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Applications for (Halo)peroxidases

Abstract

Peroxidases are enzymes which are able to catalyse a variety of reactions. These include the classical peroxidase reaction that is the one-electron oxidation of organic aromatic compounds, the oxidation of halide ions to the corresponding hypohalous acids and as has been more recently discovered enantio-selective oxygen-transfer reactions resembling those catalysed by cytochrome P450. In this overview the potential applications of peroxidases in a variety of products and processes are discussed together with a discussion of some problems to be expected upon introduction of these enzymes on the market.

Introduction

Many enzymes have found their application in daily life although this is mainly restricted to enzymes used in detergent such as proteases, lipases, amylases and cellulases. In addition, the food industry and biotechnological fermentation industry uses a variety of other enzymes which account for more than 50% of the current applications of industrial enzymes (Hodgson, 1994). A few examples are glucose isomerase in the conversion of glucose into fructose, pectolytic enzymes in the treatment of fruit juices to allow easier filtrations, clarification and concentration of the juice and chymosin for cheese making. The use of new enzymes in other processes is finding its way also thanks to the development of gene technology which allows us to produce enzymes in question in sufficient quantities. Research activities are now also directed to the application of oxido-reductases. In particular peroxidases using hydrogen peroxide as a cheap and clean oxidant, are a point of focus of current industrial interest. For application on an industrial scale a number of requirements have to be met which may seriously delay the introduction of such enzymes on a market. Some of these are obvious, other requirements are not realized in particular by those working in academic institutions with no direct links to industry.

Important factors which determine whether or not an enzyme will penetrate a certain market is that its application in a process should be competitive towards existing processes, be cheaper or more selective and preferably produces

less environmental stress. Also, the enzymes should be stable and resistant towards oxidative inactivation, they should have large turnover number in terms of product produced or converted per unit time per molecule of enzyme and they should have a long life time and operational stability.

Another important factor is that large, preferably kilogram quantities should be available at low cost and this will in most cases rule out standard time-consuming laboratory isolation procedures from unprocessed biological materials. There are a number of sources or approaches which have advantages or are being used as an alternative to produce more of an enzyme and these will be discussed shortly.

Enzyme production

One of the methods to produce desired enzymes is the use of cultured plant cells in which the enzyme is secreted into the culture medium. Several peroxidases may be produced in this way (Maldanoldo and Van Huystee,1980, Kwak *et al.*, 1995). To culture animal cells is another option but it is very unlikely to be applicable on a larger scale than a few mg of protein. The major problem is that Chinese hamster ovarian and other cell lines which are used as producer for recombinant enzymes or proteins grow only slowly and expensive growth media are required. Furthermore, these cells secrete only small quantities of desired enzyme and this makes them unsuitable as a source for most applications. The same arguments hold for the application of *Xenopus* oocytes. Also transgenic plants may be used (Gazarin, 1995) but growing plants is a relatively slow process and the amount of enzyme produced per biomass (typically 50 mg/kilo plant) may be too low to be economical. However, an exception may be when the enzyme is localized and concentrated in a seed or fruit and which may be harvested easily. Introduction of non-natural genes into animals is now also possible, forcing the transgenic animal to produce a desired enzyme or protein (e.g. lactoferrine) in its milk. Since there are in general viable alternatives for the use of transgenic animals a strong consumer and public opposition exists in some countries.

Gene amplification or gene introduction for novel enzymes into production hosts has boosted the introduction of recombinant enzyme systems and engineered micro-organisms as filamentous fungi, yeasts, bacteria or viruses are used as sources for industrial enzymes. In particular, filamentous fungi have been successfully employed as hosts in the industrial production of extracellular (glyco)proteins. Certain industrial strains are capable of secreting gram quantities of these proteins per litre medium. In addition, filamentous fungi are able to correctly perform post-translational modifications of eukaryotic proteins and many strains have U.S. Food and Drug Administration approval. Furthermore, large scale fermentation technology and downstream processing experience is available and the cost price may be as low as 2000 $ a kilo. Thus, nowadays it

is possible to produce enzymes in bulk. This holds for simple enzymes containing no prosthetic groups. If a prosthetic group is normally present in the enzyme, the recombinant enzymes are sometimes secreted into an inactive apoform which has to be reactivated by addition of the prosthetic group. This additional reactivation step may substantially increase the costs of the enzyme.

Considering all the arguments, it is likely that that we will see other enzyme systems appearing on the market and in the production of consumables. This in particular holds for peroxidases. In this contribution some of the proposed applications of these enzymes will be discussed in terms of their properties. This review will not cover all aspects rather a selection is made which is mainly based on the more recent patent literature. The reader is also referred the overview by Neidleman and Geigert (1986) which covers the literature until 1986 and to the review by Franssen, 1997 in these Proceedings on the potential application of haloperoxidases in organic chemistry.

Use of peroxidases to label proteins with radio-isotopes

In the field of nuclear medicine, there is considerable interest in the use of radioactively labelled proteins. Iodination of a protein is most commonly used. For his application in which a peroxidase is used to iodinate proteins, commercial kits are available, e.g. that from Biorad which uses lactoperoxidase and glucose oxidase. Since this method is mild and results in a stable attachment of the label it is particular useful to label monoclonal antibodies. However, there are some disadvantages of using ^{125}I and ^{131}I in particular for patient application. Bromination with ^{77}Br is another option, an advantage is that the binding strength of the C-Br bond is stronger than that of the C-I bond and this bond strength is an important factor for the in vitro stability of the radiolabelled compound. Again, catalytic halogenation by a haloperoxidase at neutral pH may be used and a procedure has been developed using the heme-containing bromoperoxidase from the algae *Penicillus capitatus* (Manthey *et al.*, 1984). Care must be taken to control the hydrogen peroxide concentration since otherwise a sharply decreased activity is found. An alternative (Lambert and Slegers, 1994) is bromination by the vanadium bromoperoxidase from *Ascophylum nodosum.* This enzyme has also a pH optimum around neutral but its is considerably more stable (De Boer *et al.*, 1987) than its heme-containing counterpart.

Analytical applications

Peroxidases are used these days in large number of diagnostics to determine the concentration of organic metabolites. The metabolite is converted by a specific enzyme to another product in a reaction in which hydrogen peroxide is generated in a stoichiometric amount. The peroxide is quantitatively determined using a peroxidase which in most cases is horseradish peroxidase. The metabolites may be sugars (glucose, galactose, lactose, maltose, saccharose) or other

compounds such as phenol, cholesterol, urate, acetate, lactate, pyruvate and L- and D-amino acids.

The peroxidase may also be used in immuno-assays (ELISA's, monoclonal antibodies) to quantitatively detect and determine a certain antigen. In these cases the peroxidase is coupled to the antibody which will have a specific interaction with the antigen and which may be detected now by a colour reaction or chemiluminescence which is highly sensitive. A patent (Wever *et al.*, 1995) using the vanadium chloroperoxidase to determine Cl^- in a liquid has also appeared. In the method the halide is oxidized in the presence of hydrogen peroxide to hypochlorous acid which is detected colorimetrically. Since the vanadium peroxidase has a very high affinity for halides the assay allows detection of Cl^- in the μM concentration and is more sensitive than existing methods. The amounts of peroxidases which are needed in these diagnostics are relatively small and are estimated to be in the order of 50 kilo/year world wide. Thus, most industrial companies will produce these enzymes using classical large scale purification methods.

Peroxidases as a pharmaceutical/antimicrobicidal agent

It is well established (Klebanoff, 1968, Albrich *et al.*, 1981, Weiss, 1989) that the products of haloperoxidases which are formed by oxidation of a halide or a pseudohalide (Cl^-, Br^-, I^-, SCN^-) are bactericidal. This bactericidal effect may be direct but it may also be mediated by singlet oxygen formation (Kanovsky, 1984).

$$H_2O_2 + X^- + H^+ \longrightarrow \underset{\text{bactericidal}}{HOX} + H_2O \qquad (1)$$

$$H_2O_2 + HOX \longrightarrow \underset{\text{bactericidal}}{{}^1O_2} + H^+ + Cl^- + H_2O \qquad (2)$$

Several patents have appeared now in which applications of peroxidases are claimed. Most of these patents deal with mammalian peroxidases (myeloperoxidase, eosinophil peroxidase and lactoperoxidase) since these enzymes catalyse the formation of bactericidal products in vivo and are implicated in defense systems of the host. One of the first patents in this area was that by Kessler and Rosenbaum (1984) in which the peroxidases were used for killing bacteria in the treatment of dental diseases in situ such as in the oral cavity or as a denture cleaner (mouthwashes, toothpastes). Montgomery (1994) propose a similar system: an orally activated antimicrobial dentifrice that includes SCN^- and lactoperoxidase. These inventions are based upon the natural system present in

saliva and in which the salivary peroxidase generates HOSCN using hydrogen peroxide formed by metabolic active bacteria in the oral cavity.

$$\underset{\substack{\text{(generation}\\\text{by bacteria)}}}{H_2O_2} + \underset{\text{(saliva)}}{HSCN} \xrightarrow{\text{salivary peroxidase}} \underset{\text{(inhibitory)}}{HOSCN} + H_2O \qquad (3)$$

During glycolysis of bacteria and in particular of *Streptococcus mutants* which is present in tooth plaque, acid is generated and as a consequence of the pH drop decay of teeth occurs. Hypothiocyanate (HOSCN) formed by the peroxidase inhibits this decay process by inhibiting bacterial glycolysis. There is indeed a toothpaste on the market (Zendium) in the Netherlands which contains glucose oxidase to increase the generation of hydrogen peroxide from carbohydrates and reinforces the natural system. A more recent version of the toothpaste does also contain lactoperoxidase. Other oral administrations are mouthwash or lozenge.

There is also a claim that some of these products which have antibacterial properties may be used to attack human tumour cells, notably in bone marrow which has been removed from a patient undergoing radiotherapy (Beggs and Davis, 1991, Beggs *et al.*, 1991). It is possible to attach a (halo)peroxidase to an antibody which is capable of binding to the target site (tumour cell) and as a result hypohalite may be generated at the target site resulting in specific killing of the target cell.

Along the same lines Allen (1992) proposed a method and compositions for the treatment of infection and control of bacterial flora using haloperoxidases. These enzymes will selectively bind to and inhibit the growth of microbes. According to the patent the methods and composition are highly useful in the therapeutic or prophylactic antiseptic treatment of human or animal subjects since their use can be designed to be highly effective in combating bacterial or fungal infections. The ability to selectively inhibit the growth of target microbes results from the fact that the haloperoxidases selectively bind to microbes. The target bound haloperoxidase catalyses halide oxidation and facilitates the disproportionation of peroxide to singlet molecular oxygen at the surface of the target microbe. This results in selective killing of the microbe with a minimum of damage to other systems. The binding of the peroxidase to the target may be related to the hydrophobic properties and the strong positive charge of some of the peroxidases. According to this patent suitable haloperoxidases are myeloperoxidase, eosinophil peroxidase, lactoperoxidase, chloroperoxidase and the heme-containing chloroperoxidase from the fungus *Caldariomyces fumago.*

A patent has also appeared directed on prophylactic and therapeutic applications of peroxidases (M. Pourtois *et al.*, 1992) for the manufacture of medicaments for the prevention and treatment of enveloped virus infections and, in particular, of herpes simplex and human immunodeficiency virus infections. Peroxidases of the medicaments include horseradish peroxidase, lactoperoxidase and myeloperoxidase. The authors claim also that the medicament is a topical

medicament (cream, gel, bandage, pad), an oral dentifrice or an injectable composition.

A problem with all these applications is that expression of the mammalian peroxidases in suitable hosts is difficult and only possible at high costs. Expression of active myeloperoxidase has been reported to occur only in Chinese hamster ovarian cell line (Jacquet *et al.*, 1991) and the procedure is probably too expensive for a realistic application. It is possible to express both the heme-containing chloroperoxidase from the fungus *Caldariomyces fumago* and horseradish peroxidase. However, reactivation of the inactive apoprotein and incorporation of the prosthetic group in a peroxidase is not straightforward (Tam and Welinder,1996). An option is to use lactoperoxidase which as a side product of cheese production (whey) is relatively cheap.

A major problem in using heme-containing peroxidases is that they are not stable towards oxidative inactivation by elevated concentration of hydrogen peroxide or their products. Also during catalysis inactive intermediates may accumulate inhibiting the action of these enzymes. A solution to this is to keep the concentration of hydrogen peroxide at a low level. However, this requires careful control of the reaction conditions and this limits the usefulness of heme-containing haloperoxidases in most applications. Accordingly, sources were screened for other haloperoxidases whose activity is substantially unaffected by relatively high concentrations of either hydrogen peroxide or hypohalous acid (Geigert *et al.*, 1990). Indeed, these investigators discovered that dematiaceous hyphomyctes secrete non-heme haloperoxidases which were reported to be very stable (Hunter *et al.*, 1990). For some of the fungi reported it was shown later that secreted peroxidases belonged to the class of vanadium peroxidases (Van Schijndel *et al.*, 1993, Vollenbroek *et al.*, 1995). Subsequently a patent (P. Barnett *et al.*, 1995) was filed in which these exceptionally stable haloperoxidases were used in a antimicrobial composition, comprising a vanadium haloperoxidase. In the patent it is claimed that the antimicrobial compositions of the invention may be employed to provide hygiene benefits for hard surface cleaning and fabric washings, but also to provide hygiene and cleaning in industrial/institutional applications such as in hospitals. The antimicrobial compositions can also be successfully used in deodorants in view of their ability to combat bacteria which cause malodour.

An entirely different application has been proposed by Wever *et al.* (1995) and this is the use of haloperoxidases as an additive in antifouling paint. In the patent it is claimed that the peroxidase will inhibit the growth of organisms on the paint and thus acts as an antifouling agent on ship or yachts. The principle of the invention is that sea water contains about 1 mM Br^- and 500 mM Cl^- and if sufficient peroxide is present the antifouling paint will continuously generate HOX as a bactericidal agent. A limiting factor is the concentration of H_2O_2 in sea water which is about 1 μMM.The peroxide is generated by photo-oxidation processes of water initiated by the UV light of the sun. Also peroxide may be generated as a result of biological activity. This idea is actually borrowed from nature. In some seaweeds a vanadium bromoperoxidase is pre-

sent on the surface of the plant (Wever *et al.*, 1991) which may be present to prevent bacterial growth on the surface by generating the bactericidal HOBr. Another application of the vanadium haloperoxidases suggested is use as a paint preservative in water based paints.

Application in bleaching/detergents

Industrial enzymes in detergent formulations make up a substantial part of the biocatalysts market and it is likely that peroxidases with their degrading and bleaching properties will also be used in detergents in the future. Several patent applications have appeared mainly generated by research groups within Novo-Nordisk A/S and there considerable activity in this area. One of the first patent patent in which this novel lead has been disclosed is that by Kirk *et al.* (1989). The claim is made that an enzyme exhibiting peroxidase activity is capable of exerting a bleaching effect on fabrics. The main advantage would be that by using the detergent additives of the invention amounts of hydrogen peroxide or its precursors can be reduced and yet provide a satisfactory bleaching effect. Thus, the amount of unspent bleaching agents released into the environment is reduced and further the inventors claim that the overall performance of detergents compositions in which the bleaching agents are included is improved. A more specific application (Damhus *et al.*, 1991 and Pederson, 1992) is that of the inhibition of dye transfer during washing or rinsing of fabrics by addition of enzyme exhibiting peroxidase activity to the wash liquor. Since the peroxidase will oxidize or bleach the bleeding dye during the wash the peroxidase will inhibit the transfer of a textile dye from a dyed fabric to another fabric when these fabrics are washed an/or rinsed together in wash liquor. This problem is most noticeable when white or light coloured fabrics are washed together with fabric with a more intense colour from which the dye is leached during washing. Peroxidases are derived from a strain of *Coprinus* or *Bacillus pumilus.* Also a microperoxidase which is a hemopeptide derived from cytochrome *c* may be used for this application (Pedersen *et al.*, 1993). Similarly, these new hemopeptides with peroxidase activity may be used for waste water treatment and for paper pulp bleaching.

A related application is the use of peroxidases in removing excess dye from newly dyed or printed textiles by using wash liquor containing oxidase or peroxidase and suitable oxidant, to reduce back-staining and to improve waste water quality (Pedersen and Schmidt, 1992). The advantage of this treatment is that it bleaches any dye leaching from the material so it prevents redeposition of dye (backstaining) even when relatively small amounts of water are used. It reduces time, energy and water, produces less polluted wash water and improves dye fastness. Bleaching of foodstuffs is also a possibility and there is a patent on the decolourisation of fish roe by treatment with hydrogen peroxide and peroxidase (Novo Nordisk, 1994).

Some of the fungal and bacterial peroxidase have been sequenced and cloned and expression systems have been developed. The tertiary structure of one of these enzymes (*Coprinus cinereus*) has been determined (Petersen *et al.*, 1994) and protein engineering has become a major tool now in developing new variants of *Coprineus cinereus* peroxidase. The native *Coprinus* peroxidase is susceptible towards high concentrations of hydrogen peroxide, resulting in the formation of compound III and inactivation. This problem has been tackled and Pedersen *et al.* (1995) have produced some variants with improved hydrogen peroxide stability at alkaline conditions (pH 7–10). Methods to express the mutants in filamentous fungi especially *Aspergillus oryzae* and *A. niger* (Welinder and Andersen, 1993) are also available. Presently there seems to be no major obstacle for introducing these peroxidases in detergent formulations. However, despite all the research efforts by the industrial enzyme producers and the scientific progress made, a washing powder containing a peroxidase has not yet appeared on the market.

Use of peroxidases in polymerisation and depolymerisation processes

Peroxidases are able to oxidize a large number of organic compounds by one-electron oxidation steps. The radicals produced may dimerise or polymerise and the products formed are in general much less soluble in water. This property may be used to remove carcinogenic aromatic amines and phenols from industrial aqueous effluent. This idea was put forward by Klibanov *et al.* (1983) using horseradish peroxidase that will oxidize phenols to phenoxy radicals. These radicals will react with other aromatic rings causing enzymatic precipitation of aromatic organic material. Peroxidases may also be used to remove colour from bleach plant effluent (Paice and Jurase, 1984). Instead of horseradish peroxidase chloroperoxidase (Pickard *et al.*, 1991) may also be used. Also degradation by peroxidases of environmental pollutants is a suggested use.

Other applications in which a peroxidase is involved in polymerisation processes are the preparation of a glue or binder from lignine sulphonate (Yde, 1994) using a a peroxidase and hydrogen peroxide at alkaline pH to give high molecular weight polymers which may be used in the manufacture of wood composites (wood fibre boards, plywood and laminated wood beams). Its main advantage is that very high molecular weight lignine is prepared without the use of organic solvents. Similarly, the treatment of pulp with a phenol oxidizing enzyme as derived from *Coprinus* or *Bacillus pumillus* after completion of grinding and refining of logs gives paper or paperboard of improved strength (Hansen and Nielsen, 1993). Peroxidases may also be used in the tanning of hides resulting in an increased degree of fixation (Ingvorsen *et al.*, 1993). A process somewhat related to this is the use (Maat and Roza, 1995) of a peroxidase in dough to improve specific volume, staling resistance and crumb structure of bread. Oxidative coupling may also be used in colouring process of hair exposing it to a solution containing soybean peroxidase, hydrogen peroxide and a aromatic

compound (Procter & Gamble, 1975). Finally, by treating aqueous coffee with a peroxidase enzyme in the presence of a peroxide an instant coffee with an improved quality is obtained (Unilever, 1989). By this treatment the coffee beverage has obtained a pleasant, mild aroma with a hint of Mocha. These examples show that it just a matter of time and peroxidases will have found their way in daily life.

Acknowledgements

This work was supported by grants from the Netherlands Foundation for Chemical Resrach (SON) and was made possible by financial support from the Netherlands organisation for Scientific Research (NWO) and the Netherlands Technology Foundation (STW).

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