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# THE FUNCTION OF FREE CARBOXYL GROUPS IN THE ACTION OF PEROXIDASE AND INDOLE-3-ACETIC ACID OXIDASE

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**Key Word Index**—*Inonotus radiatus*; *Arachis hypogea*; *Trametes versicolor*; peroxidase; enzyme immobilization; active site; indolyl-3-acetic acid oxidase.

**Abstract**—The extracellular peroxidase from cultures of *Inonotus radiatus* and of peanut (*Arachis hypogea* L.) cells as well as the mycelial peroxidase from *Trametes versicolor* were used for studies of immobilizing this protein either by its free amino or its carboxyl groups. The immobilization process was carried out either on keratin proteins derived from feathers or on polyamide coated over silica gel. Coupling was established either through the free amino or carboxyl groups. In general the indolyl-3-acetic acid oxidase activity of fungal peroxidases exceeds that of peanut peroxidase. When the peroxidase of the three sources was immobilized on the matrices by the free amino groups, little if any effect on the IAA oxidase activity could be measured. However, immobilization through the carboxyl groups resulted in a drastic reduction of indole-3-acetic acid oxidase activity. Since identical amounts of peroxidase were linked in all cases, the loss of indolyl-3-acetic acid oxidase activity implies that the carboxyl group is essential for the active site.

## INTRODUCTION

Plant peroxidase (EC 1.11.1.7) is an enzyme that has been reported to occur in the cytoplasm as well as in the cell wall [1]. On various occasions peroxidase activity has been measured in the intercellular fluid [1–3]. Regardless of the location from which the peroxidase was obtained both cationic and anionic forms occur [3–8]. Despite these reports on peroxidase, its function has not yet been clarified [1]. In terms of substrate specificity, peroxidase may catalyse the oxidation of a variety of hydrogen donors, including indolyl-3-acetic acid (IAA). Therefore, it has been suggested that peroxidase and IAA oxidase are one and the same molecule [8–12]. However, the specific arrangement and composition of the active site for IAA oxidase is still not fully described. In an effort to shed further light on this problem the free amino and carboxyl groups were examined for their function by immobilizing them on matrices.

## RESULTS AND DISCUSSION

Optima pH for the reactions of three plant peroxidases were established (Fig. 1). Peroxidase from *Trametes versicolor* has a distinct pH optimum at pH 3.0 but peroxidase from *Inonotus radiatus* possesses two pH optima, one at pH 3 and the other at a higher pH (Fig. 1). The pH optimum for peanut peroxidase was about 5.6 (Fig. 1). Following these observations, in further experiments with soluble and immobilized enzymes the activities of fungal peroxidases were studied at pH 3.0 but that of peanut peroxidase at pH 5.6. Later, the peroxidase preparations were immobilized on two kinds of matrix. However in both cases the matrices were prepared by coating silica gel activated with keratin. Amino groups of keratin react with glutaraldehyde or carbodiimide which allows further binding of peroxidase molecules by amino or carboxyl groups respectively. The second material used for silica gel coating was polyamide-6. Polyamide is an artificial copolymer with only free amino groups which are coupled to glutaraldehyde or carbodiimide before the peroxidase molecules are immobilized in a similar manner as in the case of the keratin activated matrix. Initially, the matrices were checked for the degree of protein immobilization (Table 1). The yields of protein immobilization either on keratin or polyamide were similar (about 30–40%). There

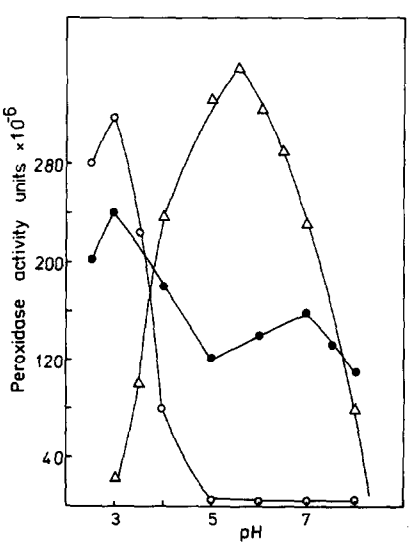


Fig. 1. Activity changes (in katals) of peroxidase in relation to pH. Peroxidase preparations were adjusted to the appropriate pH using 0.05 M citrate–phosphate buffer. (●) Peroxidase from *Inonotus radiatus*; (○) peroxidase from *Trametes versicolor*; (△) peroxidase from peanut culture cells.

Table 1. The yield of protein immobilized on keratin or polyamide matrix

| The source of peroxidase preparation and kind of coupling | Keratin matrix | | Polyamide matrix | |
|---|---|---|---|---|
| | Protein coupled (mg/g of matrix) | Yield of protein coupled (%) | Protein coupled (mg/g of matrix) | Yield of protein coupled (%) |
| *Inonotus radiatus* by $NH_2$ | 6.7 | 33 | 6.8 | 34 |
| *Inonotus radiatus* by COOH | 7.5 | 37 | 8.5 | 42 |
| *Trametes versicolor* by $NH_2$ | 7.8 | 39 | 6.0 | 30 |
| *Trametes versicolor* by COOH | 6.2 | 31 | 7.8 | 39 |
| Peanut cells by $NH_2$ | 7.0 | 35 | 7.5 | 37 |
| Peanut cells by COOH | 6.3 | 31 | 5.8 | 29 |

The amount of protein used was 20 mg/g of matrix.

were also no great differences, whether peroxidase proteins were immobilized using their free amino or carboxyl groups.

Comparing the soluble peroxidase preparations from the three sources, it was observed that the preparations of IAA oxidase activity to peroxidase activity differs (Tables 2 and 3). Both fungi sources possess a much higher ratio than peanut peroxidase. The proportion for material obtained from *Inonotus radiatus* was 97.6 and from *Trametes versicolor* 46.5 (Tables 2 and 3). The above relation of IAA oxidase to peroxidase in a preparation from peanut was much lower at about 0.043 (Tables 2 and 3). This great diversity between fungal and plant peroxidases is supposed to have a parallel with the proportion of cationic to anionic peroxidases in these materials. Fungal peroxidases contain mostly anionic peroxidase forms while peanut peroxidases consist of highly active cationic rather than anionic forms of this enzyme [2, 3, 6, 13, 14]. After immobilization, the activities of the peroxidases was decreased as is usually observed [15, 16] (Table 2). When peroxidase were coupled to the supports by amino groups, the peroxidase and IAA oxidase activities initially decreased, but then were stabilized (Table 2) except in two cases. From Table 2 it can be seen

Table 2. Peroxidase and IAA oxidase activities in soluble and immobilized by $NH_2$ groups to keratin or polyamide

| Peroxidase preparation and kind of matrix | Soluble peroxidase ($10^{-6}$kat/ml) | Immobilized peroxidase ($10^{-6}$kat/g of matrix) | | Soluble IAA oxidase ($10^{-9}$kat/ml) | Immobilized IAA oxidase ($10^{-9}$kat/g of matrix) | | IAA oxidase/peroxidase | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 7 days | | 0 | 7 days | Soluble | Immobilized 0 | Immobilized 7 days |
| *Inonotus*-keratin | 1.0 | 0.300 | 0.090 | 97.6200 | 45.780 | 35.400 | 97.6200 | 152 | 393 |
| *Inonotus*-polyamide-6 | 1.0 | 0.016 | 0.013 | 97.6200 | 35.220 | 37.080 | 97.6200 | 2201 | 2852 |
| *Trametes*-keratin | 1.0 | 0.096 | 0.075 | 45.5000 | 54.600 | 45.120 | 46.5000 | 568 | 601 |
| *Trametes*-polyamide-6 | 1.0 | 0.040 | 0.030 | 46.5000 | 14.880 | 16.320 | 46.5000 | 372 | 544 |
| Peanut-keratin | 1.0 | 0.220 | 0.185 | 0.0432 | 0.162 | 0.138 | 0.0432 | 0.736 | 0.745 |
| Peanut-polyamide-6 | 1.0 | 0.110 | 0.014 | 0.0432 | 0.010 | 0.009 | 0.0432 | 0.098 | 0.685 |

All results were recalculated on $10^{-6}$ kat/ml of soluble peroxidase preparations.

Table 3. Peroxidase and IAA oxidase activities in soluble and immobilized by COOH groups to keratin and polyamide

| Peroxidase preparation and kind of matrix | Soluble peroxidase ($10^{-6}$kat/ml) | Immobilized peroxidase ($10^{-6}$kat/g of matrix) | | Soluble IAA oxidase ($10^{-9}$kat/ml) | Immobilized IAA oxidase (units per/g of matrix) | | IAA oxidase/peroxidase | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 7 days | | 0 | 7 days | Soluble | Immobilized 0 | Immobilized 7 days |
| *Inonotus*-keratin | 1.0 | 0.280 | 0.170 | 97.620 | 0.000 | 0.000 | 97.620 | 0.000 | 0.000 |
| *Inonotus*-polyamide-6 | 1.0 | 0.017 | 0.013 | 97.620 | 11.100 | 8.400 | 97.620 | 652 | 646 |
| *Trametes*-keratin | 1.0 | 0.032 | 0.024 | 46.500 | 0.600 | 0.450 | 46.500 | 18 | 18 |
| *Trametes*-polyamide-6 | 1.0 | 0.017 | 0.010 | 46.500 | 5.220 | 1.920 | 46.500 | 307 | 192 |
| Peanut-keratin | 1.0 | 0.110 | 0.089 | 0.043 | 0.000 | 0.000 | 0.043 | 0.000 | 0.000 |
| Peanut-polyamide-6 | 1.0 | 0.144 | 0.096 | 0.043 | 0.006 | 0.006 | 0.043 | 0.041 | 0.062 |

All results were recalculated on kat $10^{-6}$/ml of soluble peroxidase preparations.

that IAA oxidase coupled to keratin matrices remains more active than that bound to polyamide. The increase of the coefficient of IAA oxidase to peroxidase activity in the amino immobilized forms of these enzymes indicate the greater stabilization of IAA oxidase activity (Table 2). The differences between peroxidase preparations immobilized by amino or carboxyl groups seem to be valuable for explaining the IAA oxidase activity centre in the materials used. After immobilization of proteins by carboxyl groups (Table 3) the peroxidase activities remained similar to that observed in Table 2. However, in the same experiments (Table 3) the IAA oxidase activities are drastically decreased and in some cases activity is lost totally. It should be stressed that identical amounts of proteins were immobilized with the matrices by the amino or carboxyl groups since the yields of coupling were similar (Table 1). Therefore it is concluded from the data in Table 3 that changes of the proportions of IAA oxidase to peroxidase expresses the requirement of carboxyl groups for IAA oxidase activity. It is therefore possible to suggest that the activity centre of IAA oxidase needs carboxyl groups in the catalytic reaction of IAA oxidation.

## EXPERIMENTAL

*Source of peroxidase.* Pure cultures of two strains of white-rot fungi *Inonotus radiatus* Sow. ex. Fr. P. Karst, and *Trametes versicolor* L. ex. Fr. Quel, were kindly supplied by the Museum of Fungi in Paris. The mycelium of *Inonotus radiatus* was grown in stationary cultures in Roux flasks containing 150 ml of 3% malt extract. The culturing time was 14 days. At this time the mycelium was removed by filtration. The protein from the filtrate was salted out at 80% $(NH_4)_2SO_4$ saturation. The sediment was dissolved in 0.02 M NaPi buffer pH 7 and dialysed against the same buffer. *Trametes versicolor* was grown in submerged conditions in 10 l. flasks containing 5 l. of Lindeberg mineral medium [17]. Peroxidase synthesis in the mycelium was induced by 0.2 mM vanillic acid. All procedures of mycelium growing and peroxidase isolation by affinity chromatography method were described in a previous study [18]. The peanut cells were cultured in suspension medium as described [19] and the extracellular peroxidase was isolated as usual [14].

*Determination of peroxidase and indolyl-3-acetic acid activity (IAA oxidase).* Peroxidase activity (EC 1.11.1.7) was determined by the method described earlier [18] using guaiacol as hydrogen donor. Peroxidase activity was defined in katals at 22–24°. Fungal peroxidase activity was determined using 0.05 M Na citrate–NaPi buffer, pH 3 (in the case of peanut peroxidase pH 5.6). The activity of IAA oxidase was determined according to the procedure of ref. [20]. IAA oxidase activity was expressed in katals. Fungal IAA oxidase activity was determined using 0.05 M Na citrate–NaPi buffer, pH 3 and in the case of IAA oxidase from peanut the pH was 5.6.

*Protein determination.* Protein was determined by the method of ref. [21].

*Immobilization of peroxidases.* Peroxidase was immobilized on two kinds of matrix and using $NH_2$ or COOH free groups of proteins. The matrices used were linked to silica gel as a base (0.2 mm, 70–230 mesh, Merck, GFR). Silica gel was coated by feather keratin or by polyamide-6. The preparation of these matrices was described elsewhere [22]. These matrices were activated with glutaraldehyde (Merck, GFR) or with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (Sigma), as described in ref. [23]. Following the activation of the matrices, preparations of the three peroxidase were immobilized in the proportions of 20 mg protein per 1 g of each matrix. The immobilization was performed in the conditions described earlier [18, 19].

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