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Communication

Carbohydrates Stimulate Ethylene Production in Tobacco Leaf Discs[1]

III. Stimulation of Enzymic Hydrolysis of Indole-3-Acetyl-L-Alanine

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ABSTRACT

The sucrose-stimulated *in vivo* hydrolysis of indole-3-acetyl-L-alanine (IAAla) in tobacco (*Nicotiana tabacum* L.) leaf discs was confirmed by *in vitro* analysis of an IAAla-hydrolyzing enzyme isolated from the same tissue. The enzymic activity could be stimulated by either aging of the tissue or by application of external IAA or sucrose. A combination of the above three treatments yielded maximal activity.

It is well established that IAA conjugates possess biological activity due to their slow hydrolysis to free IAA (2, 3, 5, 13, 18). While hydrolases responsible for hydrolysis of ester-linked IAA conjugates have been isolated from various plants (11, 12), an hydrolase responsible for hydrolysis of the amide-linked conjugate, IAAla,[2] was detected so far in extracts prepared from bush bean leaves (6) and from carrot cell cultures (7).

Recent studies of auxin metabolism in tobacco leaves (1, 17, 18) have provided evidence that sugars, particularly sucrose and galactose, are capable of stimulating the hydrolysis of naturally produced or exogenously applied IAA conjugates. The evidence for the stimulatory effect of sugars reported in these studies was indirect and based on measurements of increased ethylene induction by IAA conjugates, as well as on enhanced transformation of the conjugates during incubation (1, 18). The present study demonstrates that the stimulatory effect of sucrose on hydrolysis of IAAla in tobacco leaf discs results directly from increased activity of the IAAla-hydrolyzing enzyme.

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[2] Abbreviation: IAAla, indole-3-acetyl-L-alanine.

MATERIALS AND METHODS

Plant Material and Treatments

Tobacco (*Nicotiana tabacum* L. cv 'Xanthi') plants were grown in a greenhouse under LD conditions (18 h light) at 20 to 30°C. Leaves were washed and sterilized as described previously (18). Discs 2.5 cm in diameter were excised from fully expanded mature leaves and placed on filter paper in Petri dishes each containing 4.5 mL of 50 mM Na-phosphate buffer (pH 6.1). Where indicated, 50 mM sucrose and/or 0.1 mM IAA were included. The Petri dishes were incubated in darkness at 30°C for 2 or 3 d.

Synthesis of Indole-3-[1-^{14}C]Acetyl-L-Alanine

Labeled IAAla (370 MBq/mmol) was synthesized by the mixed anhydride procedure (23), as modified by Hangarter *et al.* (14). Separation of the resultant [1-^{14}C]IAAla from [1-^{14}C]IAA was carried out by TLC on silica gel GF-254 plates, using chloroform-methanol-acetic acid (70:25:5, v/v) as the solvent system. A single peak of [1-^{14}C]IAAla was obtained at R_F 0.56, while [1-^{14}C]IAA appeared at R_F 0.78. The peak of labeled IAAla was eluted with ethanol and rechromatographed in the same solvent system. The final yield of IAAla was approximately 25%.

Extraction and Assay of IAAla-Hydrolase Activity

The conjugate-hydrolyzing enzyme was extracted and assayed according to Cohen and Bialek (6), with slight modifications. Tobacco leaf discs (3 or 5 g), preincubated in sucrose and/or IAA, were hand-homogenized with 10 or 15 mL, respectively, of 10 mM Mes buffer (pH 6.0), containing 0.5 mM PMSF and 10 mM DTE. The extract was filtered through Miracloth and was centrifuged at 12,000*g* for 30 min. The crude enzyme supernatant was precipitated by $(NH_4)_2SO_4$ at 75% of saturation. The pellet obtained after centrifugation at 10,000*g* for 20 min was resuspended in 5 mL of Mes-NaOH buffer (pH 6.0), containing 0.5 mM PMSF, 10 mM DTE, and 0.1% Triton X-100, and this was employed as the enzyme solution. Protein levels in the extract were estimated by the

method of Bradford (4), using Coomassie blue G-250 (Sigma) reagent.

The activity assay was carried out with 2 mL of the enzyme preparation, incubated at 37°C with 2 nmol (74 Bq) of [1-^{14}C]IAAla for 1 h. The reaction was terminated by acidifying the reaction mixture to pH 2.5 with HCl and the auxins were then extracted with 2 mL ethyl acetate. The ethyl acetate fraction was dried *in vacuo* and resuspended in 0.5 mL of 80% (v/v) ethanol. Aliquots of this solution were fractionated by TLC on silica gel GF-254 plates, using chloroform-methanol-acetic acid (70:25:5, v/v) as the solvent system. Authentic IAA (Sigma) and IAAla (a gift from Dr. J. D. Cohen) were added to each chromatogram. Radioactivity in the IAAla and in the IAA regions was determined by liquid scintillation counting. The amount of radiolabeled IAA released was calculated from the ratio of counts in the two regions. Radioactivity of boiled controls was subtracted in all cases. The enzyme activity was expressed as pmol IAA released from 1 nmol IAAla per mg protein during 1 h of incubation.

Assay of Auxin Activity

The auxin activity of various IAA-amino acid conjugates (a gift from Dr. J. D. Cohen) was estimated by measuring their effect on ethylene production in the presence of sucrose. Samples of eight tobacco leaf discs were placed in 50-mL flasks on a medium containing 0.1 mM of each of the IAA conjugates and 50 mM sucrose. The IAA conjugates were dissolved in 70% ethanol, and the final ethanol concentration in the medium did not exceed 0.5%. $Hg(ClO_4)_2$ and KOH solutions were introduced into the flasks for absorption of the evolving ethylene and CO_2, respectively (18). The flasks were sealed and incubated in darkness at 30°C. The ethylene absorbed during the indicated incubation periods was released and assayed by a Packard gas chromatograph as detailed previously (18).

RESULTS AND DISCUSSION

Amide-linked IAA conjugates differ greatly in their biological activity (5, 10, 14, 16). This is probably due to the different susceptibilities of the various conjugates to the hydrolytic enzyme(s) present in the tissues (6, 7, 11, 12). In order to determine the most active substrate for the enzymic assay, we have measured the biological activity of various amino acid conjugates in the presence of sucrose by following their capability to induce ethylene production in tobacco leaf discs. Inasmuch as ethylene production, which is considered an IAA-stimulated response in leaves, was related to the ability of the IAA conjugates to undergo hydrolysis (1, 13, 14, 16), it could be taken as a measure of their biological auxin activity. Results depicted in Table I demonstrate that among the conjugates tested, IAAla showed the highest auxin activity, which exceeded that of free IAA and the other conjugates by several-folds. Therefore, IAAla was employed as the substrate in the *in vitro* hydrolase assay. These findings confirm previous reports in other test systems showing that IAAla was one of the most active IAA conjugates in exhibiting various auxin activities (3, 10, 14, 16). Cohen and Bialek (6) also used IAAla as a substrate, because of this reason.

Table I. *Biological Activity of Synthetic IAA-Amino Acid Conjugates as Measured by Ethylene Production in Tobacco Leaf Discs*

Leaf discs were incubated for 3 d with buffer, IAA, or IAA-amino acid conjugates at concentrations of 0.1 mM in a medium containing 50 mM sucrose, and their ethylene production was monitored daily. Results represent total ethylene accumulation during 3 d ± SE of three replicates.

Treatment	Ethylene Production	Stimulation
	$\sum$ *nL g^{-1} fresh wt*	%
Buffer	574 ± 84	100
IAA	2731 ± 117	476
IAAla	3910 ± 230	682
IAAsp[a]	905 ± 58	157
IAGly	1176 ± 48	205
IALeu	664 ± 112	116
IAThr	962 ± 201	167
IAPhe	641 ± 67	112

[a] IAAsp, indole-3-acetyl-DL-aspartic acid; IAGly, indole-3-acetyl-L-glycine; IALeu, indole-3-acetyl-L-leucine; IAThr, indole-3-acetyl-L-threonine; IAPhe, indole-3-acetyl-L-phenylalanine.

Table II. *Effect of Aging, Sucrose, and IAA on the Development of IAAla Hydrolase Activity in Tobacco Leaf Discs*

Enzyme was extracted from fresh leaf discs (3–5 g) or from discs aged by incubation with or without 50 mM sucrose and/or 0.1 mM IAA for 48 h, and assayed as described in "Materials and Methods." Results are the average of three different experiments ± SE.

Treatment	Incubation Time	IAAla Hydrolase Specific Activity
	h	*pmol IAA·mg^{-1} protein·h^{-1}*
None	0	1.08 ± 0.12 (100%)
Buffer	48	1.63 ± 0.06 (151%)
Sucrose	48	3.20 ± 0.29 (300%)
IAA	48	3.01 ± 0.17 (279%)
Sucrose + IAA	48	5.76 ± 0.34 (533%)

It was reported previously that the activity of synthetic IAAla, as measured by induced ethylene production, was greatly enhanced by sucrose in various leafy systems (1). This sucrose-stimulated biological activity of IAAla may stem from enhancement of its enzyme-catalyzed hydrolysis in the tissue (1, 3, 18). To test this possibility, a cell-free extract, capable of catalyzing the release of free IAA from synthetic [^{14}C]-IAAla, was prepared from tobacco leaf discs. Enzymic activity in fresh leaf discs was low, but it was increased by 50% when the discs had been aged for 48 h (Table II). A threefold increase in enzymic activity, as compared with fresh discs, was obtained with extracts prepared from discs which were preincubated with either sucrose or IAA for 48 h. A combined treatment of sucrose and IAA was the most effective, resulting in a more than fivefold enhancement of the enzyme activity after 48 h (Table II). These results confirm previous data (1, 18) showing that (a) IAAla, when applied alone, stimulated ethylene production only in 3- or 4-d-old discs, and (b) simultaneous application of sucrose plus IAAla to tobacco leaf discs caused a significant increase in ethylene production, which peaked at the second and third days of incubation. It seems therefore that the observed *in vitro* activity of IAAla

(Table II) bears relevance to its *in vivo* hydrolysis (1, 18), since both activities were maximal under the same conditions. Hence, the auxin activity of IAAla, as demonstrated by ethylene production (Table I; 18), can be attributed to increased activity of IAAla hydrolase.

The results indicate that tobacco leaf discs contain an IAAla hydrolyzing enzyme, the activity of which is stimulated either by aging of the tissue or by external IAA or sucrose. The combination of these three factors is required for exhibition of maximal enzymic activity (Table II). The stimulatory effect of IAA on enzyme activity may occur through substrate induction, while the enhancing effect of sucrose is still obscure. However, the fact that enzymic activity was further stimulated by sucrose, supports the postulated hypothesis that endogenous carbohydrates can control IAA levels in plant tissues, thereby affecting IAA-mediated processes (1).

The physiological significance of the sucrose effect in stimulating the hydrolysis of IAA conjugates is not yet clear. It was found that the level of soluble carbohydrates increased in the early stages of senescence (15, 20, 21), while that of insoluble carbohydrates declined (15). This initial rise in soluble carbohydrates may induce increased activities of IAA conjugates-hydrolyzing enzymes, thereby increasing the levels of free IAA, which in turn stimulate ethylene formation. Indeed, an increase in free IAA levels was found during senescence of tobacco (9), cotton (8), oat (19), and bean (22) leaves. Moreover, in bean leaves, the increase in free IAA levels was accompanied by a decrease in the level of IAA conjugates (22). These findings indicate further that endogenous carbohydrates can control IAA levels in leafy tissues through induction of enzymes capable of hydrolyzing IAA conjugate moieties.

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