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PEROXIDASE CATALYSED INCORPORATION INTO POLYMERS AS A MAJOR PATHWAY OF HORDENINE METABOLISM IN BARLEY CELL SUSPENSION CULTURES

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; cell suspension cultures; polymerization; metabolism; hordenine; phenylethylamines; peroxidase.

Abstract—Metabolism of hordenine in barley cell suspension cultures mainly leads to insoluble polymeric material. Polymerization proceeds exclusively via the phenolic hydroxyl group with the dimethylaminoethyl side chain of the hordenine molecule remaining intact. Peroxidase activity from barley cells was shown to polymerize 4-hydroxyphenylethylamines *in vitro* and due to its affinity to these substrates this enzyme is likely to be responsible for a major part of hordenine metabolism *in vivo*.

INTRODUCTION

Hordenine (*N*,*N*-dimethyltyramine) is only found in barley roots during the first month after germination [1, 2]. Its metabolism [3] has been the subject of renewed interest and tracer experiments on hordenine degradation in barley have been performed with intact plants [4, 5] as well as with cell cultures [6]. The use of cell suspension cultures for studies on the catabolism of secondary plant products without the problems of microbial interference [7] has made possible the identification of a number of hordenine catabolites. This led to the proposal of a degradative pathway which is similar to that of related amines in animal systems and involves *N*-demethylation, oxidative deamination and further side chain shortening by hydroxylation and decarboxylation reactions [6].

The quantitatively major pathway of hordenine metabolism in cell cultures, however, is identical with that found in intact plants [3, 5] and leads to high rates of incorporation of labelled hordenine into insoluble polymeric material. Comparative studies with [*N*-methyl-^{14}C]hordenine and [β-^{14}C]hordenine as substrates indicate a preferential incorporation of the *N*-methyl group into polymeric material, while both substrates liberated less than 1% $^{14}CO_2$ [6]. But it is not known whether the intact methyl group is transferred to an acceptor, perhaps a free hydroxyl group of the polymer, or whether incorporation takes place after oxidation of the methyl group.

Recent investigations with barley plants led to the proposal of an almost complete release of the α-carbon of hordenine as CO_2 [4] with subsequent incorporation of a C_6–C_1 unit containing the β-carbon into lignin [5]. Since these results are in contrast with earlier findings [3] and do not consider the metabolism of the methyl carbons, the experiments presented here were designed to elucidate the metabolism of all side chain carbons of the hordenine molecule. Dual isotope-labelled substrates were used to decide which carbons are incorporated into lignin together with the phenyl moiety of the molecule.

RESULTS

Choice of cell lines and growth conditions

Previous experiments [6] had been carried out with cells in the stationary phase of growth. However, preliminary feeding experiments indicated that the rate of polymerization of hordenine was higher when the substrate was added at the time of cell transfer to fresh medium [8]. Therefore, a series of tests was carried out in which the incorporation of [*N*-methyl-^{14}C]hordenine into insoluble polymers was measured over 5 days of exponential (plus beginning of the linear) growth and during the stationary growth phase. Expressed on either a fr. wt or dry wt basis, results clearly demonstrated that polymerization rates were equal or higher (up to five-fold) during exponential growth and that further experiments should be carried out over this part of the growth cycle.

Besides two lines of barley which differ in their growth characteristics and the content of 2,4-D in the medium, parsley and tobacco cultures were also included in these investigations. Although they do not produce any hordenine, parsley cultures metabolize hordenine in the same way as barley cultures [6]. Cell cultures of *Nicotiana sylvestris* and *Nicotiana tabacum*, however, which incorporated the methyl group of nicotine into insoluble materials [9] only metabolized *N*-methyl labelled hordenine at low rates and were omitted from further investigations.

Polymerization of hordenine in barley cell suspension cultures

In addition to already available [*N*-methyl-^{14}C]hordenine and [β-^{14}C]hordenine, [*N*-methyl-^{3}H]hordenine was synthesized to answer the following questions by double labelling experiments. (1) Is the methyl-carbon incorporated into polymers as the intact methyl group or as a partially oxidized unit with loss of

one or more hydrogen atoms? (2) Can it be confirmed that the methyl-carbon is incorporated into polymers in preference to the β-carbon? (3) Has the side chain to be degraded before it is incorporated?

Both [*N*-methyl-^{14}C, ^{3}H]-and [β-^{14}C, *N*-methyl-^{3}H]hordenine were fed (2×10^{-5} M) to cell cultures on day 3 after transfer to fresh medium. The changes in fr. wt (dry wt = 4–6% of fr. wt), amount of free hordenine and percent label in insoluble polymers during the 5 days of incubation are presented in Fig. 1. The recovery of label was *ca* 80%, but no $^{14}CO_2$ could be trapped. The data clearly show an increase in the incorporation of label into polymeric material which corresponds to the decrease of hordenine in the cells. It should be noted, however, that the amount of label in polymers only increased two-fold after the first 24 hr of incubation. The ethanol extract contained only traces of labelled compounds other than hordenine.

The final incorporation rates after 5 days (Table 1) were high and identical for both barley cell lines. However, the most important results in answering the questions raised above were the observed $^{3}H/^{14}C$ ratios. With both substrates and in all cell cultures investigated these ratios did not change. Thus, not only the methyl groups remained intact when incorporated into insoluble polymers but also the complete side chain of the hordenine molecule appeared to remain intact. At least no measurable difference between the incorporations of *N*-methyl hydrogens and the β-carbon were detected. Intermediary divergence of metabolic routes could be ruled out since the $^{3}H/^{14}C$ ratios in the insoluble material, as well as in the ethanol extract, did not change during the 5 day incubation period. On the other hand, TLC controls of ethanol extracts (solvent systems D and F) confirmed *N*-demethylation as the minor pathway in hordenine degradation by identification of *N*-methyltyramine as a degradation product (cf. ref. [6]). The amounts of labelled *N*-methyltyramine were so small, however, that no significant change of $^{3}H/^{14}C$ ratios in ethanol extracts could be observed when [β-^{14}C, *N*-methyl-^{3}H]hordenine was used as substrate.

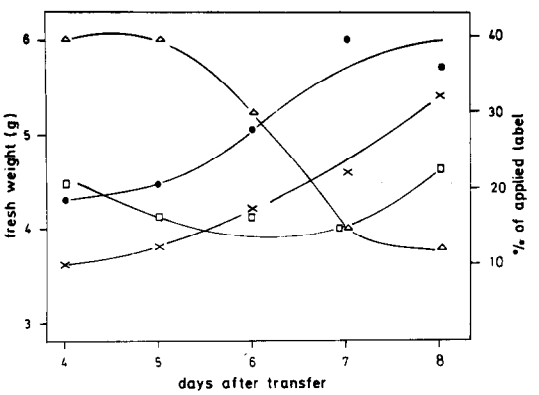

Fig. 1. Kinetics of hordenine metabolism in barley cell cultures. [β-^{14}C, *N*-methyl-^{3}H]Hordenine (2×10^{-5} M) was applied to cell cultures of barley (line D) on day 3 after transfer of cells into fresh medium. Distribution of applied label (%) was recorded in medium (□), cell extract (△) and insoluble cell wall polymers (●), and compared with the increase in fr. wt (×).

Polymerization of other phenylethylamines

To investigate whether slight structural changes would affect the observed polymerization rates for hordenine some related phenylethylamines were applied to cell cultures under the same conditions as described for hordenine. The results (Table 1) cannot be explained by differences in uptake mechanisms, since 50% uptake was reached within 1.5 (hordenine), 3.5 (tyramine) and 11 hr (dopamine). Although these data differ from those found in the stationary growth phase [6], uptake clearly did not limit metabolism during the total incubation time of 120 hr.

Only *O*-methylhordenine uptake seemed to be limited, reaching 20% after 3 hr and then staying at this level for the rest of the 24 hr test period. But, even if corrected for its low uptake, *O*-methylhordenine gave polymerization rates which were significantly lower than those of all other substrates. The 4-methoxy group obviously hinders polymerization. In contrast, tyramine, but more especially dopamine with its vicinal hydroxyl groups, were good substrates for polymerization reactions. These data confirm earlier results [6, 10]. Among the cell cultures used for this experiment parsley showed the lowest, but still good incorporation rates with all substrates tested while the two barley cell lines behaved almost identically.

Table 1. Incorporation of phenylethylamines into insoluble polymers*

Substrates (2×10^{-5} M)	% label in insoluble polymers		
	Barley line A	Barley line D	Parsley
[*N*-methyl-^{14}C, ^{3}H]Hordenine	43.2	40.1	11.6
$^{3}H/^{14}C$ ratio: 2.15	2.2 ± 0.1	2.0 ± 0.05	2.1 ± 0.2
[β-^{14}C, *N*-methyl-^{3}H]Hordenine	43.5	36.5	10.3
$^{3}H/^{14}C$ ratio: 2.9	2.8 ± 0.15	2.7 ± 0.2	2.9 ± 0.1
[β-^{14}C]Tyramine	40.9	44.7	24.8
[β-^{14}C]Dopamine	47.5	50.4	40.4
O-[*N*-methyl-^{14}C]Methylhordenine	1.0	1.2	0.5

*Substrates were applied to cell cultures of barley (lines A and D) and parsley on day 3 after transfer of cells into fresh medium. Percent of applied label in insoluble cell-wall polymers was determined after 5 days of incubation. $^{3}H/^{14}C$ ratios are means of 4–8 determinations with maximal deviations indicated.

Although *O*-methylhordenine was not a substrate for polymerization reactions, the other known catabolic pathway, i.e. *N*-demethylation, was not enhanced. *N*,*O*-Dimethyltyramine was identified by TLC (solvent systems C, D and F) but the amounts were as low as those of *N*-methyltyramine from hordenine degradation and could not be quantified. The bulk of the recovered amines in ethanol extracts as well as in the medium always consisted of unchanged substrate.

Peroxidase assays

Since free phenolic hydroxyl groups rather than different side chains seemed to be essential for efficient polymerization, the phenolase (EC 1.14.18.1) and peroxidase (EC 1.11.1.7) contents of the cell cultures were investigated. Peroxidases are present in a lot of cell cultures and their effects on degradative pathways have been elucidated in a number of cases [7]. From the various substrates used for measurement of peroxidase activity, *o*-dianisidine was chosen because it was the most sensitive compound and gave a linear response in tests with commercially available horseradish peroxidase over a wide range of activity.

Similarly, a phenolase assay was established with mushroom tyrosinase as test enzyme and catechol as test substrate.

At different growth stages representing the start and the end of each of the incubation periods used in the feeding experiments, medium, crude extract and washed pellet were assayed for peroxidase and phenolase activity. Figure 2(A) shows that peroxidase activity was already detectable in the early growth stages but that its amount increased in all fractions during the growth cycle. There was still an increase in enzyme activity both in the crude extract and the pellet when fr. wt remained constant. Until day 8, the activity in the crude extract was higher than in the medium. The point where the activity in the medium became higher, roughly coincided with the end of the linear growth phase.

The assays for phenolase activity were negative throughout the whole of the growth cycle, even when the amount of protein in the test was increased 200-fold over that necessary for easy detection of peroxidase activity.

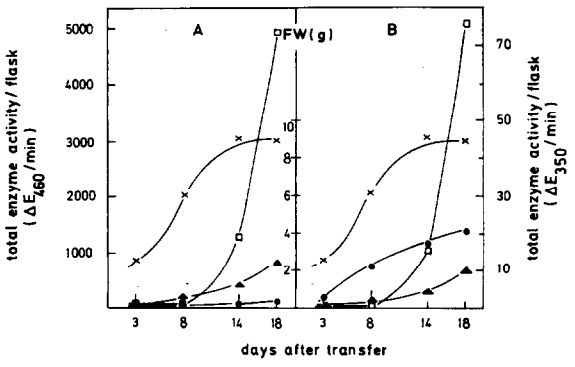

Fig. 2. Peroxidase activities in barley cell cultures. Peroxidase activities in medium (□), crude extract (▲) and pellet (●) fractions of barley line D cell cultures (cf. Experimental) are presented along with fr. wts (×). Substrates were: (A) *o*-dianisidine (0.83 mM, A_{460}); (B) hordenine (16.7 mM, A_{350}).

The activity of mushroom tyrosinase was not inhibited when added to cell extracts.

Since *o*-dianisidine is an artificial substrate, the question remained whether the enzyme activity with the physiological substrates hordenine and tyramine would follow the same pattern as found with the artificial substrate. Tyramine had earlier been shown to polymerize in the presence of horseradish peroxidase and hydrogen peroxide [6], but the rates had not been determined. A simple assay was developed where polymerization rates were followed by measuring the increase in *A* at 350 nm, and the data obtained with hordenine as substrate are plotted in Fig. 2(B). In the cases of crude extract and medium, the curves were almost identical to those found with *o*-dianisidine. Peroxidase activity in the pellet, however, was much more pronounced when hordenine was the substrate. It increased earlier and was always higher than the activity found in the crude extract. This cell-wall- or membrane-bound enzyme activity polymerized hordenine significantly better than *o*-dianisidine. It should be pointed out that this can, of course, only be said relative to crude extract and medium activities since the absolute values are not comparable because different chromophores were measured at different wavelengths, and since their identities were not known, a quantification was impossible. Nevertheless, it was possible to compare the relative rates of polymerization of the various substrates to *o*-dianisidine (Table 2). While phenylethylamines were very poor substrates for horseradish peroxidase, they were more readily accepted by cell culture preparations, especially the pellet, which polymerized dopamine 47-times and hordenine 633-times better than horseradish peroxidase, when amounts of enzyme were taken which gave identical *o*-dianisidine polymerization rates.

Two other assay techniques confirmed these results. [^{14}C]- or [^{3}H]phenylethylamines were incubated with crude extract or horseradish peroxidase for 30–60 min and the reactions stopped by the addition of either 4 M hydrochloric acid or borate buffer, pH 10. When the reaction was stopped with hydrochloric acid the complete incubation mixture was subjected to TLC in a system in which the substrate was clearly separated from polymeric material which usually remained at the origin. When the reaction was stopped by addition of borate buffer (0.5 M, pH 10) the mixture was extracted with 5 ml ethyl acetate, thus separating unreacted substrate from polymeric material which remained in the aqueous phase. Due to the long incubation times and the high conversion rates necessary for detection, these two assays were not linear, but they again clearly demonstrated that tyramine and hordenine, but not *O*-methylhordenine, were substrates

Table 2. Relative (%) polymerization rates *in vitro*

Substrate	Cell culture preparations: Crude extract + medium	Pellet	Horseradish peroxidase
o-Dianisidine	100	100	100
Catechol	22	—	13
Dopamine	41	149	3.2
Tyramine	1.9	—	0.18
Hordenine	1.1	39	0.06

for horseradish peroxidase and cell culture preparations and, based on *o*-dianisidine as standard, cell culture preparations were much more specific for phenylethylamines (Table 2, Fig. 2).

DISCUSSION

The results presented here clearly demonstrate that exogenously supplied phenylethylamines are predominantly polymerized to insoluble material by plant cell cultures. Though the highest incorporation rates were found during exponential growth, polymerization occurred at all growth stages investigated, but preferential incorporation of the methyl-carbon could not be confirmed.

The fact that *ca* 40% of the radioactivity from both [*N*-methyl-^{14}C, ^{3}H] and [β-^{14}C, *N*-methyl-^{3}H]hordenine was incorporated into insoluble material with unchanged $^{3}H/^{14}C$ ratios (Table 1) can only be interpreted as incorporation of the complete, undegraded side chain of the hordenine molecule. If the methyl- and the β-carbon were incorporated, subsequently, via different routes, the $^{3}H/^{14}C$ ratios should have differed at some point in the 5 day incubation period and would not have given the same incorporation rates as methyl-labelled hordenine alone. Though higher incorporation rates were observed in all cultures, the results agree with the early investigations on hordenine metabolism in plants [3], but they are in sharp contrast to a $^{14}CO_2$ release of up to 90% from the labelled α-carbon as described in ref. [4]. Under different experimental conditions, the same authors found significant degradation of [β-^{14}C]hordenine to *p*-hydroxybenzoic acid, which they proposed was then incorporated into lignin [5]. However, the recovery of reasonable amounts of hordenine and tyramine in these experiments did not agree with the described release of 90% of the α-carbon as CO_2 [4].

The latter finding might be explained by the lack of controls for microbial contamination of the plants and growth system used, an omission which was earlier shown to give misleading results [14]. Similarly, an accidental microbial contamination rapidly produced $^{14}CO_2$ from [β-^{14}C]hordenine while uninfected cell cultures did not release any significant amounts of $^{14}CO_2$ [6].

Production of *p*-hydroxybenzoic acid from [β-^{14}C]hordenine [5] had been demonstrated in barley cell cultures [6], but only as the minor route of hordenine catabolism. The present results confirm that both direct incorporation of the hordenine molecule as well as incorporation of all phenolic pathway intermediates can be performed by barley cell cultures and, probably, by plants too.

How hordenine and related phenylethylamines are attached to lignin or other cell-wall components cannot yet be decided. Since cell cultures of parsley polymerize hordenine as well, acceptance of phenylethylamines for lignification processes does not seem to be specific for the hordenine-producing barley cultures.

On the other hand, the incorporation of [*N*-methyl-^{14}C]nicotine into polymeric material was not found in barley and parsley cell cultures [Meyer, E., unpublished]. Therefore, nicotine metabolism in tobacco cultures might proceed in a different way. The finding of CO_2 release and polymerization at the same time and the lack of polymerization with [2′-^{14}C]nicotine [9] mitigate against polymerization of intact nicotine molecules or at least larger fragments, as has now been demonstrated for hordenine.

Peroxidase activity has often been described in cell cultures [15], and its excretion into the medium was observed as well [7, 16] as its localization in cell-walls [17, 18]. Even during the early growth stages peroxidase activity was calculated to be already high enough to polymerize most of the phenylethylamines fed. This might be one explanation why polymerization rates were not higher during the late growth stages when peroxidase activity was still increasing (Fig. 2). When different enzyme sources were compared with their reaction with a number of substrates, it was shown (Table 2) that peroxidase activity with the highest specificity for phenylethylamines was located in the pellet fraction and that it differed very significantly from horseradish peroxidase, and also from peroxidase activity in crude extracts or the medium when artifical and native substrates were compared.

Figure 2(B) shows the kinetics of peroxidase activity with hordenine as the substrate and the predominant role of pellet activity up to day 14 is obvious. Only then is the medium activity high enough to compete, but it might only lead to the formation of soluble polymers. This would explain the higher rates of hordenine incorporation during exponential growth when hordenine specific activity in the medium is almost zero.

Therefore, it can be concluded that part of the applied hordenine is probably (co)polymerized during uptake by a cell-wall located, rather specific peroxidase while the part of the substrate, which remains unchanged, is stored in a compartment where peroxidases are absent, e.g. the vacuole. Upon excretion into the medium more hordenine can be (co)polymerized when it crosses the cell-wall. Since this process is probably slower than uptake, the lower incorporation rates after the first 24 hr can be explained (Fig. 1). The absence of polyphenol oxidase activity from the cell cultures investigated might be an effect of the growth conditions used, as has been discussed in ref. [19].

To what extent (co)polymerization is due to the exogenous application of hordenine cannot be decided, though disposal of exogenous compounds as copolymerates of lignin has found increasing importance [20, 21]. It is possible, however, that endogenously synthesized hordenine is catabolized via a different pathway when it disappears from barley seedlings within 30 days after germination [1, 2]. Then, demethylation, deamination, etc. [6] might become more important.

EXPERIMENTAL

Materials. Labelled compounds were obtained from Amersham Buchler, Braunschweig ([β-^{14}C]tyramine, [*N*-methyl-^{14}C]nicotine), New England Nuclear [^{3}H]formaldehyde) and Commissariat a l'Energie Atomique, Gif-sur-Yvette, France ([^{14}C]formaldehyde) [β-^{14}C]Dopamine was generously supplied by Dr. Brian Ellis, University of Guelph, Canada. *N*-Methyltyramine was a gift from Hoffmann-La Roche, Basel. Other compounds were commercial products.

Application of labelled substrates to cell cultures. Barley cell suspension cultures were newly started from the same strain of callus cultures as described previously [6].

Parsley and tobacco cell cultures were all grown in B5-medium [11] but with different hormone composition: parsley with 1 mg/l. 2,4-D; *Nicotiana sylvestris* with 0.5 mg/l. 2,4-D, 0.5 mg/l. NAA, 0.5 mg/l. IAA and 0.2 mg/l. kinetin; and *Nicotiana tabacum* with 1 mg/l. 2,4-D and 1 mg/l. kinetin.

Addition of substrate soln (2×10^{-5} M, usually containing 0.5 μCi ^{14}C) to the medium was carried out as described in ref. [6]. At the end of the incubation period, cells were separated from the medium by filtration, homogenized and exhaustively extracted with several portions of hot 80% EtOH. When metabolites were to be identified, the EtOH extract was evaporated and phenolic amines extracted with Et_2O from the aq. residue at pH 10–11. The insoluble residue was freeze-dried and powdered for combustion analysis of incorporated radioactive label.

Syntheses. [β-^{14}C]Hordenine, [*N*-methyl-^{14}C]hordenine and [*N*-methyl-^{3}H]hordenine were synthesized according to refs. [6, 12]. For synthesis of [*N*-methyl-^{3}H]hordenine, 2.74 mg tyramine base (2×10^{-5} mol), 1.8 mg $NaHCO_3$ and 20 μl HCOOH (85%) were stirred with 5 μl formaldehyde (35%) and 12 μl [^{3}H]formaldehyde (1%, 333 μCi) (altogether 6×10^{-5} mol) in 2 ml dimethylformamide at 180° in an oil bath under reflux for 3 hr. 40 ml 0.2 M borate buffer, pH 10, was added prior to extraction with 6 × 50 ml EtOAc. The concd extract was subsequently chromatographed in solvent systems A–C on Si gel plates. Hordenine bands were eluted with MeOH. After this purification, a radiochemically pure product free of other UV detectable compounds was obtained with sp. act. 11.9 ± 0.5 mCi/mmol in a yield of 60% as judged by both recovery of radioactivity and UV spectra. For application to cell cultures MeOH was removed and hordenine redissolved in diluted HCl.

O-[*N*-methyl-^{14}C]Methylhordenine was synthesized in a similar way using *O*-methyltyramine and [^{14}C]formaldehyde. TLC was performed in solvent systems D–F. Yield was 45% and sp. act. 0.85 mCi/mmol.

Determination of radioactivity. Radioactivity was measured by LSC with internal standardization (toluene standard, NEN), especially in double labelling expts. TLC plates were scanned for radioactivity on a TLC-scanner (Berthold, Wildbad).

Cell residues were burnt in O_2 in a combustion apparatus (Berthold, Wildbad) and $^{14}CO_2$ and $^{3}H_2O$ absorbed in 1 ml ethanolamine. The procedure was standardized using strips of [^{3}H]- or [^{14}C]methylmethacrylate (New England Nuclear).

Chromatography. Solvent systems for TLC on Si gel were: (A) Et_2O–MeOH–NH_3 (15:4:1, prep.; 15:2:1, analytical); (B) MeOH–HOAc (4:1); (C) EtOH–ethylmethylketone–NH_3 (12:10:3); (D) BuOH–NH_3 (4:1); (E) BuOH–HOAc–H_2O (4:1:1); and (F) Et_2O–Me_2CO–MeOH–NH_3 (9:8:2:1).

Precoated plates (Merck) were used for analytical purposes while 0.5 mm preparative glass plates were prepared with Si gel HR (Merck).

Enzyme assays. Cells were thoroughly ground in double amounts of 0.1 M citrate–Pi buffer, pH 6.8, with quartz sand. Centrifugation at 30000 *g* yielded a clear supernatant, the 'crude extract', and the 'pellet' which was resuspended in *ca* three vols. of buffer and centrifuged again. The washed 'pellet' was suspended in buffer up to a total vol. of 10 ml. For some assays this suspension was diluted 1:10.

Tyrosinase assays were performed in a total vol. of 3 ml 0.1 M citrate–Pi buffer, pH 6.8, with 1 mM catechol as substrate. 50 μl of a soln of commercial mushroom tyrosinase (0.1 mg/ml) was used to check the assay, when ΔA was recorded at 460 nm and 25°.

The standard assay for peroxidase was adapted from ref. [13]: enzyme soln [medium, crude extract or soln of horseradish peroxidase (0.01 mg/ml) and 0.83 mM o-dianisidine (or 16.7 mM catechol, dopamine, tyramine or hordenine)] were mixed with 0.1 M citrate–Pi buffer, pH 6.8, and preincubated at 25° to check for tyrosinase activity and/or endogeneous H_2O_2. Then the assay was started by the addition of H_2O_2 to a final concn of 13.3 mM in 3 ml and ΔA was recorded at 460 nm (*o*-dianisidine, catechol, dopamine) or 350 nm (tyramine, hordenine).

When pellet activity was assayed, the enzyme soln was replaced by an aliquot of the pellet suspension and the complete mixture shaken in a 25° water bath for 3 min. The pellet solids were quickly removed by filtration and A of the clear soln of the chromophore measured after exactly 4 min. From this ΔA/min was calculated.

Other assays were performed in a total vol. of 100 μl with 30 mM H_2O_2, 25 mM tyramine or dopamine, hordenine or *O*-methylhordenine which included amounts of radioactive label. These mixtures were incubated with either crude extract or horseradish peroxidase soln (0.1 mg/ml) for 30–60 min at 25°.

When the reaction was stopped with 25 μl 4 M HCl, the complete mixtures were subjected to prep. TLC in solvent systems E (dopamine) or C (other substrates). Peak areas of the scanner diagrams were calculated by the triangulation method and compared to those of incubation mixtures without H_2O_2. When peroxidase activity was present, the peaks corresponding to the amine substrate decreased in favour of non-identifiable material remaining at the origin.

When identical reaction mixtures were stopped by the addition of 300 μl 0.5 M borate buffer, pH 10, followed by immediate extraction with 5 ml EtOAc, only unreacted amine was recovered in the organic phase, while polymeric material remained in the aq. phase. After this was proven by TLC, peroxidase activity was determined by measuring the decrease of radioactivity in the organic phase compared to identical incubation mixtures without H_2O_2.

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