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PEROXIDASES FROM PHAEOPHYCEAE: A VANADIUM(V)-DEPENDENT PEROXIDASE FROM *ASCOPHYLLUM NODOSUM**

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Key Word Index—*Ascophyllum nodosum*, Phaeophyceae, peroxidase, iodide oxidation, vanadium(V)

Abstract—A peroxidase isolated from *Ascophyllum nodosum* was completely inactivated by dialysis in pH 3 8 citrate–phosphate buffer containing EDTA The enzyme was slowly reactivated by adding vanadium(V) in suitable buffers The vanadium(V) effect is specific to this peroxidase and is not shared by other proteins, including lactoperoxidase Even small traces of vanadium(V), which can occur as impurities in the salts of most other metals, are highly effective The K_A was *ca* $3\,5 \times 10^{-8}$ M vanadium(V)

INTRODUCTION

Peroxidases, which are common in the Phaeophyceae [1, 2], catalyse the specific oxidation of iodide as found with iodide peroxidase (EC 1 11 1 8) However, iodide peroxidases, like lactoperoxidase, are known to be hemoproteides In contrast to lactoperoxidase, the peroxidases from *Ascophyllum nodosum* are cyanide-insensitive [3] In the one isoenzyme (A n I) isolated [4], the absorption spectrum lacked the Soret band, indicating that A n I is not a hemoproteid

In the course of studies of peroxidases in Phaeophyceae, it was observed that, apart from the pH, the composition of the various buffer solutions also has an effect on the activity of the peroxidases in the algal extract It seemed possible that the binding of metal ions, by forming complexes with buffer ions, could be the cause of this phenomenon By adding metal ions to enzyme preparations of *Ascophyllum nodosum* (L) Le Jolis inactivated by dialysis, it was possible to establish whether peroxidase activity could at least be partly regenerated

RESULTS AND DISCUSSION

The pre-purified extract from *A nodosum* (= PEX-A) was inactivated by dialysis against pH 3 8 citrate–phosphate buffer with EDTA present Under the same conditions, no loss of activity could be found with lactoperoxidase Immediately after adding various metal ions to inactive PEX-A, it was not possible to observe any peroxidase activity In citrate–phosphate buffers, the test remained negative, even after 24 hr, whereas in tests with the other buffers in the presence of some metal ions, peroxidase activity was detectable In the tests with pH 9 Tris–HCl, peroxidase activity tended to be especially high The most effective metal ions were those of vanadium compounds (see Table 1) In a basic medium vanadium(IV) is easily oxidized to vanadium(V), and so for this reason it was decided to restrict further studies to the effects of vanadium (V) Iodide- as well as bromide-peroxidase activity was detectable With the aid of control experiments, the possibility that peroxidases in PEX-A

Table 1 Effect of metal ions on the reactivation of peroxidase activity in the extract from *Ascophyllum nodosum*

Concn of metal ions in test	% Activity $\bar{x}$*	% Activity σ†
Blank (H_2O)	5 3	±0 4
9 1 mM V^{4+}	90 1	1 9
0 91 mM V^{4+}	104 0	2 6
0 091 mM V^{4+}	99 9	4 6
1 82 mM V^{5+}	102 2	2 4
0 182 mM V^{5+}	102 2	2 4
18 2 mM Cr^{6+}	12 2	0 4
9 1 mM Ca^{2+}	10 8	0 3
9 1 mM NH_4^+	9 9	0 1
9 1 mM K^+	8 6	0 4
9 1 mM Li^+	8 4	0 2
9 1 mM Co^{2+}	8 0	0 2
9 1 mM Mg^{2+}	7 4	0 7
9 1 mM Na^+	6 9	0 3
9 1 mM Ni^{2+}	6 4	0 1
9 1 mM Zn^{2+}	3 4	0 1
9 1 mM Mn^{2+}	2 5	0 1
9 1 mM Cu^{2+}	1 7	0 2
9 1 mM Zr^{4+}	0 0	
9 1 mM Mo^{6+}	0 0	
9 1 mM Fe^{2+}	0 0	
9 1 mM Fe^{3+}	0 0	

Reactivation in pH 9, 0 05 M Tris–HCl Activity in percentage, relative to the activity of aliquot amounts of the PEX-A before dialysis Peroxidase activity was determined by the oxidation of I^-

*$\bar{x}$ = Mean value

†σ = Standard deviation

*Part V in the series For Part IV see Vilter, H (1983) *Bot Mar* **26**, 451

were inhibited by contaminants in the dialysis membrane or in the buffer components could be excluded

When active PEX-A or inactivated PEX-A (inactivated by dialysis) was heated, peroxidase activity could not be detected, even after adding vanadium(V)

Vanadium(V) does not increase the activity of lactoperoxidase, nor do various protein solutions (e g albumin, γ-globulin, phosphorylase B, etc, see Experimental), nor do alginate solutions show pseudo-peroxidase activity after the addition of vanadium(V) Despite repeated dialysis against quartz-distilled water, traces of citrate, phosphate and EDTA could still be present in the inactivated tests It is conceivable that by adding metal ions a possible inhibitory effect of these substances could be removed As calcium forms better complexes with these substances, it should be in a better position to cancel such an inhibition However, this was not the case Control experiments were performed to ascertain that the concentration of Ca ions used in the experiments with inactive PEX-A did not themselves inactivate PEX-A Only one peroxidase (designated A n I) was visible with disc-electrophoretic checks on all the reactivated samples of PEX-A According to Vilter [4], this peroxidase is located in the transitional region between the cortex and medulla And so for this reason, further studies were carried out with purified peroxidase A n I The reactivation took place rather slowly (Fig 1) To preclude any possible pH effect as a time-determining factor, the test samples were buffered 24 hr before vanadium(V) was added The results of the reactivation were dependent on pH and the composition of the buffers (Table 2)

A comparison of enzymatic activity of the enzyme preparations before inactivation and after reactivation shows a seemingly higher yield of peroxidase activity This can be attributed to the fact that even in the non-dialysed preparations, peroxidase A n I is partly in-

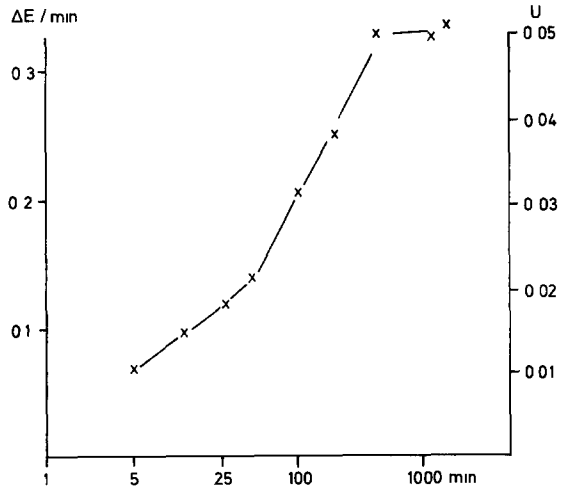

Fig 1 Reactivation of peroxidase A n I by vanadium(V) plotted against time Inactivated A n I was buffered with pH 9 Tris–HCl After 24 hr, 1 82 mM vanadium(V) was added At various time intervals, aliquot samples were taken and the peroxidase activity was determined by the oxidation of I^- ΔE/min = Change of extinction/min at a wavelength of 350 nm min = Time after the addition of vanadium(V) U = Enzyme units, determined by the formation of I_3^-, for definition see ref [1]

Table 2 Effect of buffer solutions on the reactivation of A n I

Buffer	% Reactivation $\bar{x}$*	σ†
pH 9 0 Tris–HCl	65 0	±1 8
pH 9 0 phosphate	39 6	4 7
pH 6 2 Tris–HCl	53 3	1 2
pH 6 2 citrate–phosphate	0 0	

Purified A n I was inactivated by dialysis, then buffered and 1 82 mM vanadium(V) was added Peroxidase activity was measured after 24 hr by the oxidation of I^- The degree of reactivation was compared with the activity of purified A n I (= 100%) in pH 9 Tris–HCl which contained 1 82 mM vanadium(V)

*$\bar{x}$ = Mean value

†σ = Standard deviation

activated or less active, as the activity-enhancing effect of vanadium(V) on the pre-purified or purified peroxidase clearly demonstrates

The dependence on vanadium(V) of the reactivation of A n I was checked in the concentration range $1\,82 \times 10^{-10}$–$1\,82 \times 10^{-5}$ M The results yield a Hill plot showing an apparent negative cooperativity in the region below 10^{-8} M This can be explained by background contamination of the samples with traces of vanadium(V) When working with HCl (p A) from brown glass flasks, as well as when using stainless-steel microlitre syringes, a significantly higher background effect was observed

The effectiveness of other metal ion solutions corresponded roughly to a vanadium concentration of $0\,6$–$4\,8 \times 10^{-9}$ M, and so could be attributed to traces of vanadium(V) in the substances used Above 10^{-8} M vanadium(V), the results approximated to the Hill constant, $n_H = 1$ This probably means that A n I possesses only one binding site for vanadium(V), or several individual sites which have no influence on each other Because of the results in the region above 10^{-8} M vanadium(V), it was possible to estimate the concentration which yields half-maximal reactivation, i e $K_A \sim 3\,5 \times 10^{-8}$ M vanadium(V) This almost corresponds to the vanadium concentration of salt water in the English Channel, which was estimated [5] to be *ca* 5×10^{-8} M Due to the effectiveness of even the smallest traces of vanadium(V), it seems likely that vanadium(V) is essential for peroxidase A n I activity It appears that vanadium(V) is not just a simple cofactor The fact that reactivation of A n I is time-dependent indicates that a change in conformation might be necessary for reactivation Vanadium(V) concentration has no significant effect on the Michaelis–Menten constants of the substrates KI and H_2O_2 [4]

In the triiodide test, vanadium(V) showed a pseudo-peroxidase activity However, its effect was relatively small The molar vanadium(V) concentration necessary for a positive test was more than 10^6 times greater than the molar concentration of the enzyme lactoperoxidase or peroxidase A n I when examining enzyme activity [4] As in the case of other active trace elements whose catalytic effectiveness is considerably increased in suitable complexes, it could be presumed that vanadium(V) might have a function in the active site of the enzyme complex With the amount of A n I so far obtained, it has not yet been

possible to determine whether and to what extent vanadium(V) is contained in A.n I In contrast to complexes formed between iron and cyanide, no stable complexes between vanadium(V) and cyanide are known It is then understandable that CN^- does not significantly inhibit the peroxidases of *A nodosum*, unlike the effect CN^- has on the hemoproteide lactoperoxidase [3] One must also remember that it was not possible to prove even by spectroscopical methods that A n I was a hemoproteide [4]

Vanadium is an essential trace element for animals [6], for *Chlorella* [7–10], *Enteromorpha* and *Fucus* [11]

So far, it has not been proved with certainty that under natural circumstances vanadium is a component of an enzyme Tamura *et al* [12] have reported a synthetic peroxidase only with a vanadium(IV)–porphyrin complex Nagatani and Brill [13] were unable to confirm the synthesis of a 'V-nitrogenase' in *Azotobacter* [14–16] when under a shortage of molybdenum, vanadium(V) was present According to ref [17], a certain polyphenol oxidase in *Biomphalaria glabrata* requires cations for activation, and vanadium(III) is supposed to be particularly effective

However, vanadium can also be toxic In small traces vanadate can operate analogously to phosphate on account of its comparable structure and so inhibit enzymes of phosphate metabolism [18] Except for studies of enzyme inhibition by vanadium(V), no other work appears to have been done on vanadium(V) binding to proteins [19] The low rate of reactivation of peroxidase A n I in phosphate buffer compared to Tris buffers can possibly be attributed to a competitive effect due to its structural analogy The formation of vanadate–phosphate polyanions can also be regarded as a possible cause Other reasons for the inactivation of peroxidase A n I may be found in the formation of weak complexes between vanadium(V) and EDTA [20] or citric acid [21], as well as the influence of pH on the stability of the active enzyme complex

EXPERIMENTAL

Algal material Ascophyllum nodosum was collected in the vicinity of the Station Biologique Roscoff, Brittany (France) in April For the following studies 'pre-purified extract' (= PEX) [1] as well as the peroxidase A n I [4] was isolated

Peroxidase activity was determined by the formation of I_3^- [1], and on the basis of the bromination of 1,1-dimethyl-4-chloro-3,5-cyclohexadione [3] in accordance with ref [22] The sp act of A n I was calcd based on the Lowry protein assay method to be 400 U/mg [4]

Disceleclrophoresis For procedure and the detection of peroxidases see ref [2]

Buffers pH 3 8 0 071 M Na_2HPO_4 and 0 0645 M citric acid, pH 6 2 0 13 M Na_2HPO_4 and 0 034 M citric acid, or 0 05 M tris(hydroxymethyl)aminomethane = Tris adjusted with HCl, pH 9 0 1 M Na_2HPO_4 or 0 05 M Tris adjusted with HCl

Inactivation of peroxidase activity The enzyme preparations were each dialysed twice for 24 hr against pH 3 8 citrate–phosphate buffer with 1 mM EDTA, and then twice against 5 1 H_2O

Reactivation by metal ions Equal vols of buffer solns were added to the inactive enzyme solns Ten parts of this mixture were combined with 1 part of a soln containing one of the following substances (up to 0 1 M) $VOSO_4$ $5H_2O$ purest, V_2O_5 purest, $K_2Cr_2O_7$ p A, $CaCl_2$ $2H_2O$ p A, NH_4Cl p A, KCl p A, LiCl suprapure, $CoCl_2$ $6H_2O$ p A, $MgCl_2$ $6H_2O$ p.A, NaCl p A, $Ni(NO_3)_2$ $6H_2O$ p A, $ZnCl_2$ p A, $MnCl_2$ $4H_2O$ p A, $CuCl_2$ $2H_2O$ p A, $ZrOCl_2$ $8H_2O$ p A, $(NH_4)_6Mo_7O_{24}$ $4H_2O$ p A, $FeCl_2$ $4H_2O$ p A, $FeCl_3$ $6H_2O$ p A

Peroxidase activity was investigated immediately, as well as 24 hr after the addition of the metal ions V_2O_5 was dissolved at pH 9–10 by the addition of NaOH and then adjusted to the same pH as the buffered enzyme soln with HCl To study the effect of vanadium(V), pH 9 Tris–HCl was used from tris(hydroxymethyl)aminomethane (certified primary standard, Serva) as well as HCl (suprapure, Merck) All the glass apparatus used was boiled and subsequently rinsed out several times with quartz-distilled H_2O The solns were pipetted by transfer pipettes with plastic disposable tips

Vanadium(V)-dependent pseudoperoxidase activity tests with standards Solns of the following substances, 1 mg/ml respectively, were prepared in pH 9 0 05 M Tris–HCl Vanadium(V) (0 1 ml 0 02 M) was then added per 1 ml soln and 24 hr later peroxidase activity was checked Lactoperoxidase from cow milk (Boe), ferritin from horse spleen (S), triose phosphate isomerase from rabbit muscle (Boe), tyrosinase from mushroom (S), glycero-3-phosphate dehydrogenase from rabbit (Boe), albumin from bovine serum (S), albumin from hen's egg (S), catalase from bovine liver, 39 000 U/mg (S), chymotrypsinogen from bovine pancreas (S), γ-globulin from rabbit serum (S), thyroglobulin from pig (S), phosphorylase b from rabbit muscle (Boe), aldolase from rabbit muscle, 9 U/mg (S), cytochrome *c* from horse heart (S), sodium alginate (R) (Boe = Boehringer, S = Serva, R = Riedel de Haën)

Thermal denaturation The enzyme solns were heated in sealed test-tubes for 30 min at 100°, after which enzyme activity was tested

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REFERENCES

1 Vilter, H, Glombitza, K-W and Grawe, A (1983) *Bot Mar* **26**, 331
2 Vilter, H and Glombitza, K-W (1983) *Bot Mar* **26**, 341
3 Vilter, H (1983) *Bot Mar* **26**, 429
4 Vilter, H (1983) *Bot Mar* **26**, 451
5 Biggs, W R and Swinehart, J H (1976) in *Metal Ions in Biological Systems* (Sigel, H, ed), Vol 6, pp 141–196 Dekker, New York
6 Hopkins, L L (1974) in *Trace Element Metabolism in Animals, Proceedings of the 2nd International Symposium* (Hoekstra, W G, ed) pp 397–406 University Park Press, Baltimore
7 Meisch, H-U and Bielig, H-J (1975) *Arch Microbiol* **105**, 77
8 Meisch, H-U, Brenzschawel, H and Bielig, H-J (1977) *Arch Microbiol* **114**, 67
9 Meisch, H-U and Brenzschawel, H (1978) *Arch Microbiol* **116**, 91
10 Meisch, H-U and Bauer, J (1978) *Arch Microbiol* **117**, 49
11 Fries, L (1982) *Planta* **154**, 393
12 Tamura, M, Shimidzu, N and Hayashi, K (1977) *Biochem. Biophys Res Commun* **75**, 1029

13 Nagatani, H H and Brill, W J (1974) *Biochim Biophys Acta* **362**, 160
14 McKenna, C E, Bennemann, J R and Taylor, T G (1970) *Biochem Biophys Res Commun* **41**, 1501
15 Burns, R C, Fuchsman, W H and Hardy, R W F (1971) *Biochem Biophys Res Commun* **42**, 353
16 Bennemann, J R, McKenna, C E, Lie, R F, Taylor, T G and Kamen, M D (1972) *Biochim Biophys Acta* **264**, 25
17 de Aragoa, G A and Bacila, M (1976) *Comp Biochem. Physiol* **53B**, 179
18 Simons, T J B (1979) *Nature (London)* **281**, 337
19 Chasteen, N D (1983) *Structure Bonding* **53**, 107
20 Ringbom, A, Siitonen, S. and Skrifvars, B (1957) *Acta Chem Scand* **11**, 551
21 Gmelin, L (1967) *Handbuch der Anorganischen Chemie, Vanadium, Teil B*, 8th edn Verlag Chemie, Weinheim
22 Hager, L P, Morris, D R, Brown, F S and Eberwein, H (1966) *J Biol Chem* **241**, 1769