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EFFECTS OF CINNAMIC ACID DERIVATIVES ON INDOLE ACETIC ACID OXIDATION BY PEROXIDASE*

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Key Word Index—Indole acetic acid; horseradish peroxidase; phenolic derivatives; caffeic acid; ferulic acid.

Abstract—The influence of cinnamic acid derivatives on the H_2O_2-independent oxidation of indole acetic acid by horseradish peroxidase was examined. Cinnamic acid derivatives show a sharp increase from a slight stimulation of the oxidative reaction to a complete inhibition in a very narrow concentration range. This threshold effect occurs not only for the diphenols caffeic and dihydrocaffeic acids but also for the monophenols ferulic and sinapic acids. The latter phenols are not demethylated to caffeic acid by horseradish peroxidase during the reaction. They are both inhibitors on their own account. We, therefore, conclude that the inhibition is not only due to the radical scavenging properties of the inhibitors; direct interactions between the inhibitors and peroxidase also play a role in the unusual behaviour of the inhibitors.

INTRODUCTION

The H_2O_2-independent oxidation of indole acetic acid (IAA) by horseradish peroxidase (HRP) is thought to be involved in the regulation of plant development as an antagonist of ethylene as well as in the oxidative degradation of IAA. The major products of this oxidation are 3-hydroxymethyl oxindole, 3-methylene oxindole, indole-3-methanol and superoxide [1, 2]. Scatole-3-methylene and other C-centered radicals have been identified as intermediates of the reaction sequences [3]. H_2O_2 which is also generated enables the HRP–H_2O_2-catalysed integration of IAA into tRNA [4], in the course of which the above mentioned intermediates bind covalently to certain cellular nucleophils. As a consequence, these new reactive products are able to react with glutathione, DNA or unsaturated fatty acids, thus leading directly to damage, mutagenesis or wound healing on surfaces by connecting the radical intermediates [5]. The mechanism of H_2O_2-independent oxidation of IAA by HRP is completely different from the typical H_2O_2-driven peroxidative cycle of IAA as outlined in Scheme 1.

Autoxidation of IAA· leads to other radicals, hydroperoxides and singlet oxygen and links up to the peroxidative degradation of IAA with H_2O_2 as the electron acceptor, especially under high concentrations of HRP and IAA. Until now, the physiological significance and the inhibitory or stimulatory mechanisms(s) of this oxidation have been incompletely understood. There is no doubt about the catalytic role of the HRP-compound III and the radical mechanism of this reaction. In general, monophenols are known to stimulate the IAA oxidation, whereas diphenols are thought to be inhibitors [6–8]. For the mechanism, direct interaction and competition between the enzyme, the inhibitor or stimulator and IAA have been discussed, as well as the functioning of the inhibitors as radical scavengers. But it has also been shown that different results concerning stimulation or inhibition can be due to different concentrations of the tested phenolic derivative. Low concentrations of ferulic acid or *p*-coumaric acid seem to possess cofactor functions whereas high concentrations show an inhibitory behaviour [7]. Recently, the threshold effect of caffeic acid has been published [9] ascribing the inhibitory effect of caffeic acid to the scavenging properties of this diphenolic cinnamic acid derivative. Because of our results, not only for caffeic acid, but also for other cinnamic acid derivatives and the bee glue propolis [10], we do not agree with the assumption that phenolic derivatives function purely as radical scavengers. There must also be direct structural interactions with the horseradish peroxidase.

RESULTS AND DISCUSSION

Whereas monophenols are said to stimulate IAA oxidation, diphenols are well known inhibitors. In the past, most experiments were kinetic measurements of this reaction [2, 11, 12] which prevented the recognition of the threshold effect of some inhibitors. We firstly saw this effect with different extracts of propolis [10] and tried to elucidate this phenomenon. Therefore, we tested different concentrations of several phenolic derivatives.

*Dedicated to Prof. Dr. Dres. G. C. Hubert Ziegler on occasion of his 70th birthday.

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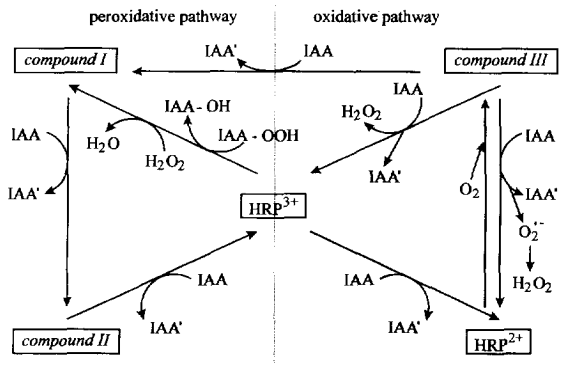

Scheme 1. Oxidative and peroxidative pathways of IAA oxidation by horseradish peroxidase.

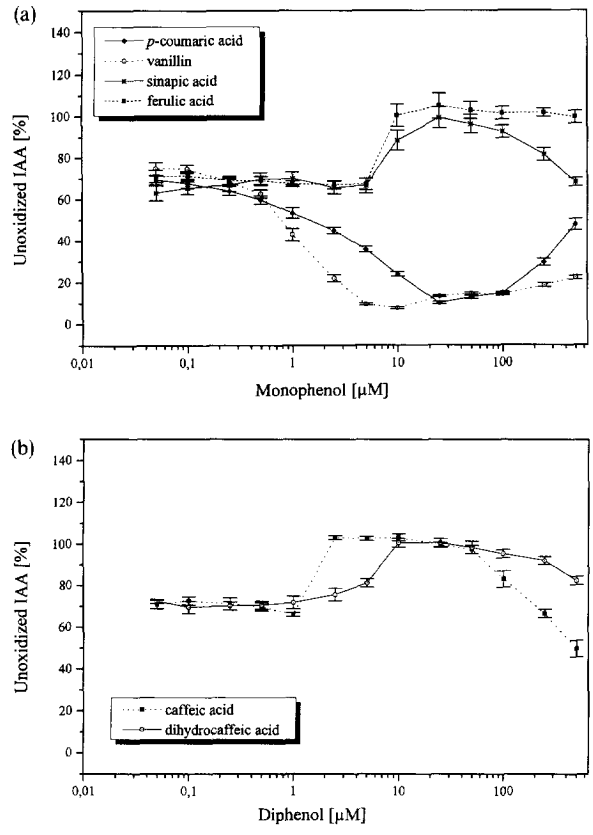

Fig. 1. Amount of non-oxidized IAA (%) after an incubation for 30 min at 37 ° with 0.3 U HRP and different concentrations of monophenols (Fig. 1a) or diphenols (Fig. 1b). The 100% values of 0.3 mM IAA range between 0.65 and 0.75 *A*. The decrease of some curves at higher concentrations of the phenolic derivative is due to the chemical reaction between this phenolic derivative and the IAA.

Chemical reaction

First it should be mentioned that a fast chemical reaction occurs between IAA and phenolic derivatives, especially caffeic acid. This chemical reaction, which seems to lower the inhibitory effect, is noticeable as a drop of the inhibitory curves at higher concentrations of the inhibitor (above 100 μM).

Effects of single phenolic derivatives

For different concentrations of cinnamic acid we can confirm the report [7] which says that derivatives without a phenolic group have no influence on the oxidation of IAA by HRP (data not shown). The monophenols vanillin and *p*-coumaric acid stimulated the oxidation of IAA as was expected. In this reaction, vanillin is a better cofactor than *p*-coumaric acid (Fig. 1a). In contrast, the monophenol ferulic acid did not stimulate the reaction (Fig. 1a) but showed the typical threshold effect observed with the diphenols caffeic acid and dihydrocaffeic acid (Fig. 1b). After an incubation time of 30 min, the turning point from a slight stimulation to a complete inhibition was situated between 9 and 10 μM for ferulic acid, between 2 and 2.5 μM for caffeic acid and between 5 and 10 μM for dihydrocaffeic acid. The inhibitory curve for sinapic acid also showed a sharp increase, which was not as steep as for the other phenolic derivatives (Fig. 1a). In contrast, both the cinnamic acid and the monophenolic isoferulic acid had no influence on the oxidation of IAA (data not shown).

Combined effects

Different combinations of monophenols and diphenols are presented in Fig. 2. The combination of the cofactor vanillin with the inhibitor caffeic acid (Fig. 2a) or ferulic acid (Fig. 2b) increased the threshold concentrations of both inhibitors. After an incubation time of 30 min, the reaction mixture with caffeic acid and vanillin indicated a change between 2.5 and 5 μM of each phenolic derivative, whereas the reaction mixture without vanillin and with only caffeic acid changed between 1 and 2.5 μM (Fig. 2a).

Combination of two inhibitors

An enhancing effect was observed with equal concentrations of two inhibitors as for example caffeic acid, ferulic acid or sinapic acid. After an incubation for 15 min ferulic acid had a turning point between 7.5 and 9 μM and caffeic acid between 1.8 and 2.1 μM. The combination of equal concentrations of both inhibitors in the reaction mixture led to a threshold concentration between 1.5 and 2 μM of each phenolic derivative. This result corresponded to a total concentration of both phenolics between 3 and 4 μM for the turning point, this clearly lying below the theoretic combination of the single threshold concentrations of both caffeic acid and ferulic acid (Fig. 3). Similar results were observed with the combination of ferulic acid and sinapic acid (data not shown).

Incubation time

After different incubation times (15 and 30 min), the turning point of the reaction with ferulic acid and caffeic acid were shifted to higher concentrations of the inhibitors. After 15 min, e.g. about 2.0 μM of caffeic acid

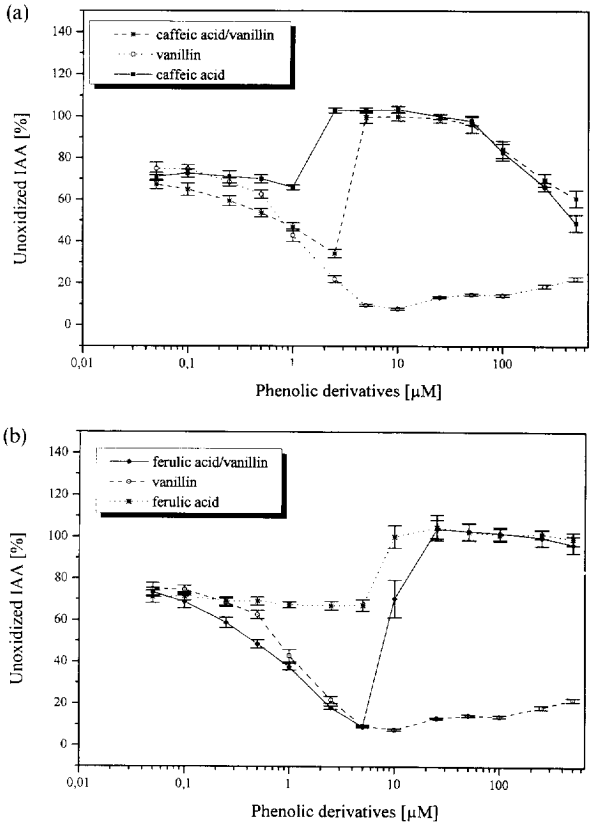

Fig. 2. Combined effects of a stimulating monophenol and an inhibiting monophenol or diphenol on the oxidation of IAA by HRP after 30 min at 37 °. The phenolic derivatives have equal concentrations in the reaction mixture. The initial amount of non-oxidized IAA is 0.3 mM and has an extinction of 0.70 ±0.05 *A*.

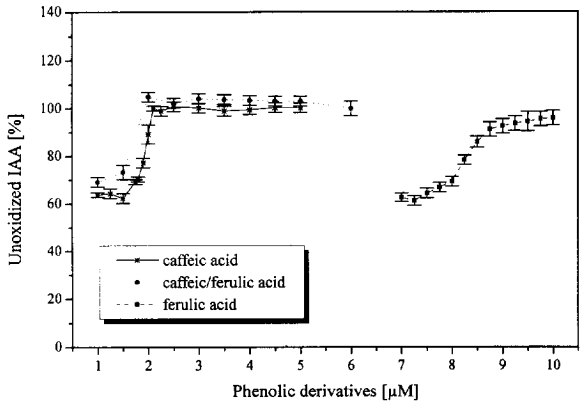

Fig. 3. Enhancing effect with the combination of ferulic acid and caffeic acid after an incubation for 15 min at 37 ° with 0.3 U HRP and 0.3 mM IAA. 100% of IAA corresponds to an extinction of 0.65 ± 0.03 *A*.

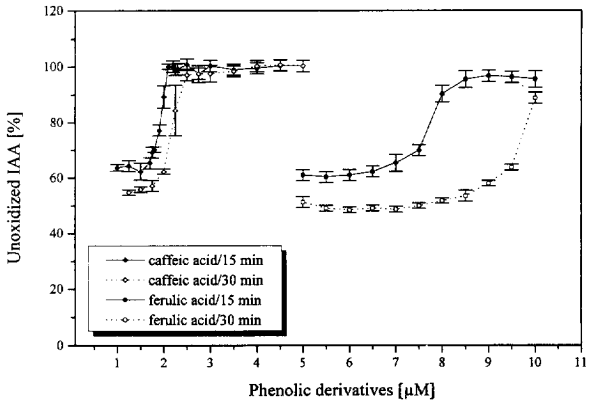

Fig. 4. Shifting of the threshold-concentration of caffeic acid or ferulic acid in relation to the incubation time. The 100% values for IAA ranges between 0.65 and 0.70 *A*.

correspond to the 'threshold-concentration', whereas after 30 min about 2.25 μM were needed (Fig. 4).

An early study concerning IAA oxidation and phenolic derivatives ascribed inhibitory effects to scavenging of intermediates and stimulatory effects of promotion of compound III formation as rate limiting steps [13]. The inhibitory mechanism was also thought to be based on competition between IAA and inhibitor for compound III leading to an inactive inhibitor–enzyme complex [11]. Lee and Chapman [12] mentioned for the first time slow inhibitor oxidation during a lag phase to about 50% before the initiation of IAA oxidation, and fast degradation of the resting inhibitor, showing the role of compounds I and II for inhibitor degradation. IAA initiates formation of compound III as the key derivative for compound I and II which in turn control oxidation of both IAA and inhibitor [14, 15]. The higher affinity of the inhibitor toward different forms of HRP compared with IAA seems to be the main inhibitory mechanism, Krylov and coworkers [9] rejected all hypotheses about direct interactions, ascribing inhibitory mechanisms only to scavenger capacities of the inhibitor. Threshold effects, inhibitor degradation, lag phase shifting due to pipetting sequence and variations of concentrations were all considered [9, 16]. However, we do not believe that phenolic inhibitors function merely as scavengers, even if most of our results as well as most of the publications on this field seem to agree with this inhibition theory. Some of our results also indicate that direct interactions between the inhibitors, IAA and the HRP exist.

One confirmation for this suggestion is the fact that a certain structure of inhibitor seems to be necessary for maximal inhibitory capacity. Thus, caffeic acid seems to possess the ideal configuration followed in its inhibitory efficacy by ferulic acid, dihydrocaffeic acid and sinapic acid. Further supporting evidence for this observation comes also from experiments with different extracts of bee glue propolis. The ethanolic extract of propolis is almost as effective in scavenging free radicals as the aqueous extract in all the tested biochemical model systems [10, 17]. In contrast, the aqueous extract is without exception a five- to 10-fold better inhibitor of different enzymes like

diaphorase, lipoxygenase, xanthine oxidase or myeloperoxidase than the ethanolic extract. Testing IAA oxidation by HRP, the ethanolic extract behaves in the same manner as in all the other tested enzymatic assays. This means that the ethanolic extract has a turning point which is 10-fold higher than that of the aqueous extract, thus indicating the role of direct structural interactions with HRP.

EXPERIMENTAL

Assay of IAA oxidase activity. H_2O_2-Independent oxidation of IAA by HRP was measured according to ref. [18]. The reaction mixture (1 ml) contained 0.3 mM IAA, different concns of phenolic derivatives (0.05–500 μM) dissolved in 10 mM NaOH or in 2 vol% EtOH and 150 mM Na citrate-Pi buffer (pH 5.6). The enzymatic reaction was initiated by 0.3 U HRP (1 unit produces an increase in A (ΔA_{436}) of 1 per min at pH 7 and 25°, calculated from the initial reaction rate using guaiacol as substrate). After an incubation for 15 min or 30 min at 37°, the non-oxidized IAA was complexed by the addition of 4 ml of a $FeCl_3$–H_2SO_4 reagent consisting of 15 ml 0.5 M $FeCl_3$, 500 ml H_2O and 300 ml 36 M H_2SO_4. The unstable red chelate dye was quantified photometrically after 14 min (530 nm) at the point of maximal dye intensity.

Assay of HPLC. HPLC was performed using a Beckman 342 Gradient LC System with detection at 280 nm. The ODS-hypersil column (250 mm × 4.8 mm i.d., particle size 5 μM) had a temp. of 40°. The mobile phases used were Na-Pi buffer (50 mM, pH 2) and MeOH (HPLC grade) with a flow rate of 1 ml min^{-1}. The gradient programme started at 0% (v/v) MeOH and 100% (v/v) Na-Pi buffer. It increased for the first 5 min to 25% (v/v) MeOH and then rose to 35% (v/v) MeOH during the following 15 min. After another 3 min 50 vol% MeOH was reached and held constant for 5 min. At the end of the programme, the MeOH concn was lowered to 0% (v/v) during the last 3 min. With this programme the phenolic derivatives can be separated from IAA and from each other. The solns of IAA, HRP and the phenolic derivatives were concd as depicted above for the enzymatic assay. All chemicals were of the highest grade of purity available. HRP was purchased from Boehringer (Mannheim), the other chemicals were obtained from Sigma (Deisenhofen), Merck (Darmstadt) or Roth (Karlsruhe).

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