Phytochemistry, Vol 22, No 5, pp 1103–1105, 1983
Printed in Great Britain

0031-9422/83/051103-03$03 00/0

CYTOKININ CATABOLISM AND CYTOKININ OXIDASE

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(*Received* 7 *October* 1982)

Key Word Index—*Zea mays*, Gramineae; kernels, cytokinin oxidase, N^6-(Δ^2-isopentenyl) adenine, adenine; HPLC

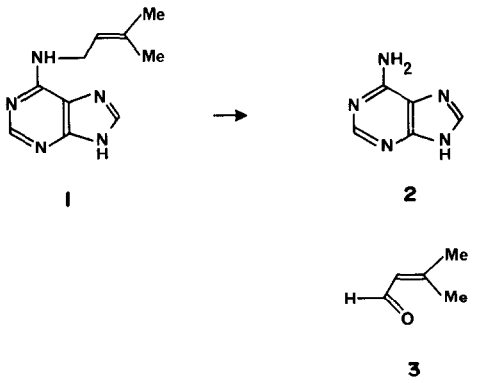
Abstract—Cytokinin oxidase has been highly purified from mature *Zea mays* kernels. Adenine has been unambiguously identified, by HPLC co-chromatography, UV and mass spectroscopy, to be the oxidation product of N^6-(Δ^2-isopentenyl)adenine by this enzyme. No other products were detected The results are in agreement with metabolic studies and suggest that cytokinin oxidase plays an important role in the catabolism of exogenously applied cytokinins.

INTRODUCTION

The exogenous application of cytokinins to plant tissues has, in almost every case, led to the production of adenine (Ade), adenosine (Ado) or Ade nucleotides [1–4] In many of these experiments they were the major catabolites This complete and irreversible destruction of the cytokinin structure is seen as a manifestation of the plant's ability to control total cytokinin activity. A number of important studies have been performed to investigate the enzymatic oxidation of the cytokinins

Xanthine oxidase has been shown to oxidize zeatin (Z) and N^6-(Δ^2-isopentenyl)adenine (2iP) to their 8- and 2,8-dihydroxy derivatives [5]. 2-Hydroxy-Z has been isolated as a minor component of immature *Zea mays* kernels [6], but has never been identified as a metabolite of exogenously supplied material Xanthine oxidase, itself, has yet to be found in plant tissues [7].

Of much greater importance, an enzyme has been characterized which cleaves the N^6 side chain of the cytokinins (Scheme 1) This enzyme, so called 'cytokinin oxidase', has been demonstrated in tobacco pith tissue (Wisconsin-38) [8] and *Zea mays* kernels [9]. The enzyme was shown to require molecular oxygen [9] and to be active with four substrates, namely. 2iP (**1**), N^6-(Δ^2-isopentenyl)adenosine (2iPA), Z and zeatin riboside (ZR). Side chain saturation, the relocation of the double bond from Δ^2 to Δ^3 and substitution of other functional groups



1 2 3

Scheme 1. Side chain cleavage of 2iP

all caused decline or total loss of enzyme activity. Those compounds that serve as substrates (i.e. 2iP, Z and ZR) are rapidly metabolized to Ade and Ado [1–4] whilst benzyladenine (BA) and dihydrozeatin (DHZ) are much more resistant to side chain cleavage [10, 11]. An interesting comparative study with bean leaves [12] shows the order of stability of exogenously applied cytokinins to be dihydrozeatin *O*-glucoside (DHZOG) > zeatin *O*-glucoside (ZOG) > DHZ > Z We now know that the *O*-glucosides are not substrates for cytokinin oxidase [13]

The mechanism of the oxidative cleavage is not known, but 3-methyl-2-butenal (**3**) has been conclusively identified by mass spectroscopy as a product of cytokinin oxidase action on 2iP (**1**) [14]. The other expected product of this reaction is Ade (**2**) and this was identified by co-chromatography on paper [8, 9, 15]. However, there was some confusion in the interpretation of the UV spectrophotometric assay [9] since the products gave a shift in λ_{max} values to 248 nm instead of the expected 260 nm. The existence of an unstable intermediate was postulated [9], possibly an imine, which was thought to break down to Ade during the processing of the sample for PC.

Other workers [16] have suggested that the oxidation products were wrongly identified. By analogy with permanganate oxidation products 6-(2,3,4-trihydroxy-3-methylbutylamino)-purine has been proposed as a product of cytokinin oxidase action on Z. This compound, which co-chromatographs with Ade on paper and on Sephadex LH-20 [16], has been isolated from immature *Zea mays* kernels [6]. It is, however, a minor component and has never been demonstrated as a metabolite. This contrasts markedly with the almost universal appearance of Ade, Ado and Ade nucleotides as catabolites of exogenously applied cytokinins.

We have, therefore, reinvestigated the degradation of 2iP by cytokinin oxidase from *Zea mays* and have positively identified the sole purine product of this reaction as Ade.

RESULTS AND DISCUSSION

Since the side chain aldehyde (**3**) (Scheme 1) has already been conclusively identified as a product of cytokinin oxidase action on 2iP (**1**) [14] and because the confusion centres around the reported shift in UV absorbance [9], it

was considered important to establish beyond doubt that Ade (**2**) is the other product [8-^{14}C]2iP was incubated with highly purified cytokinin oxidase from *Zea mays* kernels The extraction procedure was essentially that of Whitty and Hall [9], but the enzyme was further purified by preparative polyacrylamide disc gel electrophoresis [13] The incubation was analysed by gradient HPLC (System 1) [17], at time zero, and after 10 hr Extensive degradation occurred after 10 hr, all the radioactivity co-chromatographs with reference Ade and residual 2iP (Fig 1) The Ade fraction was subjected to further HPLC using a different gradient system and again all the activity was found in the Ade fraction This material gave a UV spectrum identical to authentic Ade λ_{max} 259 5 nm Mass spectroscopy (probe sample) showed unambiguously that the radioactive material was Ade Ions at *m/z* 137 and 110 are present due to the ^{14}C-label in the substrate The intensity of these peaks is approximately in the proportions predicted by the specific activity of the starting material No other products were observed

It is probable that the less rigorously purified enzyme preparation used by Whitty and Hall [9] accounted for their anomalous UV data Further oxidation of Ade by other, contaminating, enzymes could give, for instance hypoxanthine and the allantoides, both of which have lower λ_{max} values It should be pointed out that the permanganate-produced trihydroxy compound [16] has a UV spectrum similar to that of the cytokinins [6] and, therefore, could not possibly account for the shift to 248 nm [9]. With our much purer enzyme preparation this shift was not observed Instead the λ_{max} value moved from 265 to 259 nm as the reaction proceeded

It is interesting that Hall *et al.* [18], using chicken bone marrow and calf intestinal mucosa preparations, found that cleavage of 2iPA gave inosine The identification of inosine was based purely on the λ_{max} shift to 248 5 nm The results and arguments presented here shed some doubt on these conclusions

The results in this paper and the identification of the side chain aldehyde [14] show, conclusively, that the cleavage of the side chain is the mechanism of cytokinin oxidase action The parallel between these results and the numerous metabolic studies strongly suggests that cytokinin oxidase plays a central role in the catabolism of exogenously applied cytokinins

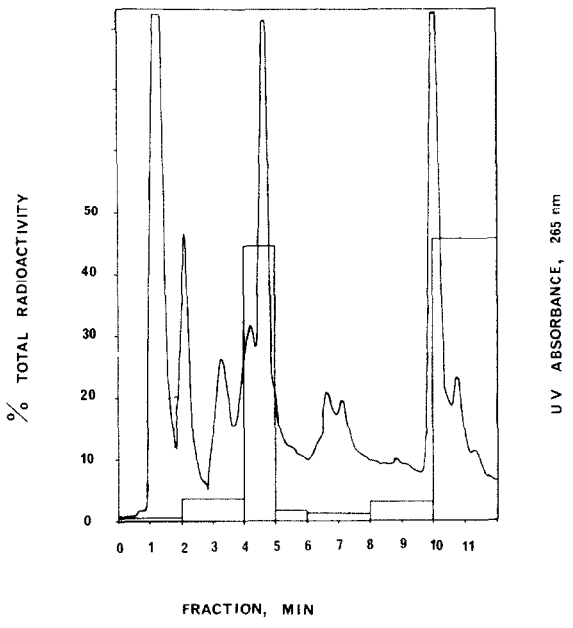


Fig 1 HPLC UV trace showing products of the incubation of cytokinin oxidase with 2iP after 10 hr (System 1) The superimposed bar diagram shows the percentage total radioactivity The UV absorbing peak at fraction 5 min co-chromatographs with reference Ade and the peak at fraction 11 min with reference 2iP

EXPERIMENTAL

Plant material Zea mays var Earliking was grown under glass The mature cobs were harvested and used immediately or were frozen at −20° until required

Chemicals [8-^{14}C]N^6-(Δ^2-isopentenyl)adenine (11 7 mCi/mmol) was synthesized by condensing [8-^{14}C]6-chloropurine with 3-methylbut-2-enylamine [19]

Cytokinin oxidase purification The enzyme was purified by the method of ref [9] and is described in detail elsewhere [McGaw, B A and Horgan, R , in preparation] The enzyme was ppted by Me_2CO fractionation between 40 and 60% and this sample was applied to a Sephadex G-150 column The active fractions from this were bulked and run on a hydroxylapatite (HAP) column The enzyme activity bound weakly to the HAP and the bulked active eluates were condensed using dialysis tubing and a satd soln of polyethylene glycol (MW 6000) before running on 7% polyacrylamide disc gels The active region of the gels was cut out and extracted into buffer This preparation, of sp act 10 7 μM 2iP/mg protein hr, was used for the incubation expts

Oxidation of 2iP by cytokinin oxidase 2iP (11 7 mCi/mmol), 64 nM, was incubated at 37° with 100 μl of the enzyme preparation At time zero and time 10 hr, 10 and 90 μl, respectively, were withdrawn and subjected to reversed phase HPLC using a 150 × 4 5 mm id column of Apex ODS eluted at a rate of 2 ml/min with a gradient system of 0–30% MeCN with H_2O at pH 7 0 (triethylammonium bicarbonate) over a 30 min period using an exponent of 1 (System 1) Fractions were collected for 1 or 2 min and 0 5 ml of these were counted in a liquid scintillation spectrometer The active region (10 hr, fraction 5 min), which co-chromatographed with reference Ade, was evaporated to dryness and reapplied to the same HPLC column using a gradient system running from 0 to 5% MeCN (10 min) (System 2) The only radioactive peak (fraction 9 min) co-chromatographed with reference Ade This fraction was evaporated to dryness and used for UV and MS analysis UV λ_{max}, H_2O, pH 7 0, was 259 6 nm The MS was obtained (direct probe) at a source temp of 180° (rel int) *m/z* 137 (14 70), 135 (100), 110 (4 96), 108 (29 15) 81 (12 97), 54 (19 15) and 53 (11 00) The ion at *m/z* 137 represents $[M + 2]^+$ due to the [8-^{14}C]-label The spectrum was otherwise identical to an Ade reference from a commercial source

Acknowledgements – We are grateful to Jim Heald for MS and to Phil Williams for technical assistance This research forms part of a project financed by the Agricultural Research Council

REFERENCES

1 Entsch, B , Letham, D S , Parker C W , Summons, R E and Gollnow, B I (1979) in *Plant Growth Substances* (Skoog, F ed) p 109 Springer, Berlin
2 Summons, R E , Parker, C W , Letham, D S , Gollnow, B I and Macleod, J K (1980) *Planta* **147**, 422
3 Horgan, R , Palni L M S , Scott, I M and McGaw, B A

(1981) in *Metabolism and Molecular Activities of Cytokinins* (Guern, J and Péaud-Lenoel, C, eds) p 56 Springer, Berlin
4 Laloue, M, Terrine, C and Guern, J (1977) *Plant Physiol* **59**, 478
5 Chen, C-M, Smith, O C and McChesney, J D (1975) *Biochemistry* **14**, 3088
6 Letham, D S (1973) *Phytochemistry* **12**, 2445
7 Brown, E G (1975) *Biochem Soc Trans.* **3**, 1199
8 Paces, V., Werstiuk, E and Hall, R H (1971) *Plant Physiol* **48**, 775
9 Whitty, C. D and Hall, R H. (1974) *Can J. Biochem* **52**, 781
10 Parker, C W, Wilson, M M, Letham, D S, Cowley, D E and Macleod, J K (1973) *Biochem Biophys Res Commun* **55**, 1370.
11. Palmer, M V, Horgan, R. and Wareing, P F (1981) *Planta* **153**, 297
12 Palmer, M V, Scott, I M and Horgan, R (1981) *Plant Sci Letters* **22**, 187
13 Scott, I. M, McGaw, B A, Horgan, R and Williams, P E. (1982) in *Plant Growth Substances* 1982 (Wareing, P F, ed) p 165. Academic Press, London
14 Brownlee, B G, Hall, R H and Whitty, C D (1975) *Can J Biochem* **53**, 37
15 Paces, V and Kamínek, M (1976) *Nucl Acids Res* **3**, 2039
16 Van Staden, J, Drewes, S E and Hutton, M. J (1982) *Physiol Plant* **55**, 143
17 Horgan, R and Kramers, M R (1979) *J Chromatogr* **173**, 263
18 Hall, R H, Alam, S N, McLennan, B D, Terrine, C and Guern, J (1971) *Can J Biochem* **49**, 623
19 Hall, R H and Robins, M. J. (1968) in *Synthetic Procedures in Nucleic Acid Chemistry* (Zorbach, W W and Tipson, R S, eds) Vol 1, p 11 John Wiley, New York