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CHARACTERIZATION OF A PEROXIDASE FROM *SPHAGNUM MAGELLANICUM*

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Abstract—The peroxidase from *Sphagnum magellanicum* is strongly inhibited by CN^- and N^{3-} and is rather heat stable. The pH optimum is 5.0. The peroxidase rapidly degrades the common hydroxycinnamic acids and sphagnum acid to non-phenolic products; *t*-cinnamic acid itself is not attacked. The significance of these reactions is discussed with respect to earlier investigations on the level of cinnamic acids in *Sphagnum magellanicum*. The peroxidase consists of five acidic and five basic isoenzymes. This band pattern does not change during the colouring of the moss, so that the peroxidase from the green and the red moss is identical.

INTRODUCTION

While peroxidase (EC 1.11.1.7.) is one of the best investigated enzymes of higher plants, it has rarely been studied in bryophytes [1–3]. Our interest in this enzyme results from our previous work on the phenolic constituents and cell wall pigments of *Sphagnum magellanicum* (see [4]). Since peroxidase has been shown to degrade various plant phenolic compounds [5, 6], it was decided to test *Sphagnum magellanicum* for peroxidase activity and to gain information on its properties. Peroxidases from peat mosses have already been investigated [3, 7] but only superficially.

RESULTS AND DISCUSSION

Enzyme preparation

Although *Sphagnum magellanicum* contains several phenolics, interference of these compounds with enzyme proteins [8] was minimal. Thus, a chromatographic investigation of the crude enzyme solution showed only traces of phenolic compounds or of sphagnum acid, the main phenolic constituent of the cell wall [9, 10]. Since homogenization in the presence of PVP did not effect a worthwhile purification, enzyme solutions were prepared without adding agents for the removal of phenolics. The only purification step employed was a $(NH_4)_2SO_4$ precipitation and subsequent gel filtration. The resultant solutions were free of phenolics and served as enzyme sources for the assays.

Examination of the peroxidase character

In order to exclude a H_2O_2-mediated oxidation of *o*-dianisidine in the standard test, the assay was carried out with heat-denaturated enzyme and in the absence of enzyme. In both cases, no oxidation occurred. On the other hand, the dependence of the test on H_2O_2 was demonstrated by incubation in the presence of catalase. A concentration of 8 μg catalase/ml test volume totally suppressed the reaction.

In order to confirm the peroxidase character of the isolated enzyme, the effect of CN^- and N^{3-} on the activity was studied. Both ions are known to react with the heme iron of peroxidases. In the presence of 10^{-3} M KCN and 10^{-1} M NaN_3, the *Sphagnum* enzyme was completely inhibited. The oxidation of *o*-dianisidine may therefore be considered as a peroxidase-catalysed reaction.

Enzyme properties

In the standard assay, maximal activity was at pH 5.0 and half maximal activity *ca* pH 4.0 and at 5.9. Above pH 6.5 the enzyme is completely inactive. Quite different behaviour has been reported for the peroxidases from *Sphagnum medium*, *S. robustum* and *S. girgensohnii*, which exhibit a pH optimum at 7.4 [7]. It is not clear whether this discrepancy is due to the *Sphagnum* species used, or can be attributed to the uncommon test system employed by Mattison [7]. The fact that the pH optimum of the *Sphagnum magellanicum* enzyme resembles those of higher plant peroxidases [6, 11–13] supports the second alternative.

The effect of temperature on the stability of the enzyme was studied by measuring the initial velocity of the *o*-dianisidine reaction at pH 5.0. The enzyme activity increased up to 52°, before increasing thermal denaturation caused a decline. This behaviour indicates considerable heat stability, a phenomenon which has also been reported for other plant peroxidases [14–16]. With regard to the storage temperature, the enzyme only retained full activity if frozen in liquid N_2 and stored in the frozen state. Linearity was observed between peroxidase activity and protein concentration, up to a concentration of 30 μg protein.

Higher plant peroxidases frequently possess phenolase and (or) IAA-oxidase activity [12, 17, 18]. Both activities were also found in the enzyme preparation from *Sphagnum magellanicum* though high protein concentrations were necessary to measure a distinct turnover of the

Table 1. Chromatographic properties of sphagnum acid and its peroxidase-induced degradation product

Property	Sphagnum acid	Degradation product
R_f in *n*-BuOH–HOAc–H_2O (3:2:95)	0.72	0.60
Fluorescence quenching of the adsorbent by irradiance with $UV_{254\,nm}$	intensive	faint
Fluorescence by irradiance with $UV_{360\,nm}$	faint	intense bright blue
Staining with Pauly's reagent	red	colourless

substrates. Reaction mixtures without the enzyme or with heat-denaturated enzyme showed no activity.

Degradation of sphagnum acid and common cinnamic acids

Among the physiological substrates of plant peroxidases, phenolic substances are preferentially attacked [5, 6]. Since sphagnum acid, *p*-hydroxy-(β-carboxymethyl)cinnamic acid, represents the main phenolic component extractable from *Sphagnum magellanicum*, its reaction with the moss peroxidase was of interest. As shown by the changes of the UV spectrum, sphagnum acid was rapidly degraded by the enzyme. The extinction immediately decreased after adding H_2O_2 while the absorption maximum shifted from 283 to 286 nm. These alterations were completed within 30–60 min, depending on the amount of enzyme used. Analogous curves were obtained with horseradish peroxidase. The chromatographic analysis of the incubation mixture showed that sphagnum acid was quantitatively converted to a non-phenolic substance, the main properties of which are compared with those of sphagnum acid (Table 1).

Besides sphagnum acid, *t*-cinnamic, *p*-coumaric, caffeic and ferulic acids were tested as substrates because these acids are possible intermediates in the biosynthesis of sphagnorubine and related wall pigments of Sphagna [4, 19, 20]. The enzymic reaction was likewise evaluated by the changes of the UV spectrum and chromatography of the incubation mixtures. Whereas *t*-cinnamic acid remained unchanged, the hydroxycinnamic acids were spontaneously attacked by the peroxidase being transformed to non-phenolic products. It is evident that the reaction needs the presence of a free *p*-hydroxyl group. Comparable results are reported by Berlin and Barz [6] for the oxidative decarboxylation of benzoic acids. These authors point out that the conversion of protocatechuic acid can mainly be attributed to a phenolase activity coexisting with the peroxidase in the enzyme preparation. Similarly, caffeic acid is easily accessible to a phenolase attack and since the *Sphagnum* enzyme preparation also shows phenolase activity, the conversion of caffeic acid will predominantly be catalysed by the latter enzyme.

Isoenzyme patterns

The isoenzyme composition of the isolated peroxidase was investigated by acid and alkaline disc electrophoresis and isoelectric focussing in polyacrylamide gel. For the detection of the active enzyme bands, benzidine in 80% methanol was preferred to *o*-dianisidine [6] because the bands were stained more slowly and remained sharp for a longer time. A comparative analysis (Fig. 1) shows that

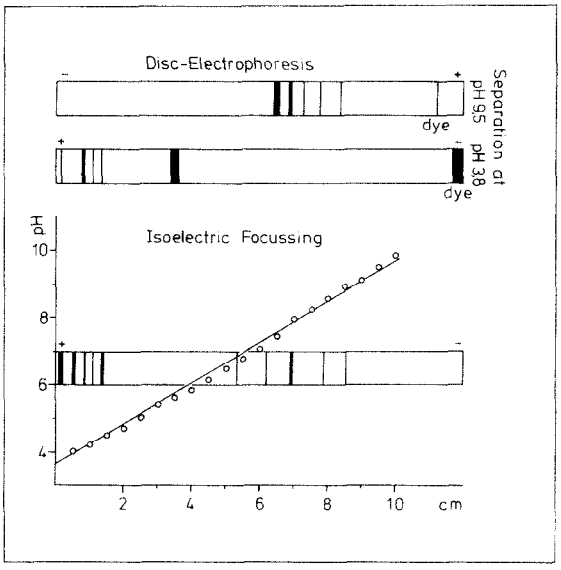

Fig. 1. Isoenzyme pattern of the *Sphagnum magellanicum* peroxidase as revealed by disc electrophoresis and isoelectric focussing in polyacrylamide gel.

the peroxidase consists of five acidic and five basic isoenzymes. With respect to the results of the alkaline electrophoresis, the isoperoxidase spectrum of *Sphagnum magellanicum* essentially agrees with that of *Sphagnum centrale* which was found to consist mainly of six acidic bands [3]. This finding was used by the authors as a criterion for a phylogenetic distinction between Sphagna and certain Bryales, for example *Pleurocium* and *Brachythecium*, which additionally possess highly active basic isoperoxidases. Basic bands, however, were also detected in *Sphagnum magellanicum*. Therefore, one should not base an evolutionary trend on the isoenzyme spectrum of a single separation method.

The characterization of the *Sphagnum magellanicum* peroxidase would be incomplete without some remarks on the properties of the enzyme from reddened tissue. The colouring of the moss was induced by a cold treatment of cultured material according to Rudolph [4]. As revealed by alkaline disc electrophoresis, the pattern from the cold-treated plants agreed with that of the control plants in every stage of the pigmentation. We may therefore conclude that the peroxidase from reddened *Sphagnum magellanicum* is identical to the enzyme from the green moss.

EXPERIMENTAL

Plant material. Sphagnum magellanicum, collected in the Kaltenhof bog near Kiel, was cultured under definite conditions according to ref. [4]. Cultured material was subjected to cold treatment which is also described in ref. [4]. For the enzyme preparation, the apical parts of the gametophytes (capitula) were used.

Enzyme preparation. All operations were carried out at 4°. Me_2CO powder was exclusively taken as source of the enzyme. The powder was prepared by homogenization of the capitula with 20-fold wt of ice-cold Me_2CO in a blendor at −10° for 3 min. Acetone powder (0.5 g) was homogenized in a mortar with 5 g quartz sand and 10 ml K-Pi buffer (0.1 M, pH 7.5). In the cold

treatment expt, 0.1 g acetone powder was stirred with 2 ml buffer for 1 hr. The homogenate was centrifuged for 30 min at 50000 *g*. The supernatant was filtered through quartz wool. To the crude extract, solid $(NH_4)_2SO_4$ was added to 80% saturation. After stirring for 30 min, the ppt. was collected by centrifugation for 20 min at 28000 *g*, dissolved in 1 ml extraction buffer (diluted 1:2) and desalted in a 5 ml syringe by the Sephadex G 25 centrifugation technique. For polyacrylamide gel electrophoresis, the Sephadex gel was equilibrated in the electrode buffer (diluted 1:10). In the cold treatment expt, the enzyme solns were brought to a constant vol. (2 ml) before electrophoresis.

Enzyme assays. The incubation mixture for the standard assay of peroxidase activity consisted of 2.8 ml acetate buffer (0.05 M, pH 5.0), 0.05 ml of a 0.5% *o*-dianisidine soln in ethylene glycol monomethyl ether and 0.1 ml of appropriately diluted enzyme soln. The reaction was started by adding 0.05 ml 0.1 M H_2O_2 soln. Linear increase of the extinction was measured at 460 nm and 25°. Enzyme activity was expressed as $\Delta E \cdot min^{-1} \cdot ml^{-1}$ of undiluted enzyme. Phenolase activity was assayed according to ref. [17] with DOPA as substrate. Authentic tyrosinase (1 mg in 25 ml 0.01 M K-Pi buffer, pH 7.5) was used for a control assay. For the measurement of IAA-oxidase activity, the assay of Mäder *et al.* [12] was used in a slightly modified form. 0.3 ml 5×10^{-4} M $MnCl_2$-, 0.3 ml 2.5×10^{-4} M 2,4-dichlorphenol- and 0.3 ml $5{,}7 \times 10^{-4}$ M IAA soln (each soln in 0.1 M K-Pi buffer, pH 5.8) were added to 0.6 ml of enzyme soln. The mixture was incubated at 30° for 1 hr, 1 ml was then removed and mixed with 2 ml Salkowski reagent prepared according to ref. [21]. After 1 hr the extinction was measured at 530 nm against a blank containing 1 ml H_2O and 2 ml Salkowski reagent. The initial absorption was determined in a sample without the enzyme.

Protein determination. Protein was precipitated by 10% TCA and determined by the Lowry method according to ref. [22]. BSA served as standard.

Analytical gel electrophoresis and isoelectric focussing. Disc electrophoresis and isoelectric focussing in polyacrylamide gel were performed as described by ref. [23]. Acid isoenzymes were electrophoretically separated at pH 9.5 in gel system No. 1. The separation of basic isoenzymes was carried out in the medium-porous gel of system No. 5 at the pH of system No. 7 (pH 3.8). For anionic separation, bromphenol blue served as tracking dye whereas for cationic separation pyronine Y was used. In the latter case, 10 ml of a 0.005% soln were mixed with the electrode buffer. Electrophoresis was carried out at 30 V per gel for 3 hr under cooling (4°). For isoelectric focussing, a 5% gel was prepared by chemical polymerization of the separation gel stock solns Nos. 2a and 3 of the gel electrophoresis system No. 1 (2 and 5 ml, respectively) with 0.005 ml TEMED, 4.5 ml H_2O and 0.3 ml ampholines, pH 3–10. Electrofocussing was carried out at 325 V for 3 hr using the electrode solns of the isoelectric focussing system No. 1 in ref. [23]. The pH gradient was measured on the surface of the gels with a microelectrode. Isoelectric points were determined by extrapolation of the isoenzyme bands to the pH gradient.

Peroxidase-catalysed degradation of aromatic acids. 2 mM solns of *t*-cinnamic, *p*-coumaric, caffeic, ferulic and sphagnum acids were prepared in ethylene glycol monomethyl ether. 0.05 ml of each soln were added to 1.80 ml acetate buffer (0.1 M, pH 5.0) and 0.1 ml of the peroxidase soln (conc soln of the *Sphagnum* enzyme and horseradish peroxidase: 1 mg/10 ml H_2O, diluted 1:10). The UV spectrum of the mixture was measured at 25°. The reaction was started by adding 0.05 ml 0.1 M H_2O_2 soln and the change of the absorption was recorded immediately. The measurement of the UV spectrum was repeated at 5 min intervals. When no further spectral alterations were observed, the incubation mixture was acidified with 0.1 N HCl to pH 2 and extracted ×5 with 0.5 ml Et_2O. From the combined Et_2O and aq. phases, the solvents were evapd, the residues dissolved in 0.2 ml MeOH and these solns chromatographed on cellulose UV_{254} layers in *n*-BuOH–HOAc–H_2O (3:2:95). Reference samples were prepared in the same way from incubation mixtures lacking the substrate and the enzyme, respectively.

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