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Vanadate substituted phytase: Immobilization, structural characterization and performance for sulfoxidations

Isabel Correia [a,*], Seda Aksu [b], Pedro Adão [a], João Costa Pessoa [a], Roger A. Sheldon [b], Isabel W.C.E. Arends [b,*]

[a] Centro Química Estrutural, Instituto Superior Técnico, TU Lisbon, Av. Rovísco Pais, 1049-001 Lisboa, Portugal
[b] Biocatalysis and Organic Chemistry, Department of Biotechnology, Delft University of Technology, Julianalaan 136, 2628 BL Delft, The Netherlands

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Abstract

A cross-linked enzyme aggregate (CLEA) of 3-phytase (EC 3.1.3.8) was synthesised, which was incubated with vanadate and tested as a biocatalyst in the asymmetric sulfoxidation of thioanisole using hydrogen peroxide as the oxidant. The results show that the 3-phytase-CLEA demonstrates a similar efficiency (*ca.* 95% conversion) and asymmetric induction (*ca.* 60%) as the free enzyme. Moreover, the 3-phytase-CLEA can be reused at least three times without significant loss of activity. The activity of the 3-phytase in the presence of organic solvents is however still limited. Studies were undertaken to elucidate the role of vanadate on the active site and on the effect of organic solvents on the conformation of the enzyme. The incorporation of vanadate in the active sites of two different phytases could be followed using ^{51}V NMR and circular dichroism (CD) spectroscopies. ^{51}V NMR spectra show the incorporation of vanadate into the active site at pH 5.0 and 7.6, and suggest coordination to oxygen functions at two different binding sites, which probably explains the poor enantioselectivity found in the catalytic studies. After addition of H_2O_2, only at pH 5.0 and with the 3-phytase a V-phytase-peroxide complex could be observed, which is the active species responsible for the oxidation reactions. CD studies showed that the α-helical content of the enzyme decreased upon coordination of vanadate, but in the concentration range used in the catalytic studies (<30 μM) the secondary conformation of the enzyme was unchanged. Acetonitrile decreases the α-helical content of both phytases from 59% to 51% and from 42% to 34%, in the 3- and 6-phytases, respectively, this being in agreement with the activity loss in the catalytic experiments.

Keywords: Phytase; CLEA; Asymmetric sulfoxidation; Circular dichroism; ^{51}V NMR

1. Introduction

Catalytic oxidative transformations for the direct synthesis of optically active intermediates could be a valuable instrument in fine chemicals industry. Ideally enzymes can be used as catalysts: with their high regio- or enantioselectivity, high chemical yields can be obtained, the use of additional reagents circumvented and waste production reduced [1]. However, their inactivation and inability to perform in organic solvents are sometimes major drawbacks [1–5]. In order to facilitate the use of enzymes, simple and efficient methods for enzyme immobilization need to be developed, which increase the operational stability and the possibility of catalyst recycling. A major breakthrough in this field was the development of cross-linked enzyme aggregates (CLEA®) [6]. For some enzymes, this immobilization process increases their tolerance to the presence of organic solvents; in others it simply allows its immobilization.

A class of enzymes which could be stabilized as CLEAs, as demonstrated in this paper, are the phytases [7]: enzymes that belong to the family of histidine acid phosphatases [8].

* Corresponding authors. Tel.: +351 218419302; fax: +351 218464455 (I. Correia), Tel.: +31 15 2784423; fax: +31 15 278 1415 (I.W.C.E. Arends).
E-mail addresses: icorreia@ist.utl.pt (I. Correia), I.W.C.E.Arends@tudelft.nl (I.W.C.E. Arends).

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They are primarily found in microorganisms and plants, catalyzing the hydrolysis of phosphate from phytic acid, which is the major phosphorus storage form in plants [9]. Phytases are used in animal feed to increase its nutritional value, by liberation of inorganic phosphate from phytate [9–11]. They also provide a way of reducing the level of phosphate pollution. Because poultry and pig feed are commonly pelleted, the commercially available phytases are able to withstand quite high temperatures [9,10]. Amongst the best-known and original commercial phytases added to animal feed is Natuphos® (DSM, the Netherlands), a recombinant phytase produced by *Aspergillus niger*. Novozymes has also launched in the market a new phytase, Ronozyme® produced by an *Aspergillus Oryzae* strain transformed with the phytase gene of the basidiomycete strain *Peniophora lycii* [10]. They belong to two different classes: Natuphos is a 3-phytase (E.C. 3.1.3.8), which initially removes orthophosphate from the 3-position of phytic acid, and Ronozyme is a 6-phytase (E.C. 3.1.8.26), which acts on the 6-position of phytate [9].

We became interested in phytases because of their potential to act as peroxidases, as demonstrated for vanadate substituted phytase, which could act as an oxygen transfer catalyst in sulfide oxidations [18,19]. When considering enzymes that can be applied in catalytic oxidative transformations, vanadium haloperoxidases show considerable potential. Vanadium haloperoxidases show structural homology with the histidine acid phosphatases (*i.e.* phytases) and are able to carry out phosphatase reactions by replacement of the vanadate with phosphate [12]. Vanadium is a known phosphatase inhibitor and since phosphate and vanadate have many similar physico-chemical properties, vanadate is able to behave as a transition state analogue, forming a trigonal bipyramidal complex with the nucleophilic histidine present in the active site of acid phosphatases [13,14]. In 2002 it was shown that an acid phosphatase incubated with vanadate was able to perform as a peroxidase [15]. Although much research effort has been devoted towards the use of (heme)-peroxidases in industrial oxidative processes, their application is hampered because of inactivation by excess hydrogen peroxide and limited substrate scope [16]. Phytases are histidine acid phosphatases that have a broad substrate scope and perform well under oxidative conditions [17]. Therefore, the application of vanadium acid phosphatase as a peroxidase seemed a suitable way to overcome these problems. A semi-synthetic vanadium peroxidase was prepared by simply incubating a phytase with vanadium(V). It showed the ability to oxidise enantioselectively prochiral sulfides to sulfoxides, in high yields and selectivity [18,19]. The synthetic peroxidase was also screened for alcohol oxidations but it showed no activity under the conditions used.

In this paper we report the ability of a vanadium-phytase-CLEA to perform as a heterogeneous and recyclable catalyst in the sulfoxidation of a model substrate, methylphenyl sulfide. The catalyst shows high conversion, reasonable selectivity, and good recyclability under aqueous conditions. However in the presence of more than 10% organic solvent, the activity decreases. Therefore, studies were undertaken to elucidate the role of vanadate on the active site and on the effect of organic solvents in the conformation of the enzyme. ^{51}V NMR was used to study possible vanadium-peroxo intermediates for both 3- and 6-phytase. Circular dichroism was used for the determination of the enzyme conformation in different solvents and at different temperatures.

2. Experimental

2.1. Materials

4-Nitrophenyl phosphate (disodium salt) was purchased from Merck and (−)-ethyl-L-lactate from Fluka. All other chemicals used were at least of analytical grade. Phytases of *A. niger* (3-phytase) were generously donated by DSM and BASF and the 6-phytase of *P. lycii* was obtained from Novozymes. They were used as received (as enzyme solutions).

2.1.1. Vanadate stock solution

An "activated" solution of monomeric orthovanadate was prepared using a method similar to that described by Gordon [20]. In short, this method involves adjusting the pH of a stock solution (100 mM) of sodium orthovanadate above 10 and heating near 100 °C until the solution is clear and colorless. With this processes it is ensured that most of the vanadate is in the monomeric form, which is prevalent in alkaline solutions.

2.2. CLEA preparation

As a general procedure the 3-phytase (1 mL of enzyme solution) was dissolved in 4 mL of TRIS buffer (200 mM, pH 8.0) and then the pH adjusted to 7.0 by addition of NaOH 1 M. This solution was added dropwise to a saturated $(NH_4)_2SO_4$ solution (20 mL) or to (−)-ethyl-L-lactate (20 mL) to precipitate the enzyme. One milliliter of glutaraldehyde (25% w/v in water) was added to the mixture, which was stirred at room temperature for 3 h to allow the cross-linking. After centrifugation (at 8000*g*), the supernatant was decanted and the residue was washed three times with acetate buffer (100 mM, pH 5.0, 5 mL each time), centrifuged (at 8000*g*) and decanted. The final enzyme preparation was kept in 10 mL of the acetate buffer. If necessary, the solid was dispersed by magnetic stirring.

2.3. Enzyme activity measurements

2.3.1. Native enzyme and CLEA-phytase

Although there is a phytase activity protocol, the enzymatic activity of the phytases was measured based on an adaptation of a Sigma protocol for pH 2.5 acid phosphatases [21], since it is more simple and suitable for the determination of the catalyst activity. The activity was measured

in a mixture containing 0.5 mL of 4-nitrophenyl phosphate (15.2 mM), 0.5 mL of acetate buffer (100 mM, pH 4.5) and 0.1 mL of the enzyme (conveniently diluted). After 10 min of incubation at 37 °C the reaction was stopped by addition of 4 mL of NaOH 0.1 M. The formation of *p*-nitrophenol was monitored by UV–visible spectroscopy at 410 nm. A blank, which contained everything except the enzyme, was prepared and incubated for 10 min; after the addition of NaOH, 0.1 mL of enzyme were added and its absorbance measured at 410 nm. The activity was calculated using the expression: U/mL enzyme $= [(A_{410} - A_{410\text{blank}}) \times V_t \times df]/[t \times \varepsilon \times V_{enz}]$, where V_t and V_{enz} are the total and the enzyme volumes, d*f* is the dilution factor, *t* is the incubation time and ε the extinction coefficient of *p*-nitrophenol at 410 nm (18.3 $mM^{-1}\,cm^{-1}$). One unit will hydrolyze 1.0 μmol of *p*-nitrophenyl phosphate per minute at pH 4.5 at 37 °C.

2.4. Determination of protein content

The Bradford method [22] was used to determine the protein content of the phytases. Bovine serum albumin (BSA) was chosen as the standard protein. A calibration curve was measured by visible absorption at 595 nm. The protein content of the enzyme preparations of 3-phytase from *A. niger* was 120 mg/mL and for 6-phytase from *P. lycii* 10 mg/mL.

2.5. Enzymatic oxidation of thioanisole

As a general procedure, the phytase-CLEA was incubated at 37 °C with vanadium (15 μM) in acetate buffer (pH 5.0, 0.1 M), for 1 h. Then the substrate, thioanisole (5 mM dissolved in DME (10%)) was added. The reaction was initiated by the addition of H_2O_2 (5.5 mM), and usually the oxidant was added at 5 μL/min with a Metrohm Dosimat®. The typical total reaction time was 2 h. The internal standard (1,3,5-trimethoxybenzene) was added at the beginning of the reaction. The reaction was stopped by addition of excess anhydrous Na_2SO_4. The organic compounds were extracted with ethyl acetate. The solutions were centrifuged; the organic phases dried over $MgSO_4$ and samples for chiral HPLC were taken. Control samples were measured in each sample set containing everything except the catalyst (enzyme + vanadate).

2.6. Analysis

A calibration curve was made with standards containing different molar ratios of 1,3,5-trimethoxybenzene (the internal standard – IS) and thioanisole, prepared in the HPLC eluent and analyzed on chiral HPLC using a Chiralcel OD column (Daicel Chemical Industries, Ltd., 250 × 4.6 mm). The eluent used was *n*-hexane:isopropanol – 75:25. Reaction samples were diluted with a *n*-hexane: isopropanol mixture of 75:25 (v/v) and dried over Na_2SO_4. After centrifugation, the samples were analyzed on the same chiral HPLC system with an eluent flow rate of 0.5 mL min^{-1}, and detected on a Waters 486 tunable absorbance detector at 215 nm.

2.7. UV–visible (UV–vis) spectroscopy

The enzymatic activity and the determination of the protein content were monitored on a Varian Cary 3 Bio, equipped with a Cary temperature controller.

2.8. CD spectroscopy

The CD spectra were measured using a cylindrical 1 mm Hellma Quartz Suprasil cuvette, on a Jasco J-720 spectropolarimeter in the wavelength range 185–260 nm (whenever possible) at room temperature (except for the temperature dependent studies). The measuring conditions were: band width, 0.5 nm; response, 4 s; data pitch, 0.2 nm; scanning speed, 50 nm/min; number of accumulations, 5. The baseline obtained with the buffer was subtracted from the sample spectra. Molar ellipticities (in deg cm^2 $dmol^{-1}$) were plotted against wavelength. The samples were prepared in acetate buffer (10 mM, pH 4.5). The protein concentrations in the samples were 0.12 and 0.10 mg/mL, for the 3- and 6-phytase, respectively. Samples were incubated with different vanadate concentrations for 30 min at 25 °C before their spectra were measured. For the temperature dependence studies the samples were incubated for 30 min at 40, 60 or 80 °C. Subsequently, either the CD spectra were measured directly at the same temperature using a thermostatized sample compartment, or the samples were first allowed to renature for 30 min at 25 °C and then the CD spectra were measured at 25 °C. Temperature was controlled with a Haake DC10 thermostat.

The analysis of the circular dichroism spectra for the determination of protein secondary structure was carried out using the CDPro suite of programs [23]. The calculations were made using the three programs included in this software: SELCON3, CONTINLL and CDSSTR. Different bases for the reference set of proteins were used and the best results (lower rmsd values) were obtained using the set based on 43 soluble proteins (with λ between 190 and 240 nm).

2.9. ^{51}V NMR spectroscopy

^{51}V NMR spectra of the phytase-vanadate solutions were recorded on a Varian Unity INOVA 300 spectrometer at probe temperature, equipped with a 5-mm broadband "switchable" probe with *z*-gradient operating at 105 MHz and at 25.0 ± 0.5 °C. A single-pulse sequence with standard acquisition parameters for ^{51}V was used. The spectral parameters were as follows: sweep width 100 kHz, pulse width 3 μs, acquisition time 0.655 s, recycle delay 1 s and line broadening 50 Hz. A $VOCl_3$ solution was used as external reference. The signals were integrated using the NUTS PC-based NMR spectral analysis program (Acorn

NMR Inc., Fremont CA, USA). In the catalytic tests, the concentration of vanadate was *ca.* 15 μM, which is not detectable in the ^{51}V NMR spectra. A higher concentration was used for this purpose: 5 mM. The vanadate-phytase systems were studied at two pH values: 5.0 (acetate buffer, 0.1 M) and 7.6 (HEPES buffer, 0.1 M, to avoid complexation of the buffer components with vanadate) [24]. Before spectrum measurement the enzyme and the vanadate were incubated during 12 h, in order to achieve equilibrium. In the assays with H_2O_2, the oxidant was added immediately before the measurement of the spectrum, to avoid decomposition.

3. Results and discussion

3.1. Preparation of CLEA

The preparation of the CLEA is a simple process that involves two steps; the 1st is the aggregation and precipitation of the enzyme induced by the addition of salts, organic solvents, or non-ionic polymers to an aqueous solution of the enzyme [7]. These super aggregates are held together by non-covalent bonding and can be redissolved when dispersed in water. Cross-linking with glutaraldehyde produces the CLEA in which the structure of the aggregates and, therefore, the activity is retained. The 3-phytase-CLEA could be prepared with two different precipitants: ammonium sulfate and ethyl lactate. The effect of the concentration of cross-linker was screened (10, 20, 50, 80 and 120 mM), and it turned out that concentrations of glutaraldehyde >50 mM were needed (data not shown) when using $(NH_4)_2SO_4$ as the precipitant, to prevent dissolution during the washing procedures. In the latter case, excellent activity was achieved and this preparation was used in the oxidation studies described in this paper (Table 1). When using ethyl lactate as precipitant, a CLEA with better aggregation properties, but lower catalytic activity was obtained.

Table 1
Conditions and results obtained in the oxidation of thioanisole with the 3-phytase from BASF

Entry	T (°C)	t (h)	V^{5+} (μM)	% DME	Conversion (%)	ee (%)
Free enzyme[a]						
1	25	2	15	10	96	37
2	40	2	15	10	99	28
3	60	2	15	10	89	7
4	80	2	15	10	92	3
				% co-solvent		
CLEA[a]						
5	25	3	20	0	35	46
6	25	3	20	10	99	41

[a] Acetate buffer (0.1 M pH 5.0), 3-phytase: 50 U, thioanisole: 5 mM, H_2O_2: 5.5 mM.

Table 2
Conditions and results obtained in the oxidation of thioanisole with V^V-3-phytase-CLEA[a]

Entry	CLEA (mL)[a]	V^{5+} (μM)	t (h)	% DME	Conversion (%)	ee (%)
1	1	15	2	10	98	56
2	1	15	2	30	86	42
3	1	15	2	40	25	28
				pH		
4	1	15	4.5	3.6	96	60
5	1	15	4.5	4.2	93	62
6	1	15	4.5	5	92	57
7	1	15	4.5	5.6	98	57
				30% solvent		
8	1	15	3.5	MeOH	58	19
9	1	15	3.5	EtOH	55	22
10	1	15	3.5	MeCN	3	1
11	1	15	3.5	DME	86	42
		V^{5+} (μM)				
12	1	10.5	3.5		92	57
13	1	21	3.5		93	48
14	1	42	3.5		96	50
15	1	84	3.5		89	45
16	1	15	3		85	40
17	0.5	15	3		69	40
18	1	15	3	[b]	70	40
19	0.5	15	3	[b]	52	42

[a] Standard conditions: acetate buffer (0.1 M pH 5.0) with 10% DME, 5.5 mM H_2O_2 and 5 mM thioanisole, $T = 25$ °C; 3-phytase-CLEA: 90 U.
[b] After incubating the CLEA 1 h at 37 °C with vanadate the supernatant was removed and replaced by buffer.

3.2. Catalytic studies

In earlier publications several phytases have been tested as catalysts in the enantioselective oxidation of thioanisole with H_2O_2 as oxidant, after incubation with vanadate [18,19]. Therefore we only performed few catalytic sulfoxidation studies with the free enzymes for comparison. The 6-phytase showed no activity even after three days. For the 3-phytase from BASF the best results were obtained

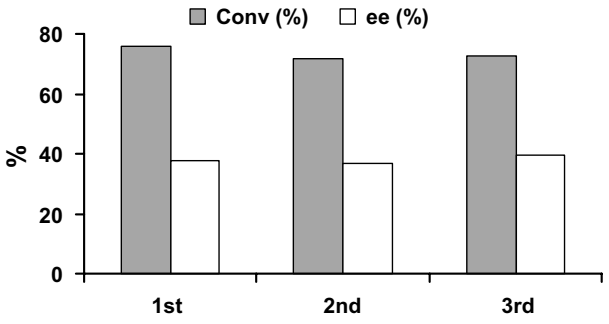

Fig. 1. Variation of the conversion and enantiomeric excess (ee) before (1st) and after (2nd/3rd) the recycling of the V-3-phytase-CLEA: $T = 25$ °C, $t = 2$ h, $[V^V] = 10$ μM, 3-phytase = 90 U.

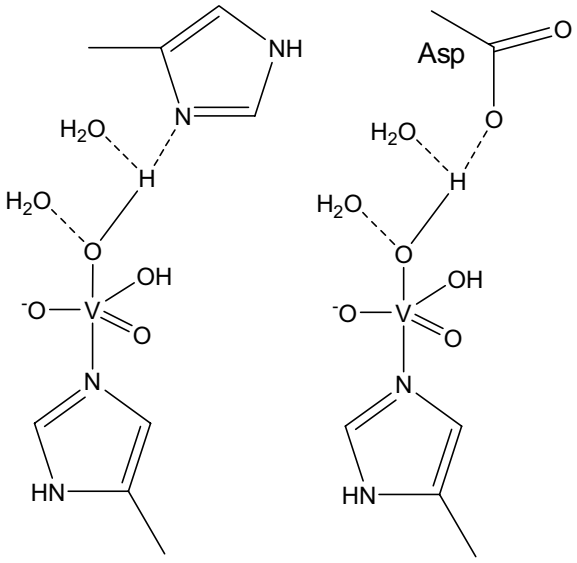

Fig. 2. Active site of the vanadium haloperoxidases [26] and vanadate inhibited acid phosphatases [12].

using 10% DME co-solvent to solubilise the substrate, resulting in high conversion and 37% ee (see Table 1). An increase in temperature had a negative effect on the enantioselectivity. At 80 °C, nearly all enantioselectivity had disappeared (ee of only 3%). The CLEA prepared from this enzyme showed similar efficiency and selectivity to the free enzyme (see entries 1 and 6, Table 1). The highest enantioselectivity was obtained in the absence of co-solvent.

The method used in the CLEA catalyst screening was similar to the one applied to the free enzyme. Generally, the CLEA was incubated for 1 h at 37 °C with the vanadate solution, and then mixed with the substrate and internal standard (IS) solution. Taking into account the results from the free enzyme [18,19], most of the oxidations were carried out in acetate buffer 0.1 M at pH 5.0. Since the substrate and IS are not water-soluble a small amount of an organic solvent was added (usually 10%). Therefore, strong stirring was important to mix both phases. When the reaction was carried out only in buffer (see Table 1, entry 5) although the enantioselectivity was higher, the conversion was quite low. The method of addition of the oxidant was varied, and it turned out that the best results were obtained using a Metrohm Dosimat® to slowly add the H_2O_2 solution (5 μL/min).

Table 2 presents the results obtained with the CLEA prepared from the 3-phytase from DSM. The CLEA is quite active and not very sensitive to most of the experimental conditions such as pH and V^{5+} concentration. In the vanadate concentration range studied, although the conversion does not seem to be affected by the increase of vanadate concentration, the selectivity decreases slightly when the vanadate concentration is increased; 15 μM seems a good compromise between high conversion and selectivity.

Organic solvents substantially deactivate the immobilized enzyme (see entries 8–11, Table 2), and MeCN has the strongest effect, inactivating almost completely the CLEA. But with 10% of dimethoxyethane (DME) the enzyme is still very active and the substrate is soluble in the reaction media. The formation of over oxidation products, like sulphone, is negligible (<1%). The maximum enantiomeric excess obtained was 62% (for the S–sulfoxide, entry 5). This is probably due to the inherent selectivity of the enzyme, but we have also tested if free vanadate is catalyzing the non-enzymatic reaction: some assays were conducted in which the CLEA was incubated with vanadate for 1 h at 37 °C, after which the supernatant was removed and replaced by an equivalent amount of buffer (entries 18–19). Comparison of entries 16 and 18 (or 17 and 19) show that there is no influence of the free vanadium since the ee is 40% in both essays. Therefore, we conclude that only

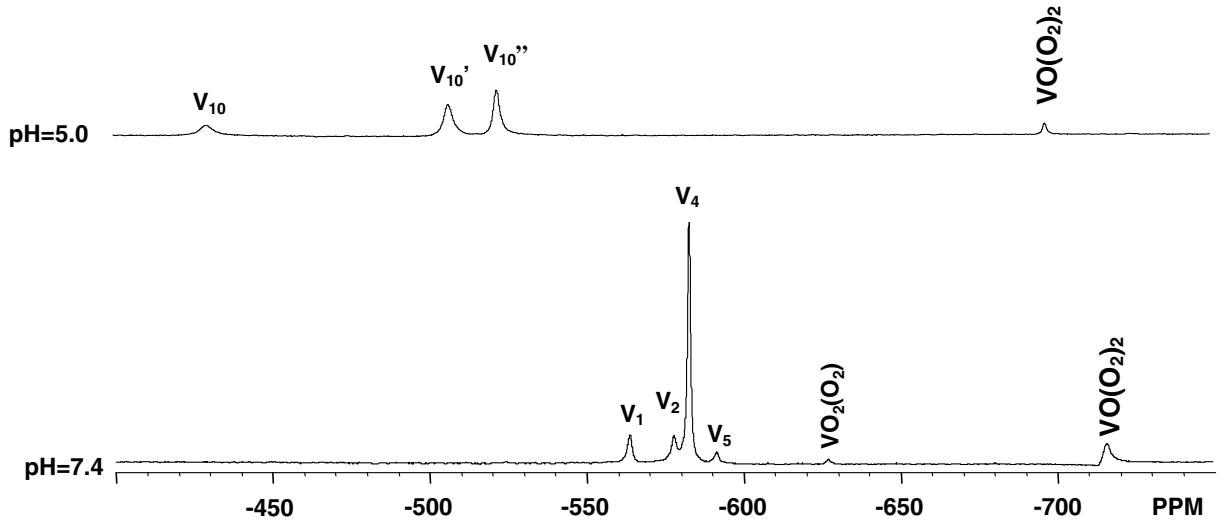

Fig. 3. ^{51}V NMR spectra and assignment of the peaks for solutions containing 5 mM of vanadate and 10 mM of H_2O_2 at two pH values indicated in the figure.

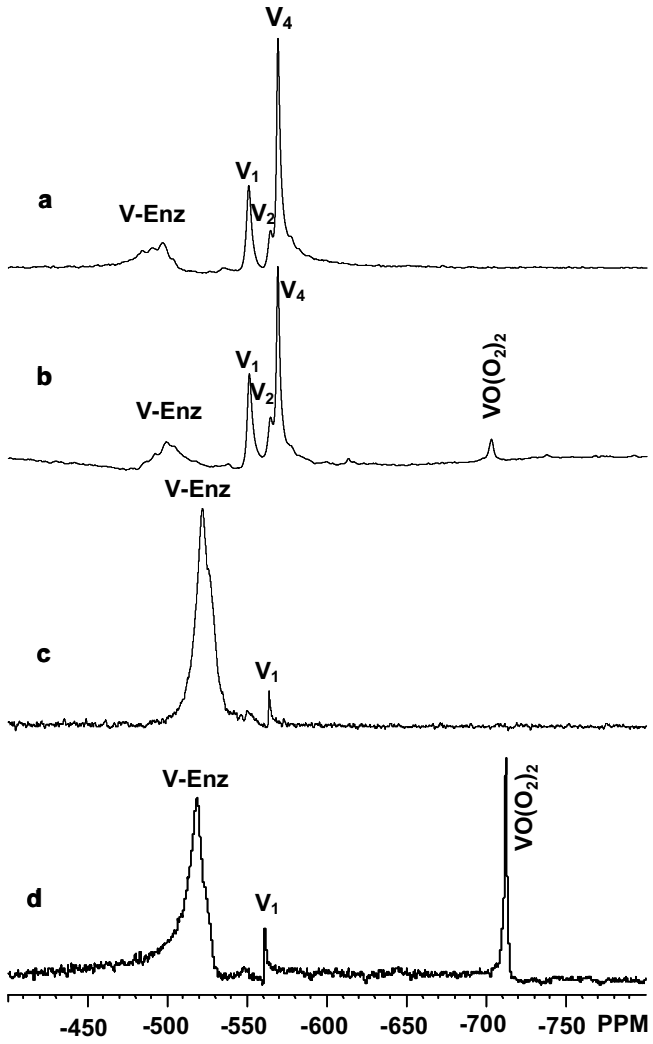

Fig. 4. ^{51}V NMR spectra and assignment of the peaks for solutions containing 5 mM of vanadate and the phytases (2 mM) at pH 7.6: (a) 3-phytase; (b) 3-phytase with 5.5 mM H_2O_2; (c) 6-phytase and (d) 6-phytase with 5.5 mM H_2O_2.

enzyme-bound vanadate activates the peroxide in our reaction.

One of the advantages of the CLEA is the possibility of recycling after use. To test this parameter, after the 1st catalytic cycle the CLEA was centrifuged and washed twice with distilled water. Acetate buffer and vanadate were then added to the CLEA, and left overnight at 37 °C and reused. This procedure was done twice. Fig. 1 shows that the CLEA does not lose activity or selectivity even after being used three times.

3.3. Structural characterization

The structural characterization of the catalytic system is an important tool to gain insight into the catalytic mechanism. It is well known that vanadate can act as analogue for the conformation of the phosphate group at the transition state expected for phosphor-transfer [25]. Researchers have used vanadate as a probe to study the catalytic mechanism and substrate binding modes of many phosphor-transfer enzymes [13,14]. In vanadium haloperoxidases and vanadium inhibited acid phosphatases, vanadate is covalently attached to the protein by an apical histidine and surrounded by a strong hydrogen-bonded network to water molecules and amino-acid side chains. Since there are close homologies between the active centres of the vanadium haloperoxidases and the vanadate-inhibited phosphatases [12,26] (see Fig. 2), we have used vanadate as a spectroscopic probe to gain insight into the active site structure. ^{51}V NMR studies were carried out with vanadate incubated 3-phytase from *A. niger* (DSM), and also with the 6-phytase from *P. lycii*, at two pH values: 5.0 and 7.6.

3.4. ^{51}V NMR

^{51}V NMR spectroscopy is a technique to study the diamagnetic vanadium(V) due to its favourable nuclear properties: nuclear spin = 7/2, small quadrupole moment (-0.05×10^{-28} m^2) and 99.76% natural abundance [24]. It has been widely used in the study of systems with biological relevance, such as vanadium enzymes, where the resonance lines are broad, but still within the narrowing limit, *i.e.*, only the central transition is observed [27–30]. In this work ^{51}V NMR spectroscopy was used to study the species present, namely the possible formation of vanadium-peroxo intermediates for both 3- and 6-phytase. The 3- and 6-phytases were incubated with vanadate (protein concentrations of 2 mM), with and without H_2O_2, and we also compare our spectral results with those obtained previously with an acid phosphatase incubated with vanadate [24].

In biological terms the pH value of interest is 7.6, but since the catalytic tests were done at pH 5.0 (the optimum pH for the 3-phytase), we prepared samples at both pH values. Aqueous vanadate solutions at pH 7.6 and 5.0 show typical resonance patterns, which depend, among other factors, on the vanadate concentration and ionic strength [31]. In most of the pH range vanadate forms different oligomers. At pH 7.6 the main species are mono (V_1), di (V_2) tetra (V_4) and pentavanadate (V_5). Mono and divanadate can be found in different protonation states and therefore its chemical shift changes with pH. Tetra and pentavanadate are cyclic oligomers and usually appear at −582 and −591 ppm, respectively. At pH 5.0 decavanadates are the main oligomeric species present in solution ($H_nV_{10}O_{28}^{6-n}$). There are three types of vanadium atoms experiencing different environments in the decavanadate, resulting in three resonances (usually designated by V_{10}, V'_{10} and V''_{10}). Due to protonation processes their chemical shifts also change with pH. When hydrogen peroxide is added to a vanadate solution, peroxovanadate species form, and new resonances appear in the ^{51}V NMR spectra [32]. Fig. 3 shows the spectra and assignment of the peaks for mixtures containing vanadate and H_2O_2 (1:1 mixtures) at the two pH values. At pH 7.6 the main diperoxovanadate present is the monoprotonated form; at pH 5, almost exclusively

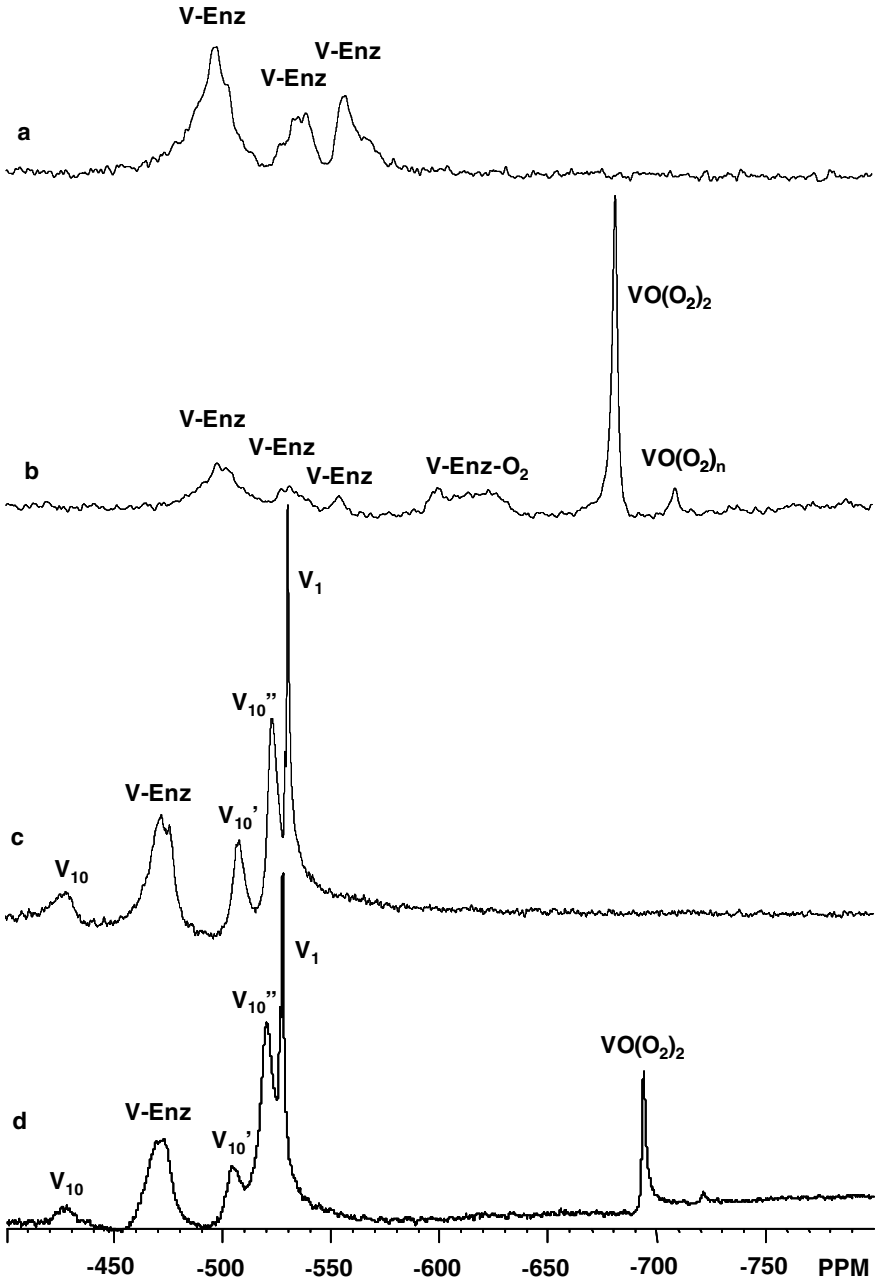

Fig. 5. ^{51}V NMR spectra and assignment of the peaks for solutions containing 5 mM of vanadate and the phytases (2 mM) at pH 5.0: (a) 3-phytase; (b) 3-phytase with 5.5 mM H_2O_2; (c) 6-phytase and (d) 6-phytase with 5.5 mM H_2O_2.

the diprotonated form is present. The peroxovanadate signals (chemical shift changes with pH due to protonation processes) decay within <24 h, since peroxovanadates are unstable, and vanadate catalyzes the decomposition of H_2O_2 [34]. Table 3 contains data obtained in this work and, for comparison, data from the literature [24,40].

When vanadate is incubated with the enzymes, the signals broaden (*ca.* twofold), basically due to the higher viscosity of the samples and to the interaction of vanadate with the enzyme, which slows down the tumbling motion and thus increases the relaxation rates, and new peaks appear. Figs. 4 and 5 show the spectra at pH 7.6 and 5.0, respectively. At pH 7.6 the 6-phytase forms a complex with vanadate, which results in a peak of high intensity at −522 ppm (the V_1 present in solution is <2%), with a shoulder at *ca.* −525 ppm. These chemical shift values are typical of $V^VO_2^+$ complexes surrounded by O-functions [27], and probably correspond to two different binding sites in the enzyme. A trigonal bipyramidal coordination geometry is expected for the vanadium in the active site of the enzyme (see Fig. 2) and this binding mode should give a peak below −470 ppm [24]. Therefore we conclude that there is a strong distortion towards a square pyramid in the active site. When hydrogen peroxide is added to this solution a new

Table 3
^{51}V NMR data (ppm) and assignment of the vanadate-enzyme systems studied in this work and data from the literature

System	V-enzyme	V-enz-O_2	V_1	V_2	V_4	V_5	V_{10}	V'_{10}	V''_{10}	$VO_2(O_2)$	$VO(O_2)_2$	Reference
Na_3VO_4 5 mM + H_2O_2 5 mM, pH 7.6			−564	−578	−582	−591				−627	−715	Fig. 3
Na_3VO_4 5 mM + H_2O_2 5 mM, pH 5.0							−430	−507	−522		−697	Fig. 3
Na_3VO_4 + 6-phy, pH 7.6	−522, −525sh		−563									Fig. 4
Na_3VO_4 + 6-phy + H_2O_2 10 mM, pH 7.6	−520, −523sh		−561								−712	Fig. 4
Na_3VO_4 + 6-phy, pH 5.0	−472, −475		−530				−429	−508	−523			Fig. 5
Na_3VO_4 + 6-phy + H_2O_2 10 mM, pH 5.0	−467, −472		−528				−423	−504	−521		−694	Fig. 5
Na_3VO_4 + 3-phy, pH 7.6	−498		−550	−564	−568							Fig. 4
Na_3VO_4 + 3-phy + H_2O_2 5 mM, pH 7.6	−498		−550	−564	−568						−702	Fig. 4
Na_3VO_4 + 3-phy, pH 5.0	−498, −539, −557											Fig. 5
Na_3VO_4 + 3-phy + H_2O_2 5 mM, pH 5.0	−497, −530, −553	−599, −623									−681 (−709)	Fig. 5
KVO_3 + Pp, pH 7.4	(−487), (−515), −539sh, −542		−557	−569	−574							[24]
KVO_3 + Pp+ H_2O_2 5 mM, pH 7.4	(−505), (−515), −542		−557	−569	−574							[24]
Vanadate + Ala-His + H_2O_2 pH 7.4		−627 to −683								−624	−725	[40]

The Na_3VO_4 concentration is 5 mM in all measurements.
Pp = bovine prostatic acid phosphatase, parenthesis = minor signals, sh = shoulder, Ala-His = alanyl-histidine.

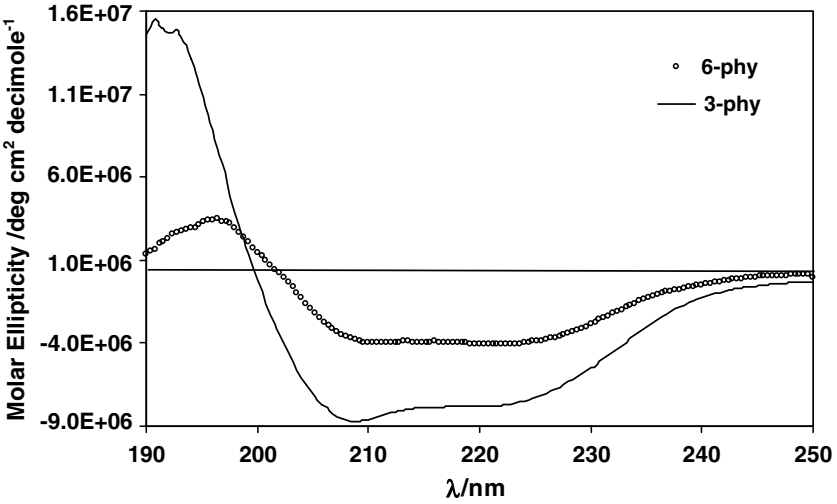

Fig. 6. Far-UV CD spectra of the 3- and 6-phytases in acetate buffer (10 mM, pH 4.5).

peak is observed at −713 ppm, which corresponds to the $VO(O_2)_2$ species, since it appears at the same chemical shift as free diperoxovanadate. This peak disappears within hours. Therefore, there is no evidence of the formation of the V-enzyme-peroxide complex at this pH. Addition of vanadate to the 3-phytase yields a new broad signal at −498 ppm, besides the signals assigned to free V_1, V_2 and V_4. Again similar conclusions can be taken, regarding the active site geometry. In the presence of H_2O_2 a new peak appears at −702 ppm, which disappears with time and is assigned to free $VO(O_2)_2$. From these results we can conclude that at the physiological pH no detectable amount of V-phytase-peroxide complex is formed for both phytases.

At pH 5.0, the situation changes considerably. With the concentration necessary to measure the ^{51}V NMR spectra decavanadate species form in the case of V-6-phytase (which decompose slowly). In the concentration range used in the catalytic experiments (μM) we do not expect the decameric species to be very important [31], although there are reports of the presence of V_{10} species at concentrations as low as 10 μM, as observed by UV–vis spectroscopy [32]. In Fig. 5c, it can be seen that under the same experimental conditions (concentration and V-enzyme ratio), the percentage of the V-6-phytase complex at pH 5.0 is much lower than at pH 7.6 (49% *vs.* 95%). Again there are two different resonances assigned to the V-enzyme complex (not well resolved), and the peaks appear at lower field, deshielded. Upon addition of H_2O_2 in the diperoxovanadate region only one new peak appears at −694 ppm, which is again assigned to free diperoxovanadate.

For the 3-phytase the situation is different: at pH 5.0 no free vanadate oligomers are observed; three peaks are present and all of them are assigned to vanadate-3-phytase complexes; the most intense peak being at −498 ppm. The addition of one equivalent of H_2O_2 results in two new broad resonances at *ca.* −599 and −623 ppm, which must correspond to V-enzyme-peroxide complexes, since these signals disappear with time (data not shown) and are shielded [33,34]. The $VO(O_2)_2$ species is also present (at −681 ppm), and a small peak at −709 ppm is assigned to other oligomeric peroxovanadate species.

Therefore, only at pH 5 and for the 3-phytase the V-phytase-peroxide peaks are observed. Two main conclusions can be taken concerning the catalytic experiments: (1) the 6-phytase is not able to perform as a peroxidase since, although vanadate is coordinated to the enzyme in the active site, it is not able to form a complex with the peroxide; (2) there are non-specific binding sites for vanadate, which might be responsible for the low enantioselectivity obtained in the sulfoxidation reactions with the 3-phytase. Furthermore, the chemical shifts are in agreement with those observed for other structurally related enzymes, and suggest coordination to predominantly oxygen functions, such as aspartic acid and water molecules [24]. Although a competitive inhibitor effect of vanadate, or of vanadate oligomers, could eventually explain the effects observed we did not find any evidence with the experiments done.

3.5. CD spectroscopy

Circular dichroism is a spectroscopic tool that may be used to study the conformation of biomolecules such as proteins [23,35]. Different secondary structures give characteristic CD spectra, and it is considered that the spectrum is a sum of the spectra of the individual secondary structures present in the protein. The far-UV CD spectra obtained for the two phytases in acetate buffer at pH 4.5 are shown in Fig. 6. Both spectra exhibit two maxima of negative ellipticity at *ca.* 208 and 222 nm, characteristic of α-helical proteins. We did the analysis of the spectra using the CDPro set of programs [36], which allowed us to estimate the amount of the different types of secondary structures present in both enzymes (see Table 4, entry $t = 25$ °C).

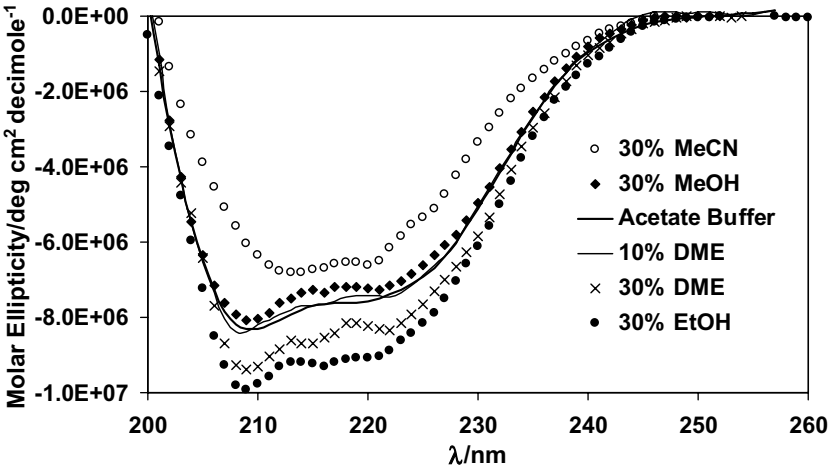

Fig. 7. Far-UV CD spectra of the 3-phytase in acetate buffer (10 mM, pH 4.5) containing 10% or 30% of an organic solvent, as indicated in the figure.

The values presented are the best fits between experimental and calculated spectra. As expected, α-helix is the most abundant type of secondary structure present: values of *ca.* 60% and 50% were obtained for the 3- and 6-phytases, respectively, when dissolved in acetate buffer at pH 4.5. We also used the CD-technique to study the stability of the enzyme as a function of temperature and solvent, namely because the negative effect of the solvent could not be avoided by using a CLEA-phytase.

Thermostability is considered an important criterion for industrial application of phytases, as the enzymes should be able to withstand high temperatures for several minutes, which are reached during the feed pelleting process [37]. To study the influence of temperature we also used CD spectroscopy. Phytases were incubated for 30 min at 25, 40, 60 and 80 °C, and then the CD spectra were measured. Subsequently, the proteins were allowed to renature for 30 min at 25 °C, and then the spectrum was measured again. These experiments show that for both enzymes the denaturation process is irreversible, since the spectrum does not return to the initial state (data not shown). Moreover, the two phytases showed different temperature behavior: for the 6-phytase, after incubation at 40 °C the spectra are roughly the same as the one obtained at 25 °C; for the 3-phytase, the spectra at these temperatures differ, showing lower α-helical content at higher temperature. In general, with increasing temperature the α-helical content decreases and the β-sheet and unordered secondary structural elements increase. Table 4 contains the results obtained from the CDPro suit of programs [36]. For the spectra measured at 80 °C it was not possible to determine the secondary structure content since the fitting was unsatisfactory for both enzymes, but a qualitative observation shows that the enzymes still keep part of their secondary structure at this temperature. The detrimental effect of temperature on the structure agrees with the catalytic oxidation studies, which show loss of selectivity and (in a minor scale loss of activity) upon increasing the temperature (see Table 1).

Table 4
Secondary structure content of 3- and 6-phytase at pH 4.5 determined by CD spectroscopy with the CDPro suite of programs [36]

	α-Helix	β-Sheet	Turns	Unordered
3-Phytase				
$T = 25$ °C	61.3	11.3	9.1	18.3
$T = 40$ °C	49.1	9.1	18.2	23.1
$T = 60$ °C	23.9	30.0	23.4	22.4
6-Phytase				
$T = 25$ °C	49.0	14.0	15.1	19.3
$T = 40$ °C	48.0	16.7	14.2	21.1
$T = 60$ °C	15.9	34.4	22.5	27.2

Another parameter that was studied was the influence of different solvents in the secondary structure of the enzymes. Most catalytic experiments were done with 10% DME to allow the dissolution of the substrate. We also observed (see above) that when a higher percentage of an organic solvent was used, in some cases, the activity sharply decreased. Therefore, we measured the CD spectra of both phytases in the presence of 10% DME and 30% DME, EtOH, MeOH, and MeCN. Fig. 7 shows the spectra obtained for the 3-phytase.

A rough estimate of the α-helical content can be obtained from the mean residue ellipticity at 222 nm $\{[\theta]_{222}\}$, using the expression: fraction of α-helix $= -([\theta]_{222} + 3000)/33000$ [38]. It is quite clear from the figure and from the estimated data that in the presence of 30% EtOH the α-helical content of the enzymes has increased, since, in particular, the band at 208 nm has higher intensity. This effect for ethanol has been observed previously for an acid phosphatase from *Penaeus penicillatus* [39]. In this case increasing amounts of ethanol increased both the α-helical and the unordered content of the enzyme. On the other hand, acetonitrile has a very strong effect on the secondary structure, decreasing the α-helical content in both enzymes. This is in agreement with an almost complete loss of enantiomeric excess in the catalytic experiments done with 30% MeCN (Table 2, entry 10).

Table 5
α-Helical content of 3- and 6-phytase with increasing vanadate concentration and with different percentages of organic solvents[a]

V(V) concentration (μM)	3-Phytase	6-Phytase	Solvent	3-Phytase	6-Phytase
0	55.0	39.4	Buffer pH 4.5	59.0	42.4
10	55.2	40.7	10% DME	59.5	41.7
25	55.8	39.6	30% DME	65.4	43.2
50	51.3	36.8	30% MeOH	57.5	42.4
100	52.5	33.5	30% EtOH	69.2	46.0
400	46.0	32.3	30% MeCN	50.8	33.6

[a] Estimated with the expression: fraction of α-helix $= -([\theta]_{222} + 3000)/33000$ [38]. Conditions 25 °C and pH 4.5.

The effect of the vanadate ion concentration was also studied with this technique. When increasing amounts of vanadate were added to the solutions of the enzymes, the intensity of the negative Cotton effect decreased for concentrations higher than 50 μM, which clearly indicates changes in the protein secondary structure, with loss of helical stability. The α-helical content was estimated [38] and Table 5 shows the results obtained for both phytases. We can conclude that for the vanadium concentration range used in the catalytic studies (⩽30 μM) the enzymes maintain their secondary structure in solution.

4. Conclusions

A simple process could be used to immobilize the 3-phytase, which allowed the reutilization of the catalyst, without significant loss of activity. High conversions of thioanisole and moderate enantioselectivities were obtained in acetate buffer (0.1 M, pH 5.0) with 10% DME. However after immobilization the catalyst is still deactivated in the presence of higher amounts of organic solvents. The results of circular dichroism studies are consistent with the catalytic data, namely that some of the organic solvents used, change the secondary structure of the enzymes: 30% of ethanol or DME increase the α-helical content of the phytases and acetonitrile has an opposite effect. Apparently, immobilization through CLEA stabilization is not able to reverse the solvent effect.

The ^{51}V NMR studies showed that vanadate is indeed incorporated in the active site of both phytase enzymes investigated. The 6-phytase, which is inactive in the sulfoxidation of thioanisole, showed higher affinity for vanadate at pH 7.6 than at pH 5.0. The situation was the opposite for the 3-phytase. Overall, the vanadium NMR data corroborate the hypothesis that vanadium is covalently coordinated in the active site of the enzyme to an apical histidine and to oxygen donors. Also there seem to be at least two sites available for coordination. Upon addition of H_2O_2 two peroxide-vanadate-phytase complexes are formed at pH 5.0 in the case of 3-phytase. For the 6-phytase no such peroxide-vanadate-phytase complexes are observed, in agreement with the inactivity of the 6-phytase for the oxidation of thioanisole.

5. Abbreviations

phy	phytase
CLEA	cross-linked enzyme aggregate
CD	circular dichroism
DME	dimetoxyethane

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