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OCCURRENCE, TITRATION AND ENZYMATIC DEGRADATION OF 3- (3-INDOLYL)-ACRYLIC ACID IN *LENS CULINARIS* MED. EXTRACTS

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Abstract—Indolylacrylic acid (AIAcryl) was identified as the main auxin in lentil root extracts. A sensitive and specific method has been developed for the quantitative determination of AIAcryl at a concentration as low as 10^{-7}M. The AIAcryl level in lentil roots increases with increasing age of the seedlings. The compound is enzymatically destroyed by a crude lentil root extract and the reaction shows two optimum pH. Purified horseradish peroxidase also destroys AIAcryl. AIAcryl destruction by fractions obtained from Sephadex filtration of the crude lentil extract parallels the occurrence of IAA-oxidase and peroxidase activities in the fractions.

INTRODUCTION

THE INDOLACETIC acid (IAA)—like substance[1] of lentil root was recently identified[2] as 3-(3'-indolyl)-acrylic acid (AIAcryl) and shown to occur in other Leguminosae. Further evidence for the natural occurrence of this compound, together with a spectrophotometric method for its estimation are described in this paper. Furthermore, in view of the great interest with regard to auxin catabolism in growth regulation,[3] *in vitro* assays of AIAcryl destruction were carried out using crude and purified lentil root extracts. Some results are presented here.

RESULTS

Occurrence and Titration of AIAcryl

AIAcryl from ether and ethyl acetate extracts of *Lens culinaris* seedlings (cultivated either in aseptic or non-aseptic conditions) was identified by its chromatographic behaviour and other characteristics compared with those of pure AIAcryl and IAA. On paper chromatograms in isopropanol–ammonia–water (IAW), AIAcryl has an R_f (0·29) very close to that of IAA (0·33); isobutanol–methanol–water (IMW), however, gives a reasonable resolution of the two compounds: AIAcryl, R_f, 0·68; IAA, 0·51. AIAcryl and IAA can be readily distinguished by the different colours given with various reagents: Ehrlich's gives a green colour with AIAcryl, whereas IAA gives a violet; dimethylaminocinnamaldehyde[4] with AIAcryl gives a brown colour and with IAA, violet-blue. AIAcryl is readily separated from indolyl-lactic acid (ILA). In the IAW and IMW solvents, ILA has R_f values of 0·36 and 0·22 respectively. ILA, like IAA, gives a violet colour with Ehrlich's reagent.

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[1] G. COLLET, J. DUBOUCHET and P. E. PILET, *Physiol. Plantarum* 2, 157 (1964).
[2] M. HOFINGER, *Arch. Intern. Physiol. Biochim.* 77, 225 (1969).
[3] P. E. PILET and TH. GASPAR. *Le Catabolisme Auxinique*, p. 148, Monographie de Physiologie végétale No. 1 (edited by MASSON), Paris (1968).
[4] J. HARLEY-MASON and A. A. ARCHER, *Biochem. J.* 69, P60 (1958).

A method for estimating AIAcryl in low concentration (5×10^{-7}M) was developed, based on the green color given with *p*-dimethylaminobenzaldehyde. Using this method it was found that a minimum of 20 hr (at 2°) is needed for extracting all the AIAcryl with ethyl acetate (Fig. 1). It was also shown that the AIAcryl content (per g of fresh root) rises as the seedlings' age increases (Fig. 2). It should be noted that a seed extract shows no noticeable trace of AIAcryl.

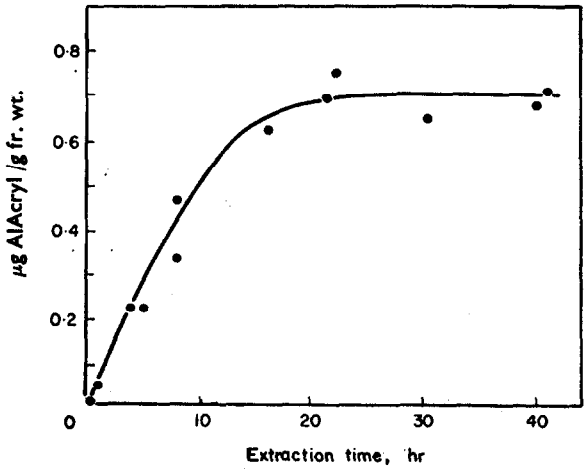

FIG. 1. RELATIONSHIP BETWEEN TIME OF EXTRACTION (hr) AND QUANTITY (μg) OF AIACRYL EXTRACTED FROM 3-day-old SEEDLINGS.

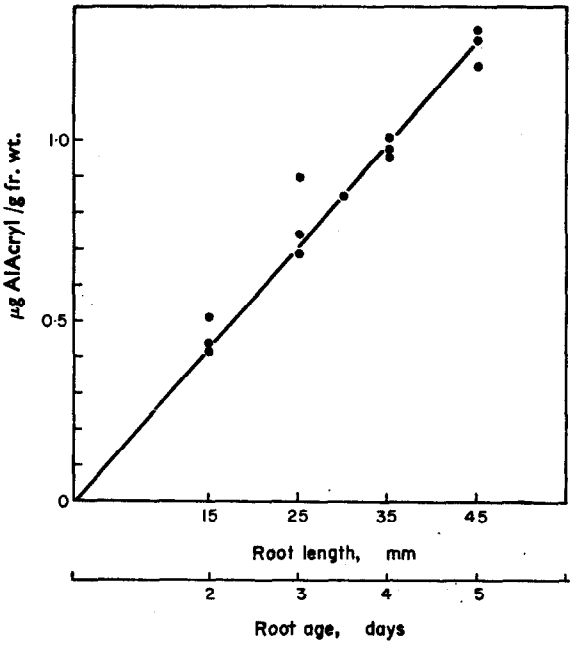

FIG. 2. AIACRYL CONTENT OF LENTIL ROOT WITH INCREASING AGE.

AIAcryl Degradation by a Lentil Roots Extract

It was found that crude buffer (pH 7·8) extracts of lentil roots can destroy AIAcryl. Using a universal buffer mixture,[5] two pH optima were found for this degradation, pH 5·5 and 7·8. At pH 7·8, the rate of AIAcryl destruction is about twice as fast as at pH 5·5. Since no destruction occurs with a boiled extract at any pH, the degradation is enzymic.

Sephadex Separation of the Enzymes Responsible for AIAcryl Destruction

A crude buffer extract of lentil roots was separated on Sephadex G 200 and peroxidase and AIAcryl destruction activity analysed in each fraction at pH 5·5 and 7·8 (Fig. 3). At

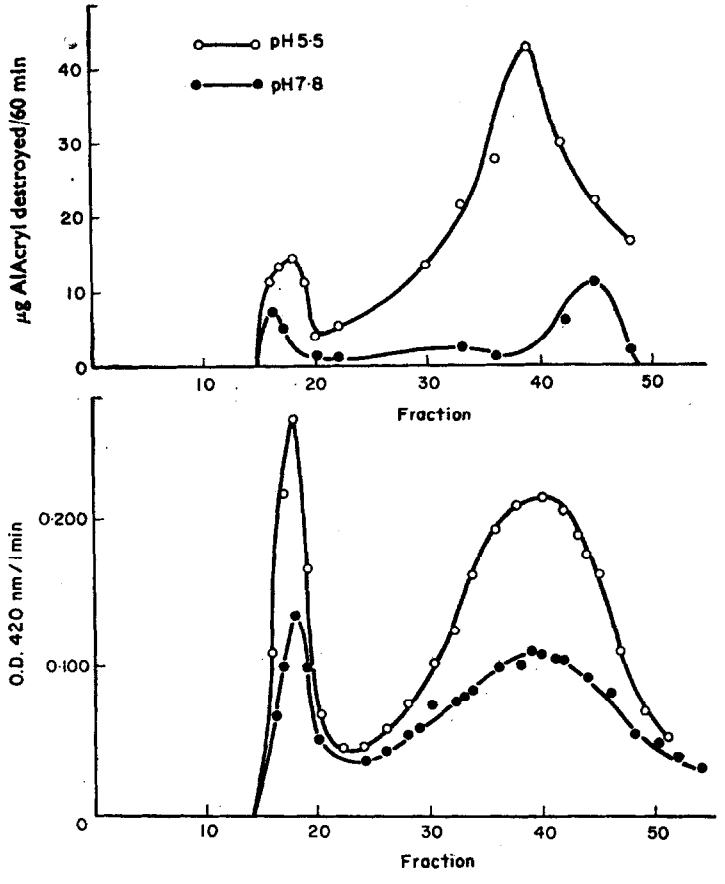

FIG. 3. AIACRYL DESTRUCTION AND PEROXIDASE ACTIVITIES IN FRACTIONS OBTAINED FROM SEPHADEX G 200 OF A CRUDE LENTIL ROOT EXTRACT.

pH 5·5, two peaks were found for peroxidase, superimposable on the two peaks for AIAcryl degradation (in presence of H_2O_2). Two peroxidase peaks can also be distinguished at pH 7·8 although the activities are lower. At pH 7·8, AIAcryl destruction is also slower, and the two peaks which are observed do not correspond to those of peroxidase.

[5] *Biochemists' Handbook* (edited by C. LONG), E. & F. N. Spon Ltd., London (1961).

AIAcryl Degradation by Purified Horse-radish Peroxidase (HRP)

AIAcryl is destroyed by HRP at pH 5·5 in the presence of H_2O_2. 2·4-dichlorophenol (DCP) activates the destruction in the presence of H_2O_2, whereas manganese ($MnCl_2$) alone stimulates the AIAcryl degradation by HRP, although this is more effective when H_2O_2 is also present.

DISCUSSION

Surprisingly, AIAcryl has not been identified in plant extracts before now. Two reasons may be given; first, AIAcryl seems to be limited in the Leguminoseae,[2] second, it is easily masked in most chromatograms by IAA or other indolic compounds. AIAcryl was listed[6] as a possible compound in *Vicia faba*, but the characteristics given (R_f, colour reactions, auxin activity, etc.) did not correspond to those of pure synthetic AIAcryl given above.

The presence of AIAcryl in relatively large quantities and its large auxin activity[2] seem to indicate that this new natural compound is the main auxin controlling lentil root growth. Moreover, the increasing AIAcryl level in growing lentil roots parallels the growth curve[7] in this tissue. This discovery raises the question of the validity of numerous earlier observations of the relationship[3] between IAA catabolism and growth in this tissue.

The preliminary experiments reported here indicate that AIAcryl is destroyed by lentil extracts and by purified horse-radish peroxidase. However, the action of the latter enzyme is apparently not the same as its action against IAA, as can be seen from the results of adding varying reagents (DCP, H_2O_2, $MnCl_2$), on the reaction. It is encouraging however, that the destruction of AIAcryl and IAA have the same general pattern and parallel the activity of the two peroxidases,[8] after gel filtration of a crude enzymic extract of the lentil roots. It seems most probable that variations in AIAcryl catabolism thus correspond to those in IAA. The lack of correspondence between AIAcryl destruction and peroxidase at pH 7·8 can be explained by presuming different isoperoxidases[8] which would have a varying affinity for AIAcryl.

EXPERIMENTAL

AIAcryl extraction. 3 G of *Lens culinaris* Med. roots (grown at 27° in the dark on moistened vermiculite) were harvested and ground in a mortar with sand and dry ice. The frozen powder was well-mixed with ethyl acetate (10 ml/g fr. wt.) and left in the dark at 2° for 24 hr (see Fig. 1).

The ethyl acetate extract was evaporated to dryness *in vacuo*, re-dissolved in a minimum volume of ethyl acetate and chromatographed on Whatman No. 1 paper in IsoPrOH–25% NH_3–H_2O 10:1:1, v/v. AIAcryl was located by u.v. light, the band cut out and eluted in 4 ml of MeOH.

AIAcryl estimation. Reagent: 3 G of *p*-dimethylaminobenzldehyde in MeOH (40 ml) and 6% HCl (10 ml). Method: 3 ml of methanolic AIAcryl solution were mixed with 1·5 ml of the reagent and heated at 60° for 2 hr. The mixture was cooled and its absorbancy measured at 625 nm. Absorbancy in MeOH = 8×10^4 [AIAcryl] between concentrations 0 and 10^{-5} M (Absorbance = 0·800). A concentration of 5×10^{-7} M can be easily estimated. Neither indolylacetic acid nor tryptophan react with the reagent under the described conditions.

In vitro *AIAcryl destruction.* Crude enzyme extracts of lentil roots were made by grinding 200 roots with 10 ml phosphate buffer (pH 7·8, I = 0·05, 0°); after 30 min extraction the brei was centrifuged and the supernatant used. Incubation mixture contained: 1 ml 5×10^{-4} M AIAcryl (in H_2O), 1 ml enzymic extract or HRP solution, and either 3 ml buffered H_2O_2 solution (0·02 vol.) when HRP or a purified extract was used, or buffer when a crude extract was used. 0·2 ml aliquots of the mixtures were taken at various times and added to 1 ml of the aldehyde reagent diluted with 2 ml MeOH and treated as described above.

[6] D. BURNETT, L. J. AUDUS and H. D. ZINSMEISTER, *Phytochem.* **4**, 891 (1965).
[7] TH. GASPAR, *Bull. Soc. Roy. Sci. Liège* **34**, 391 (1965).
[8] TH. GASPAR, M. HOFINGER and J. LACOPPE, *Biochim. Biophys. Acta* **191**, 463 (1969).

Separation of proteins on Sephadex. 2 ml of the lentil root crude enzyme extract were separated on a Sephadex G 200 column (40 × 2 cm) equilibrated with phosphate buffer (I = 0·05, pH = 7·8) at 2° 2 ml Fractions were collected and analysed for AIAcryl destruction and peroxidase activity (incubation mixtures: 1 ml guaiacol 1 ‰, 1 ml H_2O_2 0·2 M, 8 ml phosphate buffer, I = 0·2 and 0·1 ml of a fraction. The absorbancy is measured at 420 nm after 1 min).

Note added in proof—After submitting this paper, it was found that AIAcryl is actually present in the seedlings as an indole complex. It is released, presumably by a still active enzyme present in the seedling powder, during ether or ethyl acetate extraction. The time for extraction given in the paper, therefore possibly corresponds to the enzymic release of AIAcryl at the low temperature employed. The fact that the plant contains such an enzyme produces AIAcryl from a precursor promises further interest in this substance and its relation to growth.