METABOLISM OF CYTOKININS IN RAPE SEEDLINGS (*Brassica napus* L.)

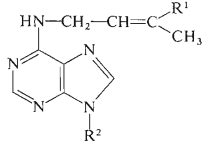
V.Pačes[a], I.Rosenberg[a], M.Kamínek[b] and A.Holý[a]

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6 and*
[b] *Institute of Experimental Botanics, Czechoslovak Academy of Sciences, 160 00 Prague 6*

Received November 25th, 1976

The cytokinin N^6-(Δ^2-isopentenyl)adenosine is in germinating seeds of rape degraded to N^6-(Δ^2-isopentenyl)adenine. In the extract of germinating seeds no significant cytokinin oxidase activity was found. Adenosine hydrolase activity is lower than that observed in other plant systems. On the basis of a comparison of our results with those obtained in other plant systems, a discussion is presented on the relation among particular metabolic pathways of cytokinins.

Metabolic conversions of cytokinins in plants have not yet been investigated in detail. Two principal degradation pathways of N^6-(Δ^2-isopentenyl)adenosine (ipAdo) (*I*) in plant cells have been found to date. Thus, in the tobacco pith tissue culture[1,2] and in immature corn kernels[3], ipAdo is converted mainly to adenosine. This degradation step is specific for cytokinin and results in its inactivation. Another enzymatic step leading to degradation of ipAdo was detected in barley leaves[4]. In this case, ipAdo is converted to N^6-(Δ^2-isopentenyl)adenine (ipAde) (*II*). This reaction is to be regarded as activation of the cytokinin since bases are generally more active than the corresponding nucleosides[5]. This metabolic step may also be assumed to be cytokinin specific[4]. The nonspecific degradation to inosine was, for example, examined in the chicken bone marrow[6]. In this case ipAdo is regarded



I, N^6-(Δ^2-isopentenyl)adenosin: $R^1 = CH_3$, $R^2 = \beta$-D-ribofuranosyl
II, N^6-(Δ^2-isopentenyl)adenin: $R^1 = CH_3$, $R^2 = H$
III, *trans*-ribosylzeatin: $R^1 = CH_2OH$, $R^2 = \beta$-D-ribofuranosyl

as a substrate of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.2). Noteworthy, ipAdo is not substrate of the barley leaves adenosine deaminase[4]. Another step in the metabolism of cytokinins was discovered by Miura and Hall[7]. These authors reported a stereospecific conversion of ipAdo to *trans*-ribosylzeatin (*III*) in corn endosperm. This modification of the side-chain results in an increase of the cytokinin activity[8,9].

In this paper, the metabolism of ipAdo in germinating rape seeds is described.

EXPERIMENTAL

Extraction of Rape Seeds

Dry rape seeds, or, seeds germinated for 3 days at room temperature in the dark (100 g) were disintegrated at 7°C for 5 min in 500 ml of medium. Acid extraction was performed with the use of 0·05M acetate buffer solution (pH 4·5). In another extraction water was used and pH 7·5 was maintained with aqueous ammonia. The resulting suspension was subjected to centrifugation (2000*g*; 30 min) and the supernatant clarified by additional centrifugation (10000*g*; 30 min).

Ammonium Sulfate Fractionation of the Extract

Solid ammonium sulfate was slowly added to the stirred clear supernatant up to a saturation level of 80%. The suspension was kept at 7°C for 16 h and subjected to centrifugation (10000*g*; 30 min). The precipitate was dissolved in 50 ml of 0·1M triethylammonium hydrogen carbonate (TEAB) (pH 7·5) and the solution desalted on a column of Sephadex G-25 (medium) in 0·05M-TEAB (pH 7·5). The eluted protein was freeze-dried.

Lyophilised protein was then fractionated as follows. Solid ammonium sulfate was portion-wise added to a stirred solution of the protein (1 g) in 100 ml of 0·05M-TEAB (pH 7·5) to the saturation level of 40%. The mixture was kept at 7°C for 15 h and subjected to centrifugation. The supernatant was saturated to the level of 60% with solid ammonium sulfate, the precipitate collected by centrifugation and the remaining supernatant saturated again (to the level of 80%). The precipitates were dissolved in a minimum volume of 0·1M-TEAB (pH 7·5), the solution desalted on a column of Sephadex G-25, and the effluent freeze-dried.

Acetone Fractionation of the Extract

Acetone fractionation was applied to the lyophilised protein from the ammonium sulfate saturation to the level of 80%. Precooled (−20°C) acetone was added to a solution of the protein (1 g) in 100 ml of 0·05M-TEAB (pH 7·5) up to the concentration level of 20% and the resulting suspension kept at −10°C for 15 h. The precipitate was collected by centrifugation, dissolved in a minimum volume of 0·1M-TEAB (pH 7·5), and the solution freeze-dried. The supernatant was treated with precooled acetone to the final concentration of 40%, the suspension kept at −20°C for 15 h and the precipitate collected by centrifugation and processed as above.

Identification of Products of Enzymatic Degradation

Conversion of N^6-(Δ^2-isopentenyl)adenosine was performed in test tubes containing 50 µl of the aqueous [8-^{14}C]N^6-(Δ^2-isopentenyl)adenosine[1] (0·3 mM; 5 µCi/µmol) and 200 µl of the enzyme solution (10 mg of the lyophilised preparation in 0·2 ml of a 0·05M phosphate buffer

solution, pH 6·8). A control experiment was performed with the enzyme solution preheated to 90°C for 10 min and cooled down.

Conversion of adenosine was performed similarly with aqueous [^{14}C—U]adenosine of the same concentration and specific activity as those of the ^{14}C-labelled aqueous ipAdo. The identification of products by paper rechromatography was described earlier[1,2,4].

RESULTS

The presence of cytokinin-metabolising enzyme activity in nonfractionated protein extracts of plants is usually difficult to detect, probably because of the low content of these enzymes. However, this low concentration is not surprising considering the low concentrations of cytokinins in most plants. In order to distinguish reactions involved in the metabolism of the N^6-(Δ^2-isopentenyl)adenosine in rape seeds, the nonfractionated total protein (obtained by saturation with ammonium sulfate) was incubated for a long period of time with the radioactively labelled substrate. The extract of dry seeds was less active than that of germinated seeds (Table I). As inferred from comparison of the two methods, the acid extraction appears more advantageous. Additional fractionation of the acid extract of germinated seeds afforded a concentrated enzyme preparation from the fraction saturated from 40 to 60% with ammonium sulfate (Table II); this enzyme preparation was concentrated enough to allow identification of products of the ipAdo metabolism. As demonstrated by paper chromatography in several solvent systems[4], ipAdo is primarily converted to isopentenyladenine (Table III) which is accompanied by adenosine and adenine as minor products. This extraction method of rape seeds gives better separation of ipAdo degrading enzyme activities than the extraction of barley leeves[4]. The adenosine-degrading hydrolase activity is precipitated to a low extent when the solution is saturated with ammonium sulfate from 40 to 60% (Table III). This activity was

TABLE I

Conversion of N^6-(Δ^2-Isopentenyl)adenosine by the Total Protein of Rape Seeds (Incubation, 16 h at 37°C)

Seed	Extraction, pH	Conversion, %	Adenine (%) in the product
dry	4·5	30	35
germinated	4·5	100	30
dry	7·5	0	0
germinated	7·5	100	0

TABLE II

Fractionation of the Protein Extracted from Germinated Rape Seeds at pH 4·5

Saturation with ammonium sulfate, %	Incubation h	Conversion of ipAdo %
0—40	5	63
0—40	16	91
40—60	5	90
40—60	16	100
60—80	5	10
60—80	16	72

TABLE III

Products of the N^6-(Δ^2-Isopentenyl)adenosine and Adenosine Conversion as Identified by Paper Chromatography

The reaction was performed with protein from the acid extract of germinated rape seeds, precipitated by saturation with ammonium sulfate (40—60%). Incubation, 16 h.

Substrate	Product, %			
	ipAde	adenosine	adenine	hypoxanthine
ipAdo	90	7	3	0
Adenosine	—	40	25	35

TABLE IV

Conversion of N^6-(Δ^2-Isopentenyl)adenosine by Protein Fractions Precipitated by Acetone

Incubation min	Conversion to ipAde, %	
	0—20% of acetone	20—40% of acetone
0	0	0
5	10	0
15	40	17
60	95	70
240	100	90

difficult to remove in investigations on the metabolism of cytokinins in the tobacco tissue culture[1,2], immature corn kernels[3] and barley leaves[4]. The total protein isolated from the acid extract of germinated rape seeds by saturation with ammonium sulfate up to 80% degrades 50% of adenosine to inosine. The remaining portion of the product contains 35% of adenine and 15% of hypoxanthine which is probably formed by deamination of adenine. In the degradation of adenosine, adenosine deaminase and adenosine hydrolase are thus almost equally involved.

A very efficient procedure for the concentration of cytokinin hydrolase consists in the acetone fractionation of germinated rape seeds. An almost complete conversion of ipAdo may be observed after 1 h of incubation with the protein fraction precipitated with acetone added to the concentration level of 20% (Table IV).

DISCUSSION

The biosynthesis[10–13], interconversions[7,14], and degradation[1–4] of cytokinins in plants have not yet been thoroughly examined. The reported interconversions of cytokinins both decrease and increase the hormonal effect. In roots of radish seedlings, the highly biologically active zeatin is metabolised to the less active 5'-ribonucleotide (probably the transport form of zeatin) and to the reserve 7-glucosylzeatin (raphanatin)[15]. The *trans*-ribosylzeatin is formed by hydroxylation from the less active ipAdo in corn endosperm and in the fungus *Rhizopogon raseolus*[7]. In barley leaves, ipAdo is somewhat surprisingly activated by degradation to ipAde[4]. It has been shown that this degradation also occurs in rape seedlings.

Specific degradation of cytokinin nucleosides to adenosine[1–3] is catalysed by cytokinin oxidase. Conversion of cytokinins to inosine is nonspecific. However, the cyto-

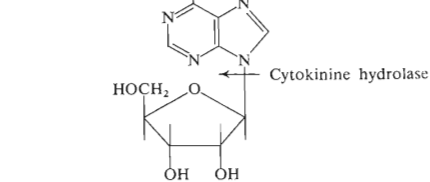


FIG. 1

Some Enzymes of the N^6-(Δ^2-Isopentenyl)adenosine Metabolism

kinins do not appear to be good substrates for plant adenosine deaminases[4]. A strong reaction of this type takes place in animal tissues[6].

Several enzymes specific for cytokinins have thus been determined (Fig. 1). On the basis of a comparison of enzyme activities observed by various authors in various experimental models, a tentative scheme of cytokinin metabolism is proposed (Fig. 2). The key role, which is in this Scheme ascribed to N^6-(Δ^2-isopentenyl)adenosine is also supported by the hypotheses on the evolution of cytokinins[14].

In view of the characteristic effect of cytokinins it would be of interest to examine if and how are the enzymes of the cytokinin metabolism localised. All plants obviously have specific mechanisms of the cytokinin inactivation. To date, specific inactivation in dry or germinated rape seeds, or, in barley leaves has not been demonstrated. The compartmentation of enzyme activities within the plant

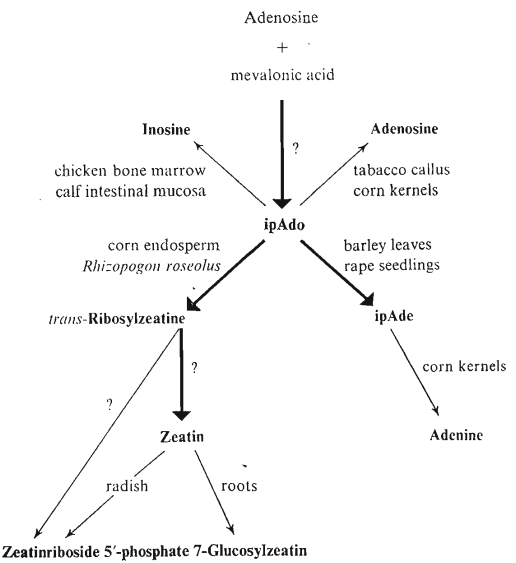


FIG. 2

Tentative Scheme of the Metabolism of Naturally Occurring Cytokinins: Key Role of N^6-(Δ^2-isopentenyl)adenosine

Thick arrows designate direction of an increased biological activity.

cells could explain the low level of these enzymes in extracts of intact cells. Actual concentration of enzymes within the cells might be locally increased by compartmentation[16,17] to such an extent that the enzymes could play their metabolic role. It is also possible that enzymes of cytokinin metabolism are distributed in various organs and tissues of particular plants. Furthermore, the occurrence of these enzymes is probably dependent on the stage of development of the plant. This could explain the low activity of ipAdo-inactivating enzyme in rape seedlings where synthesis and activation of cytokinins is expected.

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Translated by J. Pliml.