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ScienceDirect
Tetrahedron: *Asymmetry* 18 (2007) 701–704
Tetrahedron: *Asymmetry*

Enantioselective oxidation of thioanisole with an alcohol oxidase/peroxidase bienzymatic system

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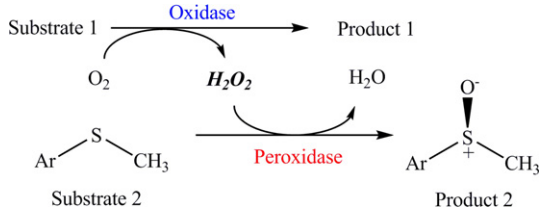
Received 21 February 2007; accepted 9 March 2007

Abstract—Optimization of a new bienzymatic couple alcohol oxidase/peroxidase for the asymmetric oxidation of thioanisole was realized. The main advantages in the application of this system is the use of inexpensive and easily available enzymes and substrates, permitting gram scale enantioselective syntheses. Methanol, as a substrate for alcohol oxidase, also facilitates the solubilization of organic substrates in the reaction medium.

1. Introduction

Asymmetric sulfoxides have proven to be useful tools in organic synthesis, where they are used as chiral auxiliaries.[1] They are also important compounds *per se*: Esomeprazol, (sold as Losec®, an anti-ulcerous compound) is one of the highest selling drugs.[2] They also have tentatively been used as prodrugs.[3] The global importance of this class of products appears in many publications, where hundreds of preparations are reported. Among them, many use biological and enzymatic methods. The most efficient biochemical systems, in terms of yields and enantioselectivity, make use of chloroperoxidase (CPO)[4] or cyclohexanone monoxygenase (CMO).[5] However, CPO is rare and expensive, while CMO uses a cofactor which must be recycled during the operation. Hence, these two systems are only suitable for small scale syntheses of chiral sulfoxides.

We have previously reported the elaboration of two bienzymatic systems, making use of an inexpensive, robust industrial peroxidase (CiP, EC: 1.11.1.7, from *Coprinus cinereus*, expressed in *Aspergillus oryzae*, marketed by Novozymes) and an oxidase producing hydrogen peroxide in situ.[6–8] The general principle of these systems is summarized in Scheme 1. The method was proven to efficiently permit the oxidation of a variety of aryl- and heteroaryl-methylsulfides into the corresponding (*S*)-sulfoxides, with high yields and enantiomeric excesses (up to >99%). Their main



Scheme 1. Synthesis of chiral aryl-methyl sulfoxides catalyzed by an oxidase/peroxidase bienzymatic system.

advantages, as compared to the direct addition of hydrogen peroxide to the reaction medium are:

- An improved operational stability of the peroxidase (this class of enzymes is inactivated by hydrogen peroxide, acting as a 'suicide substrate').
- A very significantly increased ee of the products (hydrogen peroxide directly oxidizes the sulfide, leading to racemic sulfoxide).

We previously reported the use of glucose oxidase and D-aminoacid oxidase as parts of these systems. These two enzymes offer different advantages: glucose oxidase from *Aspergillus niger* (GOD; EC. 1.1.3.4) is a well known and robust enzyme, produced on an industrial scale and at low cost. D-Glucose, its natural substrate, is also relatively inexpensive. D-Aminoacid oxidase (DAOx; EC. 1.4.3.3) from *Trygonopsis variabilis* is available from Fluka in an immobilized and easily recoverable form. In our hands, it gave sulfoxides with the highest ee. However they also have

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doi:10.1016/j.tetasy.2007.03.010

several drawbacks: (1) D-Alanine, the substrate of DAOx, is too expensive for large scale synthesis. (2) The production of water soluble gluconic acid by GOD implies that a pH-stat must be used to maintain a constant pH. (3) In some cases, the isolation of oxidized products of peroxidase might be complicated by the presence of 1 equiv of the water soluble products of oxidases (gluconate and pyruvate, respectively).

Thus we reasoned that alcohol oxidase (AOX; EC. 1.1.3.13) from the yeast *Pichia pastoris* could be an interesting alternative to these previously described systems, with the following improvements:

(1) Its best substrate is methanol, which is very inexpensive, readily available and miscible with water in all proportions. (2) Methanol can act as a useful cosolvent to assist the dissolution of hydrophobic substrates in the mainly aqueous reaction medium. (3) The product of oxidation (formaldehyde) is very volatile. (4) Although the commercial enzyme is relatively expensive (compared to glucose oxidase), the cultivation of the yeast and the preparation of cell-free extracts can be conducted on a medium scale (several litres) in most laboratories equipped for bioorganic chemistry.[9] Alternatively, frozen cells can be obtained from a laboratory equipped for scant microbiology, stored in a freezer and used when needed.

Alcohol oxidase itself has been shown to be an interesting enzyme in organic synthesis.[10,11] The coupled system alcohol oxidase/peroxidase has been widely used for analytical applications.[12] Our goal was to devise a similar system applicable to preparative asymmetric synthesis.

2. Results and discussion

We chose the classical oxidation of thioanisole into chiral methyl-phenyl-sulfoxide as a model reaction by using a limited amount of alcohol oxidase (purified enzyme, from Sigma and from Applied Enzyme Technology Ltd) and a large excess of peroxidase, in order to limit the concentration of free hydrogen peroxide in the reaction medium.

The first step was to determine the minimal amount of AOX and the suitable AOX/CiP ratio to be used for the oxidation of a given amount of thioanisole (0.25 mmol). The best results were obtained with a mixture containing 0.5 μmol of peroxidase and 4 U of AOX (Table 1, entry 7) resulting in a 90% yield in sulfoxide with 76% ee.

It is clear from these results that a large excess of peroxidase is needed for an optimal oxidation. The ee was essentially constant at 75% for the (*S*)-sulfoxide, which indicates that all the hydrogen peroxide transits through the peroxidase. This ee value is also in accordance with those reported with other systems using the same peroxidase,[6,7,13] and is probably representative of the enantioselectivity of this enzyme for this substrate.

Another important feature of this reaction to be optimized is the final concentration of methanol. Using the best conditions found above (4 U AOX, 0.5 μmol peroxidase), we varied the concentration of methanol in the reaction medium from 0.25 M to 4 M. As shown in Figure 1, the initial rate of oxidation increased with the concentration up to 1 M. After this, the rate was somewhat insensitive to the concentration, but the final yield increased to a maximum of 100% for a 4 M concentration of methanol. The exact nature of this effect was not determined, and might be complex: the final concentration of alcohol is far above the K_M of alcohol oxidase for methanol (1–2 mM). Thus, AOX works at its maximal velocity in all cases. We determined the maximal solubility of thioanisole in pure buffer as 0.5 mM. With 8% methanol (4 M), the solubility was roughly the same (0.6 M), indicating that the observed effect is not due to an increased solubility of the substrate. On an other hand, the aspect of the suspension was very different: large droplets of thioanisole were formed in pure buffer, while a fine milky suspension was observed in 4 M methanol: the reaction catalyzed by CiP is probably favoured by a better dispersion of the substrate in the aqueous medium. Under our conditions, the sulfoxide formed was completely dissolved in 4 M methanol, which may prevent an inhibition of peroxidase by the product.

Table 1. Influence of the ratio of alcohol oxidase/peroxidase used for oxidation of thioanisole (0.25 mmol in 5 mL aqueous buffer; 1 M methanol)

Entry	AOX (U)	CiP (μmol)	Yield	ee
1	0.5	0.25	43	78
2	1	0.25	60	77
3	2	0.25	58	75
4	4	0.25	48	76
5	8	0.25	26	74
6	1	0.5	60	77
7	4	0.5	90	76
8	8	0.5	55	70

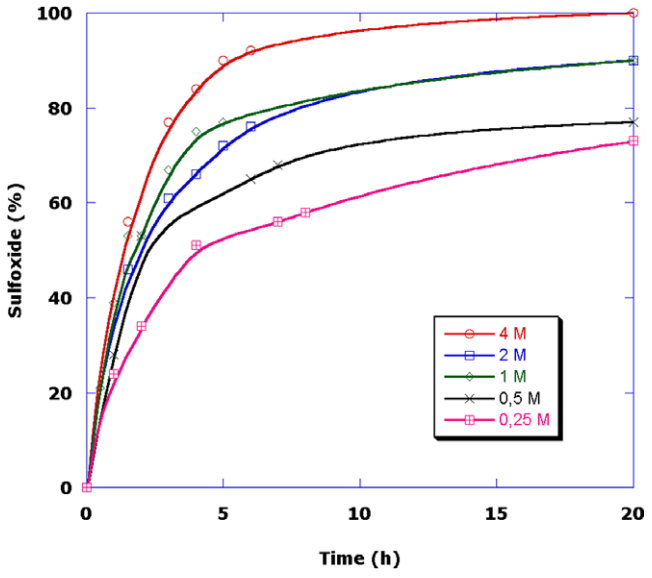

Figure 1. Effect of the concentration of methanol in the reaction medium on the oxidation of thioanisole (0.25 mmol in 5 mL aqueous buffer; 0.5 μmol of peroxidase; 4 U of AOX).

Since commercial purified AOX is somewhat expensive and sold in minimal quantities, we attempted to use a 'home made' crude extract of *P. pastoris* as a source of alcohol oxidase. The yeast was grown following standard procedures in a liquid medium containing methanol as a sole source of carbon. After centrifugation, the cells were kept deep-frozen at −18 °C. They were later resuspended in dilute buffer solution and disrupted with a French press immediately before use.[14] We measured an average activity of 19 μmol of methanol oxidized/min/mL (19 U/mL) of crude extract, which could probably be increased, if needed, by optimization of the culture conditions. The use of 2–4 U of AOX allowed us to obtain the sulfoxide with 100% yield and 75% ee.

Finally, in order to prove the usefulness of the method in preparative asymmetric synthesis, we used 10 mmol of thioanisole for a gram scale preparation of sulfoxide. After treatment of the reaction medium (see Experimental) and careful evaporation of the solvent, we obtained 1.1 g of pure sulfoxide (72% yield) as indicated by NMR, with 75% ee. The total turnover number (>700) is quite modest when compared to other peroxidases (6×10^4 for CPO[15]), but is favourably counterbalanced by the availability of CiP. Moreover, CPO gives the (*R*)-sulfoxide, while CiP gives the (*S*)-enantiomer. Thus, the two enzymes have complementary enantioselectivities.

3. Conclusion

In conclusion, an enantioselective oxidation of thioanisole was realized with a bienzymatic couple alcohol oxidase/peroxidase. Different parameters including the number of units of alcohol oxidase, the ratio alcohol oxidase/peroxidase and the concentration of methanol were optimized leading to the (*S*)-methyl-phenyl-sulfoxide with high yield and 75% ee. Similar results were obtained via the use of crude extracts of alcohol oxidase prepared from culture of *P. pastoris*. The method was shown to be efficient on a gram scale preparation. There is no doubt that the conditions optimized on the model compound thioanisole can be applied to other aryl-methyl-sulfides, as was demonstrated with the previously described bienzymatic systems.

4. Experimental

4.1. Enzymes

Purified alcohol oxidase from *P. pastoris* was obtained from Applied Enzyme Technology Ltd (Leeds, UK) with an activity of 1780 U/mL and from Sigma. *Coprinus cinereus* peroxidase was obtained from Novozymes as an aqueous solution. After dialysis against 0.5 M NaCl, the peroxidase concentration was estimated to be mM from a measure of absorbance at 404 nm ($\varepsilon = 108\ \mathrm{mM^{-1}\,cm^{-1}}$). Specific activity: 208 mmol of ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)) transformed/min/mL of solution (2 mmol of ABTS transformed/mg of protein).

4.2. *P. pastoris* production

A preculture was realized by inoculation of 100 mL of medium (phosphate buffer pH 6; 0.7% yeast nitrogen base (DIFCO); 0.5% glycerol) with *P. pastoris*. The yeast was grown at 30 °C for two days with orbital agitation. This preculture was then poured in 900 mL of medium (0.5% methanol instead of glycerol for induction of alcohol oxidase production) at 30 °C under agitation (150 rpm) for three days. The culture was then centrifuged (8000 rpm; 0 °C). The cells were recovered, freeze-dried and stored at −20 °C. Crude extracts were obtained by cell disruption of lyophilized cells (previously suspended in 0.1 M, pH 7.5 phosphate buffer) in a French press at 1100 psi. Centrifugation (8000 rpm; 0 °C) allowed us to obtain a crude extract solution with 0.664 mg of proteins/mL as determined by Bradford test, with an activity of 19.6 μmol of oxidized methanol/min/mL (115.8 U/g dried cells).

4.3. Thioanisole oxidation

4.3.1. Small scale oxidation. Thioanisole (0.25 mmol) was suspended in aqueous buffer (20 mL, phosphate buffer, 0.1 M, pH 7.5) followed by the addition of methanol (0.5–4 M), alcohol oxidase and peroxidase. At different time intervals, aliquots were extracted with ethyl acetate and the organic phase evaporated. The degree of conversion was determined by GC. Enantiomeric excess was determined by HPLC on a Chiralcel OD-H column (hexane/*i*-propanol 95:5, flow 0.7 mL/min) and by ^{1}H NMR with the addition of (*S*)-(+)-*N*-(3,5-dinitrobenzoyl)-α-methylbenzylamine as the chiral shifting agent.

4.3.2. Gram scale preparation. The initially turbid reaction medium contained 10 mmol of thioanisole (1.24 g), 30 U of AOX as freeze-dried crude cell-free extract (equivalent to 1.5 mL of extract) and 3.5 mL of CiP solution (7 μmol) in 150 mL of phosphate buffer and 12 mL of methanol (2 M). The mixture was vigorously stirred in an open vessel at 25 °C. After 24 h, the same amount of enzymes and 6 mL of methanol were added, and the mixture was stirred for another 24 h. After this additional time, the reaction solution became completely limpid. This aqueous solution was subjected to a continuous extraction with ethyl acetate for 48 h. The solvent was dried and carefully evaporated to give 1.1 g of the sulfoxide (72%; 75% ee).

Acknowledgements

We are grateful to Dr. Schneider (Novozymes) for kindly supplying *C. cinereus* peroxidase and to Dr. Drago (Applied Enzyme Technology Ltd) for supplying *Pichia pastoris* alcohol oxidase. This work was supported by scholarship to F.P. from the Mexican Government CONACyT.

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