Phytochemistry, Vol 26, No 3, pp 615-618, 1987
Printed in Great Britain

0031 9422/87 $3.00 + 0.00
Pergamon Journals Ltd

A SOLUBLE PROTEIN FACTOR FROM CHINESE CABBAGE CONVERTS INDOLE-3-ACETALDOXIME TO IAA

JURGEN HELMLINGER, THOMAS RAUSCH and WILLY HILGENBERG

Botanisches Institut der Johann Wolfgang Goethe Universitat, Siesmayerstraße 70, D-6000-Frankfurt, F R G

(Revised received 14 July 1986)

Key Word Index—*Brassica campestris* ssp *pekinensis*, Cruciferae, Chinese cabbage, indole-3-acetaldoxime, indole-3-acetaldehyde, IAA biosynthesis

Abstract—A soluble enzyme preparation from Chinese cabbage seedlings (*Brassica campestris* ssp. *pekinensis*) which catalyses the conversion of indole-3-acetaldoxime (IAOX) to IAA was partially purified by ion exchange chromatography. After purification enzyme activity was stable for more than 6 hr. Substrate kinetics showed a K_m value of 50 μM; the pH optimum was 7. The conversion of IAOX to IAA was increased by NAD, NADP or FAD, but none of them seemed to be a preferential co-substrate. Besides IAA some labelled indole-3-acetaldehyde (IAALD) could be extracted from the reaction mixture. Addition of unlabelled IAALD at 100 nmol/ml led to a significant inhibition of IAA formation while some label accumulated in the aldehyde. Indole-3-acetonitrile was never detected as a reaction product. The results are compared with those from earlier *in vivo* experiments and are discussed in view of their significance for IAA biosynthesis in the Brassicaceae.

INTRODUCTION

In vivo 3-indoleacetaldoxime (IAOX) is a precursor of 3-indolemethylglucosinolate (IMG) in the Brassicaceae [1-5]. IMG may be converted enzymatically to 3-indoleacetonitrile (IAN) by thioglucoside glucohydrolase (EC 3.2.3.1) [6-8]. IAN is hydrolysed to IAA by the enzyme nitrilase (EC 3.5.5.1) [9, 10]. These and other results led to the following postulated pathway for IAA biosynthesis restricted to the Brassicaceae: tryptophan → IAOX → IMG → IAN → IAA [11, 12]. However, the significance of the IMG pathway for auxin biosynthesis has been questioned [13] and it is indeed difficult to conceive its operation *in vivo* in view of the well characterized compartmentalization of both IMG and thioglucoside glucohydrolase [14-16]. This is different under stress conditions like physical injury or disease which may cause a disturbance of IMG sequestration, where IAA formation via IMG becomes very likely [17]. Apart from the specific role in the Brassicaceae as a precursor of IMG, several observations point to a more general metabolic function of IAOX in IAA biosynthesis in higher plants: (i) cell free preparations of *Avena* coleoptiles are able to catalyse conversion of IAOX to 3-indoleacetaldehyde (IAALD) [18]; (ii) IAOX hydrolyase converts IAOX directly to IAN, although this enzyme was characterized only for *Gibberella fujikorii* [19, 20]; (iii) the ability to convert IAOX to IAN, IAALD, 3-indoleethanol (IEOH), or IAA (or several of these metabolites) *in vivo* does not seem to be restricted to the Brassicaceae [5, Helmlinger unpublished results]; (iv) the same holds for the enzyme nitrilase [10].

We conclude that even for the Brassicaceae convincing enzymatic evidence is lacking for any of the three postulated routes from IAOX to IAA, namely the IMG pathway or the direct pathways via IAALD or IAN. The present study aimed to investigate the *in vitro* conversion of [^{14}C]IAOX at low concentration and high specific activity to one or several of the metabolites mentioned above.

RESULTS

In preliminary experiments with crude extracts from Chinese cabbage seedlings we obtained no reproducible enzymatic conversion of [^{14}]IAOX. However, after desalting the buffer soluble protein of 3-day-old etiolated seedlings on Sephadex G25 a significant amount of labelled IAA was formed from [^{14}C]IAOX. Further stabilization of the enzymatic activity resulting in excellent reproducibility of conversion rates was achieved only after batch purification on DEAE cellulose. Then, the enzyme activity was stable for at least 6 hr, when the protein concentration was kept below 150 μg/ml. A higher protein concentration resulted in some inactivation. After the ion exchange chromatography (which routinely was performed with a stepwise gradient) the specific activity was increased 4.4-fold when compared to the desalted total protein.

After incubation of the purified protein fraction with [^{14}C]IAOX the dichloromethane phase extracted at pH 3 (following an extraction at pH 7) contained IAA as the only reaction product that was dependent on a heat labile protein factor. Its identity was confirmed by TLC and HPLC (data not shown). There was no contamination with 3-indolecarbonic acid. TLC analysis showed some radioactivity in the starting zone but this/these labelled compound(s) were quantitatively the same in both test incubations and boiled extracts. In the neutral dichloromethane extracts from test incubations some IAALD was detected but not in those from the boiled controls. IAN was never detected as a reaction product.

As [^{14}C]IAA was the only enzymatic reaction product which was not extracted at pH 7 using solvent partitioning, a rapid and simple quantitative assay was developed for the quantitative determination of IAA produced from IAOX. The reaction sequence IAOX → IAA showed a pronounced pH optimum at 7 (Fig. 1). The K_m value obtained for IAOX from the Lineweaver-Burk plot was 50 μM (Fig. 2). The reduction equivalents NAD, FAD and NADP increased the conversion to some extent, but none of them seemed to be used preferentially (Table 1).

Table 1 Influence of oxidized dinucleotides on the *in vitro* conversion of IAOX to IAA

Compound	IAA formed (pmol/mg protein)	Percentage
Control	0.32 ± 0.04	100
NAD, 100 μM	0.64 ± 0.05	200
NADP, 100 μM	0.40 ± 0.03	125
FAD, 100 μM	0.52 ± 0.06	163

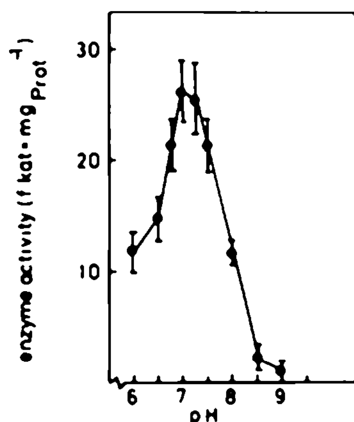

Fig. 1 pH-Dependence of the *in vitro* conversion of IAOX to IAA. From pH 7 to pH 9: 100 mM Tris-HCl buffer, from pH 6 to pH 7.5: 100 mM MES-KOH buffer.

As a direct conversion of IAOX to IAA by a single enzyme is very unlikely we assumed that two enzyme activities are involved, which, however, were not separated by the ion exchange chromatographic step. In view of the occurrence of some labelled IAALD in the test incubations it seemed reasonable to check its possible role as an intermediate. When unlabelled IAALD was present at 100 μM the conversion of [^{14}C]IAOX to IAA was inhibited significantly (Table 2). The inhibition was accompanied by an increased incorporation of label into IAALD. Furthermore, under the standard assay conditions [^{14}C]IAALD was converted to IAA. The pH optimum of this reaction was 7.5 (Fig. 3).

At pH values lower than 7 some spontaneous chemical hydrolysis of IAOX to IAALD was observed, which increased with decreasing pH (but was less than 5% at pH 5. However, this non-enzymatic aldehyde formation did not interfere with the pH optimum of IAA formation from IAOX.

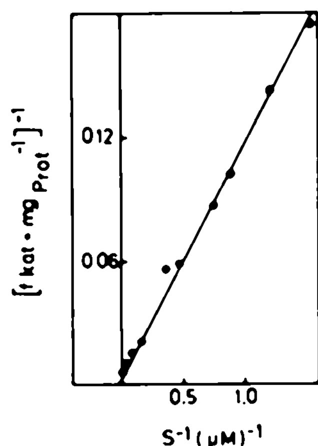

Fig. 2 Concentration dependence of the *in vitro* conversion of IAOX to IAA. Data are presented in a Lineweaver-Burk plot.

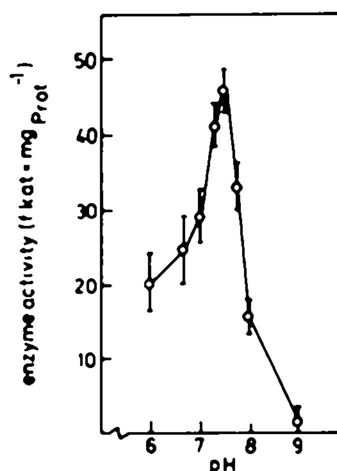

Fig. 3 pH-Dependence of the *in vitro* conversion of IAALD to IAA. The same protein preparation as that in Fig. 2 was used.

Table 2 Influence of unlabelled IAALD on the *in vitro* conversion of [^{14}C]IAOX to [^{14}C]IAALD and [^{14}C]IAA

Assay condition	IAA formed (pmol/mg protein)	Percentage	IAALD formed (pmol/mg protein)
Control + unlabelled	0.68 ± 0.07	100	0.04 ± 0.01
IAALD, 100 μM	0.16 ± 0.05	24	0.15 ± 0.06

DISCUSSION

Our recent study on the kinetics of the *in vivo* metabolism of [^{14}C]IAOX in Chinese cabbage seedlings had revealed IAN as the only lipophilic reaction product [5]. Now a soluble protein fraction has been prepared from the same tissue which is able to convert IAOX to IAA *in vitro* with no detectable formation of the nitrile. The IAA formed enzymatically has been identified by several TLC and HPLC systems, which would have clearly separated other potential reaction products (like indole-3-carbonic acid). It is noteworthy that a stable enzyme activity has been obtained only after subsequent desalting and purification by ion exchange chromatography. The pH optimum at 7 would suggest a cytoplasmic localization.

The apparent K_m (50 μM) value is rather high in view of the estimated endogenous IAOX concentration (500 pmol g fr. wt, i.e. *ca* 0.5 μM assuming even distribution in the tissue) in the same plant material [21]. However, nothing is known about an endogenous sequestration or the localization of the biosynthesis of IAOX. The K_m value compares unfavourably with the apparent K_m value for the *in vivo* conversion of IAOX to IAN (5 μM), and the difference may explain why exogenous IAOX when fed at μM concentrations is preferentially converted to IAN, IMG and desulpho-IMG [5].

Initially we were looking for IAN as the possible intermediate during IAA formation *in vitro*, because earlier work had suggested the existence of an IAOX hydrolyase in plants [22]. However, all attempts to demonstrate the formation of the nitrile *in vitro* failed. With microsomal membranes (10–50 K fraction) of the same tissue, an extremely low conversion of IAOX to IAN was sometimes detected, but it has been difficult to reproduce [21].

Several of our results point to the aldehyde as the intermediate for the *in vitro* conversion of IAOX to IAA: (i) FAD, NAD and NADP all increase the conversion of IAOX to IAA, (ii) in a trapping experiment with unlabelled IAALD, the formation of labelled IAA is inhibited while an incorporation of label into IAALD is observed, (iii) labelled IAALD itself is efficiently converted to IAA by the same partially purified protein fraction. Thus, we conclude that IAALD is the most propable intermediate in the *in vitro* conversion of IAOX to IAA. The observation that even in the presence of 100 μM unlabelled IAALD some radioactivity from [^{14}C]IAOX is accumulated in IAA points to the possibility of a channelled reaction sequence.

The first step of the proposed reaction sequence could procede either via an aldoxime hydrolase or a transoximase [18, 23, 24]. As the enzyme preparation had been desalted and partially purified by ion exchange chromatography we do not think that a transoximase reaction is likely to occur in view of the absence of other aldehyde or ketone substrates. Thus, at present we favour a hydrolytic mechanism similar to the one described by Rajagopal and Larsen [18], although their enzyme preparation from *Avena* coleoptiles showed an apparent pH optimum at 4.4.

NAD or NADP dependent enzymes catalysing the oxidation of IAALD to IAA have been found in several other plant species [25, 26]. However, all IAALD oxidases investigated so far have not been very specific. Other aromatic aldehydes like indole-3-aldehyde [27–29], benzaldehyde or phenylacetaldehyde may equally serve as substrates [29].

Kindl and Schiefer [30] presented evidence that in *Sinapsis alba* *p*-hydroxyphenylacetaldoxime is preferentially converted to tyrosolglucoside when the labelled aldoxime was fed at high concentrations (low specific activity) whereas using lower substrate concentration (high specific activity) the formation of the thiohydroxamic acid was favoured (while the formation of tyrosolglucoside was considerably reduced). These *in vivo* data may be interpreted now at the enzyme level. In our recent study on the *in vivo* metabolism of IAOX at high specific activity (1.85 MBq/μmol compared to 55 KBq/μmol used by Kindl and Schiefer [30]) the aldoxime was converted exclusively to metabolites involved in the glucosinolate pathway (IAN, IMG, desulpho-IMG). There was no indication of any significant formation of either IAALD, IEOH or IAA. However, the data of the present paper show that an enzymatic formation of IAALD from IAOX is possible though the K_m value is one order of magnitude higher than the apparent K_m value for the *in vivo* formation of IAN or IMG. Therefore, we think that the formation of tyrosol glucosides from *p*-hydroxyphenylacetaldoxime *in vivo* [30] was due to the presence of an enzyme similar to the one described in this paper. The fact that *in vivo* the aldehyde is converted to the alcohol rather than to the acid is probably due to the presence of aldehyde reductase(s) in the tissue. In this respect it may be of significance that in tobacco cells IAOX is converted to IEOH *in vivo* [31], even when fed at μ mol concentrations. However, in tobacco no competing reaction like glucosinolate biosynthesis occurs.

It has been proposed that *in vivo* the formation of IEOH is dependent on the concentration of IAA in the tissue [32]. IAA was found to inhibit IEOH oxidase as well as IAALD oxidase *in vitro* although in rather high concentrations [29, 33]. Work on the effect of IAA and other possible inhibitors as well as the effect of reduction equivalents like NADH or NADPH on the enzyme preparation described is in progress.

We conclude that the evidence for the enzymic nature of the conversion of IAOX to IAALD and IAA further supports the existence of a direct biosynthetic pathway for IAA via the aldoxime and the aldehyde. In a forthcoming paper we will report on the *in vitro* formation of IAOX from L-tryptophan by microsomal membranes from several plant species.

EXPERIMENTAL

Plant material. Seeds from *B. campestris* ssp. *pekinensis* var. Granat were surface sterilized by 0.2% $HgCl_2$ soln (w/v) and germinated in Petri dishes on filter paper soaked with 10% Knop's nutrient soln. The seedlings were cultivated in the dark for 3 days at 25°. Sterile H_2O was added daily to ensure constant humidity. For enzyme extn complete etiolated seedlings were used.

Chemicals. [^{14}C]IAOX and [^{14}C]IAALD were prepd from [^{14}C]-DL-tryptophan (Amersham) by a procedure described elsewhere [34]. Unlabelled IAALD and IAA were from Sigma. Unlabelled IAOX was synthesized from IAALD–$NaHSO_4$ as described elsewhere [35]. The substrates were stored in benzoic soln at 4°. All other chemicals were of analytical grade.

Extraction and purification of enzyme. Washed seedlings (fr. wt 12 g) were homogenized in a precooled mortar with 1 ml/g fr. wt extraction buffer (0.1 M Tris-HCl, 2 mM EDTA, 2 mM glutathione, pH 7) by grinding with acid washed sand. The homogenate was centrifuged at 50 000 *g* for 1 hr. The supernatant

(= crude extract) was desalted on Sephadex G25 (elution with 0.05 M Tris–HCl, 1 mM EDTA, 1 mM glutathione pH 7).

Protein (50 mg) from the desalted extract was bound to DEAE-cellulose (column vol 10 ml, equilibrated with 0.05 M Tris–HCl, 1 mM EDTA, 1 mM glutathione, pH 7). The column was subsequently washed with 30 ml equilibration buffer and 20 ml 0.1 M Tris–HCl buffer (1 mM EDTA, 1 mM glutathione, pH 7). Fractions with enzyme activity were then eluted by 0.2 M Tris–HCl buffer (1 mM EDTA, 1 mM glutathione, pH 7). The salt concn of the fractions was determined by conductivity measurements. Protein was assayed according to ref [36] after TCA pption.

Assay for enzyme activity. Active fractions from the DEAE eluate were pooled and used as enzyme prepn. The incubation mixture contained in a total vol of 1 ml: 50 μl enzyme prepn, 0.1 M Tris HCl, pH 7, 1 mM EDTA, 1 mM glutathione, 100 μM NAD and 500 nM [^{14}C]IAOX (1.85 M Bq/μmol). Incubation time was 6 hr at 35°. The reaction was stopped by the addition of 5 ml CH_2Cl_2 and rapid extn of the neutral organic phase by vigorous stirring. Controls were run with a boiled enzyme prepn (5 min at 100°). A sample was taken from the aq. phase and its radioactivity determined (= assay for IAA formation).

For substrate kinetics the substrate concn ranged from 1 mM to 0.5 μM. Up to 10 μM concn only labelled substrate was used. For higher concns 1 μM [^{14}C]IAOX (1.85 M Bq/μmol) was diluted with unlabelled substrate. For determination of the pH optimum mixtures of 0.1 M Tris and 0.1 M MES (both 1 mM EDTA, 1 mM glutathione) were used.

Identification of reaction products. The neutral organic fraction was dried with Na_2SO_4 and its vol reduced by rotary film evapn. It was then applied to TLC (or HPLC). TLC was performed on silica gel F254 plates (Merck) with $CHCl_3$–MeOH (24:1), which sepd the indolic compounds (R_f IAA 0.01, IAOX 0.25, IAALD 0.34, IAN 0.55). Labelled products were identified by co-chromatography with authentic standards (fluorescence quenching at 254 nm). The chromatogram was divided horizontally into 0.5 cm zones and the silica gel was scraped off with a micro spatula. The radioactivity of the individual zones was determined after vigorously stirring in 1 ml EtOH and addition of 3 ml of scintillant (Quickszint, Zinsser Analytical). The samples were counted in a liquid scintillation counter using the channels ratio method for quench correction.

HPLC sepn was achieved on a reversed phase Si C18 column (Hyperchrome NC, Bischoff) equipped with a pre-column (Guard Pak C18, Waters) and MeOH–20 mM ammonium acetate buffer (pH 3.5) (7:13) as solvent. Flow rate was 1 ml/min. Again, identification was by co-chromatography with authentic compounds and their UV-detection at 280 nm. The eluate was collected in 0.5 ml fractions which were mixed with 3 ml of scintillant. Their radioactivities were determined as already described.

For identification of IAA the aq. phase (after neutral organic extraction) was adjusted to pH 3.5 with 1 N HCl and extracted with CH_2Cl_2. This acid organic fraction was dried (Na_2SO_4), concd by rotary film evapn and analysed by TLC and HPLC, the latter allowing the sepn of IAA and indole-3-carbonic acid (R_t IAA 22 min, indole-3-carbonic acid 21 min).

Statistical treatment of data. All expts were repeated three times (= three independent enzyme extractions) and results are presented ± s.e. mean. The K_m value was calculated by linear regression analysis of the data after Lineweaver–Burk transformation.

Acknowledgements – We should like to thank the Biotronik Geräte GmbH, D-6457-Maintal-1, for providing additional HPLC equipment, and Mrs S. Ranostaj for preparing the manuscript.

REFERENCES

1. Underhill, E. W. (1967) *Eur. J. Biochem.* **2**, 61.
2. Mahadevan, S. and Stowe, B. B. (1972) in *Plant Growth Substances* 1970, p. 117. Springer, Berlin.
3. Mahadevan, S. and Stowe, B. B. (1972) *Plant Physiol.* **50**, 43.
4. Mahadevan, S. (1973) *Ann. Rev. Plant Physiol.* **24**, 69.
5. Helmlinger, J., Rausch, T. and Hilgenberg, W. (1985) *Phytochemistry* **24**, 2497.
6. Bjorkman, J. H. (1976) in *The Biology and Chemistry of the Cruciferae*, p. 190. Academic Press, London.
7. Gmelin, R. and Virtanen, A. I. (1961) *Ann. Acad. Sci. Fennicae AII* **107**, 3.
8. Searle, L. M., Chamberlain, K., Rausch, T. and Butcher, D. N. (1982) *J. Exp. Botany* **33**, 935.
9. Mahadevan, S. and Thimann, K. V. (1964) *Arch. Biochem. Biophys.* **107**, 62.
10. Thimann, K. V. and Mahadevan, S. (1964) *Arch. Biochem. Biophys.* **105**, 133.
11. Schneider, E. A. and Wightman, S. F. (1974) *Ann. Rev. Plant Physiol.* **25**, 487.
12. Sembdner, D. S., Gross, D., Liebisch, H. W. and Schneider, G. (1980) in *Hormonal Regulation of Development I. Molecular Aspects of Plant Hormones*, p. 281. Springer, Berlin.
13. Schraudolf, H. and Weber, H. (1969) *Planta* **88**, 136.
14. Grob, K. and Matile, Ph. (1979) *Plant Sci. Letters* **14**, 327.
15. Matile, Ph. (1980) *Biochem. Physiol. Pflanzen* **175**, 722.
16. Helmlinger, J., Rausch, T. and Hilgenberg, W. (1983) *Physiol. Plant.* **58**, 302.
17. Butcher, D. N., El-Tigani, S. and Ingram, D. S. (1974) *Physiol. Plant Pathol.* **4**, 127.
18. Rajagopal, R. and Larsen, P. (1972) *Planta* **103**, 45.
19. Shukla, P. S. and Mahadevan, S. (1968) *Arch. Biochem. Biophys.* **125**, 873.
20. Shukla, P. S. and Mahadevan, S. (1970) *Arch. Biochem. Biophys.* **137**, 166.
21. Helmlinger, J. (1986) Doctoral thesis, J. W. Goethe Universitat, Frankfurt, F.R.G.
22. Kumar, S. A. and Mahadevan, S. (1963) *Arch. Biochem. Biophys.* **103**, 516.
23. Yamafuji, K. (1953) *Nature* **171**, 745.
24. Omura, H. and Tsutsumi, M. (1968) *Enzymol.* **34**, 187.
25. Wightman, F. and Cohen, D. (1968) in *Biochemistry and Physiology of Plant Growth Substances*, p. 273. Runge Press, Ottawa.
26. Liu, S. T., Katz, C. D. and Knight, C. A. (1978) *Plant Physiol.* **61**, 743.
27. Rajagopal, K. (1971) *Physiol. Plant.* **24**, 272.
28. Miyata, S., Suzuki, Y., Kamisaka, S. and Masuda, Y. (1981) *Physiol. Plant.* **51**, 402.
29. Bower, P. J., Brown, H. M. and Purves, W. K. (1978) *Plant Physiol.* **61**, 107.
30. Kindl, H. and Schiefer, S. (1971) *Phytochemistry* **10**, 1795.
31. Rausch, T., Kahl, G. and Hilgenberg, W. (1986) *Physiol. Plant.* **68**, 458.
32. Percival, W., Purves, W. K. and Vickery, L. E. (1973) *Plant Physiol.* **51**, 739.
33. Brown, H. M. and Purves, W. K. (1980) *Plant Physiol.* **65**, 107.
34. Hofmann, F., Rausch, T. and Hilgenberg, W. (1981) *J. Label. Comp. Radiopharm.* **18**, 1491.
35. Rausch, T., Helmlinger, J. and Hilgenberg, W. (1985) *J. Chromatogr.* **318**, 95.
36. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.