Enantioselective sulfoxidation mediated by vanadium-incorporated phytase: a hydrolase acting as a peroxidase

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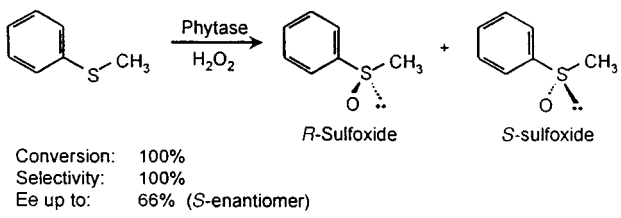
Phytase (E.C. 3.1.3.8), which *in vivo* mediates the hydrolysis of phosphate esters, catalyses the enantioselective oxidation of thioanisole with H_2O_2, both in the presence and absence of vanadate ion, affording the *S*-sulfoxide in up to 66% ee at 100% conversion.

In the last decade peroxidases, notably chloroperoxidase (CPO; E.C. 1.11.1.10) from *Caldariomyces fumago*, have been shown to catalyse a variety of synthetically useful (enantioselective) oxygen transfer reactions with H_2O_2,[1] including enantioselective oxidation of sulfides.[2] However, a major shortcoming of all heme-dependent peroxidases, such as CPO, is their low operational stability,[3] resulting from facile oxidative degradation of the porphyrin ring. In contrast, vanadium haloperoxidases, such as vanadium chloroperoxidase from *Curvularia inaequalis*[4] are non-heme enzymes and, hence, are much more stable. Unfortunately, the active site of vanadium-dependent haloperoxidases can accommodate only very small substrates, such as halide ion, which severely curtails their utility, although Andersson *et al.*[5] recently found that the vanadium-dependent bromoperoxidase from *Corallina officinalis* mediates the enantioselective oxidation of aromatic sulfides. Recently, it was established[6] that vanadium chloroperoxidases are structurally closely related to the acid phosphatases and the apoenzyme was shown to exhibit phosphatase-like activity. Moreover, vanadate and other transition metal oxoanions are known to be potent inhibitors of acid phosphatases[7] and the related phytases[8] and sulfatases.[9] Hence, we reasoned that incorporation of vanadate ion in these enzymes should produce novel, semi-synthetic peroxidases.

To test this hypothesis we investigated the oxidation of thioanisole with H_2O_2 (Scheme 1) catalysed by vanadium-incorporated phytase (from *Aspergillus ficuum*). At low vanadate concentrations (< 15 μM) we observed quantitative conversion to the sulfoxide, the *S*-enantiomer being preferentially formed in 56% ee. At higher vanadate concentrations (> 25 μM), in contrast, further oxidation to the corresponding sulfone also occurred.

In the standard procedure thioanisole (5 mM) and phytase [30 mg dry weight; 12 mg protein (> 95 % phytase); 0.18 μmol; 1400 U] were dissolved in formate buffer (7 ml; 0.1 M; pH 5.1; containing 0 to 30 μM Na_3VO_4). At this pH vanadate is predominantly present[10] as $H_2VO_4^-$. After 10 min H_2O_2 (5.5 mM) was added and the course of the reaction was followed by chiral HPLC (Chiralcel OD column; Daicel Chemical Industries, Ltd.) using 1,2,3-trimethoxybenzene as an internal standard. Of a wide variety of structurally related enzymes tested (details to be reported elsewhere) only phytase exhibited substantial peroxidase activity.



Scheme 1

We studied the influence of the vanadate concentration on the reaction rate and ee of thioanisole oxidation. As shown in Fig. 1 the reaction rate showed saturation kinetics with respect to the vanadate concentration. From these data we calculated a maximum rate of 120 μmol h^{-1} (which represents a turnover frequency of 11 min^{-1}) and a dissociation constant (K_d) for the vanadate ion of 15.4 μM, which is in the same order of magnitude as its reported K_I value for phosphatases and sulfatases.[9] The ee of the formed sulfoxide increased from 47% to a plateau (from 2.5 to 20 μM) of 56% at room temperature. At 4 °C this value was improved to 66% (see Table 1). At a vanadate concentration of 20 μM the rate of the non-enzymatic reaction was 0.5 μmol h^{-1}. Surprisingly, phytase also catalysed the enantioselective sulfoxidation of thioanisole, giving the

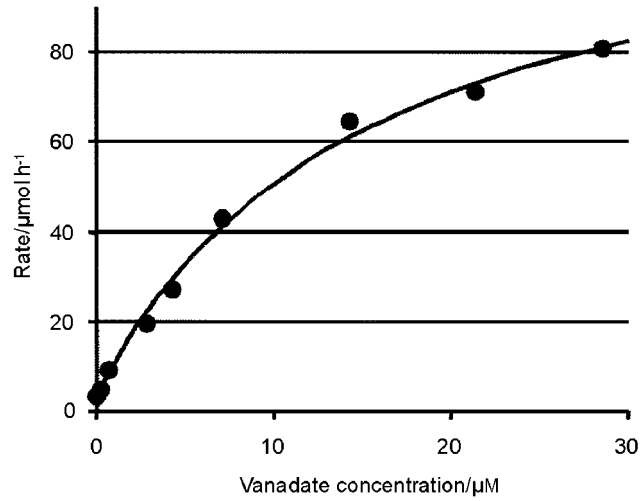


Fig. 1 Influence of the vanadate concentration on the oxidation of thioanisole

Table 1 Sulfoxidation of thioanisole mediated by phytase[a]

Phytase/mg protein	VO_4^{3-}/μM	*T*/°C	*t*/h	Conversion/ mol%	Ee (%)
0	10	25	5	10	0
12	10	25	3	100	56
12	10	4	3	100	66
0	0	25	5	10	0
1.7	0	25	5	15	25
4.2	0	25	5	23	37
12	0	25	5	47	47
16	0	25	5	81	51
12	0	4	23	44	54
12	0	40	5	68	33
0	0	40	5	34	0

[a] For experimental conditions see text.

S-sulfoxide in 47% ee, *even in the complete absence of vanadium*.

We subsequently studied the metal-free phytase-catalysed sulfoxidation in more detail (Table 1). The conversion and ee increased with increasing enzyme concentration, consistent with the reaction being enzyme catalysed. At room temperature the enzyme activity for the two enantiomers was 0.16 μmol h^{-1} (mg protein)$^{-1}$ and 0.04 μmol h^{-1} (mg protein)$^{-1}$ respectively for the *S*- and the *R*-enantiomer. In consequence, the *intrinsic* enantioselectivity of the enzyme is 58% ee (*S*) at room temperature. In the absence of enzyme, a 34% conversion to racemic sulfoxide was observed in 5 h at 40 °C. Decreasing the temperature from 40 to 4 °C reduced the rate of the blank reaction from 9.52 10^{-8} to 3.42 10^{-9} M s^{-1}, resulting in a corresponding increase in enantioselectivity from 33 to 54% ee.

The highest ees were observed in formate buffer, but a carboxylate buffer was not essential. Ammonium chloride and MES‡ were also effective, giving conversions and (ee values) after 5 h of 74% (35%) and 71% (43%), respectively, in experiments performed at 4 mM H_2O_2. This rules out the *in situ* formation of peroxycarboxylic acids (from the carboxylate buffers) as observed with lipases[11] and metal-free bacterial haloperoxidases.[12] The optimum pH was 5.1 which coincides with that of the natural reaction of phytase. The enantioselectivity decreased from 33 to 18% with increasing H_2O_2 concentration from 5.5 to 20 mM, owing to an increased contribution from the blank reaction at higher H_2O_2 concentrations.

A few experiments were performed aimed at providing insights into the origins of the observed metal-free catalysis. Reactions performed under nitrogen showed no difference with the reactions performed under air. When the reaction was performed with $H_2{}^{18}O_2$ (Campro Scientific) analysis of the sulfoxide product by GC–MS showed that the oxygen (100%) is derived from H_2O_2. Addition of the radical scavenger Trolox™-C,§ a water soluble analog of vitamin E, had no influence on the conversion or enantioselectivity, consistent with hydroxyl radicals not being involved in the enantioselective oxidation.

This leaves us with the question of the origin of the observed oxygen transfer catalysis. It is not metal-based as there is no metal ion in the active site. Although the chelating agent EDTA completely inhibited the enantioselective oxidation this is probably due to removal of calcium ions required for the stability of the enzyme. The crystal structure of phytase from *Aspergillus ficuum* was recently resolved to 2.5 Å, showing that an aspartate residue (Asp339) is located in the active site.[13] Similarly, acid phosphatases are also known to contain aspartate in the active site.[7] Hence, it is tempting to speculate that this aspartate plays a key role in the observed catalysis. Reaction of the free carboxylate group with H_2O_2 would give the corresponding peroxycarboxylic acid which could be the active oxidant. When the reaction was performed with *tert*-butyl hydroperoxide (TBHP) no catalysis was observed consistent with the above proposed formation of peroxycarboxylic acids. Appropriate site-directed mutagenesis studies should be able to confirm the key role of Asp339 in the catalytic mechanism.

In conclusion, we have demonstrated the feasibility of rationally designing a semi-synthetic peroxidase *via* incorporation of vanadium into the active site of phytase by exploiting the structural similarity of vanadate to phosphate. Surprisingly, the metal-free phytase also catalyses enantioselective oxidation and further studies are underway aimed at clarifying the mechanism and exploring the scope of this novel oxygen transfer catalysed by a hydrolase.

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Notes and References

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‡ 2-(*N*-Morpholino)ethanesulfonic acid
§ 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

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