METABOLISM OF CYTOKININS IN BARLEY LEAVES

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SUMMARY

An enzyme activity has been detected in barley leaves that converts cytokinin isopentenyladenosine to isopentenyladenine. This reaction seems to be cytokinin specific and represents an activation step in cytokinin metabolism.

INTRODUCTION

Transformations of naturally occuring cytokinins in plant cells may follow two essential pathways. A cytokinin is either converted to its more active form (1) or it is inactivated (2,3). Inactivation of cytokinin molecules is probably a specific process. Regarding the presence in plant cells of tRNA breakdown products, among which several active cytokinins are found (4), the metabolism of "soluble" cytokinins should be under precise control. A specific compartmentation, e.g. the differentiation of pools of soluble cytokinins and of cytokinin-active hypermodified bases and nucleosides released during tRNA turnover, is a possible way of the regulation. As shown recently (5,6) the cis-trans isomerisation of the most potent cytokinin found in plants so far, zeatin [N^6-(4-hydroxy-3-methylbut-2-enyl)adenine], may be also important for the control. However, the presence of another very active cytokinin, N^6-(Δ^2-isopentenyl)adenosine

in tRNA (4) and as a free nucleoside in plant tissue (7) makes the maintenance of the proper concentration of this cytokinin in plants an open question. So far an enzymic activity specifically inactivating cytokinins was detected (2) in a crude extract of cultured tobacco pith tissue (Wisconsin-38). This enzyme, cytokinin oxidase, has been recently isolated from immature corn kernels and it has been characterized in more detail (3). Cytokinin oxidase inactivates N^6-(Δ^2-isopentenyl)adenine and its ribosyl derivative, ipAdo[1], by specific conversion to adenine and adenosine, respectively. These reactions are specific for cytokinins in contrast to activities converting cytokinin nucleosides to inosine (8).

We have studied the metabolism of isopentenyladenosine in barley leaves and were able to detect an enzyme converting this cytokinin to its more active form, isopentenyladenine.

METHODS

Barley leaves were harvested from two weeks old barley plants (Ametyst var.) and were kept overnight at -20°C. All subsequent operations were performed at 4°C. 300 g of the leaves were homogenized in a Waring blender with 1 liter of 0.1 M sodium phosphate buffer pH 6.9, 0.1 M ascorbic acid (adjusted to pH 6.9 with NaOH), 100 g of polyvinylpyrrolidone (Polyclar AT, Irwin Dyestuffs Ltd) (2). The resulting suspension was filtered through cheesecloth. The filtrate was centrifuged at 10,000 g for 30 min. Solid ammonium sulphate was added to the supernatant until 20% saturation was reached. The pH was maintained at 6.9 by addition of a few drops of concentrated ammonium hydroxide and the solution was left to stand for 2 hrs. in an ice bath. The precipitate was removed by centrifugation for 30 min. at 10,000 g. The supernatant was saturated with solid ammonium sulphate to 60% and the mixture was left overnight at 4°C. The precipitate was collected by centrifugation for 30 min. at 10,000 g and dissolved in 10 ml of 0.01 M sodium phosphate buffer pH 6.9. The turbid solution

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[1] For abbreviations see Table I.

was clarified by centrifugation for 10 min. at 5,000 g and dialyzed for 48 hrs. against 5 liters of 0.01 M sodium phosphate buffer pH 6,9. The dialysate was centrifuged for 10 min at 5,000 g and 1 ml aliquots were frozen and kept in the deep-freeze. Under these conditions, the enzymic activities in the preparation were preserved for several months.

50 µl of an aquous solution of a radioactive substrate (0.3 mM; 5mCi/mmole) was added to 200 µl of the enzyme preparation. This solution was incubated for 16 hrs. at 37°C unless otherwise indicated. [8-^{14}C]N^6-(Δ^2-isopentenyl)adenosine and [^{14}C(U)]adenosine were used. 3 mM Solution of trans-zeatin riboside was used in one experiment. In the control sample, the substrate was incubated with 200 µl of the enzyme solution boiled previously for 5 min. After incubation the mixture was chilled in ice and 1 ml of cold acetone was added. After 10 min., the precipitated protein was removed by centrifugation. The supernatant was concentrated *in vacuo*, the solution was applied to chromatographic paper Whatman N° 1 and chromatographed in system A: ethanol - 0.1 M ammonium borate, pH 9,0 (1/9; v/v). Radioactivity distribution was detected in a Packard Radiochromatogram Scanner 7,200. Radioactive spots were cut out, eluted with water, concentrated and rechromatographed in system B: ethyl acetate - n-propanol - water (4/1/2; v/v; upper phase); C: butanol - concentrated ammonium hydroxide - water (86/5/14; v/v) and D: butanol saturated with water. In one case, eluted and concentrated radioactive isopentenyladenine was used for reaction with the fresh portion of the barley extract.

RESULTS

Crude protein extract of barley leaves converts the cytokinin isopentenyladenosine predominantly to isopentenyladenine (Fig. 1). Detection of the reaction products by paper chromatography (Tab. I) reveals small amount of another compound, probably adenosine (Fig. 1). However, the extensive conversion of isopentenyladenosine to adenosine by a cytokinin oxidase found in tobacco pith tissue culture (2) and in immature corn kernels (3) was not observed. Since cytokinin oxidase converts also the base, isopentenyladenine to adenine, we treated the resulting radioactive isopentenyladenine with fresh portion of the enzyme preparation for 24 hrs. No conversion to adenine was observed under these conditions, suggesting that cytokinin oxidase is not present in barley leaves.

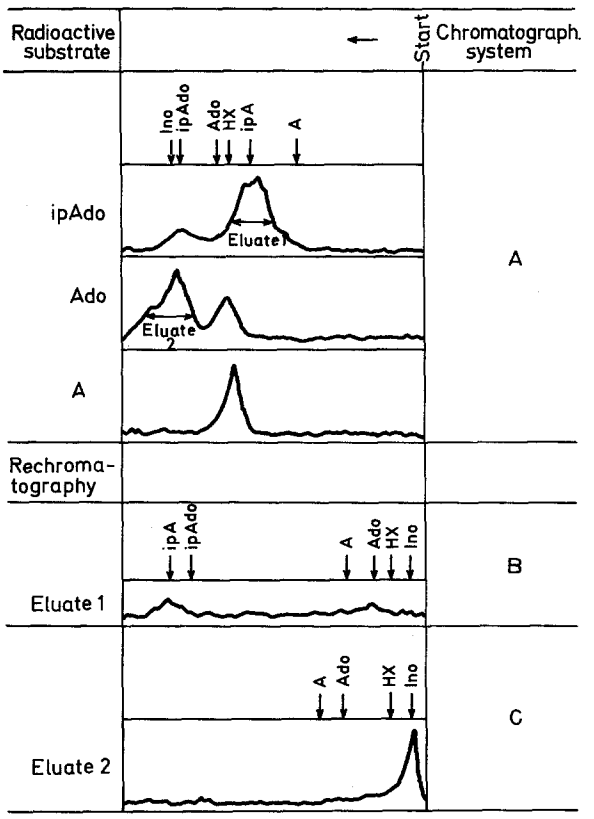


Fig.1 Conversions of Isopentenyladenosine and Adenosine by Extract of Barley Leaves
(Radioactive profiles obtained by paper chromatography)

The conversion of isopentenyladenosine to isopentenyladenine represents an activation step in cytokinin metabolism, since the base was found to be a more active cytokinin than the parent nucleoside (9). We tried to find out whether the deribosylation of isopentenyladenosine is a specific step in cytokinin metabolism. Therefore, adenosine was treated with the enzyme preparation (Fig. 1). The major degradation product arising from adenosine is inosine, formed by adenosine deaminase (adenosine aminohydrolase; EC 3.5.4.2), the enzyme common to plant tissues. Isopentenyladenosine does not seem to be

Table I. Paper Chromatography of Cytokinins and Some Related Compounds

Compound		R_f			
		System A	System B	System C	System D
N^6-(Δ^2-Isopentenyl)adenine	(ipA)	0.57	0.85	0.89	0.87
N^6-(Δ^2-Isopentenyl)adenosine	(ipAdo)	0.80	0.78	0.86	0.80
Adenine	(A)	0.42	0.27	0.35	0.25
Adenosine	(Ado)	0.66	0.18	0.27	0.18
Hypoxanthine	(HX)	0.65	0.13	0.11	
Inosine	(Ino)	0.83	0.05	0.04	0.05

Table II. Kinetics of Adenosine Degradation in Excess of nonradioactive Adenine
(Data obtained by paper chromatography in system A)

Time of Incubation (hrs.)	Ratio of Concentrations A/Ado	Radioactivity in Spot (%)		
		Ino	Ado+HX	A
2	0	47	34	19
2	10	44	21	35
6	0	60	26	14
6	10	56	18	26
16	0	60	40	0
16	10	59	27	14

degraded by this enzyme in barley leaves, in spite of its sensitivity to adenosine deaminase isolated from other sources (8). The analysis of the products of adenosine degradation by paper chromatography suggested (Fig. 1) that hypoxanthine may be also formed. Therefore, the reaction was performed in an excess of adenine, presumed intermediate of such a degradation (Tab. II). We found that hypoxanthine is indeed formed via adenine (Fig. 1 and Tab. II). This suggests that the conversion of isopentenyladenosine to isopentenyladenine may not be a cytokinin-specific step in barley leaves, since isopentenyladenosine may be a substrate for the enzyme deribosylating adenosine. However, since there is no cross competition between isopentenyladenosine and adenosine in these reactions (Tab. III), there still is the possibility of a specific cytokinin deribosylation. Isolation of all the enzymes involved in this metabolic pathway would be necessary to de-

Table III. Lack of Competition between Adenosine and Isopentenyladenosine during their Degradation in Barley Leaves Extract
(Data obtained by paper chromatography in system A)

Radioactive substrate	Cold addition	Ratio cold addition / radioactive substrate	Radioactivity in Spot (%) Ino	Ado	ipAdo	ipA
Ado	0	0	65	35	-	-
Ado	ipAdo	10	65	35	-	-
ipAdo	0	0	-	-	46	54
ipAdo	Ado	10	-	-	43	57

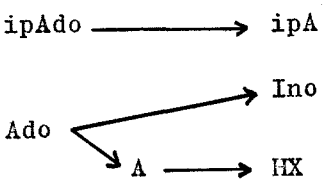
termine whether different enzymes are deribosylating adenosine and isopentenyladenosine.

To support the idea that the activity converting isopentenyladenosine to isopentenyladenine plays a role in cytokinin metabolism, the reaction was run in excess of another cytokinin, N^6-(4-hydroxy-3-methylbut-2-*trans*-enyl)adenosine (*trans*--zeatin riboside). We found that the conversion of isopentenyladenosine to isopentenyladenine is indeed inhibited by *trans*-zeatin riboside (Tab. IV). It remains to see whether ribosyl zeatin is an inhibitor or a substrate of the enzyme. Nevertheless, the result shows that the two major naturally occuring cytokinin nucleosides have an affinity for the enzyme, suggesting that the enzyme is involved in cytokinin metabolism.

Table IV. Inhibition of ipAdo Conversion by *trans*-zeatin riboside (*trans*-ZR)
(Data obtained by paper chromatography in system A)

Addition	Radioactivity in Spot (%)	
	ipAdo	ipA
0	22	78
trans-ZR	85	15

From the results presented in this paper, the following scheme for the metabolism of isopentenyladenosine and adenosine in barley leaves can be drawn:



DISCUSSION

Naturally occuring cytokinins may be metabolized via several routes. After their biosynthesis they can be either activated or converted to an inactive form. So far the activation of cytokinins by modification of the aliphatic side chain has been detected (1,10). Thus zeatin may be formed from isopentenyladenine or ribosylzeatin from isopentenyladenosine. Specific inactivation of cytokinins is achieved by cytokinin oxidase.

The conversion of isopentenyladenosine to isopentenyladenine in barley leaves may be another way of cytokinin activation. It remains to be ascertained whether the conversion is specific for cytokinins. Although we found a similar activity in the same extract, which degrades adenosine to adenine, the competition experiment suggests that the two activities might have their origin in two different enzymes. The major step in adenosine degradation in barley leaves has been shown to be its deamination to inosine. Isopentenyladenine is not a substrate for this reaction. However, we are not able to exclude the possibility that more subtle changes, namely in aliphatic side chain of isopentenyladenine or its corresponding riboside take place during the incubation. The extract of barley leaves we used in our experiments should be fractionated in order to obtain more precise data. The radiolabelled isopentenyladenosine is not available in amounts to permit such study. Therefore, the formation of even more

active zeatin derivatives or inactivation of isopentenyladenosine by other changes in the molecule are possible.

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