Oxidation of Indole-3-Acetic Acid-Amino Acid Conjugates by Horseradish Peroxidase

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ABSTRACT
The stability of 21 amino acid conjugates of indole-3-acetic acid (IAA) toward horseradish peroxidase (HRP) was studied. The IAA conjugates of Arg, Ile, Leu, Tyr, and Val were oxidized readily by peroxidase. Those of Ala, β-Ala, Asp, Cys, Gin, Glu, Gly, and Lys were not degraded and their recovery was above 92% after 1 hour incubation with HRP. A correlation between the stability of IAA conjugates toward peroxidase-catalyzed oxidation and the hydrophobicity of the amino acid moiety conjugated to IAA was demonstrated. Polar amino acid conjugates of IAA are more resistant to HRP-catalyzed oxidation.

IAA \(^1\) is the principal auxin found in the seeds and seedling shoots of higher plants (25). For IAA to function as a regulator, its own concentration in the target tissue must be controlled. One possible mechanism of such control is the formation of IAA conjugates referred to as bound auxin (6). Most of the IAA found in plant tissue is covalently linked to some other compounds (2, 6).

One class of IAA conjugates is the amide bound auxins. IAA-Asp was first reported as a metabolite of exogenous IAA in pea stem sections by Andreae and Good (1), and thereafter has been demonstrated in many species, both after feeding (15) and as a native compound (4, 21, 26, 29). IAA-Glu has also been isolated as a metabolite of exogenous IAA in some plants (22) and also as a native compound in cucumber shoot tissue (26), in etiolated pea shoots (20), and in soybean seeds (7, 21). IAA-Asp and IAA-Glu are the two amide bound auxins now known to occur naturally in plants.

The metabolic significance of chemical conjugation of IAA is not well known. IAA conjugates may act as reserve forms for the homeostatic control of IAA concentrations (5) and may function in auxin transport within the plant (6). It has frequently been suggested that they may be the products of detoxification reactions in which exogenously supplied auxins are conjugated to inactivate them biologically (1).

Cohen and Bandurski (5) suggested the protection of IAA against oxidation by peroxidase (EC 1.11.1.7) as a possible function of conjugation. This hypothesis was supported by the fact that all of the IAA conjugates tested were resistant to peroxidase-catalyzed oxidation (14). But the IAA conjugates tested were limited to IAA-β-glucosyl, IAA-Asp, IAA-Gly, IAA-β-Ala, IAA-L-Ala, and IAA-β-Ala. Whether the other amino acid conjugates would also be resistant to peroxidase-catalyzed oxidation has not been examined. The enzymic oxidation of IAA by plant peroxidase, which can act as an IAA oxidase (11, 14), has been proposed to be a major degradative pathway for the hormone in vivo (32). Thus, it will be important for understanding the regulation of IAA levels in plants to intensively study whether or not the amino acid conjugated IAA can be degraded by peroxidase.

In this study we show that HRP can degrade both IAA and its amide conjugates to varying degrees. In addition, we demonstrate that the stability of IAA conjugates toward peroxidase-catalyzed oxidation has a relationship to the hydrophobicity of the amino acid moiety conjugated to IAA.

MATERIALS AND METHODS

Synthesis of IAA-Amino Acid Conjugates. The amino acid conjugates of IAA were synthesized by the method of Mollan et al. (17). All the amino acids used were L-form except Gly. The β-Ala, Arg, Asn, Gin, Glu, Gly, Ile, Leu, Met, Phe, Tyr, and Val conjugates of IAA were crystallized by evaporation under reduced pressure. The other conjugates of Ala, Asp, Cys, cystine(Cys'), His, Hyp, Lys, Ser, and Thr were purified by silica gel TLC of the n-butanol extract fraction in isopropanol-ammonia-water (8:1:1, v/v/v). The desired product was eluted from the adsorbent with 80% ethanol and subsequently evaporated to dryness. The yields from this procedure were estimated at 10 to 30%. The purity of the products was tested, and less than 0.5% amount of IAA-p-nitrophenyl ester and IAA was detected by silica gel TLC and HPLC as impurities. HPLC was performed in a Waters model APC/GPC 244 with UV detector set at 280 nm. A reverse-phase C\(_{18}\) Bondapak column (300 × 3.9 mm i.d.) was used in methanol-sodium acetate buffer (0.02 M, pH 4.0) (1:10, v/v).

Oxidation of IAA and IAA-Amino Acid Conjugates by HRP. The reaction mixture contained 0.125 \(\mu\)g/ml HRP (type I, Sigma Chemical Co.), 0.1 mM 2,4-dichlorophenol (Tokyo Chemical Industry Co., Japan), 0.1 mM MnCl\(_2\), 50 mM sodium phosphate buffer (pH 6.0), and IAA or IAA conjugates as indicated. The final volume of the mixture was 4.0 ml. The reaction mixture was incubated in the dark in a water bath (30°C) for 60 min. One ml of the reaction mixture was transferred into a test tube containing 2.5 ml of Salkowski reagent (10), vortexed thoroughly and allowed to develop for 60 min in the dark. Absorbance at 535 nm was measured using a Pye-Unicam SP8-400 UV/visible spectrophotometer. Residual IAA or IAA conjugate was determined against a standard curve.

RESULTS AND DISCUSSION
IAA can be oxidized by plant peroxidase and this oxidation results in spectral changes (23). The oxidation products of IAA by HRP were thoroughly studied by Hinman and Lang (14),

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\(^1\) Abbreviations: IAA, indole-3-acetic acid; HRP, horseradish peroxidase.
who found that the main product was 3-methyleneoxindole in the absence of added hydrogen peroxide. Intact pea seedlings and their extracts can convert IAA to 3-hydroxy-methyloxindole (30) and this compound can be enzymically dehydrated to 3-methyleneoxindole in extracts of wheat seedlings and wheat germ (3).

Figure 1 shows marked spectral changes in the reaction mixtures containing IAA and IAA-Leu but little spectral change in the mixture containing IAA-Gly after 60 min incubation. The UV absorption spectra of IAA and IAA-Leu changed under the experimental condition to that of 3-methyleneoxindole which has a characteristic peak at 253 nm (14, 27). These spectral changes in IAA and the IAA-Leu conjugate resulted from HRP-catalyzed oxidation (5). It can be supposed from the result that HRP can degrade IAA and also IAA conjugates depending upon the amino acid moiety (see below). The results obtained differ from that of Cohen and Bandurski (5), who found that HRP is unable to catalyze the oxidation of IAA when IAA is conjugated either by an ester bond to inositol or by an amide linkage to an amino acid.

Figure 2 shows the patterns for the oxidation of IAA and IAA conjugates by HRP with time. IAA was rapidly degraded and IAA-Val was also degraded as rapidly as IAA. The small absorbance at 535 nm for IAA after 1 h is probably due to degradation product(s). IAA-Glu was not oxidized. The susceptibility of IAA conjugates to oxidation thus depends upon the amino acid moiety.

Which IAA conjugates are susceptible to HRP-catalyzed oxidation and which ones are not? Table I indicates that IAA can be rapidly degraded by HRP and that the oxidation of IAA conjugates depends upon the amino acid moiety of the conjugates. Recovery of IAA conjugates ranged from 15 to 102% after the reaction mixtures were incubated with HRP while IAA was recovered at 6.7%. The IAA conjugates of Arg, Ile, Leu, Tyr, and Val were oxidized extensively under the conditions employed, and their recovery was below 31%. But the IAA conjugates of Ala, β-Ala, Asp, Cys, Glu, Gly, and Lys were not oxidized. Their recovery was above 92%. The recovery of other conjugates ranged from 52 to 82%. Inactivity of the HRP toward Gly, Asp, Ala, and β-Ala conjugates of IAA was first reported by Cohen and Bandurski (5). Our results (Table I) are consistent with their report. This is the first report on the other 17 IAA-amino acid conjugates.

IAA-Asp and IAA-Glu, which have been identified as naturally occurring IAA conjugates, were not degraded by HRP. Feung et al. (8) showed that treatment of cultured tissue of *Parthenocissus tricuspidata* with IAA resulted in the formation of IAA-Ala, IAA-Asp, IAA-Glu, IAA-Gly, and IAA-Val. IAA-Lys has also been isolated from certain strains of *Pseudomonas savastanoi* (16). Of these conjugates, IAA-Val was most susceptible to HRP degradation. It is not clear why IAA-Val which is most susceptible conjugate to HRP oxidation can be isolated while the other conjugates which are less susceptible than IAA-Val to HRP oxidation are not isolated when plant tissues are pretreated with IAA. Vijayaraghavan and Pengelly (31) once suggested that the enzymes of conjugate synthesis are less specific for particular amino acids than the enzymes of conjugate hydrolysis. It is possible that the other conjugates can occur but may not be identified because of the unavailability of, or failure to use, satisfactory techniques.

Hargarter and Good (12) have shown that the hydrolysis of amide conjugates of IAA is specific for amino acid moiety. In the case of an amide conjugate very resistant to HRP, it might be broken down by a hydrolase to free IAA which might enter a noncarboxylative pathway (18, 24) or the IAA oxidase pathway (11, 14). The biological activity of conjugates would be expected to reflect such specificity. There has been considerable variation in the reported biological activities of the amino acid conjugates.

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**Figure 1.** Absorption spectra of the reaction mixtures before and after incubation with HRP. The reaction mixtures contained 0.125 μg/ml HRP, 0.1 mM 2,4-dichlorophenol, 0.1 mM MnCl₂, and 0.2 mM IAA (1), 0.2 mM IAA-Leu (2), or 0.2 mM IAA-Gly (3) in 50 mM sodium phosphate buffer (pH 6.0). The spectra shown by B were taken 60 min after the reactions were begun and the spectra A at zero time.

**Figure 2.** Oxidation of IAA, IAA-Val, and IAA-Glu by HRP. The reaction mixture contained 0.125 μg/ml HRP, 0.1 mM 2,4-dichlorophenol, 0.1 mM MnCl₂, and 0.1 mM IAA, 0.1 mM IAA-Val, or 0.1 mM IAA-Glu in 50 mM sodium phosphate buffer (pH 6.0). Samples were taken several times during the reaction, as shown. Residual IAA and IAA conjugates were developed with Salkowski reagent and the absorbance was measured.
jugates might suggesting the gates less polar biological activities in active polarity of stability largely inactive the basic amino acids were nearly as those were effective in supporting callus growth. Feung et al. (9) reported that several amino acid conjugates of IAA were highly active in *Avena* coleoptile and soybean callus bioassays and that the highly active conjugates in both assays were those of Ala, Cys, Cys', Glu, Gly, Lys, and Met. Most of these showed high stability toward HRP in the present study. Interestingly, the basic amino acid conjugate IAA-Lys which is very active in bioassays (9) showed high stability, but IAA-Arg which is inactive showed very low stability toward HRP (Table I).

The different activities of the different conjugates may be largely due to the different susceptibilities of the various conjugates to the enzymes present in the plant tissues. The chemical properties of the conjugates may have some effect on their biological activities (12, 13). For instance, the more polar conjugates might be expected to behave in a different way from the less polar conjugates. No clear pattern of biological activity versus polarity has been reported.

Table I includes a comparison of IAA conjugate stability with that of IAA toward HRP-catalyzed oxidation. IAA-Val was nearly as labile as IAA itself. The hydrophobicity (19, 28) of the amino acid side chains can be considered as a possible factor affecting the degree of the oxidation of IAA-amino acid conjugates by HRP. Figure 3 shows the relationship between the stability of IAA-amino acid conjugates toward HRP and the hydrophobicity of amino acid moieties. Generally, polar amino acid conjugates of IAA are more resistant to HRP-catalyzed degradation, suggesting that the higher the hydrophobicity of an amino acid, the lower the stability of the IAA conjugate toward the enzyme. This is consistent with the suggestion that the active site of HRP includes tryptophan and tyrosine, rather hydrophobic amino acids, as well as a heme (33). A hydrophilic substrate should display less affinity than a hydrophobic substrate for an active site rich in hydrophobic amino acids. However, there is significant deviation from the generalization in the cases of IAA-Phe and IAA-Arg. The hydrophobic phenylalanine conjugate showed greater stability than expected, while the hydrophilic arginine conjugate showed lower stability. Factors other than hydrophobicity, such as steric hindrance and orientation of bound ligands, could possibly play a role in the interactions of IAA conjugates with peroxidase binding sites.

**Table I. Oxidation by HRP of IAA and IAA Conjugates Incubated for 1 h at 30°C**

The mixture contained 0.125 µg/ml HRP, 0.1 mM 2,4-dichlorophenol, 0.1 mM MnCl₂, and 50 mM sodium phosphate buffer (pH 6.0). IAA and IAA conjugates included as noted. Total volume was 4.0 ml and indole recovery was estimated by a colorimetric assay. The values given are the average of triplicates which agreed within ±4%.

<table>
<thead>
<tr>
<th>IAA Conjugates</th>
<th>Amount Added (nmol)</th>
<th>Recovery (%)</th>
<th>Oxidation (a)</th>
<th>Increase in Stability of IAA Conjugate (b)</th>
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<tbody>
<tr>
<td>Ala</td>
<td>542</td>
<td>97</td>
<td>3</td>
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<td>β-Ala</td>
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*a* 100 minus recovery percentage. *b* Recovery percentage of IAA conjugate minus recovery % of IAA.

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