme immobilization: PUR-foam coimmobilized peroxidases with glucose oxidase were prepared as described in chapter 4 with the following enzyme solutions: CPO solution (1 ml from Chirazyme Labs) mixed with GOX solution (1 ml); phytase (0.85 g) dissolved in formate buffer (1 ml; 0.1 M; pH 5.1) and GOX solution (1 ml); SBP (0.75 g) dissolved in GOX-solution (2 ml).

Glucose assay: Determination of the glucose concentration was based on a two-step enzymatic assay. First, glucose is oxidized by glucose oxidase under formation of H₂O₂ that is subsequently used by soybean peroxidase to oxidize ABTS. The glucose assay solution was prepared by mixing glucose oxidase (200 μl), ABTS (28.8 mg), and soybean peroxidase (60.0 mg) with citrate buffer (85 ml; 90 mM; pH 5.0) followed by saturation with oxygen for 15 min. Samples for the determination of the glucose concentration (50 μl) were diluted with water (450 μl). An aliquot of this solution (50 μl) was added to the glucose assay (1.40 ml). The mixtures were incubated for 1 h at room temperature and their coloring was monitored at 414 nm. Glucose samples (5 to 25 mM) were treated in the same manner and used for calibration. No accurate calibration was obtained at glucose concentrations below 5 mM.

Oxidation procedure: In the standard procedure substrate (75 μmol) and wet CPO/GOX-PUR (40 mg) were mixed in citrate buffer (5 ml; 0.1 M; pH 5.0) and stirred at room temperature in a sealed vial under 1 atm. oxygen. After 15 min. glucose (4 mM) was added and the course of the reaction was monitored by HLPC. After a sample was taken the vial was purged with oxygen. Deviations from this standard procedure are mentioned when appropriate.
Abstract

Chloroperoxidase catalyzes the oxidation of various substrates with molecular oxygen as the primary oxidant, in the presence of dihydroxyfumaric acid as a sacrificial reductant. For example, indole is oxidized to 2-oxindole with up to 77% selectivity and thioanisole to the corresponding R-sulfoxide (ee >99%). A mechanism is proposed which involves initial formation of hydrogen peroxide via autoxidation of dihydroxyfumaric acid. Chloroperoxidase subsequently uses the hydrogen peroxide for the selective oxidation of the substrate, via an oxygen transfer mechanism. In contrast, horseradish peroxides primarily catalyzes the oxidation of dihydroxyfumaric acid via a classical peroxidase mechanism and oxidations of added substrates are aselective.
1 Introduction

Chloroperoxidase-catalyzed (asymmetric) oxidations are generally assumed to proceed via an oxygen transfer reaction between the active enzyme intermediate (an iron(IV)oxo porphyrin radical cation, compound I) and the substrate. In contrast, a classical peroxidase reaction involves two separate electron transfer reactions from two substrate molecules to compound I (scheme 1). The peroxidase high-valent iron oxo intermediate is formed upon oxidation of the native enzyme with $\text{H}_2\text{O}_2$. In the formation of the analogous active species in the monoxygenase catalytic cycle, reduction with a cofactor and oxidation with molecular oxygen are involved (scheme 2; see for an overview Griffin, 1992).

Scheme 1. The two reaction modes of Compound I: oxygen transfer (left side), and electron transfer (right side) [$P=$ protoporphyrin IX].

Scheme 2. The two ways of Compound I formation: the peroxidase route (left side), and the monoxygenase mechanism (right side) [$P=$ protoporphyrin IX].

A serious shortcoming of chloroperoxidase (CPO) and all heme-containing peroxidases is their low operational stability, which is caused by rapid deactivation by hydrogen peroxide (Van Deurzen, 1997c). The lifetime of the catalyst can be extended by maintaining a low $\text{H}_2\text{O}_2$ concentration, either by continuous addition or, preferably, by feed-on-demand. To avoid high local concentrations, $\text{H}_2\text{O}_2$ can be generated in situ, for example by the oxidation of glucose mediated by glucose oxidase (chapter 2).

One intrinsically attractive approach to circumvent deactivation is to replace hydrogen peroxide with the combination of molecular oxygen and a sacrificial reductant, i.e. to use a peroxidase in a monoxygenase reaction mode. It was already shown in 1957 that horse radish peroxidase (HRP), in the presence of oxygen and dihydroxyfumaric acid (DHF) as a cosubstrate (sacrificial reductant), catalyzes the hydroxylation of a number of aromatic compounds (Manson, 1957; Buhler, 1961). Two different mechanisms were proposed to account for these results. Both mechanisms involve hydroxyl radicals as the oxidizing species, but differ in the way in which these are generated: a) by an oxidized species of HRP that results from the oxidation of the native enzyme by superoxide anion (Dordick, 1986; Schmahl, 1989), or b) non-enzymatically from DHF radicals which are, in turn, formed in a classical peroxidase reaction (Durlat, 1992; Courteix, 1995a,b).

Our interest in the use of DHF/$\text{O}_2$ was two-fold: as a model to test the possibility of using CPO/$\text{O}_2$ for selective oxygen transfer reactions and to gain insight into the mechanism of these transformations.
2 Results

2.1 Phenol hydroxylation

We first investigated the hydroxylation of phenol, since most of the published work on the oxygen/DHF system was focused on this reaction. In the absence of any catalyst phenol was slowly converted to a mixture of hydroquinone and catechol. We found that, apart from the known catalysts HRP and lignin peroxidase (LiP), the reaction was catalyzed by CPO, soybean peroxidase (SBP) and lactoperoxidase (LPO) with initial turnover frequencies in the range of 128 to 496 min\(^{-1}\) (table 1). The catalytic effect of microperoxidase-11 (MP-11) was much less and catalase was inactive. The catalyst also influenced the hydroquinone/catechol-ratio (H/C), but there was no effect on the final conversion of phenol.

<table>
<thead>
<tr>
<th>Peroxidase</th>
<th>Initial rate(^a) ((\mu)mol min(^{-1}))</th>
<th>Initial TOF(^a) (min(^{-1}))</th>
<th>H/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPO</td>
<td>1.18</td>
<td>236</td>
<td>0.4</td>
</tr>
<tr>
<td>-</td>
<td>0.072</td>
<td>-</td>
<td>0.9</td>
</tr>
<tr>
<td>SBP</td>
<td>1.74</td>
<td>128</td>
<td>1.3</td>
</tr>
<tr>
<td>HRP</td>
<td>1.36</td>
<td>195</td>
<td>1.4</td>
</tr>
<tr>
<td>LPO</td>
<td>1.58</td>
<td>496</td>
<td>0.4</td>
</tr>
<tr>
<td>MP-11</td>
<td>0.18</td>
<td>7</td>
<td>2.0</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.073</td>
<td>-</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\(^a\) In duplicate at 5 ml scale, samples were taken every minute and quenched with an equal volume of DMSO.

2.2 Oxidation of indole and thioanisole

We next turned our attention to the oxidation of indole and thioanisole, because CPO is unique in its selective \(\text{H}_2\text{O}_2\)-supported oxidation of these reactants. CPO also mediated these reactions with oxygen and DHF, but the turnover numbers were reduced by a factor ten. Whereas indole is cleanly oxidized to 2-oxindole by CPO and \(\text{H}_2\text{O}_2\), the selectivity dropped to 20-70% (depending on the medium, \textit{vide infra}) in the oxygen/DHF system. Thioanisole afforded the \(R\)-sulfoxide (ee > 99%) analogous to the \(\text{H}_2\text{O}_2\)-supported oxidation. When CPO was replaced by HRP, aerobic oxidation of indole gave only a trace of oxindole and no sulfoxide was formed from thioanisole.

2.3 Effect of superoxide dismutase

Because the previously proposed mechanisms for aerobic hydroxylation all involve superoxide anion (or its protonated form \(\text{HO}_2^+\)) in some way, we investigated the effect of superoxide dismutase (SOD). In agreement with literature (Dordick, 1986) we found that the HRP-catalyzed aerobic hydroxylation of phenol was inhibited by SOD. Surprisingly, the CPO-catalyzed aerobic hydroxylation of phenol, as well as the oxidation of indole and thioanisole was not influenced by the addition of superoxide dismutase (figure 1).
2.4 Effect of radical scavengers

In order to investigate the possible role of hydroxyl radicals in the CPO-catalyzed aerobic oxidations, tert-butyl alcohol was added as a radical scavenger. tert-Butyl alcohol is only moderately reactive towards hydroxyl radicals (rate constant $k = 2.5 \text{ to } 4.2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ (Howard, 1972)), but has the advantage that it is inert towards CPO (Van Deurzen, 1997b). Hence, any effect is due only to its radical scavenging properties and not to enzyme inhibition.

The hydroxylation of phenol was inhibited by tert-butyl alcohol (figure 2), whereas the sulfoxidation of thioanisole was not influenced. The effect of tert-butyl alcohol on the aerobic oxidation of indole was two-fold. The relative conversion decreased by a factor of 10, whereas the selectivity increased from 25% (0% $t$-BuOH) to 60% (20% $t$-BuOH). Mannitol had a similar effect (table 2).

<table>
<thead>
<tr>
<th>Radical scavenger</th>
<th>Relative conversion (%)</th>
<th>Selectivity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>10 v% tBuOH</td>
<td>13</td>
<td>55</td>
</tr>
<tr>
<td>20 v% tBuOH</td>
<td>4</td>
<td>60</td>
</tr>
<tr>
<td>0.12 M mannitol</td>
<td>52</td>
<td>35</td>
</tr>
<tr>
<td>0.25 M mannitol</td>
<td>21</td>
<td>73</td>
</tr>
<tr>
<td>7.5 mM Trolox</td>
<td>124</td>
<td>61</td>
</tr>
<tr>
<td>17.3 mM Trolox</td>
<td>112</td>
<td>77</td>
</tr>
</tbody>
</table>

Trolox™-C (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a water soluble analog of $\alpha$-tocopherol (vitamin E), is a more efficient hydroxyl radical scavenger (rate constant, $k = 6.9 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, (Bielski, 1995)) than tert-butyl alcohol. Trolox inhibited the CPO- and HRP-catalyzed aerobic hydroxylation of phenol (figure 3) consistent with the
Intermediacy of hydroxyl radicals. In contrast, both the initial rates and final conversions of indole and thioanisole oxidation increased in the presence of Trolox. Moreover, in the case of indole the selectivity increased up to 77%.

![Figure 2. Effect of tert-butyl alcohol on the CPO-catalyzed aerobic oxidation of phenol (○); indole (●); thioanisole (●).](image)

![Figure 3. Effect of Trolox on the CPO-catalyzed aerobic oxidation of: phenol (○); indole (●); thioanisole (●) and the HRP-catalyzed aerobic oxidation of phenol (●).](image)

### 2.5 Spectral data

The question whether reduction of the native peroxidase to ferrous enzyme plays any role in the O₂/DHF driven oxidations still is unresolved. In agreement with the literature (Morris, 1966) the reduction of native CPO with sodium dithionite was facile and could easily be monitored by the shift of the Soret band from 403 to 409 nm. However, treatment of CPO with DHF under argon or DHF and O₂ did not induce any change in the UV spectrum. Under the same conditions HRP was converted into its compound III, as reported (Dordick, 1986).

### 2.6 The role of hydrogen peroxide in the catalytic cycle

Solutions of DHF under 1 atm. O₂ were found to undergo facile autoxidation at room temperature (half-life time: t₁/₂ = 30 min), whereas DHF is stable in the absence of oxygen. The addition of catalase or superoxide dismutase decreased the rate of DHF autoxidation. During autoxidation, even in the presence of SOD and catalase, hydrogen peroxide was detected with a titanium(IV) chloride reagent (Svahl, 1996), which is a very sensitive test. The hydrogen peroxide concentrations are low, since they cannot be detected with the less sensitive chromium pentoxide test (Svahl, 1996) or by standard titrations. In apparent contradiction with the detection of H₂O₂, catalase had no effect on the aerobic oxidation of indole catalyzed by CPO. Similarly, the addition of catalase did not totally stop the HRP-catalyzed aerobic hydroxylation of phenol (Halliwell, 1977; Dordick, 1986; Courteix, 1995b).

To investigate the role of hydrogen peroxide in the catalytic cycle, competition experiments between substrate (indole) and reductant (DHF) were carried out in a hydrogen peroxide driven oxidation. CPO and HRP, which both catalyze the oxidation of indole by hydrogen peroxide (Van Deurzen, 1996; Holmes-Siedle, 1957) were used as catalysts. The plots
of DHF concentration versus time (figure 4), show that DHF is oxidized by \( \text{H}_2\text{O}_2 \) in the absence of any enzyme. When a mixture of indole and DHF is allowed to react with \( \text{H}_2\text{O}_2 \) in the presence of CPO the indole is oxidized preferentially. DHF is oxidized only at low indole concentrations. In contrast, HRP catalyzes only the oxidation of DHF and no indole was converted.

![Graph showing DHF concentration over time](image)

**Figure 4.** Amount of DHF during competition experiments in the presence of indole (0.11 mmol): no oxidant (●●●); \( \text{H}_2\text{O}_2 \) without catalyst (●●); CPO with \( \text{H}_2\text{O}_2 \) (▲▲); HRP with \( \text{H}_2\text{O}_2 \) (●●●).

### 2.7 Other reductants

For the HRP-catalyzed aerobic hydroxylation of phenol it is reported that only DHF acts as a sacrificial reductant (Dordick, 1986; Durlia, 1992). Other reductants, such as ascorbic acid or NADH, did not give rise to hydroxylated products. To our surprise we found that CPO catalyzes the aerobic oxidation of thioanisole and indole also in the presence of ascorbic acid or 1,2-diphenylhydrazine, although, analogous with HRP, ascorbic acid does not support the hydroxylation of phenol by CPO. On the other hand, phenol is hydroxylated in the presence of 1,2-diphenylhydrazine and CPO, but not HRP (table 3). The CPO-catalyzed oxidation of thioanisole in the presence of ascorbic acid was later confirmed by Pasta et al. (1999).

Strong reducing agents, such as sodium dithionite, copper(I) chloride, and chromium(II) chloride, that are known to reduce the native peroxidases to their ferrous form, did not afford any detectable amounts of oxidized products.

<table>
<thead>
<tr>
<th>Table 3. Ability of different reductants to support aerobic oxidations catalyzed by peroxidases.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
</tr>
<tr>
<td>CPO</td>
</tr>
<tr>
<td>Indole</td>
</tr>
<tr>
<td>Thioanisole</td>
</tr>
<tr>
<td>HRP</td>
</tr>
<tr>
<td>Phenol</td>
</tr>
<tr>
<td>Dihydroxyfumaric acid</td>
</tr>
<tr>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>1,2-diphenylhydrazine</td>
</tr>
</tbody>
</table>
3 Discussion

On the basis of our results a monooxygenase catalytic cycle, involving initial reduction of the native iron(III) enzyme to its ferrous form, can be ruled out. Thus, native CPO was not reduced by DHF and sodium dithionite did not support aerobic oxidation. However, Pasta et al. (1999) still believe in a monooxygenase catalytic cycle for the CPO-catalyzed oxidation of thioanisole in the presence of ascorbic acid, as they observed reduction of CPO to its ferrous form in the presence of an excess of ascorbic acid under anaerobic conditions.

We propose a mechanism for the CPO-catalyzed aerobic oxidations which involves initial formation of hydrogen peroxide via autoxidation of dihydroxyfumaric acid towards diketosuccinic acid (DKS, scheme 3). Detection of H$_2$O$_2$ in aerated DHF solutions confirmed the formation of hydrogen peroxide. CPO subsequently utilizes the hydrogen peroxide ($K_m = 1.7 \mu$M; $k = 2.3 \cdot 10^5 \text{M}^{-1}\text{s}^{-1}$ (Sun, 1994)) in the selective oxidation of the substrate via an oxygen transfer mechanism. This mechanism cannot be operative with HRP, because it is predominantly present as its inactive compound III (section 2.5). Consequently, only non-enzymatic oxidation is observed in the presence of HRP. Catalase is a relatively poor scavenger of H$_2$O$_2$ at low concentrations due to its very high $K_m$ (1.1 M; Garrett, 1995), and, therefore no influence of catalase on the CPO-catalyzed aerobic oxidations was observed.

**Scheme 3.** Autoxidation of dihydroxyfumaric acid, yielding H$_2$O$_2$.

Hydroxyl radicals are likely reactive species in the non-enzymatic oxidation. They can be formed during the autoxidation of DHF, for example by bimolecular termination of HO$_2^*$ radicals (reaction 1). Superoxide dismutase (SOD) suppresses reaction 1 by catalyzing the conversion of HO$_2^*$ to H$_2$O$_2$ and O$_2$ (reaction 2). Hence, the CPO-catalyzed aerobic oxidation of indole and thioanisole is not inhibited by SOD, consistent with the proposed oxygen transfer mechanism. By the same token, the increase in selectivity observed in the CPO-catalyzed aerobic oxidation of indole (table 2) in the presence of hydroxyl radical scavengers (Trolox or tert-butyl alcohol) is consistent with hydroxyl radicals being involved in competing aselective oxidation of indole. Since indole itself is an efficient hydroxyl radical scavenger ($k = 3.2 \cdot 10^{10} \text{M}^{-1}\text{s}^{-1}$ (Buxton, 1988)) selectivities will probably never reach 100%.

Similarly, the CPO-catalyzed aerobic oxidation of thioanisole was not inhibited by Trolox or tert-butyl alcohol. The rate acceleration observed in the presence of Trolox suggests that it either accelerates hydrogen peroxide formation, by scavenging HO$_2^*$ radicals, and/or it protects the enzyme from deactivation. In contrast, phenol hydroxylation with DHF/O$_2$ in
the presence of CPO and HRP was inhibited by radical scavengers (Trolox or tert-butyl alcohol) consistent with the involvement of hydroxyl radicals as previously proposed.

A mechanism for aromatic hydroxylation via hydroxyl radicals is described by Walling (1975a,b) for Fenton’s reagent (scheme 4). Hydroxyl radicals add very rapidly to aromatics (rate constant, $k = 10^9$ to $10^{10}$ M$^{-1}$s$^{-1}$) resulting in the formation of hydroxy-cyclohexadienyl radicals which are subsequently oxidized to the corresponding phenols by iron(III).

\[
\begin{align*}
2 \text{HO}_2^- & \rightarrow \text{O}_2 + 2 \text{HO}^- \\
\text{Reaction 1}
\end{align*}
\]

\[
\begin{align*}
2 \text{HO}_2^- + \text{SOD} & \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \\
\text{Reaction 2}
\end{align*}
\]

Scheme 4. Mechanism of aromatic hydroxylation by hydroxyl radicals.

In the case of the DHF/O$_2$ system hydroxyl radicals are formed non-enzymatically from DHF and oxygen (see above). The dihydroxycyclohexadienyl radicals are oxidized by the oxidized forms of the peroxidases, such as compound I (in the case of CPO), compound II, or compound III (in the case of HRP). Since the addition of hydroxyl radicals to phenol is a very rapid process, the peroxidase mediated oxidation of the intermediate dihydroxycyclohexadienyl radicals is expected to be the rate limiting step. This accounts for the different hydroquinone/catechol-ratios for different peroxidases shown in table 1. Hydrogen peroxide is formed from DHF and O$_2$ in the presence of SOD, therefore CPO-catalyzed hydroxylation of phenol is not inhibited by SOD. In contrast, HRP compound III is formed from native HRP and superoxide anion. Addition of SOD inhibits HRP compound III formation and therefore aerobic hydroxylation catalyzed by HRP.

Hence, we conclude from our observations that the role of the peroxidases in the aerobic oxidation of phenol is limited to the oxidation of the intermediary dihydroxycyclohexadienyl radicals to the corresponding cation.

4 Concluding remarks

In this chapter the replacement of hydrogen peroxide with the combination of oxygen and a sacrificial reductant to circumvent the deactivation of peroxidases has been discussed. A combination of molecular oxygen and a sacrificial reductant (dihydroxyfumaric acid, ascorbic acid or 1,2-diphenylhydrazine) can replace hydrogen peroxide as the oxidant in CPO-catalyzed oxidations. However, the goal of improving the operational stability of CPO was not achieved, since the TTNs obtained in an aerobic oxidation were 10-fold lower compared to the H$_2$O$_2$ driven oxidations. Moreover, the regioselectivity for the formation of 2-oxindole during the oxidation of indole was decreased to 70%. The enantioselectivity for the formation of the $R$-enantiomer of thioanisole sulfoxide was maintained at 99%.

The reaction does not involve a monoxygenase pathway, i.e. initial reduction of native iron(III) enzyme to its ferrous form, but rather *in situ* formation of H$_2$O$_2$, via free radical autoxidation of DHF, followed by CPO-catalyzed oxygen transfer from H$_2$O$_2$ to the substrate.
Selective oxidations with molecular oxygen

An inevitable consequence of the homolytic pathway for $\text{H}_2\text{O}_2$ formation is the occurrence of side reactions of substrates with reactive radicals ($\text{HO}_2^\cdot$, $\text{HO}_2^\cdot$, etc.) in competition with CPO-catalyzed oxygen transfer. This leads to lower selectivities (and turnover numbers) compared to those observed with CPO/$\text{H}_2\text{O}_2$. The improvement of selectivities in the presence of radical scavengers is consistent with this notion. In contrast to CPO, HRP predominantly catalyzes the $\text{H}_2\text{O}_2$ oxidation of DHF, rather than added substrate, via a classical peroxidase pathway.

Experimental part

General experiment with molecular oxygen: The substrate (50 $\mu$mol indole or thioanisole, or 100 $\mu$mol phenol) was dissolved in acetate buffer (10 ml, 0.1 M pH 5.0). Enzyme solution (100 $\mu$l; concentration of CPO: 30.6 $\mu$M; MP-11: 1.0 mg/ml; HRP: 5.5 mg ml$^{-1}$; LPO: 5.0 mg ml$^{-1}$; SBP: 8.1 mg ml$^{-1}$) was added to the reaction mixture, followed by 5 min of stirring under 1 atm. O$_2$ at room temperature. The reaction was started by the addition of 2.5 eq of reductant (dihydroxyfumaric acid, ascorbic acid, or diphenylhydrazine). Samples for monitoring the reaction were taken every 5 or 15 min, respectively, for phenol and the other substrates and analyzed by HPLC.

Competition experiments: Competition experiments were carried out at 0 °C under continuous sparging with argon. Indole (0.11 mmol) was dissolved in acetate buffer (20 ml, 0.1 M pH 5.0) and stirred for 10 min. Enzyme solution (200 $\mu$l; concentrations see above) and dihydroxyfumaric acid (0.17 mmol) were added. After 5 minutes of stirring the addition of $\text{H}_2\text{O}_2$ (1.0 M in water; saturated with argon) was started, at a rate of 1 $\mu$l min$^{-1}$; total volume 0.11 ml. Samples were taken every 15 min and analyzed directly for the DHF concentration.

UV-spectra of reduced CPO: A acetate buffer solution (0.1 M pH 5.0) was cooled to 4 °C, saturated with argon and stored under argon. A cuvet was charged with 2.5 ml buffer and 0.5 ml of CPO solution and sparged with argon for 3 min. CPO was reduced by adding 200 $\mu$l sodium dithionite solution (200 mg in 3 ml, saturated with argon) followed by 2 min of sparging with argon. In the same way CPO was treated with 200 $\mu$l of a saturated DHF solution. After the spectrum was recorded, argon was replaced by oxygen.
Abstract

Glycosylated enzymes, viz. aminoacylase from *Aspergillus melleus*, chloroperoxidase from *Caldariomycetes fumago*, and phytase from *Aspergillus ficuum*, were covalently immobilized into polyurethane foams with an unprecedented high enzyme loading of up to 190 mg protein per g dry foam. The immobilization efficiency (retained activity) ranged from 100% at a low loading to 60% at high loadings. In contrast to many other immobilization methods, no leaching of the enzyme from the support took place under reaction conditions. In short, an universal method for the immobilization of enzymes from fungal sources was developed, affording a highly active, stable and reusable biocatalyst.

* In cooperation with Martin Bakker from the Laboratorium for Organic Chemistry and Catalysis, Delft University of Technology.
1 Introduction

Enzymes are gaining in importance as catalysts in (industrial) organic synthesis (Johnson, 1998). For optimum performance they are often used in immobilized form (Katchalski-Katzir, 1993). A milestone in the use of immobilized enzymes in biotransformations was the Tanabe process for the production of L-amino acids (scheme 1) using an immobilized aminoacylase developed by Chibata and coworkers (1972, 1978).

![Scheme 1. Production of L-amino acids via an aminoacylase-catalyzed resolution of N-acetyl-D/L-amino acids.](image)

Scheme 2. Covalent attachment of enzymes into polyurethane foams. (R is the prepolymer based on toluene-2,6-diisocyanate)

In comparison with their native form immobilized enzymes offer several advantages, such as enhanced stability, easier product recovery and purification, the possibility of continuous processes and repetitive enzyme use. However there are also disadvantages, such as the additional costs for the carrier, loss of activity due to immobilization, a reduction of the activity per unit volume, and the occurrence of diffusion limitations.

The immobilization of enzymes can be classified into three approaches: support binding methods (covalent bonding, adsorption), crosslinking methods (e.g. crosslinked enzyme crystals) and the entrapment method (in a gel lattice, microcapsules). The covalent attachment of enzymes into polyurethane foams via reaction with isocyanate groups was developed and patented by Wood (1982). The method involves mixing an isocyanate prepolymer with an enzyme solution. The curing reaction of the prepolymer is accompanied by the reaction of isocyanate groups with amine and/or hydroxyl groups of the enzyme, which attach it to the polyurethane matrix (scheme 2). Water, which is introduced with the enzyme solution, reacts with the isocyanate groups with the formation of CO₂. Evolution of the latter causes the curing polymer to become porous, forming a spongellike matrix. The physical properties of the polyurethane foams can be influenced by the addition of different amounts of additives, such as initiators, surfactants, and blowing agents (Lejeune, 1997).

Covalent attachment into polyurethane foams has been used to immobilize enzymes, e.g. β-D-galactosidase (Hu, 1993), cellulase (Chakrabarti, 1988), amyloglucosidase (Storey, 1990b), glucose isomerase (Storey, 1990a), phosphotriesterase (LeJeune, 1997) and lipases (Van Rantwijk, 1998). The amounts of enzyme that have been loaded into polyurethane foam in published examples is in the order of 1 mg protein (g foam)⁻¹, which would be undesirably low for many applications. However, for the immobilization of cell suspensions, loadings up to 300 mg cell suspension (g foam)⁻¹ have been reported (Hu, 1994; O'Reilly, 1989). The maximum loading of purified protein into the polyurethane foam was
never published or investigated. We expected that glycosylation of the enzyme would facilitate its attachment to the polymer backbone, due to the large number of superficial hydroxyl groups. This chapter describes the polyurethane foam immobilization of glycosylated enzymes from fungal sources with a very high loading (up to 190 mg protein per gram dry foam) with no leaching of the enzyme from the carrier being observed during the reaction. The method is suitable for the immobilization of a variety of glycosylated enzymes (figure 1) and affords highly active, stable and reusable biocatalysts.

Figure 1. Indication of the amount of enzymes which can be immobilized into polyurethane foams: starting materials (upper photo) and final PUR-foams (lower photo); immobilized enzymes (from left to right): phytase, soybean peroxidase, and aminoacylase.
2 Results and discussion

2.1 Immobilization method

We used the technique developed by LeJeune (1996) for preparing the foam immobilized enzymes. This technique involves mixing an equal volume of enzyme solution with the prepolymer (Hypol 3000: a water activated derivative of toluene-2,6-diisocyanate) together with an emulsifier (Brij52: diethylene glycol monocetyl ether) improve the distribution of the enzyme in the polymerization mixture. Cobalt(II) chloride was used to initiate the polymerization reaction. Under these conditions foams were formed with an open pore structure and with a narrow pore size distribution. Variation of the prepolymer, the amount of water or different surfactants could effect the physical properties of the foam, such as porosity, density, and surface area. Photographs and scanning electron micrographs of foams with different properties were reported earlier (LeJeune, 1997).

2.2 Immobilization of glycosylated enzymes

Owing to the large number of reactive carbohydrate hydroxyl functionalities, glycosylated enzymes are eminently suited to immobilization via reaction with isocyanate groups in a polyurethane prepolymer. Hence, we have studied the immobilization of three representative glycosylated enzymes: aminoacylase (EC 3.5.1.14), chloroperoxidase (EC 1.11.1.10), and phytase (EC 3.1.3.8). These enzymes are known to be heavily glycosylated, having a sugar content of respectively 18% for chloroperoxidase from C. fumago (Keningsberg, 1987), 25% for phytase from A. ficuum (Kostrewa, 1997), and 46% for aminoacylase from A. melleus (Gentzen, 1980). The hydrolysis of N-acetyl-L-methionine was used as an assay for aminoacylase (scheme 1; R = -CH₃CH₂S-CH₃) because this allowed us to compare our results with the commercial Tanabe process. Chloroperoxidase, which is known to be an efficient catalyst for a wide variety of synthetically useful (enantioselective) oxygen transfer reactions (Hager, 1998; Van Deurzen, 1997a), and vanadate substituted phytase (chapter 8) were studied with the sulf oxidation of thioanisole as a model reaction.

The amount of protein that could be attached into the polymer was first investigated, because this could possibly put an upper limit on the activity of the immobilized enzyme. The amount of enzyme that had been fixed to the polymer was determined indirectly, by subtracting the amount of protein found in the wash solvent from the amount of protein added to the polymerization mixture.

Aminoacylase was captured very efficiently; even at a loading of 190 mg g⁻¹ no residual protein could be detected in the wash solvent. This resulted in a straight line in figure 1. Chloroperoxidase was immobilized to a maximum of 25 mg g⁻¹, at this loading 82% of the available protein was fixed to the carrier (table 1). The fraction of phytase that became attached to the carrier was rather less and it decreased slightly to 46% when the fraction of protein in the polymerization mixture was increased to 200 mg g⁻¹ (figure 2).

The enzyme loading into the polyurethane foam reflects the carbohydrate content of the enzyme and decreased in the order aminoacylase > phytase > CPO. We also observed that the fraction of CPO and phytase that is bound decreased when the loading was increased, indicating that saturation of the available isocyanate groups took place. This could be caused by the considerable amounts of adjuvants (stabilizers e.g. salts, other proteins,
surfactants, polymeric sugars) and impurities that are present in CPO and in particular in phytase. We note that stable immobilization of the crude aminoacylase preparation, which contains large amounts of adjuvant, could not be achieved (data not shown) In contrast, with phytase the crude enzyme powder could be used but the use of partly purified phytase led to an increase in yield from 60% to 85%.

![Figure 2. Amount of protein attachment to the polyurethane foam during the immobilization of aminoacylase (○) and phytase (●).](image)

![Figure 3. Immobilization efficiency for the PUR-foam immobilization of aminoacylase (○), CPO (●), and phytase (△) as a function of the enzyme loading.](image)

Besides the protein loading and the immobilization yield (Y\text{immob}: the fraction of protein added to the polymer that becomes bound into the foam) one more parameter is used to describe the immobilization process: the immobilization efficiency (E\text{immob} or retained activity) represents the residual activity of the bound enzyme (E\text{immob} = U_{\text{found in foam}}/U_{\text{bound into foam}}).

The immobilization yields and efficiencies obtained with the three glycosylated enzymes in this present study are summarized in table 1. This table also gives an overview of the results of foam immobilized enzymes reported in the literature. The enzymology of the immobilized enzymes is also included in this table.

Table 1 clearly shows that the glycosylated enzymes were efficiently immobilized into polyurethane foams. At low loadings (< 10 mg g\text{-1}) aminoacylase, CPO, and phytase showed an immobilization efficiency of 100%. All other enzymes given in the table were less efficiently immobilized. Two more extracellular glycosylated enzymes were used in literature studies, viz. amyloglucosidase (sugar content 19%) and β-D-galactosidase (sugar content 8%). The low immobilization yield and enzyme loading obtained for these enzymes could result from the fact that both enzymes are glycoside hydrolyzing enzymes which are able to cleave their own sugar chains that bound them to the polymeric backbone. Furthermore, the purity of the enzymes was not defined.
### Table 1. Immobilization of different enzymes into polyurethane foam.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Reference</th>
<th>Immobilization</th>
<th>Protein properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Enzyme loading(^a)</td>
<td>Yield(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg g(^{-1})foam)</td>
<td>(%)</td>
</tr>
<tr>
<td>Aminoacylase</td>
<td></td>
<td>8</td>
<td>100</td>
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<tr>
<td><em>A. melleus</em></td>
<td></td>
<td>187</td>
<td>100</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
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<td>100</td>
</tr>
<tr>
<td><em>C. fumago</em></td>
<td></td>
<td>24</td>
<td>82</td>
</tr>
<tr>
<td>Phytase</td>
<td></td>
<td>23</td>
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</tr>
<tr>
<td><em>A. fuscum</em></td>
<td></td>
<td>92</td>
<td>46</td>
</tr>
<tr>
<td>Amyloglucosidase</td>
<td>Storey,</td>
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<td>30</td>
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<tr>
<td><em>A. niger</em></td>
<td>1990b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>Hu,1993</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td><em>A. oryzae</em></td>
<td></td>
<td>0.73</td>
<td>45</td>
</tr>
<tr>
<td>Glucose isomerase</td>
<td>Storey,</td>
<td>(72 U)</td>
<td>47</td>
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<tr>
<td><em>S. rubiginosus</em></td>
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<td></td>
<td></td>
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<tr>
<td>β-Glucosidase</td>
<td>Chakrabarti,</td>
<td>2.6(^d)</td>
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<tr>
<td><em>A. niger</em></td>
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<td>Lipase</td>
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<td><em>H. lanuginosa</em></td>
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<td>Phosphotriesterase</td>
<td>LeJeune,</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>1996</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Units reported in literature were when possible converted into mg using the activity of commercially available enzyme samples;

\(^b\) Yield: fraction of protein that was bound to the foam;

\(^c\) Efficiency: (U/mg bound enzyme)/(U/mg free enzyme);

\(^d\) This 2.6 mg commercial preparation contained only 0.04 mg protein;

\(^*\) Immobilization efficiency did not change with increasing enzyme loading;

\(^f\) No information about the degree of glycosylation was published.

In the case of phytase, an immobilization efficiency of more than 100% was obtained. This suggests that the immobilized phytase had an enhanced activity in comparison with the native form. This is probably due to the selective immobilization of phytase. An efficiency above 100% was also reported for glucose isomerase (Storey, 1990). For the three glycosylated enzymes used in this study the immobilization efficiency was monitored as a function of the protein loading (figure 2).

As shown in figure 2, the immobilization efficiency decreased with increasing protein loading. One possible reason for this could be stacking of the enzymes on the carrier, which decreases the accessibility of the enzymes close to the carrier. The sugar tails of glycosylated enzymes serve as a spacer and, therefore, would reduce the stacking effects. Aminoacylase, which has the highest degree of glycosylation (46%), showed the smallest influence of the enzyme loading on the immobilization efficiency. The lower degree of glycosylation for phytase (25%) and CPO (18%) resulted in a lower efficiency at a high protein loading. The fast decrease in efficiency for CPO could only partly be explained by its lower sugar content compared to the other two enzymes. Furthermore, an increased
concentration of protein could possibly cause more binding of hydroxyl and amino groups in the protein with the isocyanate and decrease their activity. Diffusion limitations could account for the rest, since CPO is by far the most active enzyme studied. The $k_{cat}$ for the sulfoxidation of thioanisole is 900 min$^{-1}$ (Van Deurzen, 1997b), whereas phytase has a maximum turnover frequency of only 11 min$^{-1}$ (chapter 8). The low efficiency reported for β-galactosidase (Hu, 1993) at a low loading (0.73 mg g$^{-1}$) is in agreement with the observed influence of the degree of glycosylation; the sugar content for the β-galactosidase from \textit{Aspergillus oryzae} is only 8% of its molecular weight (Ogushi, 1980).

Generally speaking, covalent immobilization results in a loss of enzyme activity. This could either result from a loss of functional groups (amino groups that react with aldehyde functions from a crosslinking reagent or with isocyanate groups) or from structural changes by multi-point attachment. As outlined in scheme 2, isocyanate groups can react with both amino and hydroxyl groups of the enzyme during polyurethane foam immobilization. During polymerization the isocyanate groups show a higher reactivity towards amino groups compared to hydroxyl groups, however, the amino groups are not accessible to a growing polyurethane polymer. The glycosylated enzymes are completely covered with a mantel of carbohydrate moieties which prevent access to the amino groups of the protein. For example in the case of CPO, the 13 amino groups (Van Deurzen, 1995) are shielded by at least 120 hydroxyl groups on the surface (Kenigsberg, 1987). Our concept is based on this and, hence, we reasoned that glycosylated enzymes are predominantly bound into the PUR-foam via their sugar hydroxyl groups.

In short, the sugar tails protect glycosylated enzymes in two ways towards deactivation during covalent immobilization into PUR-foams. First, the sugar hydroxyl groups compete for the isocyanate groups, and, therefore less functional amino groups are attacked. Second, the sugar tails serve as a spacer between enzyme and polymeric matrix and avoid deactivation that results from structural changes upon multi-point attachment.

In contrast to the glycosylated enzymes discussed above, entrapment of lipases in polyurethane foam seems to result in their deactivation. It seems likely that deactivation is caused by carbamoylation of the active serine by the isocyanate function in the prepolymer (Van Rantwijk, 1998).

### 2.3 Performance of PUR-foam immobilized enzymes

PUR-foam immobilized enzymes are both easy to prepare and facile to handle and therefore they bear the potential for being applied on industrial scale. This was also mentioned by Storey (1990a) with regard to their coimmobilization of cellulase, β-glycosidase and glucose isomerase for the production of high fructose syrup from cellulose. As already explained above, the enzyme is directly bound into the polymeric matrix, without any protein modification step. However, better results were obtained with purified enzymes. Immobilization of enzymes as a suspension is possible with a high enzyme loading and a good immobilization efficiency. In the case that only part of the enzyme is bound to the carrier, the enzyme found in the wash solvent is fully active and easy to recover and reuse via membrane filtration. The single purification step in the immobilization of aminoacylase was membrane filtration, which would be compatible with large scale applications.
Manipulating the physical properties of the foam makes the biocatalysts suitable for different purposes and reactor designs. Crushing the polymer under liquid nitrogen reduced their particle size and resulted in a biocatalyst, which settled on the bottom of the reaction vessel (left side of figure 4). Cutting the foam into cubes afforded a biocatalyst, which floated on the surface of the liquid (right side of figure 4). Cylindrical foams are easy to use in packed bed reactors making them suitable for application in continuous processes. The catalyst productivity of the PUR-foam immobilized aminoacylase in the conversion of racemic $N$-acetyl-methionine to L-methionine was $3.4 \text{ g g}^{-1}\text{catalyst d}^{-1}$ compared to $2.15 \text{ g g}^{-1}\text{catalyst d}^{-1}$ for the commercial DEAE-sephadex immobilized aminoacylase (Chibata, 1972).

![Figure 4. Behavior of PUR-foam biocatalyst in different forms: crushed foam settles on the bottom (left side); cubes float on top of the reactor (right side).](image)

The prepared PUR-foam immobilized enzymes are stable and reusable biocatalysts. As shown in figure 5 no leaching of the enzyme from the polymeric matrix was observed under catalytic conditions, since the oxidation of indole stopped when the PUR-foam immobilized CPO was removed from the reaction mixture. As figure 5 shows the biocatalyst is still active after drying overnight over $\text{P}_2\text{O}_5$. These dried biocatalysts are applied in organic solvents (chapter 5).

The stability of the PUR-foam immobilized aminoacylase was studied in more detail. PUR-foam immobilized aminoacylase was reused 12 times in the hydrolysis of N-acetyl-D/L-methionine (30 minutes reaction time). The activity of the PUR-foam immobilized aminoacylase did not change during these 12 reaction cycles, demonstrating that it is a perfectly reusable biocatalyst.
3 Concluding remarks

This chapter describes the immobilization of glycosylated enzymes into polyurethane foams to render them stable and applicable in different reactor designs. Three glycosylated enzymes from fungal sources, viz. aminocyclase from A. melleus, chloroperoxidase from C. fumago, and phytase from A. ficuum, were successfully immobilized via covalent attachment to PUR-foams. The immobilization method was very efficient, yielding an enzyme preparation with a maximum protein content of 190 mg protein per g dry foam by an immobilization efficiency of 60% in the case of aminocyclase. This efficiency increased to 100% at a lower protein content. Chloroperoxidase was immobilized to a maximum of 25 mg per g, at this loading 82% of the available protein was fixed to the carrier. The immobilization efficiency for CPO decreases rapidly from 100% to 50% by increasing the protein fraction to 25 mg per g probably due to diffusion limitations.

We propose that glycosylation protects the enzyme from deactivation during immobilization. The large number of carbohydrate functionalities will react with the isocyanate groups during polymerization and avoid modification of the essential amino residues. On the other hand, the sugar tails serve as a spacer between enzyme and the polymeric backbone and save the enzyme from deactivation caused by structural changes upon multi-point attachment.

This PUR-foam immobilization method is applicable for large scale purposes and no modification of the protein is needed. Further, we demonstrated that the enzymes can be immobilized without purification. Moreover, enzyme suspensions can be used to immobilize large amounts of enzyme in high concentrations. However best results were obtained with purified protein.

This method yields a stable and reusable biocatalyst, which can be used in different reactor designs. The foam immobilized enzymes can be crushed using liquid nitrogen and dried over P₂O₅ without any loss of activity, yielding a catalyst which forms a suspension in solution. Alternatively, foam-cubes float on top of the reaction mixture.
Experimental part

**Enzyme immobilization:** To prepare the foam immobilized enzymes, CPO was concentrated to 35.5 mg ml\(^{-1}\) and aminoacylase was dissolved in TRIS buffer (pH 7.5; 50 mM containing 1 mM of CoCl\(_2\) for enzyme stabilization) and purified and concentrated using an ultrafiltration membrane. The enzyme solution (2.0 ml) was mixed with Brij52 (50 μl; 4% solution) and CoCl\(_2\) (125 μl; 1 mM solution) in a 50 ml PE centrifugation tube. Hypol 3000 (2.0 g: preheated to 35°C to limit handling problems due to its high viscosity) was subsequently added. The mixture was stirred vigorously for 30 s to achieve a homogeneous distribution of enzyme within the prepolymer. Polymerization took place at room temperature. When the increase in volume ceased (1 to 2 min) the tubes were stored in ice to minimize enzyme deactivation caused by the increased temperature during the exothermic polymerization reaction. The foams were stored for at least 2 hours before use. The foam cylinder was cut into round slices of 3 mm thickness (d=25mm), and washed and squeezed 3 times in 50 ml of buffer (50 mM citrate pH 5.0, 50 mM Tris pH 7.0, and 0.1 M formate pH 5.0 for respectively amino acylase, CPO, and phytase). Wash solvents were analyzed for protein concentration and enzyme activity. The foam slices with immobilized enzyme were stored at 4°C. For activity analysis a slice was crushed under liquid nitrogen and dried over nightover P\(_2\)O\(_5\).

**Aminoacylase activity** was measured using the hydrolysis of N-acetyl-L-methionine as an assay. N-Acetyl-L-methionine (15.7 mM) was dissolved in TRIS buffer (5 ml; 50 mM; pH 7.5) and the pH was adjusted to 7.5 with NaOH (1M). The reaction was quenched by adding HCl (1M; 5ml) after 1 h and the conversion was determined by HPLC.

**Chloroperoxidase activity** was measured using the oxidation of thioanisole with TBHP in tert-butyl alcohol/water mixtures (Van Deurzen, 1997b) as a test reaction. Citrate buffer (7 ml; 0.1 M; pH 5.0; containing 10% (v:v) tert-butyl alcohol) and thioanisole (12 mM) were added to an appropriate amount of enzyme preparation. After 15 min stirring at room temperature tert-butyl hydroperoxide (45 mM) was added and the course of the reaction was followed by HPLC.

**Phytase activity** was measured using the peroxidase activity in the presence of vanadate (chapter 8). Formate buffer (7 ml; 0.1 M; pH 5.0; containing 10 μM Na\(_2\)VO\(_4\)) and thioanisole (5.0 mM) were added to an appropriate amount of enzyme preparation. After 15 min stirring at room temperature hydrogen peroxide (5.5 mM) was added and the course of the reaction was followed by HPLC.
Abstract

Chloroperoxidase from *Caldariomyces fumago* was successfully used, to catalyze enantio- and regioselective oxidations with tert-butyl hydroperoxide as oxidant in predominantly organic media. CPO was immobilized via covalent attachment into polyurethane foam as well as lyophilized with a surfactant or polymer. Dried PUR-foam immobilized CPO was used in selective oxidations in organic solvents, such as isoctane and chloroform. For example, indole was oxidized to 2-oxindole in virtually quantitative yield and thioanisole was converted into the corresponding R-sulfoxide (ee >99%) with TBHP as the oxidant.

The complex of CPO with ethyl cellulose was soluble in dichloromethane and chloroform, but optimum catalytic properties were observed in less polar solvents in which the complex was not soluble. For example, using 1-octanol as solvent thioanisole was oxidized to the corresponding R-sulfoxide (ee >90%) and indole to 2-oxindole in virtually quantitative yield. The enzyme activity increased with increasing water activity and no enzyme activity was found at a water activity below 0.5. High activities were observed in hydrophobic solvents, such as *n*-hexane and isoctane.
Chapter 5

1 Introduction

The practical application of peroxidases in organic synthesis is limited by the low solubility of most substrates in aqueous buffer solution. Mixtures of aqueous buffer solution with a water-miscible organic solvent, such as tert-butyl alcohol (Van Deurzen,1997b) or acetone (Allain,1993) were used to increase the solubility of apolar substrates. However, the development of the application of peroxidases in predominantly organic media is still in a preliminary phase.

There are two approaches to perform reactions with peroxidases in organic media: by using an immobilized enzyme or by modifying the enzyme to render it soluble in organic solvents. Most studies of peroxidases in organic media have been performed with horseradish peroxidase (HRP) and both approaches were reported for this enzyme. For example, HRP was immobilized on silica and ion exchange resins for application in effluent treatment (Peralta-Zamora,1998), attached to poly(styrene/acrolein) latexes (Basinska,1995) and entrapped in sol-gel matrices (El-Essi,1997). Alternatively, the enzyme was solubilized in benzene by means of chemical modification with polyethylene glycol (Takahashi,1984; Ozaki,1998) or via a surfactant-HRP complex (Kamiya,1997). In the latter case a water-in-oil emulsion of an HRP-solution in toluene containing the surfactant (dioleyl glutamate ribitol amide) was lyophilized, resulting in a light brown, benzene-soluble solid. Besides working in pure organic solvents, the solubility problem can be solved by the use of a two-phase system in the form of a reversed micellar system (Gebicka,1997; Setti,1995).

The only example of immobilization of CPO involved adsorption on talc (Aoun,1998a,b). The resulting immobilized CPO was used for regioselective bromohydroxylation of alkenes in aqueous solution. On the other hand, CPO was used in a two-phase system for the enantioselective oxidation of racemic alcohols (Kijunen,1999). We studied the application of immobilized CPO as an enantioselective catalyst in predominantly organic solvents. Two immobilization methods were used: lyophilization of the enzyme in the presence of polymers or surfactants and covalent bonding into polyurethane foams. tert-Butyl hydroperoxide (TBHP) was used as the oxidant for these oxidations, because it is miscible with organic solvents.

2 Results and discussion

2.1 Immobilization into polyurethane foam

The immobilization of CPO into polyurethane foam (chapter 4) did not influence its enantioselective properties. CPO-PUR catalyzed oxidation of thioanisole with hydrogen peroxide, in a mixture of citrate buffer and tert-butyl alcohol (50:50 v/v), afforded the R-sulfoxide with an ee of >99% analogous to results obtained with native CPO under the same conditions (Van Deurzen,1997b). The high enantioselectivity of CPO-PUR was maintained when the reaction was performed in water saturated isooctane and with TBHP as the oxidant. In contrast, with HRP the enantiomeric purity of the formed sulfoxide decreased from 58% to 28% in aqueous buffer solution, as well as in benzene or chlorobenzene, upon polyethylene glycolation (Ozaki,1998). Moreover, the enantiomeric preference shifted from the S-enantiomer to the R-enantiomer.
2.2 Solubilization of chloroperoxidase

Initially, we attempted to solubilize chloroperoxidase via extraction from aqueous solution into organic media containing surfactants as described for α-chymotrypsin (Paradkar, 1994; Meyer, 1996). We used Aerosol OT (AOT), sodium dodecylsulfate (SDS), and sodium octadecylsulfate (SOS) as ion pair reagent and isooctane, toluene, and MTBE as the organic phase. However, in no case was solubilization of CPO into the organic phase observed. Therefore, CPO-complexes were prepared by lyophilization of mixtures of enzyme and additives: CPO-SOS and CPO-EC (EC = ethyl cellulose).

The CPO-SOS complex was insoluble in all of the organic solvents listed in table 1. The CPO-EC complex dissolved in chloroform and dichloromethane (the concentration tested was 5 mg ml⁻¹). In toluene and xylene CPO-EC formed two liquid phases and the enzyme-complex was dissolved in the bottom layer. However, the soluble CPO complex did not show higher activity than the undissolved complex in the oxidation of thioanisole. Therefore, we conclude that solvent parameters had a much larger effect on the catalytic properties of the CPO-complex compared to possible diffusion limitations resulting from the solid-liquid interface around the undissolved complexes.

2.3 Choice of the oxidant

We compared the use of H₂O₂ and TBHP as the primary oxidant in CPO-catalyzed oxidations in organic media. Figure 1 shows the course of the reaction of the oxidation of thioanisole catalyzed by CPO-SOS in water saturated isooctane. When TBHP (250 mM) was used as the oxidant, thioanisole sulfoxide was formed at a rate of 146 min⁻¹ with an ee of 70%. However, after 1 hour the oxidation rate declined and only racemic sulfoxide was formed, indicating that the catalyst was deactivated within one hour. Addition of a second amount of TBHP (250 mM) after 2 hours gave a small increase in oxidation rate but only racemic sulfoxide is formed confirming that the catalyst was no longer active.

![Figure 1. Oxidation of thioanisole catalyzed by CPO-SOS using different oxidants: 250 mM TBHP (●); 2x 250 mM TBHP (○); 250 mM TBHP with 250 mM vitamin E (▲); 250 mM H₂O₂ added in ethyl acetate (■).](image_url)
When hydrogen peroxide was used as the oxidant (250 mM; added in ethyl acetate) only a trace of racemic thioanisole sulfoxide was formed in the first 10 minutes of the reaction. In the same time substantial evolution of oxygen took place suggesting that the H$_2$O$_2$ was extensively decomposed by the catalase activity of CPO. In contrast, with TBHP in organic media no decomposition took place. Moreover, TBHP is miscible with organic solvents and further studies were performed with this oxidant.

2.4 Solvent effects

The catalytic properties of the CPO-SOS and CPO-EC complexes (5 mg ml$^{-1}$) were studied in a range of organic solvents using the oxidation of thioanisole with TBHP as a model reaction (table 1). The water activity ($a_w$) of the solvents was set at 1.0 by saturation with aqueous buffer solution (0.1 M citrate pH 5.0) before use. The solvent had a dramatic effect on the catalytic properties of CPO-complexes, the highest initial rates being observed in apolar solvents, such as $n$-hexane and isooctane. The use of polar solvent, such as chloroform and dichloromethane, resulted in low initial rates and dramatically low ees of the formed sulfoxide. When the reaction was carried out in aromatic solvents, e.g. toluene and xylene, moderate ees and initial rates were observed. The reactions in MTBE and 1-octanol were highly enantioselective but the initial rate was moderate in 1-octanol and low in MTBE. Since, the best results were obtained in 1-octanol, $n$-hexane, and isooctane, these solvents were used for further studies of the CPO-complexes.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Rate of the Non-enzymatic reaction (mM h$^{-1}$)</th>
<th>TOF Initial (min$^{-1}$)</th>
<th>ee$^b$ (%)</th>
<th>TOF Initial (min$^{-1}$)</th>
<th>ee$^b$ (%)</th>
</tr>
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<tbody>
<tr>
<td>Isooctane</td>
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<td>70</td>
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<td>n-Hexane</td>
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<td>60</td>
<td>37</td>
<td>72</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>0.36</td>
<td>61</td>
<td>90</td>
<td>50</td>
<td>91</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>0.19</td>
<td>46</td>
<td>13</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>0.11</td>
<td>27</td>
<td>74</td>
<td>17</td>
<td>74</td>
</tr>
<tr>
<td>MTBE</td>
<td>0.12</td>
<td>25</td>
<td>90</td>
<td>13</td>
<td>89</td>
</tr>
<tr>
<td>Chloroform</td>
<td>0.38</td>
<td>6</td>
<td>34</td>
<td>3</td>
<td>34</td>
</tr>
</tbody>
</table>

* a) concentration tested was 5 mg ml$^{-1}$; 
* b) the ee was determined after 30 min; the R-enantiomer was predominantly formed in all media.

2.5 Influence of α-tocopherol on the oxidations in organic media

The uncatalyzed background reaction cannot be the cause of the low ee observed in the oxidation of thioanisole catalyzed by CPO-complexes in organic media with TBHP as the oxidant (table 1). Even in the case of isooctane, in which the highest rate for the background reaction was observed, the latter contributed for only 4% to the formation of the sulfoxide. A possible explanation is that the generation of tert-butoxy radicals, via competing one-electron oxidation of the resting state of CPO, leads to the formation of racemic sulfoxide as
Selective oxidations in predominantly organic media

shown in scheme 1. To test this hypothesis we investigated the effect of the radical scavenger \( \alpha \)-tocopherol (vitamin E) on the CPO-SOS mediated oxidation of thioanisole.

\[
\begin{align*}
\text{CPO-Fe}^{\text{III}} + \text{RO}_2\text{H} &\rightarrow \text{CPO-Fe}^{\text{IV}}-\text{OH} + \text{RO}^* \\
\text{RO}^* + \text{RO}_2\text{H} &\rightarrow \text{RO}_2^* + \text{ROH} \\
\text{R}'\text{SR}'^* + \text{RO}_2^* &\rightarrow \text{R}'(\text{R}'')\text{S}-\text{OOR} \\
\text{R}'(\text{R}'')\text{S}-\text{OOR} &\rightarrow \text{R}'(\text{R}'')\text{SO} + \text{RO}^*
\end{align*}
\]

**Scheme 1.** Oxidation of sulfides via the radical pathway.

As shown in table 2 the addition of \( \alpha \)-tocopherol had a positive effect on both the initial rate and the ee of the product, particularly in apolar solvents. For example, in isooctane the initial TOF and the ee were increased from 146 to 302 min\(^{-1}\) and from 70% to 94%, respectively. In the mean time the catalyst lifetime was increased to around 3 h (figure 1). The important influence of the addition of the radical scavenger showed that radicals are the most important intermediates responsible for the deactivation of the catalyst and the formation of racemic sulfoxide.

**Table 2.** Influence of \( \alpha \)-tocopherol on the oxidation of thioanisole catalyzed by CPO-SOS in organic solvent with TBHP as oxidant\(^a\).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>TOF(^{\text{initial}}) (min(^{-1}))</th>
<th>ee(^a) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Vit. E</td>
<td>200 mM Vit. E</td>
</tr>
<tr>
<td>Isooctane</td>
<td>146</td>
<td>320</td>
</tr>
<tr>
<td>(n)-Hexane</td>
<td>138</td>
<td>302</td>
</tr>
<tr>
<td>Toluene</td>
<td>74</td>
<td>93</td>
</tr>
<tr>
<td>Xylene</td>
<td>62</td>
<td>123</td>
</tr>
<tr>
<td>1-Octanol</td>
<td>61</td>
<td>109</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>46</td>
<td>38</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>27</td>
<td>60</td>
</tr>
<tr>
<td>MTBE</td>
<td>25</td>
<td>68</td>
</tr>
<tr>
<td>Chloroform</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

\(a\) reactions performed at 1 ml scale (5 mg ml\(^{-1}\) CPO-SOS; 100 mM thioanisole; 200 mM TBHP) samples were taken every 5 min;

\(b\) turnover frequency based on the number of CPO-molecules present in the complex;

\(c\) average ee over the first 30 min of the reaction; the R-enantiomer was predominantly formed in all media.

**2.6 Kinetic analysis**

The two complexes, CPO-SOS and CPO-EC were used to determine the kinetic parameters for the oxidation of indole in water-saturated 1-octanol and isooctane. In 1-octanol, the initial rate of both catalysts showed normal saturation kinetics with respect to the substrate concentration (figure 2). These data were fitted with the Michaelis-Menten equation and the kinetic parameters calculated (table 3). In isooctane substrate inhibition was observed (figure 2) and the \( K_m \) and \( K_i^S \) were determined taking substrate inhibition into account (Tipton, 1996). As shown in table 3 the highest \( K_m \) (84 mM) and the highest initial rate were
observed in isoctane (due to the low solubility of indole in isoctane the $k_{cat}$ was estimated to be 900 min$^{-1}$). In 1-octanol $k_{cat}$ and $K_m$ for CPO-SOS and CPO-EC were of the same order of magnitude and $k_{cat}/K_m$ was the same for these two complexes. However, in isoctane the $k_{cat}/K_m$ was twice as high as in 1-octanol.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Solvent</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$K_i^S$ (mM)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPO-SOS</td>
<td>1-octanol</td>
<td>75.6</td>
<td>12.3</td>
<td>-</td>
<td>6.1</td>
</tr>
<tr>
<td>CPO-SOS</td>
<td>isoctane</td>
<td>900</td>
<td>84</td>
<td>115</td>
<td>10.7</td>
</tr>
<tr>
<td>CPO-EC</td>
<td>1-octanol</td>
<td>43.5</td>
<td>8.3</td>
<td>-</td>
<td>5.2</td>
</tr>
</tbody>
</table>

The effect of the TBHP concentration on the initial rate of the oxidation of indole was studied in water saturated isoctane and 1-octanol using CPO-SOS as catalyst. There was a linear correlation between TBHP concentration and initial rate and no saturation kinetics were observed with TBHP concentrations up to 250 mM (figure 3). Again the initial rate was higher in isoctane than in 1-octanol.

![Figure 2. Effect of the substrate concentration on the oxidation of indole: CPO-SOS in 1-octanol (△); CPO-SOS (●) and CPO-EC in isoctane (●).](image1)

![Figure 3. Effect of the oxidant concentration on the oxidation indole catalyzed by CPO-SOS in: isoctane (●) and 1-octanol (△).](image2)

### 2.7 Water activity

The effect of the water activity, in the range 1.0 (saturated with water) to <0.05 (dry solvents), on the catalytic activity of the CPO-complexes was studied for the oxidation of thioanisole in hexane and 1-octanol. The organic solvent was adjusted to the desired a using the saturated salt method (Valivety, 1992). As shown in figure 4 CPO-SOS and CPO-EC showed the highest activity in water saturated solvents and no enzyme activity was observed in 1-octanol at a water activity below 0.5. In hexane, however, no influence of the water activity on the CPO-SOS catalyzed reaction was found.
Therefore, we studied the behavior of CPO-SOS in more detail. Samples of lyophilized CPO-SOS that contained 28% (w:w) water, were dried over P₂O₅ or equilibrated above a saturated LiCl-solution for two days. In both cases, no detectable loss in weight was observed. Therefore, we concluded that the water present in the enzyme preparation after lyophilization is tightly bound. The treated CPO-SOS was applied in the oxidation of thioanisole in hexane with water activities of 1.0 and 0.12 (table 4).

<table>
<thead>
<tr>
<th>Catalyst pretreatment</th>
<th>Oxidation rate (mM h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a_w = 1.0</td>
</tr>
<tr>
<td>Stored at T_room</td>
<td>32.9</td>
</tr>
<tr>
<td>Dried over P₂O₅</td>
<td>17.8</td>
</tr>
<tr>
<td>Stored over sat. LiCl solution</td>
<td>24.1</td>
</tr>
</tbody>
</table>

From table 4, we concluded that drying of CPO-SOS leads to enzyme deactivation. We propose that the deactivation resulted from denaturation upon loss of structurally important water molecules or rearrangement of these molecules. However, the amount of water that is subtracted from the enzyme preparation upon drying is not detectable. On the other hand, the activity of the pretreated CPO-SOS for the sulfoxidation in hexane at a_w 0.12 was nearly the same as at a_w 1.0. We reasoned that hexane is an apolar solvent that does not subtract water from the catalyst, and, therefore no influence of the water activity on the catalyst activity was observed in this solvent. In contrast, in 1-octanol that is a more polar solvent, exchange of water and solvent molecules took place, which caused enzyme denaturation. This resulted in lower activities at water activities below 1.0.
3 Concluding remarks

This chapter describes the application of immobilized CPO in predominantly organic media, which allows the use of higher substrate concentrations compared to conventional use of CPO in aqueous buffer solutions. Two methods for modifying CPO have been developed, viz., immobilization into polyurethane foam and complexation with ethyl cellulose. The CPO-PUR catalyzed the highly regioselective oxidation of indole to 2-oxindole in hexane, isoctane, and 1-octanol and the enantioselective oxidation of thioanisole to the R-sulfoxide (ee >99%) in isoctane, with TBHP as the oxidant. The complex of CPO with ethyl cellulose is soluble in chloroform and dichloromethane, but the best results were obtained in less polar solvents, in which it is insoluble. For example, thioanisole afforded the R-sulfoxide with an ee of 90% in 1-octanol as solvent and TBHP as oxidant.

To our knowledge, these are the first examples of selective oxygen transfer reactions catalyzed by CPO in predominantly organic media. However, both the TOF and the TTN of the catalyst were much lower in organic solvent compared to the reactions in mixtures of aqueous buffer and tert-butyl alcohol. The high oxidant concentration (250 mM) that is necessary to obtain acceptable rates caused a rapid deactivation of the catalyst. Also the lower enantioselectivities obtain by the CPO-complexes, compared to those observed with H_2O_2 in aqueous media, are due to competing free radical processes which can be suppressed by the addition of the radical scavenger, α-tocopherol. In the mean time the catalyst lifetime is prolonged. Until now no epoxidation is organic media was observed.

Experimental part

**Enzyme immobilization:** PUR-foam immobilized CPO was prepared as described in chapter 4. CPO-PUR with a protein content of 16.4 mg protein per g dry foam was used. Enzyme-polymer/surfactant complexes were prepared by dissolving polymer or surfactant (600 mg) in citrate buffer (60 ml; 5 mM; pH 5.0) and if necessary, acetone was added to obtain a clear solution. The enzyme solution (1.5 ml) was added and the mixture was frozen in liquid nitrogen and lyophilized.

**General oxidation procedure:** In the standard procedure, the substrate (0.7 mmol) was dissolved in the solvent (7 ml). Immobilized CPO (40 mg) was added and the mixture was stirred at room temperature. After 15 min, a TBHP solution in decane (280 μl; 1.4 mmol) was added and the course of the reaction was followed by HPLC. Aliquots of the reaction mixture were quenched with tri(α-buty1)phosphine.

The organic phase was adjusted to the desired a_w using saturated salt solutions: LiCl (a_w 0.12), MgCl_2.6H_2O (0.32), Mg(NO_3)_2.6H_2O (0.55), and KCl (0.86) (Valley, 1992). Molecular sieves were used to obtain dry (a_w <0.05) solvents and saturation with citrate buffer (0.1 M; pH 5.0) was used to obtain solvents with a_w 1.0.

Reactions with different oxidants were performed at 5 ml scale (5 mg ml⁻¹ CPO-complex; 100 mM substrate; 250 mM oxidant).

Kinetic measurements were performed at 1 ml scale (5 mg ml⁻¹ CPO-complex; 0-250 mM substrate; 0-250 mM TBHP) and samples were taken every 5 minutes.
Chemical and genetic modification of chloroperoxidase

Abstract

The extraction of the iron atom from the heme group of chloroperoxidase was attempted using chelating agents under reducing conditions. After a one month period of continuous extraction the enzyme solution still contained 80% of its original activity.

In an alternative approach to substituting iron with other elements the mold Caldariomyces fumago was cultured on iron-free media containing a non-native transition metal. In the case of chromium, molybdenum, and ruthenium active chloroperoxidase was produced. However, analysis of the enzyme produced in this way revealed that although it contained non-native metal atoms the iron content is still one atom per enzyme molecule, comparable to the native enzyme.

A series of CPO mutants, generated via random mutagenesis, was screened for enhanced activity in organic solvents. Three strains showed enhanced activity in the oxidation of indole to 2-oxindole in 40% tert-butyl alcohol. A maximum increase of 3.4-fold compared with the wild type CPO was observed.

* In cooperation with Gyan P. Bai from the University of Illinois at Urbana-Champaign and Ana Conesa Cegarra from the Department of Molecular Genetics and Gene Technology, TNO Nutrition and Food Research.
1 Introduction

In the preceding chapters several methods to stabilize peroxidases were described. All of these methods involved modification of external factors that influence the stability of CPO either by controlling the oxidant level or immobilizing the enzyme. An alternative approach to improve the stability of this peroxidase involves manipulation of its intrinsic properties. Heme containing peroxidases can be modified in at least two ways: the heme-iron center and the protein itself are amenable to modification by metal exchange and mutagenesis, respectively.

In 1940 Theorell demonstrated the reversible binding of heme into horseradish peroxidase. The apo-HRP was obtained by extraction of the heme group from HRP with acetone-HCl and active HRP was regenerated by mixing apo-HRP with fresh heme. Subsequently the acetone-HCl procedure was frequently used for the removal of the heme group from heme proteins, but was later replaced by the acid-butanolone method (a mixture of HCl and methylethylketone) as reported by Teale (1959). Two years after Theorell’s publication the first report concerning the reconstitution of apo-HRP with metalloporphyrins containing Cu, Co, Mn and Ni appeared (Gjessing,1942). Since then the synthesis and properties of numerous isomorphously substituted variants of HRP, in which iron is replaced by manganese (Khan,1996), magnesium (Kuwahara,1982), zinc (Kaneko,1980), cobalt (Wang,1977), ruthenium (Morishima,1986), vanadium (Tamura,1977), molybdenum and tungsten (Shiro,1988) have been reported. Apo-peroxidases are applied in analysis assays, which contain an antibody-bound heme group that is released upon binding with the antigen resulting in the reconstitution of the peroxidase followed by an colorimetric reaction (Pugia,1999).

The reconstitution of apo-hemoproteins with metalloporphyrins incorporated with metals other than iron is not limited to HRP. Metal exchange has been applied to superoxide dismutase (Brock,1976), cytochrome c (Dickinson,1975), myoglobins and hemoglobins (Yonetani,1974), and cytochrome P-450 BM-3 (Modi,1995). Also the influence of iron porphyrins other than protoporphyrin IX on HRP has been studied (Gafert,1994; Araiso,1981).

Metal exchange via extraction and replacement of the heme group is not possible with microperoxidase (MP-8). In MP-8, obtained by peptic and trypic digestion of cytochrome c, the heme group is covalently linked to the remaining 8 amino acid residues. The iron atom in MP-8 has been replaced by manganese (Llow,1998) and by $^{57}$Fe (Primus,1999). The free base of MP-8 is obtained after extraction of the iron atom with anhydrous HF at -78 °C. Metalation reactions were performed by addition of manganous or ferrous acetate at 40 °C for 2 hours followed by oxidation with air.

In contrast to the extensive studies on the reconstituted of HRP, very little has been published concerning metal exchange in CPO. Van Deurzen (unpublished data) studied the heme extraction from CPO using the acid-butanolone method and found it unsuitable for CPO. Apo-chloroperoxidase was produced via recombinant expression of the CPO gene in Escherichia coli (Zong,1995). In contrast to HRP, for which quantitative heme incorporation was obtained, the yield of active holo-CPO was only 5% when high-pressure treatment (207
MPa) was used. Hence, we concluded that metal-exchanged CPO could not be obtained via heme reconstruction.

This chapter describes two attempts to achieve this goal. The first attempt involved the extraction of iron from CPO under reducing conditions. In the second approach the mold C. fumago was grown in an iron-free medium that contained the desired non-natural metal ion. The usefulness of mutagenesis to obtain biocatalysts with enhanced properties has already been discussed for HRP and myoglobin (chapter 1). In this chapter the screening of CPO mutants with enhanced activity in organic solvents is discussed. The second generation CPO mutants were obtained via random mutagenesis of the CPO gene that has been expressed in C. fumago (Patterson, 1993; Rai, 1999).

2. Results and discussion

2.1 Metal exchange in CPO

2.1.1 Iron-extraction from CPO

The selective extraction of the iron ion from the heme group of CPO was attempted using chelating agents, e.g. EDTA (ethylenediaminetetraacetic acid) and DTPA (diethylene-triaminepentaacetic acid), at neutral pH. Sodium dithionite was used to reduce the iron to the ferrous form (Morris, 1966) to facilitate its extraction from the heme group. After continuous extraction for 1 month at 4°C the CPO solution still retained 80% of its initial activity. Hence, we concluded that iron extraction by chelating agents under the condition applied is not feasible with CPO. Probably that extraction under other conditions (pH, salt concentration and/or cosolvents) is possible.

The synthesis of metal exchanged CPO starting from native CPO has to be developed by heme exchange under high pressure (Zong, 1995) or by iron extraction under the harsh conditions as applied for microperoxidase.

2.1.2 Culture of C. fumago on different metals

As discussed above, we did not obtain metal-exchanged CPO via metal or heme replacement and we expected that the harsh conditions applied with microperoxidase would not be feasible for an efficient synthesis of CPO with non-natural metals in its active site. Therefore we turned our attention to the possibility of preparing metal-exchanged CPO by allowing the mold C. fumago to grow in iron-free media containing other metal ions.

As shown in table 1 seven non-native transition metal salts were tested together with an iron and a metal-free culture as references. To remove as much iron as possible from the C. fumago cultures, inocula were grown twice on a small scale (50 ml) before large scale roller bottles (2 L) were inoculated. The mold C. fumago was able to grow on iron-free media containing high concentrations (5 mM) of non-native metal salts. Both the inoculation cultures and the large-scale roller-bottle cultures were analyzed for their specific chlorination activity (MCD assay). Table 1 showed that all the inoculation cultures were active, whereas from the final cultures only the iron-, chromium-, and molybdenum-containing cultures showed activity. Negligible background activity was observed with 5
mM metal salt solutions consistent with the observed activity resulting from enzyme activity and not from free metal ions in the culture medium.

Table 1. Results of *C. fumago* cultures on iron-free media containing different metal salts

<table>
<thead>
<tr>
<th>element</th>
<th>salt</th>
<th>concentration (mM)</th>
<th>50 ml culture</th>
<th>roller-bottle culture</th>
<th>metal solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
<td>0</td>
<td>8.6</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>CoSO₄</td>
<td>5.2</td>
<td>4.6</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>Cr</td>
<td>Cr(NO₃)₃</td>
<td>6.0</td>
<td>2.3</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Fe</td>
<td>FeSO₄</td>
<td>5.0</td>
<td>n.d.</td>
<td>55</td>
<td>0.02</td>
</tr>
<tr>
<td>Mn</td>
<td>MnSO₄</td>
<td>5.4</td>
<td>5.1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Mo</td>
<td>Na₂MoO₄</td>
<td>5.5</td>
<td>12.6</td>
<td>6.3</td>
<td>0.10</td>
</tr>
<tr>
<td>Ru</td>
<td>RuCl₃</td>
<td>6.3</td>
<td>27.8</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>Na₂WO₄</td>
<td>5.3</td>
<td>3.0</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

a) medium consisted of fructose (100 g L⁻¹), NaNO₃ (2 g L⁻¹), MgSO₄·7H₂O (2 g L⁻¹), and K₂HPO₄ (2 g L⁻¹);
b) no metal added as a reference culture;

The final cultures showing activity in the MCD-assay were purified using acetone precipitation followed by dialysis. Enzyme samples were analyzed by UV/Vis spectrophotometry and atomic absorption spectroscopy and their specific chlorination and one-electron oxidation activities were measured using the MCD and ABTS-assay.

Table 2. Characteristics of metal exchanged CPO

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fe-CPO</th>
<th>Cr-CPO</th>
<th>Mo-CPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda_{\text{max}}$ (nm)</td>
<td>400</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>C₄₀₀ (µM)</td>
<td>43.5</td>
<td>12.9</td>
<td>10.5</td>
</tr>
<tr>
<td>Rs</td>
<td>0.87</td>
<td>0.24</td>
<td>0.23</td>
</tr>
<tr>
<td>MCD (U ml⁻¹)</td>
<td>2904</td>
<td>13.7</td>
<td>407</td>
</tr>
<tr>
<td>(U nmol⁻¹)</td>
<td>66.8</td>
<td>1.1</td>
<td>38.8</td>
</tr>
<tr>
<td>ABTS/H₂O₂ (mU ml⁻¹)</td>
<td>52.4</td>
<td>1.9</td>
<td>9.6</td>
</tr>
<tr>
<td>(U µmol⁻¹)</td>
<td>1.2</td>
<td>0.15</td>
<td>0.92</td>
</tr>
<tr>
<td>ABTS/TBHP (mU ml⁻¹)</td>
<td>2017</td>
<td>37.4</td>
<td>400</td>
</tr>
<tr>
<td>(U µmol⁻¹)</td>
<td>46.4</td>
<td>2.9</td>
<td>38.2</td>
</tr>
<tr>
<td>Fe (µM)</td>
<td>60.7</td>
<td>13.4</td>
<td>17.7</td>
</tr>
<tr>
<td>Cr (µM)</td>
<td></td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>Mo (µM)</td>
<td></td>
<td></td>
<td>0.83</td>
</tr>
</tbody>
</table>

As shown in table 2 the metal exchanged CPO still contained around 1 iron atom per enzyme molecule, which is equal to native CPO (Morris, 1966). This indicates that the inoculation cultures still contained enough iron to produce substantial amounts of iron-containing CPO. In the case of chromium less than 1 Cr ion was found per enzyme molecule and the case of molybdenum only a trace of Mo. Comparable results were obtained with a ruthenium culture (Fe: 0.68 mol mol⁻¹; Ru: 38 mol mol⁻¹). Metal-exchanged CPO samples that contained one iron atom per enzyme molecule together with the nonnative transition
metals (Cr, Mo, and Ru) showed a low specific activity in the MCD and ABTS-assay compared to native CPO. Similarly, lower activities were also found in oxygen transfer reactions, e.g. thioanisole and indole oxidation.

### 2.2 Mutant chloroperoxidase

Second generation mutants produced by random mutagenesis (Rai, 1999) were cultured in 24 well plates at the University of Illinois by Gyan P. Rai. Aliquots of the recombinant CPO secreted into the culture medium were directly used for determination of the indole oxidation activity. The activity assays were performed in aqueous buffer solution as well as in aqueous buffer containing 40% (v:v) tert-butyl alcohol in a 96 well microplate reader. Due to the fact that the enzyme concentrations in the culture media were unknown, relative indole oxidation activities (activity in 40% tert-butyl alcohol divided by the activity in aqueous solution) were used to compare the mutants with the wild type CPO. Over one hundred mutants were screened for enhanced activity for the oxidation of indole in aqueous buffer containing 40% (v:v) tert-butyl alcohol. Seven mutants were selected for further analysis.

The selected mutants were grown on a larger scale (500 ml) and purified via acetone precipitation followed by dialysis. Purified enzyme samples with a known enzyme concentration were analyzed for their indole oxidation activities in both aqueous buffer solution and aqueous buffer containing 40% (v:v) tert-butyl alcohol in a 4 ml quartz cuvet. Specific activities (activity per amount of enzyme) of mutant and wild type CPO are summarized in Table 3. From the seven mutants selected, three showed a moderate to good enhancement of activity in buffer containing 40% tert-butyl alcohol.

<table>
<thead>
<tr>
<th>Mutant ID</th>
<th>Enzyme concentration (mg ml⁻¹)</th>
<th>Activity ([dA/dt]/μg Buffer</th>
<th>Activity ([dA/dt]/μg 40% t-BuOH</th>
<th>Relative activity (-) Buffer</th>
<th>Relative activity (-) 40% t-BuOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>13.4</td>
<td>610</td>
<td>30</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1C2</td>
<td>3.4</td>
<td>683</td>
<td>43</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>2B1</td>
<td>0.8</td>
<td>264</td>
<td>22</td>
<td>0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>2B3</td>
<td>2.3</td>
<td>642</td>
<td>59</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>2C1</td>
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<td>121</td>
<td>11</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
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<td>11</td>
<td>0.6</td>
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</tr>
<tr>
<td>3B4</td>
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<td>568</td>
<td>102</td>
<td>0.9</td>
<td>3.4</td>
</tr>
<tr>
<td>3C4</td>
<td>0.8</td>
<td>587</td>
<td>33</td>
<td>1.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

A maximum 3.4-fold increase in activity indicates that random mutagenesis is a powerful tool to obtain enzymes with enhanced catalytic properties. A further increase in activity can be obtained by new mutagenesis rounds on these most active mutants, as reported for CPO mutants that were resistant to suicide inactivation (Rai, 1999).
2.3 Purification
Chloroperoxidases (wild type as well as mutants and metal-exchanged variants) from *C. fumago* cultures were purified using the method described by Zong (1997). This purification method involved precipitation with cold acetone followed by membrane filtration. As shown in figure 1, the purity of the final enzyme preparation is dependent on the amount of enzyme present in the culture liquid. The enzyme purity is given by its $R_z$: purity standard = $A_{400}/A_{280} = 1.44$ for pure CPO. Reasonable purities ($R_z$ around 1.2) were obtained if the enzyme concentration was above 75 mg L$^{-1}$ culture liquid. Below this concentration, the purity of the enzyme dropped dramatically with decreasing enzyme concentration. The commercial CPO preparation from Chirazyme Labs that is produced via this precipitation method also had a $R_z$ of 1.23.

![Figure 1. Influence of the enzyme concentration in the culture liquid on the final enzyme purity.](image)

3 Concluding remarks
This chapter describes two attempts to modify chloroperoxidase, viz. modification of the iron-protoporphyrin system and protein engineering. We did not succeed in preparing metal-exchanged CPO. Iron extraction from CPO by means of chelating agents, such as EDTA and DTPA, under reducing conditions did not lead to the formation of apo-CPO. The observed decrease in activity probably resulted from enzyme deactivation rather than from the formation of apo-CPO.

The mold *C. fumago* was able to grow on iron-free media containing non-natural metal salts. In the case of chromium, molybdenum, and ruthenium containing cultures active CPO was produced. Similar to the native CPO, the purified metal-exchanged CPO samples contained around one iron atom per enzyme molecule. The enzyme solution contained 0.61 mol mol$^{-1}$, 0.08 mol mol$^{-1}$, and 38 mol mol$^{-1}$ non-natural metal ion for respectively Cr-CPO, Mo-CPO and Ru-CPO. These metal-exchanged CPO samples had a lower specific activity for the MCD, ABTS, indole, and thioanisole oxidation compared to the iron-containing CPO. Metal-exchanged CPO that contained one atom of non-natural metal per enzyme molecule and no iron was not obtained. The method of culturing *C. fumago* on different metals possible has some utility if a method could be found to eliminate the iron-storage from the
Chemical and genetic modification of CPO

inocula (the microorganism stored so much iron inside its cells that it will produce iron containing CPO even when no iron is present in the medium).

Protein engineering was attempted by screening a series of over one hundred CPO mutants for enhanced activity in indole oxidation in aqueous buffer containing 40% tert-butyl alcohol. Seven mutants were selected for further analysis and produced and purified on a larger scale. Purified enzyme samples were analyzed for indole oxidation activity and three mutants showed an enhanced activity in aqueous buffer solution containing 40% tert-butyl alcohol. The best result obtained was a 3.4-fold increase in activity compared to the wild type CPO.

In conclusion, protein engineering is the most promising attempt to obtain CPO with enhanced stability. Tools to produce recombinant CPO are available today (chapter 7). Changing the screening conditions will result in enzymes with different fine-tuned properties. However, protein engineering cannot solve the fundamental instability of heme peroxidases due to oxidative degradation of the iron-protoporphyrin moiety. Therefore, heme and/or iron replacement is still a goal to obtain peroxidase with enhanced operational stability.

Experimental part

Strains: The wild type *C. fumago* strain as well as strains that contained second generation mutants (Rai, 1999) were received as a gift from Prof. Hager (University of Illinois at Urbana-Champaign).

Production of CPO: Fungal culturing was carried out in two-liter roller-bottles containing fructose-salt medium (500 ml; 100 g L⁻¹ fructose; 2 g L⁻¹ NaNO₃, KH₂PO₄, and MgSO₄·7H₂O; 20 mg L⁻¹ FeSO₄ or other metal salts as indicated in table 1). Cultures were inoculated with 15 cm² mycelium and grown for two weeks at 20° on roller apparatus at 1 rpm.

Purification of CPO: To the filtered medium (400 ml) cold acetone was slowly added (170 ml; 30% v/v; -20°C) at 4°C, followed by incubation at -20°C for 1 hour. Precipitated carbohydrate polymers were removed by centrifugation (4400 min⁻¹; 20 min; 0°C). To the supernatant was slowly added cold acetone (765 ml; final concentration 70% v/v; -20°C) precipitation of CPO occurred overnight at -20°C. Supernatant was decanted and the precipitated CPO was dried for 10 min and dissolved in citrate buffer (50 ml; 50 mM; pH 5.0). The CPO solution was dialyzed against citrate buffer (2 L; 50 mM; pH 5.0; three times) and concentrated to a final volume of 5 ml (Centriprep-30 concentrator, Amicon; 1800 min⁻¹; 4°C).

Oxidation of indole (96 well plate): Enzyme solution (10 µl) was mixed with the indole solution (200 µl; 0.25 mM indole in citrate buffer; 0.1 M; pH 5.0; containing 0 or 40% tert-butyl alcohol). The reaction was initiated by the addition of oxidant (2 µl; 25 mM H₂O₂ or 1.5 M TBHP) and the course of the reaction was monitored at 250 nm.

Oxidation of indole (4 ml cuvet): Enzyme solution (50 µl) was mixed with the indole solution (3.40 ml; 0.375 mM indole in citrate buffer; 0.1 M; pH 5.0; containing 0 or 40% tert-butyl alcohol). The reaction was initiated by the addition of H₂O₂ (50 µl; 35 mM) and the course of the reaction was monitored at 276 nm.

Continuous metal extraction: The dialysis buffer was prepared by dissolving DTPA (50 g; 51 mM), sodium acetate (21 g; 0.1 M), sodium dithionite (50 g; 0.1 M) and sodium hydroxide (14 g) in water (2.5 L). This buffer was adjusted to pH 5.9 with a NaOH-solution (0.1 M) and cooled to 4°C. An
Amicon stirred cell equipped with a 30 kDa cut-off membrane was charged with CPO (62 mg) and dialysis buffer (50 ml). Enzyme solution was dialyzed with 2.4 L of dialysis buffer followed by concentration to a final volume of 40 ml.
Expression of recombinant CPO in *Aspergillus niger*

Abstract

The *Caldariomyces fumago* chloroperoxidase was successfully expressed in *Aspergillus niger*. The recombinant enzyme was produced in the culture medium as an active protein in concentrations up to 10 mg L⁻¹ 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 U nmol⁻¹) 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. For example, indole was oxidized to 2-oxindole with 99% selectivity and thioanisole to the corresponding R-sulfoxide (ee >98%). Incorporation of ¹⁸O from labeled H₂¹⁸O₂ into the oxidized products was 100% in both cases.

* In cooperation with Ana Conesa Cegarra from the Department of Molecular Genetics and Gene Technology, TNO Nutrition and Food Research.
1 Introduction

Chloroperoxidase (CPO; EC 1.11.1.10) is a heavily glycosylated monomeric heme protein, with a sugar content of 18% of its molecular weight of 42 kDa (Kenigsberg, 1987). The chloroperoxidase is secreted by the filamentous fungus *Caldariomyces fumago* and was first purified and described by Morris and Hager (1966). *In vivo*, CPO catalyzes oxidative chlorination. *In vitro*, in the absence of Cl\(^{-}\), CPO catalyzes a variety of synthetically useful (enantioselective) oxygen transfer reactions (chapter 1). In catalyzing these oxygen transfer reactions CPO behaves more like the P-450 cytochromes than like a classical peroxidase, such as the peroxidases from horseradish roots, soybeans, and the fungus *Caprinus cinereus*, which mostly catalyze one-electron oxidations. Also the iron protoporphyrin is ligated to the active site of CPO through a cysteine residue (Blanke, 1988; Dawson, 1987; Liu, 1995), which is normal for P-450 cytochromes, whereas the axial ligand in peroxidases normally is a histidine residue (Smith, 1998). These features make CPO an unique member of the peroxidase family. Furthermore, the enzyme shows no sequence similarity to other extracellular heme peroxidases (Black, 1991; Godfrey, 1990; Mayfield, 1994; Sawai-Hatanaka, 1995; Zhang, 1991) nor to known microbial haloperoxidases (Bantleon, 1994; Burd, 1991; Van Schijndel, 1993), however these are vanadium and not heme containing enzymes.

Site-directed mutagenesis has proved to be a powerful tool in exploring the structure-function relationships in classical peroxidases (Smith, 1998); especially horseradish peroxidase (HRP) has been studied in great detail by Morishima and coworkers (Mukai, 1997; Nagano, 1996; Tanaka, 1996, 1997) and Smith and coworkers (Rodriguez-Lopez, 1996a, b; Sanders, 1994; Smulevich, 1994). On the other hand, Ortiz de Montellano and coworkers have used site-specific mutagenesis to turn HRP into an oxygen transfer catalyst suitable for enantioselective sulfoxidation and epoxidation reactions (section 4.3 of chapter 1). The use of such an approach in CPO could help in revealing the structural basis of the unique properties of this enzyme and to explore its 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: heavy glycosylation, disulfide-bridge formation, cleavage of N-terminal and C-terminal sequences and prosthetic group incorporation (Kenigsberg, 1987), bacterial hosts could present problems in producing the active protein. Indeed, Zong (1995), reporting the expression of *cpo*-gene in *E. coli*, showed that a non-glycosylated enzyme was secreted into the periplasm in its apo-form and only after a tedious high-pressure assisted reconstitution process could some active holo-enzyme be recovered.

Consequently, 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 (Sigle, 1993). Similarly, attempts to produce CPO in *Saccharomyces cerevisiae* and *Pichia pastoris* have been unsuccessful (Zong, 1997). Data on the use of a fungal expression system are spurious although a transformation procedure for *Caldariomyces fumago* has been described (Patterson, 1993). This system was expected to be insufficient to be used for selective expression of
Expression of recombinant CPO

recombinant CPO. However, recently a paper appeared in which this system was used to produce directed evolution mutants of CPO that are resistant to suicide inactivation by primary olefins (Rai, 1999). The authors mentioned the question of whether both wild type and mutant CPO are being produced in the C. fumago constructs. They could not answer this question unequivocally but no detectable amounts of wild type CPO contaminates were found in their most active mutant.

We have explored the possibility of producing CPO in a 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, all the necessary tools are available for the production of recombinant CPO. To date several reports on the expression of fungal metalloproteins in filamentous fungi have been published (Saloheimo, 1991; Stewart, 1996). However, the yields were still far from those typically obtained for less complex fungal proteins. Therefore, also from the fundamental point of view, there is a growing interest in understanding how filamentous fungi can achieve the (over)production of extracellular metalloproteins. Recently, the expression of two fungal heme containing peroxidases in the filamentous fungus A. niger was reported (Conesa, 1999). 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.

This chapter describes the construction of an expression vector and 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.

2 Results and discussion

2.1 Isolation of Aspergillus niger transformants producing rCPO

In a co-transformation experiment the uridine-deficient strain MGG029 was transformed with a mixture of plasmids pCPO3.1-AmdS and pAB4-1. Several uridines-phototrophic, acetamide-utilizing transformants were obtained and were transferred to both acrylamide and o-anisidine containing plates. Efficient growth and sporulation on acrylamide plates reflects multicityp integration of the transforming vector (Verdoes, 1993), and colored product formation on o-anisidine plates indicates extracellular peroxidase activity (Conesa, 1999). Four transformants, which grew vigorously on acrylamide and which developed an intense coloring with the o-anisidine assay, were selected. These four strains were cultured on maltose minimal medium for 48 hours and analyzed for cpo-mRNA and extracellular protein production. From this analysis the best producing transformant, strain [MGG029]pCPO3#5, was selected for production and purification rCPO.
2.2 Production and purification of rCPO.

To optimize the level of rCPO production the influence of the addition of external heme on the production of rCPO in A. niger was studied. This revealed (Conesa, 1999) that the level of active heme peroxidase production in A. niger can be significantly increased by heme supplementation of the culture medium. Also in this case, a 10-fold increase in rCPO production was obtained upon hemin addition up to a concentration of 500 mg L\(^{-1}\). Similar results have been obtained in previous studies by our and other groups on the expression of fungal peroxidases in Aspergillus species (Erol, 1997; Conesa, 1999; Stewart, 1996). However, despite heme supplementation, rCPO was only partially (40%) filled with heme (vide infra).

An additional 5-fold increase was achieved by decreasing the culture temperature from 30\(^{\circ}\)C to 22\(^{\circ}\)C. This could be explained by a lower protease activity in the culture medium at the lower temperature. Similarly, addition of BSA to the culture medium resulted in a more prolonged stability of rCPO during the fungal culture, which supports the hypothesis of partial degradation of rCPO by extracellular protease activity. Under these optimized conditions up to 10 mg L\(^{-1}\) rCPO could be produced.

<table>
<thead>
<tr>
<th>Table 1. Purification of rCPO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
</tr>
<tr>
<td>Culture liquid</td>
</tr>
<tr>
<td>Acetone precipitation</td>
</tr>
<tr>
<td>DEAE Sepharose</td>
</tr>
<tr>
<td>GPC</td>
</tr>
</tbody>
</table>

The rCPO, from a hemin (500 mg L\(^{-1}\)) containing culture, was purified to electrophoretic homogeneity by acetone precipitation and column chromatography as reported for CPO from C. fumago (Van Deurzen, 1994; Zong, 1997). The results obtained in the purification of rCPO, are summarized in table 1.

Purification of CPO from C. fumago via acetone precipitation followed by dialysis resulted in enzyme samples with a Rz of 1.2 only when the enzyme concentration exceeded 75 mg L\(^{-1}\) (section 2.3 of chapter 6). As indicated in table 1 this method is not suitable for an A. niger culture that contained only 1.5 mg rCPO L\(^{-1}\). However, during further purification using ion exchange resin (DEAE Sepharose) and GPC (Superose 12) the rCPO showed the same characteristics as native CPO. Finally, homogeneous rCPO was obtained in 11% yield and with a Rz of 0.54.

2.3 Characterization of rCPO

Figure 2 shows the UV-spectra of purified rCPO and native CPO (commercial preparation with Rz 1.23). As 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 native CPO. Homogeneous CPO from C. fumago has a Rz of 1.44. This indicates that even with heme supplementation the rCPO is only partly (38%) occupied with heme. This is in contrast to the observations on the production of Phanerochaete chrysosporium manganese peroxidase (MnP) in A. niger (Conesa, 1999), where the recombinant enzyme was produced
with the same heme content as the native protein. It can be argued that this difference is due to the different nature of heme attachment in the MnP (axial ligand histidine) and CPO (axial ligand cysteine) proteins.

![Figure 1. UV-spectra of rCPO (—) and native CPO (----).](image)

![Figure 2. SDS-PAGE analysis of rCPO and nCPO, partially deglycosylated with EndoH (+).](image)

SDS-PAGE analysis of rCPO showed lower migration mobility when compared to native CPO. This difference could be due to a higher degree of glycosylation of the recombinant enzyme. To test this possibility, both rCPO and native CPO were deglycosylated by treatment with endoglycosidase H: EndoH (figure 3). After deglycosylation, rCPO recovered the mobility of native CPO on SDS-PAGE, indicating that the differences in size could indeed be attributed to overglycosylation of rCPO. Overglycosylation has been reported for the expression of other heterologous proteins in *Aspergillus sp.* (Wyss, 1999; Conesa, 1999). In these reports, the excess of glycosyl groups was shown to have only a minor effect on the properties of the recombinant enzymes. Our results on the characterization of the recombinant chloroperoxidase indicate that this may also be the case for rCPO. Furthermore, studies on the expression of chloroperoxidase in *E. coli* showed that glycosylation is not a mandatory requirement for the activity of this enzyme (Zong, 1995).

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. This was not completely surprising, since native CPO is known to possess an N-terminal glutamic acid residue which is mostly cyclized into a pyrrolidone carboxylic acid. Such molecules, whose formation is induced in acidic environments, are unreactive to the Edman's reagent. As the culture medium of *A. niger* reaches pH 2, this may explain the N-terminal blockage of rCPO.

### 2.4 Catalytic properties of rCPO

To further validate the *A. niger* production system for CPO, the recombinant enzyme has to be fully active and its catalytic properties have to be comparable with those of native CPO. The specific chlorination activity of purified rCPO (MCD assay was 47 U (nmol heme)⁻¹).
which was in agreement with the activities reported in the literature: 70 (Morris, 1966); 53 (Van Deurzen, 1997b); 59 (Libby, 1996) and for different batches of native CPO determined by us: 56; 70 and 92 U nmol⁻¹. The pH-optimum for the chlorination of monochlorodimedone was measured for rCPO and native CPO. rCPO and native CPO showed the same pH-profile with a pH-optimum at 2.75. The pH-optimum for this chlorination reaction coincided with the pH of the MCD assay as described by Morris (1966). From these results we concluded that the natural chlorination activity of CPO is completely present in the recombinant enzyme.

The enantioselective sulfoxidation of thioanisole and derivatives (table 2) was used to monitor the enantioselective properties of the enzyme. As shown in table 2 results obtained in 1 ml scale experiments differed slightly from the results published for 50 ml scale experiments. Analogous to the native CPO rCPO produced predominantly the $R$-sulfoxide in up to 99% ee. Experiments with labeled $\text{H}_2^{18}\text{O}_2$ showed 100% incorporation of $^{18}\text{O}$ into thioanisole sulfoxide.

<table>
<thead>
<tr>
<th>Sulfide</th>
<th>Native CPO\textsuperscript{a}</th>
<th>Native CPO\textsuperscript{b}</th>
<th>rCPO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conversion (%)</td>
<td>e.e. (%)</td>
<td>Conversion (%)</td>
</tr>
<tr>
<td>![Sulfide structure]</td>
<td>100</td>
<td>99</td>
<td>65</td>
</tr>
<tr>
<td>![Sulfide structure]</td>
<td>83</td>
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<td>94</td>
</tr>
<tr>
<td>![Sulfide structure]</td>
<td>53</td>
<td>99</td>
<td>62</td>
</tr>
</tbody>
</table>

\textsuperscript{a} results reported by for a 50 ml scale experiment (Van Deurzen, 1997b);

\textsuperscript{b} results obtained in a 1 ml scale experiment with native CPO from Chirazyme Labs.

The regioselectivity of rCPO was studied by means of the oxidation of indole and derivatives (table 3). As shown in table 3 the conversions obtained with rCPO were 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 $\text{H}_2^{18}\text{O}_2$ showed 100% incorporation of $^{18}\text{O}$ into 2-oxindole.

The oxygen transfer properties of CPO were not changed upon expression of the enzyme in \textit{A. niger}. Recombinant CPO showed an enantioselectivity of 98% 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 these obtained with native CPO. However when a mixture of \textit{t}err\text{-}butyl alcohol and aqueous buffer (50:50 (v/v)) was used (oxidation of indoles, table 3) rCPO afforded lower yields than native CPO. A possible explanation would be an effect of the increased glycosylation of rCPO on the stability of the enzyme in mixtures of \textit{t}err\text{-}butyl alcohol and aqueous buffer.
Expression of recombinant CPO

<table>
<thead>
<tr>
<th>Indole derivative</th>
<th>Native CPO(^a) Conversion (%)</th>
<th>Native CPO(^b) Conversion (%)</th>
<th>rCPO Conversion (%)</th>
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<tr>
<td></td>
<td>(15 min)</td>
<td>(60 min)</td>
<td>(15 min)</td>
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<td></td>
<td></td>
<td></td>
<td>(60 min)</td>
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<tr>
<td>Br</td>
<td>25</td>
<td>96</td>
<td>24</td>
</tr>
<tr>
<td></td>
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<td>Cl</td>
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<td>n.d.</td>
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<td>37</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
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<td></td>
<td>19</td>
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<td>5</td>
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</table>

\(\text{a)}\) results reported for a 50 ml scale experiment (Van Deurzen, 1996);

\(\text{b)}\) results obtained in a 1 ml scale experiment with native CPO from Chirazyme Labs.

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 \(^{18}\text{O}\) from labeled H\(_2\)^{18}\text{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 (Doerge, 1991) and the oxidation of indole (Van Deurzen, 1996). Hence, we conclude that both the chlorination activity and the oxygen transfer properties of CPO are fully retained in the recombinant enzyme.

3. Concluding remarks

This chapter describes the construction of an expression vector and the expression of the *Caldariomyces fumago* cpo-gene in the filamentous fungal expression host *Aspergillus niger*. The optimized production of rCPO by *A. niger* was only 10 mg L\(^{-1}\) rCPO, at 22\(^\circ\)C and with external heme (500 mg L\(^{-1}\) hemin) added. The rCPO had a higher degree of glycosylation than the native CPO.

Analysis of the catalytic properties of rCPO revealed that the recombinant enzyme is fully active. For example in the MCD-assay, rCPO showed the same specific activity and pH-profile to the native CPO. rCPO catalyzes the oxidation of various substrates in comparable yields and selectivities as native CPO. For example, indole was oxidized to 2-oxindole with 99% selectivity and thioanisole to the corresponding R-sulfoxide (ee >98%). Incorporation of \(^{18}\text{O}\) from labeled H\(_2\)^{18}\text{O}_2 into the oxidized products was 100% in both cases.

The homologous expressing of CPO in the host *A. niger*, offers the possibility for mutagenesis studies. Site-directed mutagenesis could reveal the structural background of CPO characteristic features. On the other hand knowledge about the deactivation mechanism could pave the way for further improvement of the operational stability of CPO.
Experimental part

Strains: Escherichia coli DH5 was used for construction and propagation of vector molecules.
The Aspergillus niger strain MGG029 (prtT, gla::floet, pyrG, (Conesa,1999)) was used as recipient strain in transformation experiments.

Construction of cpo expression vector: pCf6, a cpo genomic clone, was a gift from Prof. Hager (University of Illinois). Primers CLP15F/A (GGAATTCACATGTTCCTCAGGTCC) and CLP1CTERM3 (CGCGGGATCTTAAAGGGGCGGC) were used to amplify cpo from pCf6 and introduce suitable cloning sites. The resulting PCR product was EcoRI/BamHI digested and cloned into pUC19 to render pCPO3. The cpo gene was checked by sequence analysis and excised from pCPO3 as an AffIII/HindIII fragment and cloned into the pAN52-10Not fungal expression vector (Punt,1991) at the NcoI/HindIII cloning sites, resulting in pCPO3.1. In vector pCPO3.1 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 (Kelly,1985) was introduced in pCPO3.1 at an unique NotI site to render the final cpo expression vector pCPO3.1-AmdS (figure 3).

![Diagram](https://via.placeholder.com/150)

Figure 3. cpo-Expression vector pCPO3.1-AmdS.

Transformation procedures: Fungal co-transformation was carried out as described (Punt,1992), using pCPO3.1-AmdS and pAB4-1 (Van Hartingsveldt,1987) 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 (Verdoes,1993) and for extracellular peroxidase activity on o-anisidine plates as described (Conesa,1999).

Polyclonal antisera: For preparation of polyclonal antibodies, CPO from C. funago IMI 089362 was purified according to literature (Van Deurzen,1994). A 3 mg aliquot of the purified CPO was treated with aceton-0.3% HCl to remove the heme group (Nakahara,1996), and both holo and apo-chloroperoxidase were used for rabbit immunization. Immunizations were done in duplo using 100 µg protein in Freund's Complete Adjuvant-H2O2 (1:1). Boosters were done after two and sixteen weeks after immunization 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 minimal growth medium (Bennet,1991) with 5% maltodextrine and supplemented with 0.5% casein amino acids and 500 mg L-1 hemin. Cultures were inoculated with 5·10⁶ 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, followed by incubation at -20°C for 1 hour. The precipitated impurities were removed by centrifugation (4400 min⁻¹; 20 min; 0°C). Cold acetone was 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 decanted; the precipitated protein was dried for 10 min and dissolved in phosphate buffer (300 ml; 10 mM; pH 5.2), adjusted to pH 5.8 by 10 mM H₃PO₄ and brought onto a DEAE Sepharose (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 by a gradient of 20-200 mM phosphate buffer (pH 5.8; 10 ml min⁻¹) during 4 hours. Fractions with a chlorination activity (MCD assay) above 0.25 U/ml were pooled, adjusted to pH 5.2 and concentrated over a 30 kDa membrane (Centricon-30 concentrator) at a speed of 1800 rpm. Further purification was done by gel filtration over a Superose 12 HPLC column (phosphate buffer pH 5.2; 200 mM; 0.5 ml min⁻¹).

**Molecular and protein methods:** Molecular methods were carried out essentially as described by Sambrook (1989).

Total fungal RNA was isolated using the RNAzol™ kit from CINNA/BIOTECX. For northern analysis experiments a 1 kb. SfII fragment from pCF6 containing most of the cpo coding region was used.

SDS-PAGE was performed with a BioRad MiniProteII system using the TRIS-glycine method and 10% polyacrylamide gels. For N-terminus determinations, purified CPO was run on SDS-PAGE, blotted onto a PVDF membrane and colored with coomassie blue. The rCPO protein band was excised from the PVDF membrane and N-terminus determinations were carried out at the Biocentrum Utrecht by Edman degradation.

For deglycosylation experiments, proteins were treated with EndoH endoglycosylase (New England Biolabs) following manufacturer’s instructions.

**Oxidation of sulfdes:** At room temperature sulfide (50 μmol) was dissolved in 1.0 ml of solvent (0.2 M phosphate buffer pH 5.2). Chloroperoxidase (24 U) was added to the reaction mixture, followed by 5 min of stirring. The reaction was started by the continuous addition of H₂O₂ (0.15 M) at a rate of 1eq/2h. In total 1.1 eq of H₂O₂ was added and the reaction was quenched after 2.5 h by the addition of an excess of Na₂SO₃. The reaction mixture was homogenized by the addition of isopropyl alcohol (400 μl) and analyzed by chiral HPLC.

Oxidation of thioanisole with H₂¹⁸O₂ was performed at 0.5 ml scale. Oxidation was started with the stepwise addition of H₂¹⁸O₂ (1.0%; 5 μl every 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 analyzed with GC-MS.

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

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 every 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 analyzed with GC-MS.
Abstract

A semi-synthetic peroxidase was designed by exploiting the structural similarity of the active sites of vanadium-dependent haloperoxidases and acid phosphatases. Incorporation of vanadate ion into the active site of phytase, which in vivo mediates the hydrolysis of phosphate esters, leads to the formation of a semi-synthetic peroxidase, which catalyzes the enantioselective oxidation of sulfides with H₂O₂ affording the S-sulfoxide, e.g. in 68% ee at 100% conversion for thioanisole. Under reaction conditions the semi-synthetic vanadium peroxidase is stable for over 3 days with only a slight decrease in turnover frequency. Polar water-miscible cosolvents, such as methanol, dioxane, and dimethoxyethane, can be used in concentrations of 30% (v:v) with reasonable relative activities and enantioselectivities. Amongst the transition metal oxoanions that are known to be potent inhibitors only vanadate resulted in a semi-synthetic peroxidase, when incorporated into phytase.
1 Introduction

Vanadium haloperoxidases, such as vanadium chloroperoxidase from Curvularia inaequalis (Messerschmidt, 1996; Van Schijndel, 1993, 1994) differ from heme-containing peroxidase by their active site architecture. Their active sites contain a vanadate ion instead of an iron porphyrin complex, which is susceptible to oxidative degradation. Consequently, vanadium-containing haloperoxidases are much more stable than their heme-containing counterparts. Unfortunately, the active site of vanadium-dependent haloperoxidases can accommodate only very small substrates, such as halide ion, which severely curtails their utility. Nevertheless, enantiointropic sulfoxidation was catalyzed by the vanadium-dependent bromoperoxidases from Corallina officinalis (Andersson, 1997) and Ascophyllum nodosum (Ten Brink, 1998). Recently, it was established (Hemrika, 1997; Neuwald, 1997; Stuckey, 1997) that vanadium chloroperoxidases are structurally closely related to the (membrane-bound) acid phosphatases and the apo-enzyme of vanadium chloroperoxidase was shown to exhibit phosphatase-like activity. Moreover, vanadate and other transition metal oxoanions are known to be potent inhibitors of acid phosphatases (Lindqvist, 1994; Vescina, 1996) and the related phytases (Greiner, 1997; Mahajan, 1997) and sulfatases (Stankiewicz, 1988). Hence, we reasoned that incorporation of vanadate ion into the active site of these enzymes should produce novel, semi-synthetic peroxidases.

Scheme 1. Hydrolysis of phytate.

In this chapter the feasibility of rationally designing a semi-synthetic peroxidase via incorporating vanadium into the active site of phytase (myo-inositol-hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8) is demonstrated. In this way inexpensive phytases (15 $ kg^{-1};$ Gist-brocades), which catalyze the hydrolysis of phytic acid to inositol and phosphate ion and are used commercially in animal feed (Nelson, 1971; Pasamontes, 1997; Ullah, 1996), can be converted into vanadium peroxidases that are able to catalyze enantioselective sulfoxidation with hydrogen peroxide. Due to the size of phytic acid we expected that phytase would accept larger substrates than CPO from C. fimago, the active site of which is limited to substrates with ca. nine carbon atoms.

2 Results and discussion

2.1 Vanadate-incorporated phytase

In an initial study the feasibility of designing a semi-synthetic peroxidase via incorporating vanadate ion into the active site of phytase from A. ficuum was demonstrated. To obtain insights into the interaction of vanadate with phytase, the influence of vanadate concentration on the reaction rate and ee of thioanisole oxidation was studied. As shown in figure 1 the reaction rate showed saturation kinetics with respect to the vanadate
concentration. Detailed inspection of the part of the curve close to the origin, revealed that the enzyme had peroxidase activity even when no vanadate ion was present (the rate of the uncatalyzed reaction is only 0.6 μmol h⁻¹ at this point, table 1). Hence, the observed rate of sulfoxidation (v_total) is composed of two reaction rates, one for the metal-free phytase (v₀) and one for the vanadium-incorporated phytase (v_max).

\[
v_{\text{total}} = v_0 f_{\text{mf}} + v_{\text{max}} f_{\text{V}}
\]  

(1)

In this equation f_{mf} and f_{V} are the molecular fractions of metal-free phytase (f_{mf}) and vanadium-incorporated phytase (f_{V}). The ratio between the metal-free and vanadium-incorporated phytase is determined by the dissociation constant (K_d) of the latter.

\[
K_d = \frac{[E][V_i]}{[V_i,E]}
\]

(2)

When this equilibrium was used to calculate the fractions of metal-free and vanadium-incorporated phytase, the overall rate of oxidation could be written as a function of the vanadate concentration [V_i]:

\[
v_{\text{total}} = \frac{v_0 K_d + v_{\text{max}} [V_i]}{K_d + [V_i]}
\]

(3)

The reaction rate was plotted against the amount of added vanadate (figure 1). When equation (3) was fitted to these data points we calculated a reaction rate for the metal-free phytase (v₀) of 3.5 μmol h⁻¹ (experimental: 3.2 μmol h⁻¹; table 1) and for the vanadium-incorporated phytase (v_max) a rate of 124 μmol h⁻¹ (which represents a TOF of 11 min⁻¹). The calculated apparent dissociation constant (K_{d^{app}}) of the vanadate-phytase complex was 15.6 μM. Hence, at 15.6 μM added vanadate f_V is 0.5; when the amount of vanadate incorporated into the phytase is taken into account, this corresponds with a K_d of 3 μM, which is in the same order of magnitude as the measured K_i (3.9 μM) for the phytase-catalyzed hydrolysis of p-nitrophenyl phosphate (vide infra), as well as the reported K_i for phosphatases and sulfatases (Stankiewicz,1988). At low vanadate concentrations (<15 μM) quantitative conversion to the sulfoxide was observed. At higher vanadate concentrations (>25 μM) in contrast, slow further oxidation to the corresponding sulfone also occurred. This observed sulfone formation is enzyme-catalyzed, since in the absence of enzyme at a vanadate concentration of 50 μM no sulfone was formed. Kinetic resolution of thioanisole sulfoxide subjected to oxidation catalyzed by vanadium-incorporated phytase was not observed. The ee of the formed sulfoxide increased from 47% (S) to a plateau (from 2,5 μM to 20 μM vanadate) of 56% (S) at room temperature.

The pH-optimum of this newly designed vanadium peroxidase is 5.0 (figure 7). At this pH vanadate is predominantly present as H₅VO₄⁻ (Pope,1983).
2.2 Metal oxoanion-incorporated phytase

Incorporation of a metal oxoanion in the active site of phytase presumably should result in inhibition of its hydrolytic activity. Hence, we measured the effect of vanadate, molybdate, tungstate, and selenate on the phytase-mediated hydrolysis of \( p \)-nitrophenyl phosphate. As shown in figure 2, both vanadate and molybdate are strong inhibitors, as reported for phosphatases and related enzymes (Lindqvist, 1994; Mahajan, 1997; Stankiewicz, 1988; Vescina, 1996), whereas tungstate has little influence on the hydrolysis reaction and selenate is not an inhibitor. \( K_i \) was calculated at 3.9 \( \mu \text{M} \) and 9.4 \( \mu \text{M} \) respectively for vanadate and molybdate.

![Figure 1. Influence of the vanadate concentration on the oxidation of thioanisole catalyzed by vanadate-incorporated phytase.](image1)

![Figure 2. Inhibition of the phytase-catalyzed hydrolysis of \( p \)-nitrophenyl phosphate by: vanadate (●); molybdate (▲); tungstate (◆); selenate (●).](image2)

In order to study the design of semi-synthetic peroxidases based on other transition metal oxoanions thioanisole was oxidized in the presence of phytase and the above mentioned metal oxoanions in concentrations as indicated in table 1 (approx. 2.5 times the \( K_i \)). As shown in table 1 the rate of the uncatalyzed reaction is 0.6 \( \mu \text{mol h}^{-1} \), whereas the rate of the metal-free phytase-catalyzed oxidation was 3.2 \( \mu \text{mol h}^{-1} \) giving an ee of 47%. Addition of vanadate (10 \( \mu \text{M} \)) to the reaction mixture had no significant effect on the non-enzymatic reaction, whereas the rate of the enzymatic reaction increased 13-fold in the presence of 10 \( \mu \text{M} \) vanadate. This indicated that a semi-synthetic peroxidase is formed and that the oxidation of sulfides is only catalyzed by vanadate that is coordinated to ligands.

In contrast with vanadate, molybdate at 25 \( \mu \text{M} \) concentration increased the rate of the background reaction in the absence of enzyme 10-fold to 6.5 \( \mu \text{mol h}^{-1} \) and no further increase in rate was observed in the presence of both molybdate and phytase. Moreover, the ee of the formed sulfoxide was only 21%. Hence, we conclude that thioanisole is mainly oxidized by free molybdate, with a minor contribution by metal-free phytase. Comparable results were obtained with perrhenate.

In the presence of 100 \( \mu \text{M} \) tungstate the rate of the non-enzymatic reaction was increased more than 100-fold. In the presence of both phytase and tungstate, thioanisole was oxidized at the same rate and ee as with metal-free phytase. Apparently, tungstate
Rational design of semi-synthetic peroxidases

binds very tightly to phytase (no free tungstate remained in the solution), without blocking the active site. Selenate, which had no inhibitory effect on the hydrolysis reaction, also did not influence the sulfoxidation reaction. This is in agreement with the finding that no significant inhibition of the hydrolysis activity of phytase was observed in the presence of tungstate (figure 2).

Table 1. Oxidation of thioanisole catalyzed by phytase incorporated with different transition metal oxoanions.

<table>
<thead>
<tr>
<th>Metal oxoanion:</th>
<th>Ki (μM)</th>
<th>Oxoanion concentration (μM)</th>
<th>Rate of the non-enzymatic reaction (μmol h⁻¹)</th>
<th>Overall rate of reaction in the presence of enzyme (μmol h⁻¹)</th>
<th>ee of the enzymatic reaction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoO₄²⁻</td>
<td>9.4</td>
<td>25</td>
<td>6.49</td>
<td>6.56</td>
<td>21 (S)</td>
</tr>
<tr>
<td>ReO₄³⁻</td>
<td>n.d.</td>
<td>20</td>
<td>6.00</td>
<td>5.44</td>
<td>22 (S)</td>
</tr>
<tr>
<td>SeO₄²⁻</td>
<td>n.d.</td>
<td>200</td>
<td>0.68</td>
<td>3.47</td>
<td>45 (S)</td>
</tr>
<tr>
<td>VO₄³⁻</td>
<td>3.9</td>
<td>10</td>
<td>0.62</td>
<td>42.3</td>
<td>55 (S)</td>
</tr>
<tr>
<td>WO₄³⁻</td>
<td>n.d.</td>
<td>100</td>
<td>74.5</td>
<td>3.07</td>
<td>40 (S)</td>
</tr>
<tr>
<td>Metal-free</td>
<td>-</td>
<td>-</td>
<td>0.63</td>
<td>3.15</td>
<td>47 (S)</td>
</tr>
</tbody>
</table>

The results given above indicated that phytase is only converted into an effective semi-synthetic peroxidase when incorporated with vanadate ion, with a 13-fold increase in enzyme activity.

2.3 Screening for semi-synthetic peroxidases

To investigate whether or not the phytase from *A. ficuum* is unique among the large number of phytases occurring in nature (Dvoráková, 1998), phytases from different sources were tested for their peroxidase activity in the presence of vanadate.

As shown in table 2 not only phytase from *A. ficuum* but also the phytases from *A. nidulans* and *A. fumigatus* act as vanadium peroxidases in the presence of vanadate. We also tested other hydrolases; from these the phospholipase D from cabbage and the sulfatase from *Helix pomatia* were shown to act as peroxidases when incorporated with vanadate ion. This is in accordance with sulfatases being inhibited by vanadate ion (Stankiewicz, 1988). The formed sulfoxide had an ee of 20% (not optimized). Acid phosphatases from *A. niger* and wheat germ produced the sulfoxide in 27% and 19% ee, respectively (*S*-enantiomer) when incorporated with vanadate ion. In contrast with the phytases and sulfatase, however, the acid phosphatases deactivated rapidly under the standard oxidation conditions. Besides the above mentioned hydrolases, also apo-ferritin showed peroxidase activity when incorporated with vanadate. It is known that the apo-form of the iron storage protein ferritin binds 12 to 18 vanadyl ions per protein molecule (Chasteen, 1999). Vanadium-ferritin catalyzed the oxidation of thioanisole with a moderate rate (5.8 μmol h⁻¹) affording the sulfoxide in 23% ee, the *R*-enantiomer being predominantly formed.

The results, given in table 2, demonstrate that the rational design of semi-synthetic peroxidases is not limited to phytase for *A. ficuum*. Other hydrolases which are inhibited by vanadate-ion and phytases from different sources act as peroxidases in the presence of this metal oxoanion. Surprisingly, all these semi-synthetic peroxidases preferentially form the *S*-enantiomer. When the above results are combined with those obtained with different metal
oxoanions (table 1) it is clear that the vanadate-incorporated phytase from *A. ficuum* is the best semi-synthetic peroxidase. Hence, further studies were focused on this system. Optimization of the oxidation of thioanisole led to a maximum ee of 68%, obtained in formate buffer at 4°C.

**Table 2. Peroxidase activity of vanadium-incorporated hydrolases.**

<table>
<thead>
<tr>
<th>Enzyme:</th>
<th>Source:</th>
<th>Amount (mg)*</th>
<th>Rate (μmol h⁻¹)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>-</td>
<td>0.40</td>
<td>-</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td><em>A. niger</em></td>
<td>5</td>
<td>1.74</td>
<td>&lt; 5</td>
</tr>
<tr>
<td></td>
<td>Potato</td>
<td>15</td>
<td>1.26</td>
<td>&lt; 5</td>
</tr>
<tr>
<td></td>
<td>Sweet potato</td>
<td>4.7</td>
<td>5.0</td>
<td>19 (S)</td>
</tr>
<tr>
<td></td>
<td>Wheat germ</td>
<td>32</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>Acylase I</td>
<td><em>A. melleus</em></td>
<td>85</td>
<td>0.0</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Albumin</td>
<td>Bovine</td>
<td>40</td>
<td>0.59</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>apo-Ferritin</td>
<td>Horse spleen</td>
<td>27</td>
<td>5.84</td>
<td>23 (R)</td>
</tr>
<tr>
<td>Phospholipase D</td>
<td>Peanut</td>
<td>2.8</td>
<td>0.28</td>
<td>&lt; 5</td>
</tr>
<tr>
<td></td>
<td>Cabbage</td>
<td>4.3</td>
<td>1.17</td>
<td>31 (S)</td>
</tr>
<tr>
<td>Phytase</td>
<td><em>A. ficuum</em> (analytical grade)</td>
<td>24</td>
<td>32</td>
<td>33 (S)</td>
</tr>
<tr>
<td></td>
<td><em>A. ficuum</em> (commercial grade)</td>
<td>9.5</td>
<td>36</td>
<td>36 (S)</td>
</tr>
<tr>
<td></td>
<td><em>A. fumigatus</em></td>
<td>7.8</td>
<td>16</td>
<td>16 (S)</td>
</tr>
<tr>
<td></td>
<td><em>A. nidulans</em></td>
<td>10</td>
<td>20</td>
<td>22 (S)</td>
</tr>
<tr>
<td>Sulfatase</td>
<td>Abalone entrails</td>
<td>2.2</td>
<td>0.40</td>
<td>&lt; 5</td>
</tr>
<tr>
<td></td>
<td><em>Aerobacter aerogenes</em></td>
<td>0.6</td>
<td>7.45</td>
<td>&lt; 5</td>
</tr>
<tr>
<td></td>
<td><em>Helix pomatia</em></td>
<td>28</td>
<td>2.66</td>
<td>15 (S)</td>
</tr>
<tr>
<td></td>
<td>Limpets (<em>Patella vulgata</em>)</td>
<td>10</td>
<td>0.51</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>

a) Crude enzyme preparation as purchased.
b) One Unit (U) catalyzes the conversion of one μmol substrate per minute under standard conditions: Acid phosphatase: hydrolysis of p-nitrophenyl phosphate at pH 4.8 at 37°C.; Acylase I: hydrolysis of N-acetyl-L-methionine at pH 8.0 at 37°C.; Phospholipase D: liberation of choline from L-α-phosphatidylcholine per hour at pH 5.6 at 30°C.; Phytase: liberation of inorganic phosphate from phytate at pH 2.5 at 37°C.; Sulfatase: hydrolysis of p-nitrophenyl sulfate at pH 7.1 at 37°C.

2.4 Scope of the vanadium phytase-catalyzed sulfoxidation

In order to demonstrate the potential of vanadium-incorporated phytase from *A. ficuum* as a catalyst for organic synthesis a fed batch oxidation of thioanisole was carried out (scheme 2). Fresh substrate (dissolved in DME) was added continuously to the reaction mixture, while controlling the addition of oxidant using a hydrogen peroxide-stat (set-point: 125 μM H₂O₂). As shown in figure 3 the vanadium-incorporated phytase is stable for over three days, producing around 0.5 g of thioanisole sulfoxide per day (STY is 5.0 g L⁻¹d⁻¹). The catalytic activity of the catalyst (TOF) dropped from its initial value of 7.5 min⁻¹ to 5.6 min⁻¹ after 3 days. The thioanisole sulfoxide formed had an enantiomeric excess of 50% (S-enantiomer). An excess of thioanisole accumulated in the reactor, resulting in a two-phase system of small organic droplets in the aqueous phase. However, the appearance of the
second phase had no influence on the catalytic activity of the enzyme and the reaction proceeded to 100% conversion after the addition of fresh substrate was stopped.

**Scheme 2.** Schematic figure of the sensor-controlled fed-batch oxidation of thioanisole.

![Scheme 2](image)

**Figure 3.** Fed-batch oxidation of thioanisole catalyzed by vanadium-incorporated phytase: formed sulfoxide (●); turnover frequency (▲).

As noted above, the low solubility of non-polar reactants in aqueous medium may give rise to a biphasic system. Because a homogeneous reaction system is often preferred, we explored the use of water-miscible organic cosolvents in the oxidation of thioanisole (figure 4). Polar solvents, such as methanol, dimethoxyethane (DME), and dioxane, when added in a concentration of 30% (v:v) to the reaction mixture lowered the oxidation rate as well as the ee of the formed sulfoxide. Dimethoxyethane seemed to be the cosolvent of choice, resulting in the highest ee at a reasonable relative activity. The octanol/water partition coefficient, log P (Gupta, 1997), is a good measure of the hydrophobicity/hydrophilicity of the various cosolvents. The relative activity showed linear correlation, with $r^2 = 0.950$, with log $P$ (figure 5).

**Figure 4.** Influence of organic cosolvent (30%; v:v) on the oxidation of thioanisole. Open: ee of the sulfoxide; Shaded: relative rate of reaction.

**Figure 5.** Relationship between relative activity of the catalyst and log $P$ of the organic cosolvent.
To explore the accessibility of the active site of this novel semi-synthetic peroxidase, a series of meta and para substituted thioanisoles as well as ethyl phenyl sulfide were tested. To minimize the influence of differences in solubility of the different sulfides, the oxidations were performed in buffer containing 10% (v:v) dimethoxyethane. As shown in Table 3, electron withdrawing substituents, such as nitro, chloro, and bromo, lowered the rate of oxidation, consistent with rate-limiting nucleophilic attack of the sulfur atom on an electrophilic peroxy species. In the crystal structure of the peroxide form of vanadium chloroperoxidase from *C. inaequalis*, which was recently resolved (Messerschmidt, 1997), the peroxide is bonded side-on to the vanadium atom. One of the oxygen atoms from the peroxide is hydrogen-bridged with a lysine residue (Lys353) resulting in a polarization of the O-O bond (see scheme 3). It is tempting to speculate that the sulfide will attack the slightly positively charged oxygen atom as proposed for the chlorination reaction. In the active site of phytase there is also a lysine (Lys278) present, which could be favorably juxtaposed for polarization of the O-O bond.

<table>
<thead>
<tr>
<th>Sulfide</th>
<th>Rate (µmol h⁻¹)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42.3</td>
<td>50 (S)</td>
<td></td>
</tr>
<tr>
<td>7.6</td>
<td>60 (S)</td>
<td></td>
</tr>
<tr>
<td>52.6</td>
<td>65 (S)</td>
<td></td>
</tr>
<tr>
<td>78.5</td>
<td>58 (S)</td>
<td></td>
</tr>
<tr>
<td>2.8</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>7.1</td>
<td>55 (S)</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>33 (S)</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>65 (S)</td>
<td></td>
</tr>
<tr>
<td>1.1</td>
<td>28 (S)</td>
<td></td>
</tr>
<tr>
<td>1.6</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

A methyl or methoxy substituent on the para position of thioanisole resulted in a slight increase in the rate of oxidation. Enantioselectivities were also higher (up to 65% ee for p-methyl) than those observed for thioanisole. The ee of ethyl phenyl sulfoxide (60% for the S-enantiomer) was higher than that of thioanisole sulfoxide, while the rate of oxidation was decreased by a factor 5. The higher ee observed with these larger substrates, which had a
lower sulfoxidation rate, suggests that they fit more tightly into the active site of phytase than thioanisole itself. This is in agreement with our assumption that the active site of this semi-synthetic peroxidase is able to accommodate larger substrates than CPO from *C. fumago*.

### 2.5 Sulfoxidation catalyzed by metal-free phytase

In order to gain insights into the origins of the observed catalysis, we studied the sulfoxidation catalyzed by metal-free phytase (Van de Velde, 1998) in more detail. The influence of the protein concentration on the rate of oxidation is shown in figure 6. The conversion and ee increased with increasing enzyme concentration, consistent with the reaction being enzyme-catalyzed. Both the commercial (>95% phytase) and the analytical grade (>99% phytase) from *A. ficuum* (Gist-brocades B.V.) showed the same specific activity of 18 μmol h⁻¹ (μmol protein)⁻¹ and the same ee of 47% in the standard procedure. Hence, we conclude that phytase is responsible for the observed catalysis.

![Figure 6](image_url.png)

**Figure 6.** Rate of sulfoxide formation in the metal-free phytase-catalyzed oxidation of thioanisole as function of the protein concentration: overall rate (○); rate of formation of the S-sulfoxide (△); rate of formation of the R-sulfoxide (■).

![Figure 7](image_url.png)

**Figure 7.** pH-Optimum of the phytase-catalyzed oxidation of thioanisole: metal-free phytase (○); phytase in combination with 10 μM vanadate (△).

The rates of formation of the separate sulfoxide enantiomers were derived from the overall rate and the ee and plotted as a function of the protein concentration (figure 6). The enzyme activity was 0.16 μmol h⁻¹ (mg protein)⁻¹ and 0.04 μmol (mg protein)⁻¹, respectively, for the S- and R-enantiomer. Hence, the *intrinsic* enantioselectivity of metal-free phytase at room temperature (25°C) is 58% ee, which is comparable with the ee observed for the vanadate-incorporated phytase at room temperature (55%). Hence, we conclude that the active site architecture was not influenced by the incorporation of vanadate. Decreasing the temperature to 4°C resulted in an increased enantioselectivity of the metal-free phytase-catalyzed sulfoxidation to 57% ee, as a result of two phenomena. The rate of the background reaction decreased from 0.6 μmol h⁻¹ at 25°C to 0.08 μmol h⁻¹ at 4°C. In the
same time the *intrinsic* ee of phytase improved to 66% at 4°C which is again comparable with the ee observed with the vanadate-incorporated phytase at 4°C. (68%).

As shown in figure 7 the pH optimum for both the vanadate-incorporated and the metal-free enantioselective sulfoxidation was 5.0. This pH coincides with the optimum of the natural reaction, the hydrolysis of phytic acid (Liu, 1999; Ullah, 1996).

We subsequently studied the influence of the buffer type on the rate and enantioselectivity of this sulfoxidation (table 4). Comparable ee and reaction rates were observed in carboxylate buffers (acetate and formate) and non-carboxylate buffers (MES, HEPES and TRIS). This demonstrated that a carboxylate group is not essential for catalysis and ruled out the *in situ* formation of percarboxylic acids as observed with lipases (Björkling, 1992; De Zoete, 1993) and metal-free bacterial haloperoxidases (Picard, 1997). In contrast, phosphate and citrate completely inhibited the enantioselective oxidation. Phosphate presumably occupies the active site of phytase, whereas citrate can act as a chelating agent for metal ions.

**Table 4.** Influence of the buffer type on the metal-free phytase-catalyzed oxidation of thioanisole.

<table>
<thead>
<tr>
<th>Buffer (0.1 M; pH 5.0):</th>
<th>rate (µmol h⁻¹)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>0.63</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.71</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.62</td>
<td>25</td>
</tr>
<tr>
<td>Formate</td>
<td>1.65</td>
<td>33</td>
</tr>
<tr>
<td>MES</td>
<td>1.80</td>
<td>27</td>
</tr>
<tr>
<td>HEPES</td>
<td>2.18</td>
<td>31</td>
</tr>
<tr>
<td>TRIS</td>
<td>2.08</td>
<td>34</td>
</tr>
</tbody>
</table>

![Figure 8](image)

**Figure 8.** Influence of EDTA and calcium ions on the rate of the metal-free phytase-catalyzed oxidation of thioanisole: EDTA (●); CaCl₂ (●): dotted line: rate of the uncatalyzed reaction.

To investigate the latter possibility analogous experiments were performed with EDTA. As shown in figure 8 the chelating agent EDTA inhibited the enantioselective oxidation. At EDTA concentrations above 0.5 mM the rate of the enzymatic reaction decreased to that of the uncatalyzed reaction and the ee of the sulfoxide formed dropped to around 10%. This is probably due to the removal of calcium ions required for the stability of the tertiary enzyme structure, since no transition metals were detected by X-ray analysis of crystalline phytase (Kostrewa, 1997) or in the ICP-analysis of the commercial phytase (data sheet, Gist-brocades). Analogous experiments with calcium ion concentrations up to 1.0 mM showed only a small increase in the rate and enantioselectivity of the oxidation.

### 2.6 Mechanistic aspects

It is tempting to speculate that the vanadium assisted sulfoxidation follows the catalytic cycle of the vanadium chloroperoxidase. The side-on bound peroxovanadium(V) species
undergoes nucleophilic attack by the sulfur atom of the sulfide (scheme 3). The incorporation of $^{18}$O (100%) from labeled H$_2^{18}$O$_2$ into the sulfoxide is consistent with this mechanism. As shown in table 5 tert-butyl hydroperoxide (TBHP) does not function as an oxidant in these reactions: a low rate of oxidation and no enantioselectivity was observed. This is in agreement with the results obtained with VBPO (Andersson, 1997; Butler, 1993).

Scheme 3. Proposed mechanism for the oxidation of sulfides catalyzed by (semi-synthetic) vanadium peroxidases. Numbering of amino acids residues is for VCPO from C. inaequalis.

The mechanism of (enantioselective) oxygen transfer, catalyzed by phytase in the absence of added metal ions is less obvious. Hydroxyl radicals are not involved in this enantioselective oxidation as the addition of the radical scavenger Trolox$^\text{TM}-C$, a water soluble analog of vitamin E, had no influence on the conversion or enantioselectivity. Oxygen is not involved in the reaction as reactions performed under nitrogen showed no difference with the reactions performed under air. Experiments with $^{18}$O labeled hydrogen peroxide showed that the oxygen in the sulfoxide product is derived from H$_2$O$_2$ (100%). As with the vanadium-assisted sulfoxidation the metal-free enzymatic oxidation is not supported by TBHP (table 5).

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>$\text{H}_2\text{O}_2$ rate ($\mu\text{mol h}^{-1}$)</th>
<th>ee (%)</th>
<th>TBHP rate ($\mu\text{mol h}^{-1}$)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.63</td>
<td>-</td>
<td>0.07</td>
<td>-</td>
</tr>
<tr>
<td>VO$_4^{3-}$</td>
<td>0.62</td>
<td>-</td>
<td>0.27</td>
<td>-</td>
</tr>
<tr>
<td>Phytase</td>
<td>3.15</td>
<td>47 (S)</td>
<td>0.13</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Phytase/VO$_4^{3-}$</td>
<td>42.3</td>
<td>55 (S)</td>
<td>0.11</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>
The crystal structure of phytase from *Aspergillus ficuum* (Kostrewa, 1997) showed that an aspartate residue (Asp339) and a histidine residue (His338) are located in the active site. Together with several arginine residues, these two residues will bind the vanadate ion in the same way as vanadate is bound in vanadium chloroperoxidase (Messerschmidt, 1996) and in the vanadium-inhibited rat acid phosphatase (Lindqvist, 1994). It is tempting to speculate that this Asp339 also plays a key role in the metal-free phytase-catalysis. Reaction of the free carboxylate group with $\text{H}_2\text{O}_2$ would give the corresponding peroxycarboxylic acid, which could be the active oxidant. The lack of catalysis observed with TBHP is consistent with this notion. Appropriate site-directed mutagenesis studies should be able to confirm the key role of Asp339 in the catalytic mechanism.

3 Concluding remarks

In this chapter we have demonstrated the feasibility of rationally designing a semi-synthetic peroxidase with enhanced operational stability compared to native peroxidases. A semi-synthetic vanadium peroxidase was prepared by incorporating vanadate ion into the active site of phytase from *Aspergillus ficuum*. The resulting vanadium-phytase catalyzed the enantioselective oxidation of several sulfides, affording the $S$-sulfoxide in up to 68% ee for thioanisole. Slightly higher enantioselectivities were observed with ethyl phenyl sulfide and meta and para substituted thioanisoles compared to thioanisole under the same conditions. Incorporation of other transition metal oxoanions, such as tungstate, molybdate, and selenate, did not result in peroxidase activity.

From a wide variety of hydrolases tested, the phytases from *A. fumigatus* and *A. nidulans*, sulfatase from *Helix pomatia*, and phospholipase D from cabbage also showed peroxidase activity in the presence of vanadate. However, only phytase from *A. fumicu* was able to catalyze enantioselective sulfoxidation in the absence of vanadate ion.

| Table 6. Comparison of the catalytic properties of three different peroxidases based on the oxidation of thioanisole. |
|---|---|---|
| | heme CPO<sup>a</sup> | phytase / VO<sub>3</sub><sup>b</sup> | VBPO<sup>c</sup> |
| | C. *fumago* | | A. *nodosum* |
| ee (%) | 99 (R) | 68 (S) | 83 (R) |
| TOF (min<sup>-1</sup>) | 900 | 5.5 | 0.78 |
| TTN (·) | 108,000 | 25,000 | 750 |
| STY (g L<sup>-1</sup>d<sup>-1</sup>) | 84 | 5.3 | 0.1 |
| catalyst cost ($ kg<sup>-1</sup>)<sup>d</sup> | 140·10<sup>3</sup> | 0.84 | 5·10<sup>6</sup> |

a) Results reported by Van Deurzen (1997b).  
b) Results reported by Ten Brink (1998).  
c) Costs for the catalyst to produce 1 gram thioanisole sulfoxide; CPO: 5$ mg<sup>-1</sup> (Chirazym, USA); Phytase: 15$ kg<sup>-1</sup> (Gist-brocades); Na<sub>2</sub>VO<sub>3</sub>: 0.8$ g<sup>-1</sup> (Acros); VBPO: 5$ U<sup>-1</sup> (500$ mg<sup>-1</sup> for *C. officinalis*, Sigma).

The catalytic properties of the semi-synthetic peroxidase in relation to those of vanadium-dependent BPO from *A. nodosum* and heme-dependent CPO from *C. fumago* are shown in table 6. If turnover numbers and space-time yields are compared CPO is by far the most efficient catalyst. In contrast to CPO, which maintained its high activity for only 2
hours at low H₂O₂ concentrations, the semi-synthetic peroxidase is stable for more than 3 days at relatively high H₂O₂ concentrations. Comparing the semi-synthetic vanadium peroxidase with the native BPO, the synthetic one is more efficient showing higher TOF, TTN and STY.

The catalyst cost ($ per kg of product produced) differ enormously from catalyst to catalyst. The semi-synthetic vanadium peroxidase is by far the cheapest catalyst (catalyst cost <1$ kg⁻¹) owing to the low price of phytase (15 $ kg⁻¹). The catalyst cost for VBPO is astronomical owing to the very high price of the enzyme and low turnover number. However, the price of VBPO may decrease if it were to be used on a large scale and when the recently patented (Carlton,1998) simple purification method is applied.

The best enantioselectivities were obtained with the natural enzymes CPO and VBPO. Phytase, which in its natural reaction catalyzes the hydrolysis of a large substrate molecule, could be a good catalyst for substrates which cannot be oxidized by CPO, the active site of which can accommodate only small molecules (up to 9 carbon atoms). Furthermore, site-directed mutagenesis could be used to increase the enantioselectivity of this semi-synthetic peroxidase.

Experimental part

Enzymatic oxidation of thioanisole: In the standard procedure thioanisole (5.0 mM) and commercial phytase from Gist-brocades B.V. (30 mg dry weight; 12 mg protein; 0.18 µmol; 1400 U) were dissolved in formate buffer (7 ml; 0.1 M; pH 5.0; containing 0 to 30 µM Na₃VO₄). After 10 min H₂O₂ (5.5 mM) was added and the course of the reaction was followed by HPLC.

In the case of oxidations in the presence of organic cosolvent, the formate buffer was replaced with premixed mixtures of formate buffer (0.1 M; pH 5.0; 70%; v:v) and cosolvent (30%; v:v), containing 10 µM Na₃VO₄.

For the oxidation of different sulfides, commercial phytase (30 mg dry weight) was dissolved in formate buffer (6.3 ml; 0.1 M; pH 5.0 mM; containing Na₃VO₄ final concentration 10 µM). A solution of the sulfide in dimethoxyethane (700 µl; final concentration of DME 10% (v:v); final concentration of sulfide 5.0 mM) was added. After 10 min H₂O₂ (5.5 mM) was added and the course of the reaction was followed by HPLC.

Oxidations with H₂¹⁸O₂ were performed at 1 ml scale at 4°C.

Studies on effects of buffer type: Commercial phytase from Gist-brocades (65 mg dry weight) was dissolved in 15 ml buffer (formate, acetate, citrate, phosphate, MES, HEPES and TRIS; 0.1 M; pH 5.0). Enzyme solutions were concentrated at 4°C, using Amicon Centriprep-30 tubes (cut-off 30 kDa) at 2400 min⁻¹. Concentrated enzyme solutions were diluted twice with buffer. The final concentrate was diluted to a final volume of 15 ml. Thioanisole (5.0 mM) was added to 7 ml of these enzyme solutions. After 10 min. of stirring at room temperature H₂O₂ (5.5 mM) was added and the course of the reaction was followed by HPLC.

Screening of other enzymes: Thioanisole (5.0 mM) and enzyme (amounts as indicated in Table 2) were dissolved in acetate buffer (7 ml; 0.1 M; pH 5.0) containing 10 µM Na₃VO₄. After 10 min. of stirring at room temperature H₂O₂ (5.5 mM) was added and the course of the reaction was followed by HPLC.
Preparative scale. Thioanisole (5.0 mM) and commercial phytase (71 mg dry weight) were dissolved in HEPES buffer (45 ml; 0.1 M; pH 5.0), containing 15 μM Na₂VO₄. After 15 min. of stirring at 20°C the addition of thioanisole (0.90 M solution in dimethoxyethane/water-mixture of 90:10 (v:v); 2.7 μmol min⁻¹) and hydrogen peroxide (0.30 M solution), using a hydrogen peroxide-stat (as described by Van Deurzen, 1997c), operated at 125 μM H₂O₂ were started. The formation of thioanisole sulfoxide was followed by HPLC.

Inhibition constants of phytase. p-Nitrophenyl phosphate (5.3 mM) was dissolved in acetate buffer (0.1 M; pH 5.0) containing metal oxoanion in concentrations up to 100 μM. To 2.95 ml of this solution was added 50 μl of a phytase solution (35 mg commercial phytase per ml). At 25°C the formation of p-nitrophenol was monitored at 405 nm.
Evaluation of peroxidases as industrial catalysts

1 Introduction

In this chapter the results of the research described in this thesis are evaluated. Before doing so, for the convenience of the reader the objectives of this thesis, as mentioned in section 5 of chapter 1, are described below.

The objective of this thesis was the development of catalysis by peroxidases in the manufacture of fine chemicals and chiral intermediates. Oxidation procedures that use high-valency metal compounds in stoichiometric amounts are environmentally unacceptable and economically unattractive. Peroxidases are potentially attractive catalysts that use hydrogen peroxide as a clean and inexpensive oxidant. Unlike monooxygenases, peroxidases do not require a stoichiometric amount of a cosubstrate, such as NADH, to supply reducing equivalents. Furthermore, some peroxidases, notably haloperoxidases, are able to catalyze synthetically useful oxygen transfer reactions with high selectivities. However, the application of peroxidases is seriously hampered by their low operational stability. Hence, a major theme of this thesis was the improvement of the operational stability of peroxidases.

The oxidation of indole and thioanisole catalyzed by chloroperoxidase from *Caldariomyces fumago* with H$_2$O$_2$ as oxidant was taken as the starting point of the research. The stabilization of native enzyme was attempted by lowering the oxidative degradation by replacing H$_2$O$_2$ by oxygen as the primary oxidant (section 2). Stabilization by immobilization was performed to apply peroxidases in organic solvents (section 3). The inherent instability of heme peroxidases was improved by chemical and genetic modification (section 4). Finally, semi-synthetic peroxidases were designed, that are industrially attractive candidates to replace the natural peroxidases (section 5).
2 Stabilization of native peroxidases

The major problem for the industrial application of peroxidases is their facile oxidative deactivation by their oxidant, hydrogen peroxide. Continuous and feed-on-demand addition of \( \text{H}_2\text{O}_2 \) proved to be good procedures to increase the stability, however obtained \( \text{TTN} \)s are still too low to compete with chemical methods. Replacement of \( \text{H}_2\text{O}_2 \) by molecular oxygen as the primary oxidant could circumvent deactivation. Glucose oxidase was used for the \textit{in situ} reduction of oxygen to \( \text{H}_2\text{O}_2 \) with simultaneous oxidation of glucose to gluconic acid. Molecular oxygen in combination with a sacrificial reductant was used to turn CPO into a monoxygenase-like catalytic cycle.

| Table 1. Different modes of oxidant addition in the CPO-catalyzed oxidation of thioanisole. |
|-----------------------------------------------|------------|-------|----------------|-----------------|
| **Mode of \( \text{H}_2\text{O}_2 \) addition** | **TTN \( \times 10^3 \)** | **TOF \( \text{min}^{-1} \)** | **STY \( \text{g L}^{-1}\text{d}^{-1} \)** | **Reference** |
| \( \text{H}_2\text{O}_2 \) Step-wise | 41 | 11 | 0.7 | Fu,1992 |
| \( \text{H}_2\text{O}_2 \) Continuous | 108 | 900 | 84 | Van Deurzen,1997b |
| \( \text{H}_2\text{O}_2 \) Feed-on-demand | 148 | 1375 | 57 | Seelbach,1997a |
| Glucose / \( \text{O}_2 \) | 250 | 500 | 23 | Chapter 2 |
| \( \text{O}_2 \) / Reductant | 7 | 1180 | 168 | Chapter 3 |

As shown in table 1 the \textit{in situ} generation of \( \text{H}_2\text{O}_2 \) by means of an oxidase led to an improved \( \text{TTN} \), whereas the combination of oxygen with a reductant led to a significant decrease in \( \text{TTN} \). Reduction of oxygen by an oxidase led to the homogenous generation of \( \text{H}_2\text{O}_2 \) in contrast to the addition of \( \text{H}_2\text{O}_2 \) via one entry point. The disappearance of these so-called 'hot spots' resulted in an enhanced operational stability of CPO. On the other hand the combination of oxygen and a sacrificial reductant led also to the \textit{in situ} formation of \( \text{H}_2\text{O}_2 \), however this proceeds via free radical autoxidation of the reductant. An inevitable consequence of the homolytic pathway for \( \text{H}_2\text{O}_2 \) formation is the formation of reactive radicals (\( \text{HO}^* \), \( \text{HO}^- \), etc.), that resulted in enzyme deactivation and undesired side reactions.

3. Immobilization of CPO

To render enzymes active in predominantly organic media, immobilization is a well-known technique. CPO and two other glycosylated enzymes (phytase and aminoacylase) were efficiently immobilized into polyurethane foam. CPO was immobilized at a loading of 24 mg (g dry foam)\(^{-1}\) with an immobilization efficiency of 54%. Much better results were obtained with aminoacylase: 187 mg g\(^{-1}\) with an efficiency of 60%. We propose that glycosylation protects the enzyme from deactivation during immobilization. In this way a stable and reusable biocatalyst was obtained.

Dried immobilized CPO, e.g. PUR-foam immobilized and lyophilized CPO-surfactant complex, were applied as catalyst for oxidations in predominantly organic media with tert-butyl hydroperoxide as oxidant. Best results (high TOF, \( \text{TTN} \) and ee) were obtained in apolar solvents, e.g. hexane, isoctane and 1-octanol. The use of organic solvents makes high substrate concentrations possible, thus reducing the costs of the down-stream processing.
However, TTNS obtained in organic media were much lower than those obtained in aqueous solution, probably due to the formation of tert-butyloxy radicals. Therefore, the immobilization and application of CPO in organic solvent does not improve the applicability of CPO for industrial processes.

4. Modification of CPO

Chloroperoxidase and all other heme-dependent peroxidases contain an iron-protoporphyrin IX moiety that is susceptible to oxidative destruction. Modification of either the prosthetic group or the surrounding protein could protect the enzyme against oxidative destruction.

For metal-replacement in CPO, the extraction of the iron atom from the heme group was studied with chelating agents under reducing conditions. However, the suspected iron-removal was not observed. Also a second approach to metal-exchanged CPO did not succeed. *Caldariomyces fumago* growing on iron-free medium that contained non-natural metal salts produced CPO that contained 1 iron atom per enzyme molecule together with the additional metal. A method to remove the iron reserve from the inocula had to be found to make this method applicable.

Random mutagenesis together with a well defined screening method is a powerful tool to increase the stability of enzymes by protein engineering. Second generation mutants produced by random mutagenesis of the *cpo*-gene expressed in *C. fumago* were screened for enhanced activity in mixtures of aqueous buffer solution with an organic cosolvent. From the more than one hundred mutants that were screened, three showed enhanced activity in 40% (v:v) tert-butyl alcohol, with a maximum of 3.4-fold increase. Thus, random mutagenesis leads to enzymes with enhanced catalytic properties.

However, a major drawback of the expression of the *cpo*-gene in its original organism is the possibility that both wild type and mutant CPO are being produced in the *C. fumago* constructs. Homologous expression of CPO in another host will circumvent this problem. Expression of the *cpo*-gene in the filamentous fungus *Aspergillus niger* resulted in the production of active extracellular enzyme. The catalytic properties of recombinant CPO are equal to those of native CPO, this with respect to the specific activity, pH-optimum, regio-

and enantioselectivities, and $^{18}$O incorporation.

The homologous expressing of CPO in the host *A. niger*, offers the possibility for mutagenesis studies. Directed evolution, random mutagenesis combined with a well defined screening system, could obtain mutants with enhanced catalytic properties. Site-directed mutagenesis could reveal the structural background of CPO characteristic features. On the other hand knowledge about the deactivation mechanism could point the direction to render CPO stable.

5 Semi-synthetic peroxidases

Even after genetic modification heme-dependent peroxidases still contain the intrinsically instable protoporphyrin IX moiety. Vanadium-dependent peroxidases, on the other hand, exhibit higher stabilities, but are hampered by narrow scope and/or low activities. We designed and developed a semi-synthetic vanadium peroxidase that is stable and that does not have these limitations.
Table 2. Comparison of the semi-synthetic vanadium peroxidase with heme- and vanadium-dependent peroxidases.

<table>
<thead>
<tr>
<th></th>
<th>heme CPO C. fumago</th>
<th>phytase / VO$_4$$^-$</th>
<th>VBPO A. nodosum</th>
</tr>
</thead>
<tbody>
<tr>
<td>ee (%)</td>
<td>99 (R)</td>
<td>68 (S)</td>
<td>83 (R)</td>
</tr>
<tr>
<td>TOF (min$^{-1}$)</td>
<td>900</td>
<td>5.5</td>
<td>0.78</td>
</tr>
<tr>
<td>TTN (-)</td>
<td>108,000</td>
<td>25,000</td>
<td>750</td>
</tr>
<tr>
<td>STY (g L$^{-1}$ d$^{-1}$)</td>
<td>84</td>
<td>5.3</td>
<td>0.1</td>
</tr>
<tr>
<td>catalyst cost ($ kg$^{-1}$)</td>
<td>14·10$^3$</td>
<td>0.84</td>
<td>5·10$^6$</td>
</tr>
<tr>
<td>Reference</td>
<td>Van Deurzen, 1997b</td>
<td>Chapter 8</td>
<td>Ten Brink, 1998</td>
</tr>
</tbody>
</table>

Vanadate-incorporated phytase catalyzes the enantioselective oxidation of sulfides with H$_2$O$_2$. Based on the commercial price of 15 $ kg$$^{-1}$ of phytase and a turnover number of 25,000 the calculated catalyst costs per kg product are 0.84 $ (table 2), which are compatible with fine chemicals production. Unfortunately, the inherent enantioselectivity of vanadate-phytase for sulfoxidation is only moderate (max. 68%). Moreover, for use in fine chemicals manufacture a broader scope is needed, e.g. to include enantioselective epoxidations and Bayer-Villiger oxidations. Hence, further studies have to be focused on improving the enantioselectivity and the scope of vanadate-phytase. In principle, this could be achieved by employing site-directed mutagenesis or directed evolution.

6 Concluding remarks and future prospects

Summarizing, it can be concluded that to date, peroxidases are not suitable as catalysts for the industrial manufacture of fine chemicals and chiral intermediates. A substantial increase of the total turnover number of the catalyst or a reduction of the peroxidase costs is necessary to achieve profitable industrial processes based on peroxidases. Reduction of the catalyst cost could be achieved by large-scale production, which is necessary at the time the enzyme is used in industrial applications. But the industrial application was hampered by the peroxidase costs, therefore as long as nobody starts using or producing on large-scale the catalyst cost remaining high.

On the other hand, investigations described in this thesis, have shown that at least three topics have potential for improving the industrial viability of peroxidases: the in situ generation of H$_2$O$_2$ by means of an oxidase, site-directed mutagenesis and/or directed evolution of recombinant CPO, and the semi-synthetic vanadium peroxidases.
De toepassing van peroxidases in de bereiding van fijnchimicaliën

1 Inleiding
De engelse titel van dit proefschrift ‘The application of peroxidases in the synthesis of fine chemicals’ kan het best vertaald worden met ‘De toepassing van peroxidases in de bereiding van fijnchimicaliën’. Fijnchimicaliën zijn chimicaliën die over het algemeen complex zijn, meestal in veel stappen en op kleine schaal gemaakt worden. Tot de fijnchimicaliën worden de volgende stoffen gerekend: geneesmiddelen, cosmetica, geur- en smaakstoffen, gewasbeschermingsmiddelen en voedselingsrediënten.

Fijnchimicaliën worden over het algemeen in kleine porties gemaakt in apparatuur die voor meerdere doeleinden gebruikt wordt. Dit in tegenstelling tot de productie van bulkchimicaliën (bv. benzine en plastics), die continu en in speciaal ontwikkelde produktieeenheden gemaakt worden.

Een belangrijke stap in de bereiding van chimicaliën is een oxidatietap: het inbrengen van een zuurstofatoom in de te maken stof. In de bereiding van fijnchimicaliën zijn vaak gecompliceerde oxidatie-stappen nodig. Oxidanten, die hiervoor worden gebruikt, zijn vaak zuurstofhoudende verbinding van zware metalen, zoals chroom. Helaas moeten deze oxidanten in een één-op-één-verhouding met het produkt gebruikt worden om een goed resultaat te behalen. Dit levert echter een enorme stroom afval op, die voor de bereiding van geneesmiddelen kan oplopen tot 100 kg afval per kg medicijn.
Om deze afvalstroom te verminderen, wordt er zowel binnen de industrie als aan universiteiten veel onderzoek verricht. Zware metaalverbindingen worden daarbij vervangen door zogenaamde schone oxidanten als zuurstof \((O_2)\) en waterstofperoxide \((H_2O_2)\). Het voordeel van de oxidanten is dat water \((H_2O)\) het enige afvalprodukt is van de oxidatiereactie (zie figuur 1). Reacties met \(O_2\) en \(H_2O_2\) verlopen niet spontaan, maar alleen als er een extra verbinding (de zogenaamde katalysator) toegevoegd wordt. Naast het mogelijk maken van een reactie, kunnen katalysatoren ook de selectiviteit van de reactie verhogen. Van selectiviteit is sprake als alleen het gewenste product gemaakt wordt en niet ook nog eens verschillende bijprodukten. Selectieve processen leveren minder afval op en zijn daarom milieuvriendelijker en economisch aantrekkelijker dan niet selectieve processen.

\[
\begin{array}{c}
\text{H} \\
\text{C} \\
\text{C}
\end{array} + \text{Metaal-O}_4 & \rightarrow & \begin{array}{c}
\text{OH} \\
\text{C} \\
\text{C}
\end{array} + \text{Metaal-O}_3 \\
\begin{array}{c}
\text{H} \\
\text{C} \\
\text{C}
\end{array} + \text{H}_2\text{O}_2 \xrightarrow{\text{Katalysator}} & \rightarrow & \begin{array}{c}
\text{OH} \\
\text{C} \\
\text{C}
\end{array} + \text{H}_2\text{O}
\end{array}
\]

Figuur 1. Een oxidatie met een metaalverbinding (boven) en met een katalysator en waterstofperoxide (onder).

2 Peroxidases

Eiwitten, die als katalysator werken, worden enzymen genoemd. Enzymen katalyseren de meest uiteenlopende reacties, bijvoorbeeld alle reacties in ons lichaam worden door enzymen gekatalyseerd. De enzymen die in dit promotie-onderzoek gebruikt zijn, behoren tot de groep van de peroxidases.


Peroxidases katalyseren een breed scala aan reacties en zijn daarbij heel selectief. En net als alle andere enzymen werken ze het best in een waterige oplossing en bij kamertemperatuur. Deze eigenschappen maken peroxidases heel geschikt om als katalysator voor de bereiding van fijnchemicaliën te dienen.

Bij de start van dit promotieonderzoek, was al veel bekend over de verschillende reacties, die peroxidases kunnen katalyseren. Met name chloorperoxidase (CPO) gemaakt door de schimmel *Caldariomyces fumago* is bekend om zijn veelzijdigheid en hoge selectiviteit (boven de 99%). Op laboratoriumschaal is CPO uitstekend geschikt voor het maken van bouwstenen/beginstoffen voor geneesmiddelen en andere fijnchemicaliën.
De huidige commerciële toepassing van peroxidases is veelal als toevoeging aan diverse produkten. Lactoperoxidase (uit koemelk) wordt samen met nog twee andere enzymen gebruikt in tandpasta (Zendium™) voor een antibacteriële werking en sojaboonperoxidase werd door Quest Int. ontwikkeld voor de toepassing als broodverbeteraar. Daarnaast worden diverse peroxidases toegepast in diagnostische kits (bv voor de bepaling van het bloedsuikergehalte).

De ontwikkeling van peroxidases als industriële katalysator staat nog in de kinderschoenen. De hoge prijs van de peroxidases en de geringe hoeveelheden, die gemaakt worden, zijn beperkende factoren. Dit komt, omdat ze uit natuurlijk materiaal (schimmels, planten, slachtafval) gezuiverd moeten worden. Daarnaast is de levensduur van de peroxidases te kort om economisch aantrekkelijk te zijn (met een gram peroxidases kan maar een beperkte hoeveelheid produkt gemaakt worden). De korte levensduur wordt veroorzaakt door waterstofperoxide, dat het enzym deactiveert (kapot maakt en van structuur verandert, vergelijk het met het koken van een ei). Als er meer waterstofperoxide aanwezig is, dan door de peroxidase wordt gebruikt, wordt het enzym kapot gedeactiveerd.

3 Resultaten

De doelstelling van dit promotie-onderzoek was het vinden van nieuwe methodes om de levensduur van peroxidases te vergroten. Als uitgangspunt voor het onderzoek is de levensduur van CPO tijdens de oxidatie van indool en thioanisool met H₂O₂ als oxidant gekozen. In een eerste methode is geprobeerd H₂O₂ te vervangen door zuurstof als oxidant om daarmee de deactivering van CPO te verminderen (§ 3.1). CPO is vast gemaakt aan een drager (geïmmobiliseerd) om het gebruik in oplosmiddelen mogelijk te maken (§ 3.2). De korte levensduur van peroxidases wordt voornamelijk bepaald door het actieve centrum van het enzym. Dit is een ijzer-bevattende heemgroep*, die makkelijk kapot gaat. Door CPO chemisch en genetisch te veranderen is geprobeerd om het actieve centrum sterker te maken (§ 3.3). Tot slot zijn semi-synthetische peroxidases op basis van andere enzymen ontworpen, die goede kandidaten zijn om de natuurlijke peroxidases te vervangen (§ 3.4).

3.1 Oxidaties met zuurstof

Het grootste probleem met peroxidases is dat ze makkelijk gedeactiveerd worden door hun eigen oxidant, H₂O₂. Het continu en gecontroleerd toevoegen van H₂O₂, om de concentratie zo laag mogelijk te houden, verhogen de levensduur. Maar tot nu toe is het resultaat te klein om in de huidige chemische processen te gebruiken. Het probleem van de deactivering zou verholpen kunnen worden door H₂O₂ te vervangen door een andere oxidant (O₂). Omdat peroxidases niet met zuurstof reageren zijn er twee methodes onderzocht om dit toch voor elkaar te kunnen krijgen. Een tweede enzym (glucoseoxidase) is gebruikt om glucose te oxideren met zuurstof, waarbij H₂O₂ gemaakt wordt. Daarnaast is het chemisch geprobeerd door er een extra stof (een reductant) aan toe te voegen.

* Heem is een porfirine-ligand met daarin een ijzeratoom gebonden. Deze groep komt ook in rode bloedcellen voor en zorgt voor de binding met zuurstof.
Samenvatting

<table>
<thead>
<tr>
<th>Verbeteringen in de oxidatie van thioanisool gekatalyseerd door CPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>manier van $H_2O_2$ toevogen</td>
</tr>
<tr>
<td>$H_2O_2$ Stapsgewijs</td>
</tr>
<tr>
<td>$H_2O_2$ Continu</td>
</tr>
<tr>
<td>$H_2O_2$ Gecontroleerd</td>
</tr>
<tr>
<td>glucose/O₂</td>
</tr>
<tr>
<td>$O_2$/reductant</td>
</tr>
</tbody>
</table>

a) **TTN** = *total turnover number* is het aantal produkt dat per hoeveelheid peroxidase gemaakt wordt en is een maat voor de levensduur.

b) **TOF** = *turnover frequency* is de snelheid waarmee het produkt gemaakt wordt per hoeveelheid peroxidase.

c) **STY** = *space time yield* is de hoeveelheid produkt die per dag per reactorvolume gemaakt kan worden.

Uit tabel 1 blijkt dat de eerste methode ($H_2O_2$ maken uit glucose mbv glucoseoxidase) tot een verlenging van de levensduur van de peroxidases leidt (hoogste TTN). Dit komt doordat bij deze methode de $H_2O_2$ in de oplossing gemaakt wordt en dus overal een lage concentratie heeft. Dit in tegenstelling tot de drie eerste methoden uit tabel 1, waarbij een geconcentreerde $H_2O_2$-oplossing toegevoegd wordt. Op de plaats waar $H_2O_2$ de reactor binnenkomt is de concentratie zo hoog dat er deactivering plaatsvindt.

De tweede methode ($O_2$ met een reductant) leidt echter tot een verkorting van de levensduur (laag TTN). Onderzoek wees uit dat ook hier $H_2O_2$ in de oplossing gemaakt wordt maar dan via een radicaalreactie. Het gevolg van deze radicaalreactie is het ontstaan van agressieve verbindingen, die voor deactivering van CPO zorgen.

### 3.2 Immobilisatie van CPO

Een beperking van reacties in water is dat veel stoffen er slecht in oplossen, dit leidt tot een laag rendement. Door reacties uit te voeren in een (organisch) oplosmiddel kunnen hogere concentraties produkt bereidt worden. Over het algemeen zijn enzymen alleen actief in water en moeten ze aangepast worden voor het gebruik in oplosmiddelen. Hiervoor worden enzymen vast gemaakt op een drager (geimmobiliseerd).

CPO en twee andere enzymen (aminoacylase en phytase) zijn geimmobiliseerd in PUR-schuim. Het voordeel van deze enzymen is dat ze een suikermantel bezitten. Tijdens het immobiliseren beschermt deze suikermantel het enzym tegen deactivering. Hierdoor wordt een stabiele en herbruikbare biokatalysator gemaakt. Het belangrijkste resultaat van de immobilisatie in PUR-schuim is de hoge belading (hoeveelheid enzym per hoeveelheid schuim) die behaald werd. Voor CPO is dat 24 mg enzym per gram schuim en voor aminoacylase 187 mg/g.

Geimmobiliseerd CPO, zoals het PUR-schuim en een complex van CPO met een surfactant (oppervlakte actieve stof), kan nadat het gedroogd is, gebruikt worden in een (organisch) oplosmiddel. Als oxidant werd TBHP (tert-butylihydroperoxide) gebruikt. De beste resultaten (hoge reactiesnelheid enenantioselectiviteit en een lange levensduur) werden behaald in apolare oplosmiddelen, zoals hexaan, iso-octaan en 1-octanol. De levensduur van CPO is in organisch oplosmiddel echter veel lager dan in water. Dit komt waarschijnlijk door de vorming van tert-butoxy radicalen, die CPO deactiveren. De
immobilisatie van CPO en de toepassing in organisch oplosmiddel verhoogd de industriële toepasbaarheid niet.

3.3 Chemische en genetische veranderingen van CPO
CPO en andere peroxidases hebben een ijzer-bevattende heemgroep als actief centrum, dat gevoelig is voor afbraak. Veranderingen aan deze heemgroep of aan het eiwit van het enzym zouden de levensduur kunnen verlengen. Veranderingen aan de heemgroep en het eiwit van CPO zijn onderzocht.

Het maken van CPO met een ander metaalatoom is geprobeerd door eerst het ijzeratoom te verwijderen, maar dat bleek niet mogelijk. Daarna is onderzocht of het mogelijk was om de schimmel, die CPO maakt, te laten groeien zonder ijzer, maar met een ander metaal. De schimmel maakt onder deze condities wel CPO, maar dat bezit nog steeds 1 atoom ijzer per eiwit molecuul, naast een kleine hoeveelheid van het nieuwe metaal. Om deze methode geschikt te maken zal een oplossing gevonden moeten worden, om het ijzer uit de schimmel te verwijderen.

Random mutatie, het willekeurig aanbrengen van kleine veranderingen in een eiwit, gevolgd door een goede selectie op verbeterde eigenschappen is een goede methode om enzymen te ontwikkelen met een langere levensduur. Random mutanten van CPO (gemaakt in de natuurlijke schimmel, Caldariomyces fumago) zijn geselecteerd op een verhoogde activiteit in mengsels van water met een organisch oplosmiddel. Van de meer dan honderd mutanten die onderzocht zijn, hadden er drie een verhoogde activiteit in water met 40% tert-butanol (maximaal een 3,4 maal hogere activiteit).

Een nadeel van de genetische aanpassing van CPO in zijn natuurlijke schimmel is dat de aanmaak van niet aangepast CPO mogelijk blijft. Als CPO gemaakt wordt door een andere schimmel, dan treedt dit probleem niet op. Als het cpo-gen ingebouwd wordt in de schimmel Aspergillus niger, dan gaat deze schimmel CPO maken. De katalytische eigenschappen van dit recombinante CPO zijn onderzocht en blijken gelijk te zijn aan die van het natuurlijk CPO, dat gemaakt wordt door C. fumago.

De produktie van CPO door A. niger gecombineerd met random mutatie is een goed stuk gereedschap om verbeterd CPO te maken. Daarnaast kan gerichte verandering van CPO inzicht geven in de manier waarop de deactivering van het enzym plaatsvindt. Deze kennis kan vervolgens weer gebruikt worden om CPO te verbeteren.

3.4 Semi-synthetische peroxidases
Zelfs al leveren genetische veranderingen een beter CPO, dan nog is de heemgroep in het enzym gevoelig voor deactivering. Er zijn peroxidases, die geen heemgroep bezitten, maar een actief centrum met het metaal vanadium erin. Deze peroxidases hebben een veel langere levensduur dan de heemperoxidases. Alleen zij hebben het nadeel dat hun activiteit erg laag is en het aantal reacties beperkt. Wij hebben semi-synthetische peroxidases ontworpen, die stabiel zijn en deze nadelen niet hebben.

De semi-synthetische peroxidase is gemaakt door een complex te maken van het enzym phytase met vanadium. Vanadium-phytase katalyseert de enantioselectieve oxidatie van sulfides, met H₂O₂ als oxidant. Voor het bepalen van de commerciële haalbaarheid van een proces, zijn de katalysatorkosten per hoeveelheid produkt van groot belang. Voor vanadium-
phytase zijn de kosten slechts f1,70 per kg produkt, wat commercieel aantrekkelijk is. Deze lage kosten worden veroorzaakt door de lage prijs van het enzym phytase (f30 per kg). Phytase wordt op grote schaal geproduceerd om aan veevoer te worden toegevoegd. Veevoer met phytase vermindert de hoeveelheid fosfaat in de mest.

| Table 2. Vergelijking van het semi-synthetisch vanadium-peroxidase met heem- en vanadium-peroxidases. |
|---------------------------------|---------------|---------------|---------------|
|                                | Heem-CPO      | vanadium-phytase | vanadium-peroxidase |
| (enantio)selectiviteit (%)     | 99 (R)        | 68 (S)         | 83 (R)         |
| TOF² (per min)                 | 900           | 5,5            | 0,78           |
| TTN² (-)                       | 108.000       | 25.000         | 750            |
| STY² (g / liter / dag)         | 84            | 5,3            | 0,1            |
| katalysatorkosten² (f / kg)    | 28.000        | 1,7            | 10.000.000     |
| referentie                     | Van Deurzen, 1997b | hoofdstuk 8   | Ten Brink, 1998 |

a-c) zie tabel 1
d) de prijs van de hoeveelheid enzym, die nodig is om 1 kg produkt te maken.

Echter de enantioselectiviteit van vanadium-phytase bij de oxidatie van sulfides is maar matig (68%). Ook moeten voor industriële toepassing meer reacties mogelijk zijn, dan alleen de oxidaties van sulfides. Vervolg-onderzoek zal zich dan ook moeten richten op het verbeteren van deze twee punten. Hiervoor zou gebruik gemaakt kunnen worden van genetische veranderingen van het enzym.

4 Conclusies en toekomstperspectief

Alles overziend zijn peroxidases op dit moment nog niet geschikt zijn als katalysator voor de industriële bereiding van fijnchemicië. Een sterke daling van de kostprijs van peroxidases of een aanzienlijke verbetering van de levensduur van het enzym zijn nodig om economisch rendabele processen op basis van peroxidases te ontwikkelen.

Verlaging van de katalysatorkosten kan bereikt worden door peroxidases op grote schaal te produceren. Alleen enzymen worden pas op grote schaal gemaakt als er grote vraag is. De toepassing van peroxidases op grote of industriële schaal wordt juist verhinderd door de hoge kostprijs. Kortom een kip-en-ei probleem.

Aan de andere kant heeft dit promotieonderzoek aangetoond, dat er tenminste drie mogelijkheden zijn om de industriële haalbaarheid van peroxidases te verbeteren. Het in de oplossing maken van H₂O₂, met behulp van glucoseoxidase, verhoogd de levensduur van peroxidases aanzienlijk. Genetische veranderingen aan CPO, geproduceerd door A. niger, kunnen op maat verbeterd CPO opleveren. Semi-synthetische vanadium-peroxidases op basis van goedkoop phytase zijn een commercieel aantrekkelijk alternatief voor de natuurlijk peroxidases.
Materials and methods

Enzymes

Chloroperoxidase from *Caldariomyces fumago* was isolated and purified as described in literature (Van Deurzen, 1994). The enzyme preparation (30.6 μM) contained 3000 U/ml (standard monochlorodimedone assay (Morris, 1966)) with a purity of R₂=1.3 (R₂=A₄₀₀/A₂₈₀=1.44 for pure CPO). CPO solution obtained from Chirazyme Labs (Urbana, IL) contained 11.4 mg ml⁻¹ CPPO with an activity of 22.8 kUml⁻¹ and a purity of R₂ 1.23 and was used for chapter 4 and chapter 7.

Phytase from *Aspergillus ficuum*, both commercial grade (> 95% phytase) and analytical grade (> 99% phytase) was a gift of Gist-brocades B.V., the Netherlands. Phytases from *A. fumigatus* and *A. nidulans* and acid phosphatase from *A. niger* were donated by F. Hoffmann-La Roche Ltd., Switzerland. Lactoperoxidase and horseradish peroxidase were received as a gift from Boehringer Mannheim. Soybean peroxidase was received as a gift from Enzymol International, Inc. (USA) and also from Quest International B.V. (the Netherlands).

All other enzymes were obtained from the following suppliers and used without further purification:

*Fluka*: acid phosphatase from wheat germ.

*Sigma*: acid phosphatase from potato and sweet potato, albumin from bovine serum, aminoacylase from *A. melleus*, catalase from bovine liver, apo-ferritin from horse spleen, glucose oxidase from *A. niger*, microperoxidase-11, phospholipase D from peanut and cabbage, sulfatase from *Helix pomatia*, *aerobacter aerogenes*, abalone entrails and limpets, superoxide dismutase from bovine erythrocytes.
Materials and methods

Materials

The foamable hydrophilic prepolymer (Hypol™ 3000) was graciously supplied by Hampshire Chemical Ltd. Hypol 3000 is a water-activated derivative of toluene-2,6-diisocyanate. Methyl m-bromophenyl sulfide and methyl m-chlorophenyl sulfide were donated by Prof. Dr. Brandsma of the University of Utrecht. Methyl p-chlorophenyl sulfide and ethyl p-chlorophenyl sulfide were synthesized according to literature procedures (Van Deurzen, 1997b). Racemic sulfoxides were prepared by chemical oxidation of the corresponding sulfide according to Drabowicz (1990). Racemic cis-2-heptene oxide was prepared according to literature (Furniss, 1989).

Solvents were of analytical grade and purchased from J.T. Baker and used without further purification. All other chemicals were obtained from the following suppliers:

Acros: tri(n-butyl)phosphine, DTPA [diethyleneetriaminepentaacetic acid], EDTA [ethylenediaminetetraacetic acid].

Aldrich Chemical Company: N-acetyl-L-methionine, Brij52 [diethylene glycol monocetyl ether], 5-bromoindole, catachol, 5-chloroindole, chromium(II) chloride, copper(I) chloride, dihydroxyfumaric acid, 1,2-diphenylhydrazine, ethyl phenyl sulfide, cis-2-heptene, hydroquinone, indole, 5-methoxyindole, methyl p-bromophenyl sulfide, methyl p-methoxyphenyl sulfide, methyl p-methylenphenyl sulfide, methyl p-nitrophenyl sulfide, 2-oxindole, phenol, resorcinol, sodium dithionite, thioanisole.

Campro Scientific: 18O labeled hydrogen peroxide [H218O2; 90% 18O].

Fluka: α-anisidine, cobaltous sulfate heptahydrate, D(-)-fructose, D(+)-glucose, HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid], magnesium sulfate heptahydrate, MES [2-(N-morpholino)ethanesulfonic acid], TRIS [tris(hydroxymethyl)aminomethane], Trolox™-C [6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid].

Fisher Scientific: (L)-ascorbic acid.

Johnson-Matthey: ruthenium trichloride nH2O.

Pharmacia: gel filtration low molecular weight calibration kit.

Sigma: ABTS [2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)], Bradford reagent, hemin, MCD [monochlorodimedone].

Analysis

Reversed phase HPLC was done 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 using Waters Millennium™ software. Samples were quenched with sodium sulfite, diluted with methanol and centrifuged before analysis. Eluent composition and flow rates for different substrates are summarized in table 1, together with the internal standard and wavelength used.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Eluent (%)</th>
<th>Flow (ml min⁻¹)</th>
<th>IS</th>
<th>Wave length (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetyl-L-methionine</td>
<td>7.5 ACN</td>
<td>1.5</td>
<td>-</td>
<td>210</td>
</tr>
<tr>
<td>Dihydroxyfumaric acid</td>
<td>0.25 TFA</td>
<td>1.0</td>
<td>acetate</td>
<td>280</td>
</tr>
<tr>
<td>Ethyl p-chlorophenyl sulfide</td>
<td>35 ACN</td>
<td>1.0</td>
<td>tmb</td>
<td>220</td>
</tr>
<tr>
<td>Ethyl phenyl sulfide</td>
<td>35 ACN</td>
<td>1.0</td>
<td>tmb</td>
<td>220</td>
</tr>
<tr>
<td>Indole</td>
<td>60 MeOH</td>
<td>1.0</td>
<td>t-BuOH</td>
<td>260</td>
</tr>
<tr>
<td>Methyl m-bromophenyl sulfide</td>
<td>35 ACN</td>
<td>1.0</td>
<td>tmb</td>
<td>220</td>
</tr>
<tr>
<td>Methyl p-bromophenyl sulfide</td>
<td>35 ACN</td>
<td>1.0</td>
<td>tmb</td>
<td>220</td>
</tr>
<tr>
<td>Methyl m-chlorophenyl sulfide</td>
<td>35 ACN</td>
<td>1.0</td>
<td>tmb</td>
<td>220</td>
</tr>
</tbody>
</table>
Materials and methods

Methyl p-chlorophenyl sulfide 35 ACN 1.0 tmb 220
Methyl p-methoxyphenyl sulfide 35 ACN 1.0 tmb 220
Methyl p-methylphenyl sulfide 35 ACN 1.0 tmb 220
Methyl p-nitrophenyl sulfide 35 ACN 1.0 tmb 220
Phenol 25 MeOH 1.0 t-BuOH 254
Thiocianisole 35 ACN 1.0 tmb 220

a) Completed to 100% (v:v) with water; ACN: acetonitrile; MeOH: methanol; TFA: trifluoroacetic acid.
b) IS: Internal standard added to either the reaction mixture or to an aliquot of the reaction mixture; t-BuOH: tert-butyl alcohol; tmb: 1,2,3-trimethoxybenzene.
c) Aqueous phase: 50 mM phosphate buffer pH 2.2
d) After 4 minutes the flow was increased to 3 ml min⁻¹.

Chiral HPLC was done 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 with Waters Millennium³² software. Hexane/isopropyl alcohol mixture with compositions as indicated in table 2 were used as eluent. Samples were diluted with a hexane/isopropyl alcohol mixture of 75:25 (v:v) and dried over Na₂SO₄ and centrifuged before analysis.

<table>
<thead>
<tr>
<th>Table 2. Chiral HPLC analysis using a Chiralcel OD column.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
</tr>
<tr>
<td>Ethyl p-chlorophenyl sulfide</td>
</tr>
<tr>
<td>Ethyl phenyl sulfide</td>
</tr>
<tr>
<td>Methyl m-bromophenyl sulfide</td>
</tr>
<tr>
<td>Methyl p-bromophenyl sulfide</td>
</tr>
<tr>
<td>Methyl m-chlorophenyl sulfide</td>
</tr>
<tr>
<td>Methyl p-chlorophenyl sulfide</td>
</tr>
<tr>
<td>Methyl p-methoxyphenyl sulfide</td>
</tr>
<tr>
<td>Methyl p-methylphenyl sulfide</td>
</tr>
<tr>
<td>Methyl p-nitrophenyl sulfide</td>
</tr>
<tr>
<td>Thioanisole</td>
</tr>
</tbody>
</table>

a) Hexane/isopropyl alcohol-mixtures.
b) IS: Internal standard added to either the reaction mixture or to an aliquot of the reaction mixture; tmb: 1,2,3-trimethoxybenzene.

GS analysis was performed on a Varian 3400CX GC equipped with an Varian 8200 CX autosampler with the columns and temperature programs as indicated in table 3. An aliquot of the reaction mixture was saturated with NaCl or Na₂SO₄ and extracted with dichloromethane. Normal alkenes were used as internal standard.

<table>
<thead>
<tr>
<th>Table 3. GC analysis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
</tr>
<tr>
<td>cis-2-heptene</td>
</tr>
<tr>
<td>cis-2-heptene oxide</td>
</tr>
</tbody>
</table>

a) IS: internal standard added to either the reaction mixture or to an aliquot of the reaction mixture; C₁₄: tetradecane;
b) from Chrompack, size: 50m x 0.53mm;
c) from Astec, size: 40m x 0.25mm.

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

Apparatus

96 Well plate experiments were performed with a Spectra MAX 250 microplate Spectrophotometer from Molecular Devices.

Addition of reagents was done using a Metrohm Dosimat 665.

Roller-bottle cultures were grown using a Wheaton Modular Cell Production Roller apparatus.

Centrifugation was performed either with a Bechman J2-21M/E Centrifuge or with a Megafuge 2.0R from Heraeus Instruments.

Enzyme concentration was carried out using either an Amicon Stirred cell, equipped with an ultrafiltration membrane or a Centriprep concentrator from Amicon.

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 performed using a Superose 12 HPLC column (Pharmacia, 10 x 300 mm) with a Water 590 programmable HPLC pump with detection on a Waters 486 tunable absorbance detector at 280 nm or 400 nm with Waters Millennium software. Fractions were collected using a Waters fraction collector.

Hydrogen peroxide-stat (Dulcometer Perox 20/21) was obtained from Prominent Dosiertechnik, Heidelberg, Germany. The measurement of hydrogen peroxide is based on amperometric measurement at a platina electrode, which is operated as a potentiostat. Two point calibrations were made before each reaction.

Mixing of enzyme-prepolymer mixtures was done using an IKA stirring device, equipped with a double bladed rotor (d=22mm).

UV measurements were performed on a Cary 3 spectrophotometer from Varian or on a Shimadzu UV-1200 UV-VIS spectrophotometer.

Protein concentration

Protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Bradford reagent (1.4 ml) was added to an appropriate dilution of enzyme solution (50 μl; protein concentration less than 1 mg/ml). After 30 min incubation at 40°C the absorbance at 595 nm was measured.

_Biochemistry_ 32: 241-252.


Chibata I (1978) - Immobilized enzymes, Kodansha, Tokyo.


References


References


References


References


References


References


References


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