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Proposed Enzymes of Auxin Biosynthesis and Their Regulation III. Some Properties of Pea Indolylacetaldehyde Oxidase

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Abstract. Indole-3-acetaldehyde oxidase (IAAld-oxidase) occurs in pea in two forms, of which the first, more active enzyme, has its pH optimum at 4.5, while the second, barely half as active, has a pH optimum at 7.0. Only the pH 4.5 oxidase can be resolved from the acetone powder. Besides IAAld the more stable IAld was used as substrate in testing the enzymatic activity. The pea enzyme seems not to be a dismutase since indolylmethanol or indolylethanol were not formed as products. Pyridine nucleotide coenzymes did not activate the partially purified enzyme. The pH 4.5 oxidase was inhibited by more than 50 % by IAA > L-asp > tryptophol > indoleacetylaspartic acid > 2,4-D (at 1 mM concentration). The pH 7.0 oxidase was inhibited relatively more weakly, a stronger than 50 % inhibition was caused only by NAA > L-asp. The oxidases were clearly distinguished by the response to L-asparagine (1 mM): the activity of the pH 4.5 oxidase was increased (+ 12 %), while the activity of the pH 7.0 oxidase was decreased (– 71 %). In preliminary *in vitro* experiments the phytohormones (1 mM) kinetin and GA_3 increased the conversion of IAAld to IAA, while ABA decreased it.

Indole-3-acetaldehyde (IAAld) is proposed as the key intermediate (Larsen 1944, 1949, Larsen and Rajagopal 1964, Rajagopal 1967, 1968a, Wightman and Cohen 1968, Purves and Brown 1978) and direct precursor in the biosynthesis of IAA in higher plants.

A considerable amount of information has already been collected concerning the enzymatic conversion of IAAld to IAA (Larsen 1949, Rajagopal 1968a,b, Rajagopal and Larsen 1972, Wightman and Cohen 1968, Liu *et al.* 1978, Bower *et al.* 1978), but a final knowledge of this process has not yet been achieved. It is also possible that the system metabolizing IAAld differs in individual plants (Schneider *et al.* 1972, Schneider and Wightman 1978). The basic problem consists in the recognition of the type of enzymatic reaction of the IAAld conversion, i.e. whether it is catalyzed by a dismutase (Rajagopal 1968b) or by a pyridine nucleotide dehydrogenase (Wightman and Cohen 1968) or an oxidase (Bower *et al.* 1978, Rajagopal 1971).

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In a series of investigations Purves and coworkers described the accumulation and metabolism of indole-3-ethanol (tryptophol (TOH)) in cucumber seedlings (Brown and Purves 1980, Rayle and Purves 1967). This compound seems to contribute to the regulation of the IAAld level in plants. In this regulatory system IAAld is converted by an allosteric IAAld-reductase (with pyridine nucleotide coenzymes) (Bower *et al.* 1976, Brown and Purves 1976, 1980) to TOH, which under the effect of TOH-oxidase (probably a flavoprotein) (Percival *et al.* 1973, Vickery and Purves 1972) gives back IAAld. The growth inactive TOH may be a reserve product by means of which the equilibrium of the IAAld level in plants is maintained (Brown and Purves 1980). IAAld itself is converted to IAA by means of IAAld-oxidase, regulated by excess of IAA (Bower *et al.* 1978, Brown and Purves 1980).

Our aim was to present some basic information on the IAAld-oxidase system in pea. We also investigated the regulatory effect of some natural substances, indoles, amino acids and phytohormones on this enzymic activity.

MATERIAL AND METHODS

Sterilized seeds of pea (*Pisum sativum* L. cv. Jupiter) were germinated under aseptic conditions and further cultivated at 25 °C in darkness (Sahulka 1972). For the preparation of enzyme extracts 7–9 days-old seedlings were used.

Preparation of enzyme extracts

IAAld-oxidase was prepared by the modified method of Suzuki *et al.* (1981), in two variants, using buffers of pH 4.5 and 7.0 for extraction. The shoots of etiolated plants were homogenized at 0–4 °C with a double volume (m/v) of a 100 mM phosphate buffer pH 7.0, containing 1 mmol l^{-1} of mercaptoethanol, 0.1 mmol l^{-1} of EDTA and 20 % of glycerol together with insoluble polyvinyl-polypyrrolidone (PVPP) (0.06 g per 1 g of plant material). After 2 h standing at 0–4 °C the homogenate was filtered through a cloth and centrifuged at 10 000 *g* and 0–4 °C for 30 min. The supernatant was saturated with ammonium sulphate to 80 % and the precipitates were separated by centrifugation (10 000 *g*, 30 min, 0–4 °C). The pellet was dissolved in a minimum amount of 50 mM phosphate buffer (pH 4.5 or 7.0), and dialysed at 0–4 °C for 10–12 h against

Abbreviations used: IAAld = indole-3-acetaldehyde; TOH = tryptophol (indole-3-ethanol); IAA = indole-3-acetic acid; EDTA = ethylenediaminotetraacetic acid; IAld = indole-3-aldehyde; ICOOH = indole-3-carboxylic acid; IMeOH = indole-3-methanol; NAA = α-naphtylacetic acid; 2,4-D = 2,4- dichlorophenoxyacetic acid; IAAsp = indoleacetylaspartic acid; IAN = indole-3-acetonitrile; L-asp = L-aspartic acid; L-asn = L-asparagine; trp = tryptophan; phe = phenylalanine; GA_3 = gibberellic acid; ABA = abscisic acid; TCA = trichloroacetic acid; L-glu = glutamic acid; NAD(H) = nicotinamide adenine dinucleotide; NADP(H) = nicotinamide adenine dinucleotide phosphate.

a 50 mM phosphate buffer of pH 4.5 or 7.0, respectively. The volume of the dialysate was adjusted so that 1 ml of the enzyme extract contained 0.5 mg of protein.

Determination of the enzymatic activity

a) Using indole-3-aldehyde (IAld) as substrate. The chemically unstable substrate IAAld was replaced by the much more stable IAld. It is known that IAld is oxidized to indole-3-carboxylic acid (ICOOH) at 88 % of the rate of conversion of IAAld to IAA (Rajagopal 1971). This substrate also permitted a direct determination of the effect of IAA on the oxidase activity. A solution of substrate was prepared as follows: IAld 1 mM was dissolved in a drop of ethanol, than glycerol (20 % of the total volume) and the phosphate buffer (pH 4.5 or 7.0) in a final 100 mM concentration were added. The enzyme assay, of a total volume of 3 ml, contained IAld in 0.5 mM final concentration, the enzyme (0.5 mg of proteins), all in a solution of 100 mM phosphate buffer containing 20 % glycerol. The incubation took place in a water bath at 30 °C for 1 h. The reaction was stopped by addition of 100 µl of 50 % trichloroacetic acid (TCA). ICOOH formed by the enzymatic reaction was extracted with three 3 ml portions of ethyl acetate from the acidified incubation solution. The combined extracts were evaporated and the residue was dissolved in 1 ml of ethanol. 500 µl of the solution were applied on a thin layer of cellulose (Lucefol, Kavalier, Czechoslovakia). ICOOH was separated from the remaining IAld in isopropyl alcohol – ammonia – water 10:1:2. The cellulose layer, containing ICOOH was scratched off and eluted twice with ethanol (2 ml), at 35 °C for 30 min. After filtration the eluted ICOOH was determined spectrophotometrically at 284 nm.

b) Using IAAld as substrate. The IAA formed was determined colorimetrically (Bower *et al.* 1978).

Checking of indole-3-methanol formation by the enzyme

The ethyl acetate extract from the incubation solution was analyzed for the presence of indole-3-methanol (IMeOH) by means of TLC on silica gel layers (Silufol, Kavalier, Czechoslovakia). Three solvent systems were used: chloroform-methanol-water (50:20:30); isobutanol-methanol-water (80:50:15); chloroform – 96 % ethanol (60:40). The spots on chromatograms were detected by Procházka's reagent (Hais and Macek 1958).

Interaction of some naturally occurring compounds with IAAld oxidase

With both IAAld-oxidases, having optima at pH 4.5 and 7.0, the effect of some substances was tested: a) IAA, synthetic auxins, naturally occurring indoles; b) amino acids and amides; c) kinetin, GA_3, ABA; d) pyridine nucleotide

coenzymes. Substances from the groups a) to c) were tested at 1 mM concentration, compounds of the group d) at a 0.1 mM concentration. The compounds were preincubated with the partially purified enzyme for 15 min. Suitable chromatographic systems in which ICOOH does not interfere with the investigated compounds were used.

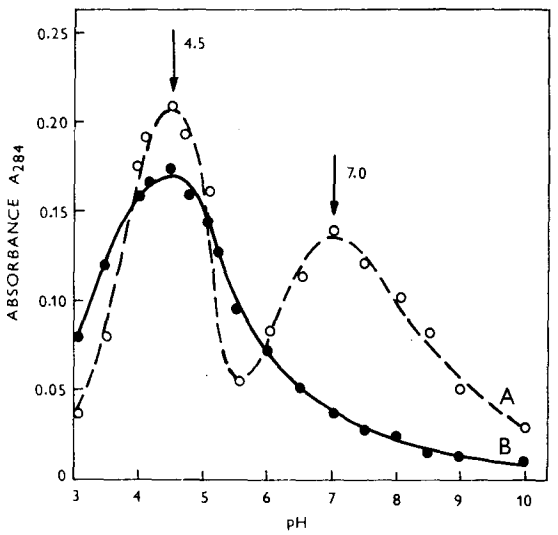

Fig. 1.: Effect of pH on the activity of indolylacetaldehyde oxidase from pea. Comparison of the activity of the enzyme isolated from fresh plant material (O - - - - O) and the enzyme isolated from acetone powder (●———●).

Determination of proteins in the enzyme extract

The proteins were determined using Coomassie Brilliant Blue G 250 according to the method of Bradford (1976).

RESULTS

Some kinetic data of the oxidases

The effect of H^+ concentration on the enzymatic activity was measured within a broad pH range, from 3.0 to 9.0 (100 mM citrate buffer, 100 mM phosphate buffer). IAld was oxidized actively at two pH intervals, with activity optima at pH 4.5 and 7.0 (Fig. 1). The activity at pH 4.5 was higher (195 %) then the activity at pH 7.0 (100 %). On extraction of the enzyme from an acetone powder the oxidase activity was found only in the pH 4.5 fraction.

The activity of both enzymes increases practically linearly up to 1 h. An almost linear increase of activity of both enzymes was observed up to 0.6 mg (0.2 mg ml^{-1}) of enzyme protein. On denaturing by boiling the enzymes lost their activity.

TABLE 1

Regulation of the activity of indolylacetaldehyde oxidase from pea at pH 4.5 and pH 7.0 with selected compounds [1 mM]: a) IAA and auxinoids, b) indole compounds, c) amino acids and d) phytohormones

a)

Inhibitor [10^{-3}M]	% inhibition IAAldox. pH 4.5	pH 7.0
IAA	−88.9	−34.0
NAA	−63.9	−77.5
2,4–D	−50.0	−30.0

b)

Inhibitor [10^{-3}M]	% inhibition IAAldox. pH 4.5	pH 7.0
IAAsp	−67.8	−31.0
TOH	−75.0	−31.0
IAN	−30.9	−20.0
IAAld	−44.5	−55.0
I-COOH	−74.6	−26.0

c)

Inhibitor [10^{-3}M]	% inhibition IAAldox. pH 4.5	pH 7.0
Aspartic acid	−81.6	−31.0
Asparagine	+12.0	−71.0
Glutamic acid	+5.0	+24.0
Glutamine	0	+15.0
L-trp	+5.6	0
D-trp	−1.1	0
L-phe	+2.2	+10.0
D-phe	0	+2.0

d)

Inhibitor [10^{-3}M]	% inhibition IAAldox. pH 4.5	pH 7.0
GA_3	+16.7	+40.0
Kinetin	+52.4	+21.3
ABA	−60.0	−72.5

Effect of pyridine nucleotide coenzymes

At pH 4.5 and at pH 7.0 no effect of an addition of pyridine nucleotides NADP and NADPH on the activity of the partially purified enzyme could be observed. This was found both by the determination of an unincreased amount of ICOOH, and on the basis of the absence of the photometric response at 340 nm.

Study of the products of the enzymatic conversion of indole-3-aldehyde in pea

For the determination of the character of the enzymatic reaction, *i.e.* whether or not it is of the oxidase or dismutase type, the end products of IAld conversion were analyzed. Only the presence of ICOOH and the remains of IAld could be chromatographically detected. (The absence of IMeOH makes the dismutase character of the investigated conversion of IAld improbable.)

Effect of individual groups of substances on the activity of IAAld-oxidase from pea

a) IAA and synthetic auxins. At 1 mM concentration IAA as well as NAA and 2,4-D inhibited the activity of both oxidases. The inhibition caused by IAA and 2,4-D was more distinct in the case of the pH 4.5 oxidase, but this was not the case for NAA (Table 1a).

b) Other indole compounds. TOH and indolelacetylaspartic acid (IAAsp) inhibited both oxidases more effectively than indolelacetonitrile (IAN) (Table 1 b). The suitability of the IAld as substrate was confirmed by the inhibitory effect of ICOOH, determined also with IAAld as substrate, which was similar for both enzymes to the inhibition caused by IAA and determined using IAld as substrate. IAAld competed with IAld for the binding sites of the enzymes.

c) Amino acids and amides. Among the amino acids investigated (Table 1c) L-asp had a distinct inhibitory effect, especially on the pH 4.5 oxidase. The influence of L-asparagine (L-asn) differentiated the two enzymes. L-glu stimulated the activity of both oxidases slightly. L-trp and L-phe and their D-isomers remained without effect.

d)Other phytohormones. *In vitro* a positive effect of GA_3 and kinetin was detected on pea IAAld-oxidase activity. On the other hand, ABA inhibited the conversion of IAAld to IAA (Table 1d).

DISCUSSION

In the literature a considerable confusion exists with respect to the pH optimum of the IAAld-oxidase activity in individual plants. In the majority of so far investigated experimental plants pH 7.0 was described as optimum (Bower *et al.* 1978, Rajagopal 1968a, 1971, Suzuki *et al.* 1981, Wightman and Cohen 1968), with the exception of oat, *Nicotiana glauca* and *Nicotiana langsdorfii,* in

which the oxidases were determined at pH 4.4 and 4.1 (Liu *et al.* 1978, Rajagopal 1971, Rajagopal and Larsen 1972). In pea and later in tissue cultures of *Nicotiana tabacum* (El Bahr *et al.* 1984) we found both types of activities simultaneously, at pH 4.5 and 7.0. The effect of inhibitors and activators is comparable to a considerable extent. We assume that the active sites in both oxidases are rather similar. In contrast to this, in addition to the different pH optimum, further differences have been observed, for example the activating and the inhibiting effect of L-asn. The stability of both enzymes towards acetone was also different. While the activity of the oxidase pH 4.5 was decreased only negligibly by acetone, the oxidase pH 7.0 almost lost its activity in acetone powder. We believe that the enzyme with pH optimum 7.0 differs from the pH 4.5 oxidase by a less strongly bound lipid component, which, however, is indispensible for the activity of the enzyme. Under the effect of acetone the lipid component of the oxidase pH 7.0 is probably eliminated. The idea of the presence of a lipid component is supported by the finding by Rajagopal and Larsen (1972) that lipase and phospholipase D affect both types of oxidases from oat. They differentiated the oat enzymes at pH 4.4 by means of the electron acceptor, *i.e.* O_2 or phenazine methosulphate.

The type of reaction of the IAAld converting enzyme system has not yet been determined accurately. In pea plants it seems that its dismutase character may be excluded, because a simultaneous formation of IMeOH beside ICOOH during the enzymatic reaction was not observed. The addition of pyridine nucleotide coenzymes did not increase the activity of the conversion, which makes the dehydrogenase character of the pea enzyme improbable (Rajagopal 1971, Wightman and Cohen 1968). For a more detailed knowledge of the oxidase character the stoichiometric determination of the oxygen consumption will be a contribution.

In the case of IAAld-oxidase a regulatory role in the IAA biosynthesis could be expected. It is a terminal enzyme of the IAA biosynthetic pathway and it follows the system regulating the IAAld level by the reversible conversion of IAAld to the growth inactive reserve product TOH (Brown and Purves 1980). IAAld oxidase is inhibited both by higher concentrations of IAA and synthetic auxins. The activity of IAAld-oxidases was further regulated by other indoles connected with the metabolism of IAA, *i.e.* IAAsp and TOH. Among amino acids L-asp is an effective inhibitor. All the inhibitors mentioned, with the exception of IAA and synthetic auxins also affect the first reaction of IAA biosynthesis, *i.e.* the transamination of L-trp (Terziivanova-Dimova and Kutáček, in press). The regulatory character of the IAAld-oxidase system is made more probable by the fact that its components with differing pH optimum are inhibited in a differential manner, *i.e.* in the majority of cases the enzyme pH 4.5 is inhibited more distinctly. However, NAA and L-asn depart from this scheme.

The regulatory character of IAAld-oxidase was also accentuated by preliminary *in vitro* experiments with different phytohormones. GA_3 (more at pH 7.0) and kinetin (more at pH 4.5) increased the conversion of IAld to ICOOH. On the contrary ABA inhibited this conversion. In this connection it will be advisable to check the allosteric character of the IAAld-oxidase.

From the presented study of pea IAAld-oxidase activity the complex character of the last enzymatic step of IAA biosynthesis is evident. We believe that information on its state and activity in a broader selection of plant species, the determination of the compartmentation of both its forms in the cell and the elucidation of the type of inhibitions observed could be important goals in advancing or understanding of IAA synthesis in higher plants.

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