Oxidative Decarboxylation of *para*-Hydroxybenzoic Acids by Peroxidases under *in vivo* and *in vitro* Conditions

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Decarboxylation, *p*-Hydroxybenzoic Acids, Peroxidases, Plant Cell Suspension Cultures

Oxidative decarboxylation of *p*-hydroxybenzoic acids in plant cell suspension cultures is catalyzed by peroxidases. This reaction has been characterized *in vivo* and *in vitro*. Decarboxylation of substituted benzoic acids yields monomeric, dimeric and oligomeric benzoquinones. All peroxidases obtained from soybean *(Glycine max)* cell suspension cultures by gel electrophoresis are equally capable to decarboxylate *p*-hydroxybenzoic acids as indicated by their rather low differences in specific activity for various benzoic acids.

Oxidative decarboxylation of *p*-hydroxybenzoic acids has repeatly been observed in higher plants as part of the biosynthesis of hydroquinone [1], methoxylated phenols [2] and ubiquinone [3]. Using plant cell suspension cultures several alternative pathways for the metabolism of hydroxy- and methoxybenzoic acids have recently been determined, such as ring-fission [4, 5], *para*-O-demethylation [6], hydroxylation [7], glycosidation [8], esterfication [8] and decarboxylation [4, 6]. In these suspension cultures, as in rat caecal microflora [9] and aseptically grown wheat seedlings [10], benzoic acids bearing an hydroxyl group *para* to the carboxyl were most actively decarboxylated to a very high extent. The expected phenolic decarboxylation products, however, could not be isolated from the cells [6]. Experiments have now been carried out to characterize this decarboxylation reaction of benzoic acids in cell suspension cultures. We furthermore report on the enzyme responsible for the rapid decarboxylation of benzoic acids and provide evidence for its oxidative nature.

Experimental

Materials

[COOH – ^{14}C]- and [CH_3 – ^{14}C]*p*-hydroxybenzoic acids were kindly supplied by Drs. K. Haider and H. Harms, Forschungsanstalt für Landwirtschaft, Braunschweig. Horseradish peroxidase and tyrosinase were purchased from Roth, Karlsruhe and Serva, Heidelberg, respectively.

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Methods

The methods applied in the *in vivo* experiments are described in earlier communications from this laboratory such as growing of the cell suspension cultures [6], inoculation of labelled compounds [4], $^{14}CO_2$ trapping and counting [4], anaerobic cell cultures [11].

Peroxidase assay:

a. The standard *o*-dianisidine (benzidine, guajacol) assay contained in a final volume of 3 ml: 50 µl *o*-dianisidine (0.05 M), 50 µl H_2O_2 (0.8 M), 10 – 100 µl enzyme, sodium acetate buffer (0.1 M; pH 5.4) to 3 ml. Linear increase of the extinction was measured at 460 nm in a Zeiss PM 4 spectral photometer. Enzyme activity was expressed as ΔE/min/ml.

b. 50 µl [COOH – ^{14}C]*p*-hydroxybenzoic acid (5000 – 50000 dpm) dissolved in ethylene glycol monomethylether, 50 µl H_2O_2 (0.8 M), 10 – 100 µl enzyme, sodium acetate buffer (0.05 M; pH 5.0) to 2.0 ml. The reaction was stopped by adding 0.5 ml H_2SO_4 (2 N). The decarboxylation rate was determined by either one of the two methods. 1. The reaction was performed in an injection vial and $^{14}CO_2$ was absorbed on strips of filter paper (placed in a small test tube) wetted with ethanolamin : ethylene glycol monomethylether (1 : 2 v/v)). The strips were counted in a toluene cocktail. 2. The enzyme reaction was performed in open test tubes and the extent of decarboxylation measured by the decrease of radioactivity in the assay mixture

Some of the results were presented at two conferences:
1. Gemeinsame Herbsttagung der Biochemischen Gesellschaft der Bundesrepublik Deutschland, der Schweiz und Österreich, Innsbruck 1973 (Hoppe-Seyler's Z. Phys. Chemie **354**, 1170 [1973]).
2. Tagung der Gesellschaft für Arzneipflanzenforschung, Tübingen 1974 (Planta Medica, Supplement, 1975).

(counted in a dioxane cocktail). The absorption method (1) is more accurate so that small differences in radioactivity can well be determined.

Enzyme purification

One part (fresh weight) of cells was homogenized with half its weight of phosphate buffer (0.2 M; pH 7.5) in an Ultraturrax at 4 °C and centrifuged for 10 min at 20000 × *g*. To the supernatant (= crude extract of Table I) solid $(NH_4)_2SO_4$ was added to 80% saturation. After stirring for 30 min the precipitate was collected by centrifugation at 20000 × *g*. The precipitate was dissolved in the required buffer and desalted on a Sephadex G 25 column. Small samples of protein for electrophoresis were desalted in a small syringe by Sephadex G 25 centrifugation method. If necessary the extracts were concentrated by filtration through a Diaflo-Ultrafiltration Cell (Amicon). The extracts were then chromatographed on a Sephadex G 200 column (18 × 900 mm) with sodium acetate buffer (0.1 M; pH 6.0). The eluates were tested for peroxidase activity by a spot test with syringaldazine/H_2O_2 [12]. For quantitative determinations the *o*-dianisidine-test was used. The main peak was concentrated, dialysed against phosphate buffer (0.01 M; pH 7.0), absorbed on a DEAE-column (20 × 500 mm) and eluted with a linear gradient of phosphate buffer (0.01 – 0.5 M; pH 7.0). Detection of peroxidase activity was again by *o*-dianisidine and syringaldazine/H_2O_2. Three peaks were obtained. The second peak (gradient 0.1 – 0.12 M) is called "soybean peroxidase" in Table I.

Isoenzyme preparation

The Sephadex G 25 extracts were taken as source of enzyme.

a. Polyacrylamide disc gel electrophoresis was performed in a BioRad electrophoresis cell modell 151 with tubes of 9 mm OD and 125 mm length using the systems of Davis for separation at pH 9.5 and of Reisfeld at pH 3.8 [13].

b. Isoelectric focusing on polyacrylamide gels were performed in the same apparatus as under a. using the chemical polymerization system of Macko and Stegemann [13]. The pH gradient was build up before the samples (100 µl) were layered into the gels under a protecting ampholine layer. The pH gradient was measured with an Ingold microelectrode.

Staining for peroxidases: 0.2 g *o*-dianisidine (benzidine, guajacol) was dissolved in 9 ml methanol and 1 ml H_2O_2 (3%). The gels were dipped into this solution until coloured bands appeared. Corresponding zones of unstained gels were cut out, homogenized in phosphate buffer (0.1 M; pH 7.5) and squeezed through a small cheese cloth. The resulting solutions were quantitatively tested for peroxidase and decarboxylation activity. For one band all measured activity values were set to 100% activity and the relative activities of all other bands calculated in such a way that the *o*-dianisidine values of all bands were also set to 100%.

Products

2,6-dimethoxybenzoquinone was synthesized from syringic acid according to the method of Walter [14]: 0.3 g syringic acid was dissolved in 8 ml methanol and a solution of 1.5 g potassium dinitrosodisulfonate in 60 ml H_2O and 2 ml 1 N sodium acetate was added under shaking. After 2 h the reaction mixture was extracted with 5 × 20 ml ether. The ether was dried over anhydrous Na_2SO_4 and evaporated. [$CH_3 - {}^{14}C$]2,6-dimethoxybenzoquinone from [$CH_3 - {}^{14}C$]syringic acid a product of peroxidase catalyzed reaction was co-chromatographed on polyamide plates in the systems a. chloroform : methylethylketone : methanol 60 : 26 : 14 and b. methanol : H_2O 50 : 50, on kieselgel G plates in the systems c. toluene : formic acid ethylester : formic acid 5 : 4 : 1, d. toluene : chloroform : acetone 40 : 25 : 35 and e. benzene : ethylacetate : methanol : petrolether 6 : 4 - 1 : 3. In all systems the R_F-values of both synthetic and the enzyme product were identical, and in all cases the same specific radioactivity was measured. UV-absorption spectra of the enzyme product and the synthetic product are identical showing maxima in methanol at 285 nm and 378 nm.

Results

Characterization of decarboxylation in vivo

We first compared the rate of decarboxylation of [$COOH - {}^{14}C$]vanillic acid under aerobic and anaerobic conditions by cell suspension cultures of soybean (Fig. 1).

Under aerobic conditions approximately 50% decarboxylation of vanillic acid could be observed within 60 min. Under anaerobic conditions, however, this reaction was almost completely inhibited. In these anaerobically kept cultures decarboxylation could again be initiated by the addition of H_2O_2. This provided evidence for peroxidases being involved in this reaction. On the other hand, the decarboxylation of vanillic acid under aerobic conditions could not be inhibited by the addition of

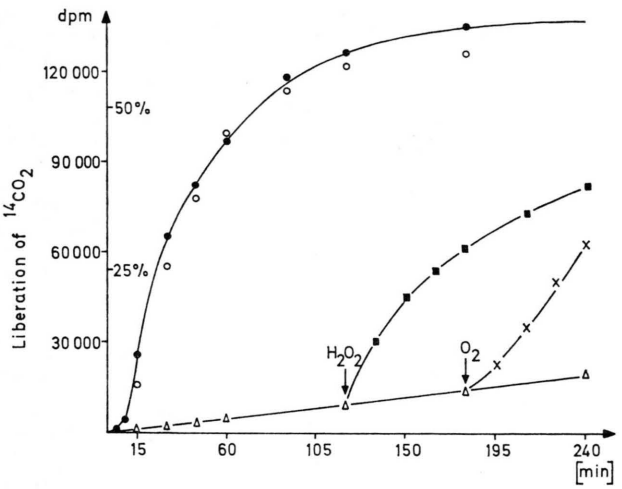

Fig. 1. Rate of decarboxylation of [COOH-^{14}C]vanillic acid under various conditions: aerobic conditions, (●—●—●); aerobic conditions plus catalase (5×10^{-7} M), (○—○—○); anaerobic conditions, (△—△—△); anaerobic conditions plus H_2O_2, (■—■—■); after changing from anaerobic to aerobic conditions, (×—×—×).

catalase to the medium. When anaerobic cultures were flushed with air decarboxylation quickly resumed, showing rapid synthesis of hydrogen peroxide by these cultures.

The very rapid beginning of decarboxylation of *p*-hydroxybenzoic acids, almost immediately after inoculation of substrate into the cell cultures [4, 6], and the aforementioned participation of peroxidases led us to the assumption that peroxidases released from the cells into the medium were responsible for this rapid reaction. Thus, transport of substrate into the cells is not necessary. It is well-known that plant suspension cultures release enzymes [15] including peroxidases [16] into their medium. Determination of the activity of peroxidases in the medium and in the cells at the beginning of our feeding experiments showed that the ratio of peroxidases in the cells to peroxidases in the medium ran up to 30 – 40 : 1. Although the absolute amount of peroxidase activity in the medium seems to be low compared with the peroxidases in the cells, it might well be sufficient for the decarboxylation of the administered vanillic acid before it is taken up by the cells. We therefore compared the rates of decarboxylation of soybean cell cultures in their original growth medium, of cell cultures washed several times with fresh medium and of medium in which cells had been grown before (Fig. 2). This experiment indicated that cell cultures in their original growth medium decarboxylated vanillic acid more quickly than the washed cell cultures and that the growth medium must have contributed to the extent of decarboxylation. It can be calculated from these results that approximately 50% of the acid were decarboxylated by peroxidases present in the medium and the other 50% by the cells. Although the amount of peroxidases in the medium should be sufficient to decarboxylate

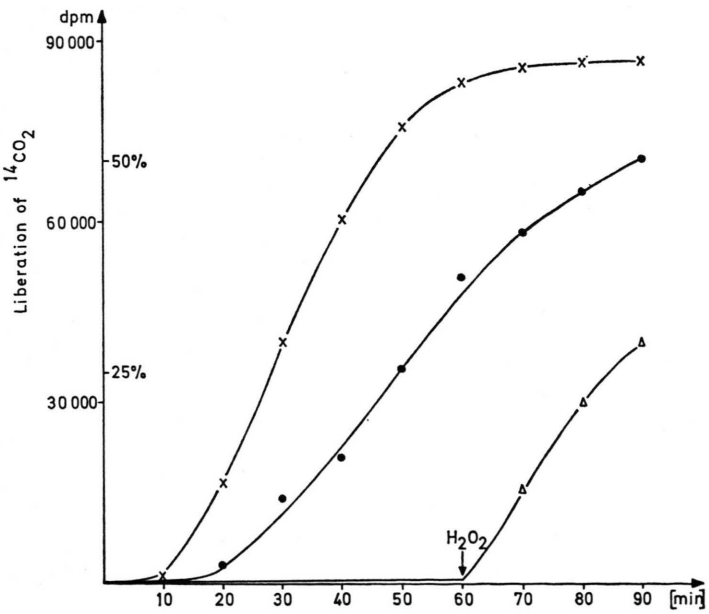

Fig. 2. Rate of decarboxylation of [COOH-^{14}C]vanillic acid by soybean cell suspension cultures (administered radioactivity 140 000 dpm): cell cultures with growth medium, (×—×—×); washed cultures with fresh medium, (●—●—●); growth medium without cells, (△—△—△).

all of the administered vanillic acid, a substantial amount is still taken up by the cells. This implies that the H_2O_2 concentration in the growth medium is the limiting factor for decarboxylation outside of the cells. The results further suggest that H_2O_2 released from the cells into the medium is not accumulated but constantly being destroyed. This is indicated by the observation that the $^{14}CO_2$-production in both the H_2O_2-enriched, cell free original growth and in cell cultures with their growth medium is identical.

Characterization of decarboxylation in vitro

Decarboxylation of *p*-hydroxybenzoic acids was found in cell suspension cultures of mungbean and soybean [4]. Crude protein extracts of these cultures are still able to decarboxylate such compounds at a low rate (Table I).

Table I. Characterization of decarboxylation as peroxidase catalyzed reaction.

Protein	Decarboxylation of vanillic acid [COOH-^{14}C]	
	$-H_2O_2$	$+H_2O_2$
Crude extract (Soybean)	+	+++
Crude extract (Mungbean)	+	+++
Sephadex-G-25 extract (Soybean)	−	+++
Horseradish peroxidase	−	+++
Soybean peroxidase	−	+++
Tyrosinase	−	−
Medium + Fe^{2+} (1 mM)	−	−
+ Fe^{3+} (1 mM)	−	−

After addition of H_2O_2 to the extracts, however, an high increase in $^{14}CO_2$-production is noticed. After ammonium sulfat precipitation and Sephadex-G 25-filtration decarboxylating activity of the protein preparation is only found after the addition of H_2O_2. Purification of the soybean peroxidases by Sephadex-G 200-chromatography and DEAE-ion exchange chromatography led to several fractions with peroxidase activity (detected by syringaldazine or *o*-dianisidine/H_2O_2). All these fractions were able to decarboxylate *p*-hydroxybenzoic acids. As expected from these results, peroxidases obtained from other cell cultures and commercial horseradish peroxidase were also able to decarboxylate these compounds. Tyrosinase showed no decarboxylating activity, but laccase can be expected to possess such activity [17, 18]. H_2O_2 without enzyme or medium containing either Fe^{2+} or Fe^{3+} ions (1 mM each) did not decarboxylate under our experimental conditions.

Fig. 3 summarizes experiments designed to measure the dependence of decarboxylation activity on the concentration of H_2O_2. The best rate of decarboxylation is achieved at a 1:1 ratio of *p*-hydroxybenzoic acid to H_2O_2. Excess peroxide inhibits decarboxylation as has been noted with other peroxidase-catalyzed reaction [17], because peroxidase itself is oxidized to oxyenzyme (Fe^{6+}), a sluggish electron acceptor [19]. In agreement with our results it has already been shown [17] that at lower concentrations of H_2O_2 (lower than the 1 : 1 ratio) an higher conversion of substrate than expected from

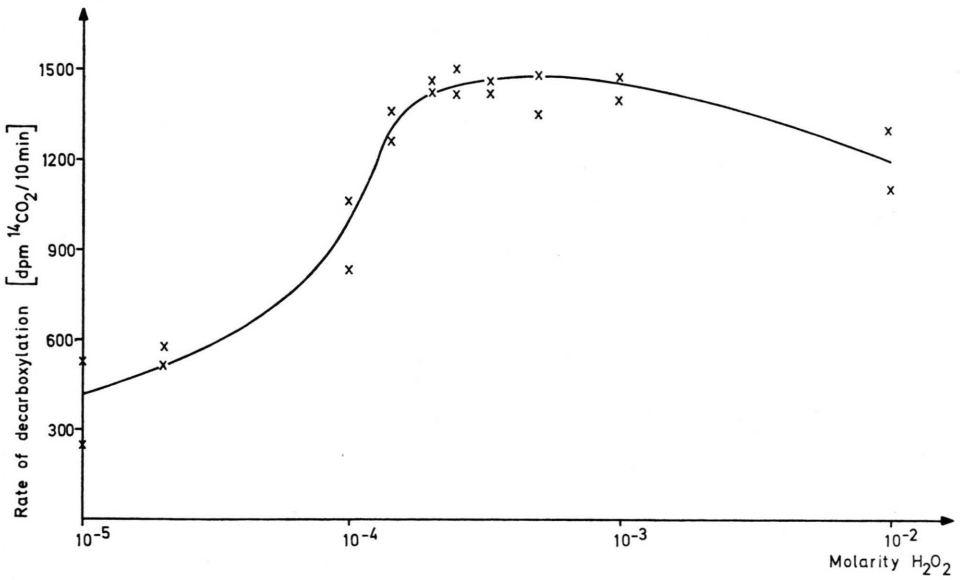

Fig. 3. Dependence of decarboxylation on H_2O_2 concentration; concentration of vanillic acid: 5×10^{-4} M.

the stoichiometry can be obtained in a peroxidase catalyzed reaction. This seems to be due to oxygen dissolved in the enzyme preparation [17].

The pH optimum of the decarboxylation reaction was found between pH 4.5 and 5.5. At pH 7.0 a very small rate of decarboxylation was measured (Fig. 4).

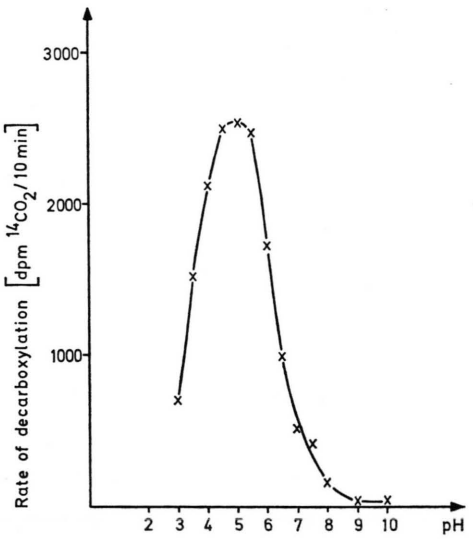

Fig. 4. Dependence of decarboxylation on pH value.

The effect of various compounds on the rate of decarboxylation is shown in Table II. The decarboxylating activity was not altered by EDTA, sodium diethyldithiocarbamate, mercury dichloride and α,α′-di-pyridil. As expected various well-known peroxidase inhibitors, namely mercaptoethanol, benzoquinone, sodium azide, potassium cyanide and catalase all strongly decreased the decarboxylation reaction. Compounds inhibiting the *o*-dianisidine test for peroxidase activity are also inhibitors of the decarboxylation reaction. However not all of these inhibitors, effective under *in vitro* conditions, are also active *in vivo*. Ascorbic acid (100 mM), for example, as well as catalase do not inhibit decarboxylation *in vivo* (Fig. 1). On the other hand, sodium diethyldithiocarbamate, without effect *in vitro*, strongly inhibits decarboxylation *in vivo*, although benzoic acids are still taken up by the cells (Berlin, unpublished).

Earlier experiments [4, 6] had shown that only benzoic acids with a free *para*-hydroxyl group are substrates for decarboxylation by cell suspension cultures. In Table III we compare the substitution pattern of suitable substrates for decarboxylation under *in vivo* and *in vitro* conditions and present the relative reaction velocities. It is evident from the data that the isolated peroxidases of soybean cell cultures show exactly the substrate specifity ex-

Table III. Substrate specifity of decarboxylation reaction under *in vivo* and *in vitro* conditions.

Substrate-[COOH-^{14}C] 0.1 mM	Rate of decarboxylation (percent of total radioactivity in CO_2)		Recovered substrate
	in vivo/ 3 h	*in vitro*/ 10 min	*in vitro*/ 10 min
Benzoic acid	0	0	100
p-Hydroxybenzoic acid	8,5	8	85
Salicylic acid	0	0	100
Protocatechuic acid	37	35	0
Vanillic acid	75	68	4
Syringic acid	75	72	22
Anisic acid	0	0	100
3,4,5-Trimethoxybenzoic acid	0	0	100

Table II. Influence of various compounds on enzymatic activity.

Compound added	Concentration [mol/l]	Relative activity [%]	Compound added	Concentration [mol/l]	Relative activity [%]
None	—	100	α,α-Dipyridil	10^{-3}	100
Benzoquinone	10^{-6}	100	EDTA	10^{-3}	100
	5×10^{-5}	39	Mercury dichloride	10^{-3}	100
Hydroquinone	10^{-6}	100	Sodium diethyldithiocarbamate	10^{-3}	100
	10^{-5}	12	Catalase	5×10^{-7}	0
Mercaptoethanol	10^{-6}	89			
	10^{-3}	72			
	5×10^{-5}	21			
Ascorbic acid	10^{-6}	100			
	10^{-5}	0			
Sodium azide	10^{-6}	100			
	5×10^{-5}	50			
	10^{-4}	10			

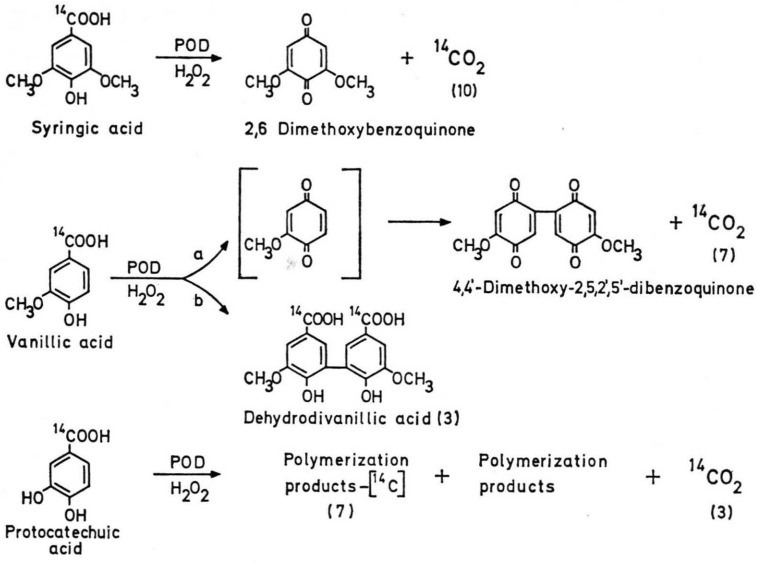

Fig. 5. Proposed pathways for the reaction of peroxidases with syringic, vanillic and protocatechuic acid.

pected from our *in vivo* experiments. Furthermore the relative reaction velocities are highly comparable.

As mentioned above, products of the decarboxylation reaction are not detectable in the cell cultures. We have, therefore, tried to isolate products formed in the decarboxylation reaction in cell free experiments. Thus, syringic acid was quantitatively decarboxylated by peroxidase treatment and the only product – besides some red polymeric material – turned out to be 2,6-dimethoxybenzoquinone as characterized by TLC and UV-absorption spectra (Fig. 5). This is not surprising because it has already been shown that the peroxidative elimination of side chains in syringly compounds leads to the monomeric benzoquinone [17, 18]. Like other benzoquinones [17, 20] this compound inhibits the decarboxylation reaction, so the rate of decarboxylation depends on the starting concentration of substrate. Such benzoquinones are not further converted by peroxidase/H_2O_2.

The highest yield of $^{14}CO_2$ from [COOH – ^{14}C]-vanillic acid ever obtained with purified enzyme ran up to 68% of total radioactivity, though only small amounts of vanillic acid could be reisolated from the reaction mixture. This indicates that peroxidases not only decarboxylate vanillic acid but also convert it to dimeric and oligomeric material (benzoquinones) with or without the carboxyl group still being present. The monomeric 2-methoxybenzoquinone has never been found. We have not tried to identify any compounds from the complex reaction mixture obtained with vanillic acid, but we can exclude (co-chromatography) the expected 3,3′-dimethoxydiphenyl-2,5,2′,5′-benzoquinone * and 4,4′-dimethoxydiphenyl-2,5,2′,5′-benzoquinone * as significant reaction products of the decarboxylation of vanillic acid. On the other hand, vanillin, the equivalent aldehyd of vanillic acid, which is also metabolized by peroxidases yields dehydrodivanillin as the main reaction product [21]. The two related compounds vanillic acid and vanillin are thus metabolized by peroxidases to very different compounds.

The complexity of peroxidase catalyzed reactions is also shown in the case of protocatechuic acid. While only 35% of radioactivity of [COOH – ^{14}C]-protocatechuic acid is released as $^{14}CO_2$, protocatechuic acid is quantitatively converted. As indicated by TLC various (unidentified) oligomeric and polymeric compounds are formed. Since it is well-known that peroxidase also exhibits phenoloxidase activity [22], our findings are best interpreted by the assumption that in the presence of H_2O_2 the "phenolase activity" is much more pronounced than the "decarboxylating activity". Peroxidase, therefore, exhibits not only a broad substrate specifity, but can also catalyze different reactions with the same substrate molecule.

Numerous reactions have been reported for peroxidases from higher plants acting on rather dif-

* Both compounds are kindly supplied by Dr. H. Harms, FAL, Braunschweig.

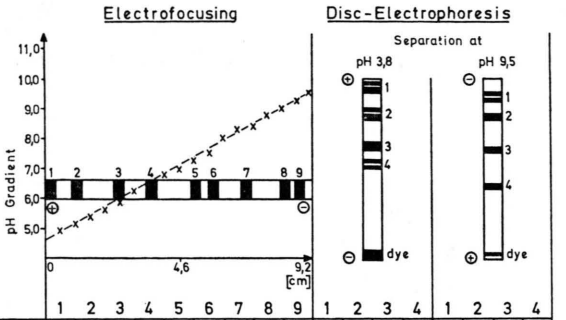

Fig. 6. Separation of soybean peroxidase isoenzymes by isoelectric focusing and gel-electrophoresis on polyacrylamide and catalytic activity of the eluted bands with various substrates (see Results and Experimental).

	1	2	3	4	5	6	7	8	9	1	2	3	4	1	2	3	4
o-Dianisidine	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
ΔE/ml	3,65	0,95	1,10	1,40	1,25	1,90	2,40	17,5	16,5	0,39	7,25	3,88	0,31	5,90	2,10	2,20	2,55
Benzidine	100	129	44	117	147	107	75	48	46	89	96	74	55	55	54	46	50
ΔE/ml	0,90																
Guajacol	100	96	83	87	100	114	100	83	67	49	136	136	93	103	72	73	69
ΔE/ml	0,48																
Decarboxylation	100	91	86	90	60	121	102	63	74	71	73	100	79	96	84	87	81
cpm/2 min	380																

ferent molecules. Despite the multiplicity of data the physiological function of peroxidases still remains unknown. A new approach to eludicate the physiological functions of peroxidases is the isolation of individual isoenzymes and determination of their substrate specifity [23]. We have therefore separated the peroxidase isoenzymes of soybean cell suspension cultures by polyacrylamide gel electrophoresis and polyacrylamide isoelectric focusing (Fig. 6). The isoenzymes obtained were eluted from the gels and the preparations tested for their activity to oxidize *o*-dianisidine, benzidine, guajacol and vanillic acid. To obtain comparable values on a relative scale we set the measured values for all substrates to 100% for one isoenzyme, set the measured values of *o*-dianisidine for all isoenzymes 100% and calculated the measured values of the other substrates in relation to 100% of the *o*-dianisidine activity. The values given in Fig. 6 show that all isoenzymes are capable of decarboxylating vanillic acid and of oxidizing the other typical substrates for peroxidases. The relative differences in specific activity of the isoenzymes in the decarboxylation reaction of vanillic acid are not sufficient to asign a physiological function with regard to this reaction to anyone of the isoenzymes. The capability to catalyze the oxidative decarboxylation of *p*-hydroxybenzoic acids rather seems to be a common feature of all peroxidase isoenzymes.

Discussion

Decarboxylation of exogenously administered *p*-hydroxybenzoic acids in plants [10] and plant cell suspension cultures is catalyzed by peroxidases. To the numerous reactions carried out by peroxidases a new one is thus added. *p*-Hydroxyl substituents seem to be of special importance for a number of peroxidase catalyzed reactions because flavonols [24], flavanones [25, 26], chalcones and aurones [26] all with a free *para*-hydroxyl group in the B-ring are substrates of peroxidases. Elimination of the carboxyl-group of aromatic acids by peroxidases has not been described hitherto, though it is known that both syringic and vanillic acid stimulate the amounts of peroxidases in the fungus *Inonotus radiatus* [27]. That provides indirect evidence for a conversion of syringic and vanillic acid by peroxidases.

Steelink and coworkers [17, 18] demonstrated that syringic compounds with different side chains all produce 2,6-dimethoxybenzoquinone as endproduct of a peroxidase catalyzed reaction. The mechanism of the cleavage reaction is not fully understood. Our results support the assumption that the different syringyl compounds are converted by peroxidases to 2,6-dimethoxybenzoquinone with syringic acid as intermediate. This assumption seems to be questioned if one compares the different products of the peroxidase catalyzed conversion of vanillic acid

and vanillin. While vanillic acid is rapidly decarboxylated to approximately 70% yielding a complex mixture of oligomeric benzoquinones with and without carboxyl groups, the aldehyd group of vanillin is retained because dehydrodivanillin is described as main product [21]. Dimerization of syringyl compounds obviously not occurs, so that peroxidase can oxidize the postulated intermediate [17] (with an α-keto group in the side chain) to the carboxyl group. On the other hand vanillyl compounds are dimerized more quickly than oxidation of the α-keto group to a carboxyl group can occur. Carbonyl groups of dimeric and oligomeric compounds are eliminated by peroxidases with more difficulty [18]. In the special case of vanillic acid decarboxylation occurs more quickly than dimerization and the following reactions.

Our results are in accord with other authors [18], who have demonstrated that syringyl compounds are degraded to 2,6-dimethoxybenzoquinone, while guajacol-like compounds are metabolized to higher molecular weight material undergoing preferential *o,o'*-coupling. Peroxidase-catalyzed decarboxylation of *p*-hydroxybenzoic acids is most likely not involved in the catabolism of aromatic acids, where secondary plant constituents are funnelled into primary cell metabolism. Ring fission of protocatechuic acid for example must be catalyzed by another enzyme. Whether 2,6-dimethoxybenzoquinone is further degraded remains to be elucidated. Feeding experiments with [$CH_3-{}^{14}C$]syringic acid, however, suggest that polymerization is more likely to occur because most of the radioactivity was incorporated into insoluble plant material [10].

The physiological functions of peroxidases in plants are still not clearly known although much work has been done in this field. Even the participation of peroxidases in lignin biosynthesis is controversial [29, 30]. Main reason for the difficulty to eludicate physiological functions must be seen in the fact that peroxidases tend to react with an endless number of physiological and unphysiological substrates. It is therefore difficult to decide whether the described decarboxylation reaction fulfills a physiological function. Recent feeding experiments have shown that flavonols, flavonones, chalcones, aurones (for some of these compounds turnover in plants has been demonstrated [31]) as well as *p*-hydroxybenzoic acids and cinnamic acids are degraded by peroxidases [32]. Such results should however be interpreted with special care, because peroxidases released into the medium and/or peroxidases in the cell walls are probably the first enzymes to act on exogenously administered substrates. This may well represent an unphysiological situation. At present it is difficult to decide whether endogeneous substrates localized in compartments will also get into contact with peroxidases. The participation of peroxidases in the regulation of phenolic and growth hormon metabolism has been postulated [33].

Physiological functions of peroxidases may be found by determination of the substrate specifity of individual isoenzymes [23]. Our results do not support this approach because all isoenzymes show comparable catalytic activity in the decarboxylation reaction. Similar results have been reported for the peroxidase isoenzymes of *Cicer arietinum* in connection with flavonol metabolism [24]. On the other hand only one isoenzyme of tobacco cell suspension cultures was reported to react with scopoletin [34], while two others are specific for ferulic acid oxidation [23]. The varying composition of brown pigments in different senescent tobacco leaves is supposed to result from the catalytic heterogeneity of peroxidase isoenzymes [35]. Some peroxidase isoenzymes also possess a significantly higher specific activity for IAA degradation [36]. Whether such demonstrated specifities of peroxidase isoenzymes are sufficient to postulate physiological functions must be proved by other methods.

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