SEPARATION OF IAA DEGRADING ENZYMES FROM PEA ROOTS ON COLUMNS OF POLYVINYLPYRROLIDONE

C. A. VAN DER MAST

Botanisch Laboratorium, Utrecht

SUMMARY

In extracts of pea roots two types of indoleacetic acid degrading enzymes are present. The first type is not retained by polyvinylpyrrolidone, shows peroxidase activity and does not denaturate in 3 M urea. The second type adsorbs to polyvinylpyrrolidone, does not show peroxidase activity and denaturates in 3 M urea. It is also destroyed by deoxycholate. Presumably it is associated with polyphenols.

Both types possibly aggregate with each other and with other proteins to form large complexes. Both depend for their action with indoleacetic acid on an added cofactor.

1. INTRODUCTION

Several studies on indoleacetic acid oxidase from various plant sources have demonstrated the complex nature of this enzyme. SIEGEL & GALSTON (1967) showed that the removal of the heme group from horse-radish peroxidase resulted in a loss of peroxidase activity. The protein nevertheless retained its indoleacetic acid oxidase activity. SEQUEIRA & MINEO (1966) and JANSSEN (1969) could isolate two separate enzyme fractions with indoleacetic acid oxidase activity.

In the present study a few experiments are described and discussed which also demonstrate the existence of several indoleacetic acid oxidising enzymes. Use has been made of column chromatography on polyvinylpyrrolidone, a material known to bind tenaciously phenolic compounds (ANDERSEN & SOWERS 1968) and that also shows adsorption chromatigraphic properties (LERNER *c.s.* 1968).

2. MATERIAL AND METHODS

Seeds of *Pisum sativum* c.v. "Vlijmsche gele krombek" were surface sterilized with 0.02% sublimate during 6 minutes, rinsed and imbibed in tap water for 2½ hours. The seeds were sown on moist filter paper and grown in darkness at a temperature of 23 °C and a relative humidity of 80%.

After about 65 hours roots of 2–3 cm length were cut and homogenized in phosphate-citrate buffer according to Mc Ilvaine, pH 5.0, which sometimes contained 6 M urea, or in Tris-HCl buffer (35 mM Tris, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$), pH 7.4. For every gram fresh weight 2 ml buffer were added.

Cell debris was removed by a 20 minutes centrifuging period at 27000 g. The supernatant was used as a crude enzyme preparation or further purified on

columns of polyvinylpyrrolidone (PVP). All operations were carried out in the cold.

In experiments with sodium deoxycholate this compound was added to the supernatant to a concentration of 0.5% and stirred during 30 minutes. Excess deoxycholate was then removed by centrifuging 5 minutes at 27000 g.

PVP (Polyclar AT powder) was purchased from the General Aniline and Film Corporation, New York. It was imbibed in distilled water and decanted frequently to remove the fines. The slurry was poured in perspex columns with a diameter of 3 cm and packed under a pressure of $2\frac{1}{2}$ atm. nitrogen. Bed length was usually about 15 cm. After bringing the column to the pH wanted with appropriate buffer, 7 ml pea root homogenate was laid on the gel bed, pushed into it and chromatographed with an elution buffer under $2\frac{1}{2}$ atm. Flow rate was normally 10–15 ml per hour, but when urea was added to this buffer it was around half this figure.

Fractions of 5 ml were obtained using a LKB fraction collector. The eluate was monitored at 280 nm.

Indoleacetic acid (IAA) degradation of the eluate was performed by adding 1 ml of the fractions to 1 ml Mc Ilvaine's buffer, pH 5.0, which contained 100 ppm IAA and 4 ppm p-coumaric acid. Incubation was carried out at 30°C for 20 minutes. Residual IAA was measured by adding 1 ml of the incubate to 4 ml Salkowski's reagent.

IAA conversion of crude homogenates was measured by adding 1 ml to 4 ml Mc Ilvaine's buffer, pH 5.0, which contained IAA and p-coumaric acid. End concentrations were the same as in the other experiments, i.e. 50 ppm IAA, 2 ppm p-coumaric acid and sometimes 3 M urea.

Peroxidase activity was determined with benzidine as reducing agent. As there is no quantitative relationship between the amount of H_2O_2 used in this reaction and the amount of IAA degradation, this test was only performed qualitatively.

Ribosomal material was detected with the orcinol test (COLOWICK & KAPLAN 1957).

Gel chromatography over Sephadex G-100 was performed using a gel bed of 34 × 1.9 cm. Elution was carried out with a 20 times diluted phosphate-citrate buffer, pH 5.0, which contained 6 M urea.

3. EXPERIMENTS

Pea roots homogenized in Tris-HCl buffer and chromatographed over PVP show four UV-absorbing peaks as shown in *fig. 1*. The first one corresponds with the high molecular weight peak obtained with gelchromatography over Sephadex G-100. Ribosomes are present in these fractions. The second peak corresponds with the low molecular weight peak obtained with the same Sephadex. The third peak elutes at a volume equal to the volume accessible to watermolecules. The PVP apparently adsorbs the enzyme molecules present in this fraction.

IAA-degrading capacity was found in the first three peaks. Addition of 1 M urea to isolation and elution buffer resulted in a threefold increase of IAA conversion in the third peak.

Assuming that phenols play a role in aggregating the IAA-degrading enzymes or in coupling them to other proteins, the following experiments were carried out at a lower pH in phosphate-citrate buffer.

Fig. 2 shows that in this buffer, probably due to the chelating action on ribosomal material, the first UV-absorbing peak is distinctly lower.

Part of the IAA-degrading enzymes are greatly retarded at this pH, possibly by hydrogen bonding with the PVP. This retarded enzyme fraction does not show peroxidase activity. The beginning of this fraction is indicated by arrows in the figures. Fraction number 8 to 12 form a transition zone with low peroxidase content.

It is also shown in this figure that addition of 6 M urea to the isolation and elution buffer abolishes all activity of non-peroxidative IAA-degrading enzymes. The remaining IAA degradation is confined to the first peak.

When a sample containing this amount of urea was run with a similar elution buffer through Sephadex G-100, this enzyme fraction eluted behind the peak of ribosomes and high molecular weight proteins which mark the void volume of the column. The active fraction corresponds with the low molecular weight enzyme as found by CRONENBERGER *c.s.* (1966) and JANSSEN (1969). Its molecular weight as determined by DETERMANN's (1967) formula gives a value of about

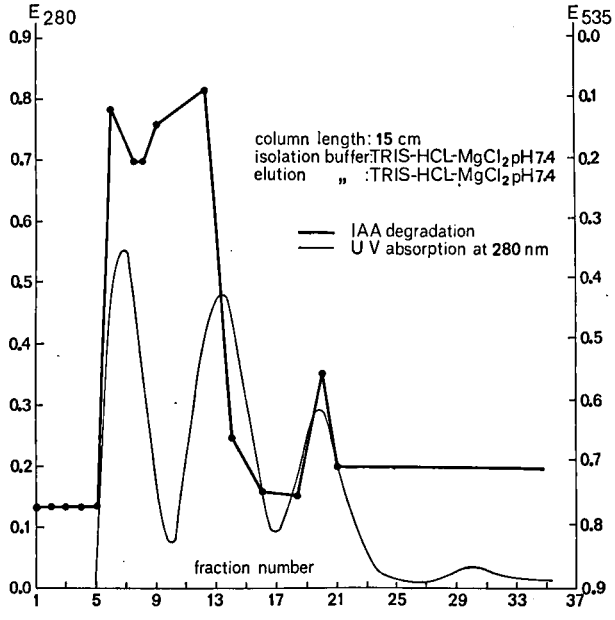

Fig. 1. UV.-absorption and IAA degradation activity in pea root homogenate chromatographed over PVP, pH 7.4.

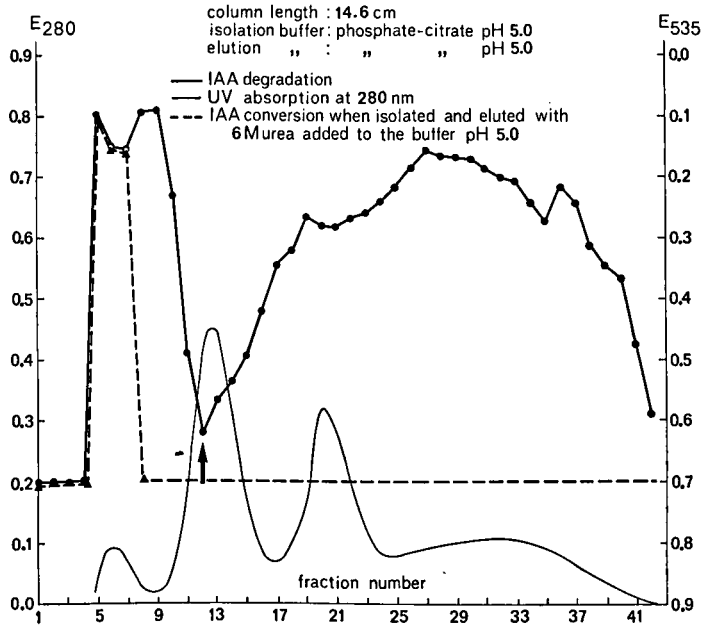

Fig. 2. UV. absorption and IAA degradation activity in pea root homogenate chromatographed over PVP, pH 5.0.

35000. This is in good agreement with the value of 32000 of the peroxidase from *Lens* roots as found by PILET & LAVANCHY (1969). This means that the molecular weight of the substances excluded from the PVP is smaller than this figure.

Addition of sodium-deoxycholate to the crude enzyme preparation instead of urea gave essentially the same result after chromatography over PVP, i.e. one peak of IAA-degrading activity, which also showed peroxidation. The effect of desoxycholate, however, is not reversible as is the case with urea, where after dilution of the homogenate to 1.2 M urea the normal rate of reaction – as compared with the control – is soon reached (*fig. 3*). If the preparation is diluted to 3 M urea the reaction proceeds slower than after purification over PVP.

If 6 M urea is added to the isolation buffer, but not to the elution buffer, retention of enzyme molecules can not take place as the bond formed with PVP is continously broken by the passage of the urea band (*fig. 4*).

This results in elution of the enzyme type that is normally adsorbed to the PVP, just prior to the emergence of the urea.

A small part of the enzyme activity is still found after the urea has passed out of the column, but this can be explained by dilution of the urea band due to the high content of dry matter in this type of column: 35 grams per 15 cm length giving a volume inaccessible to molecules of about 20 ml.

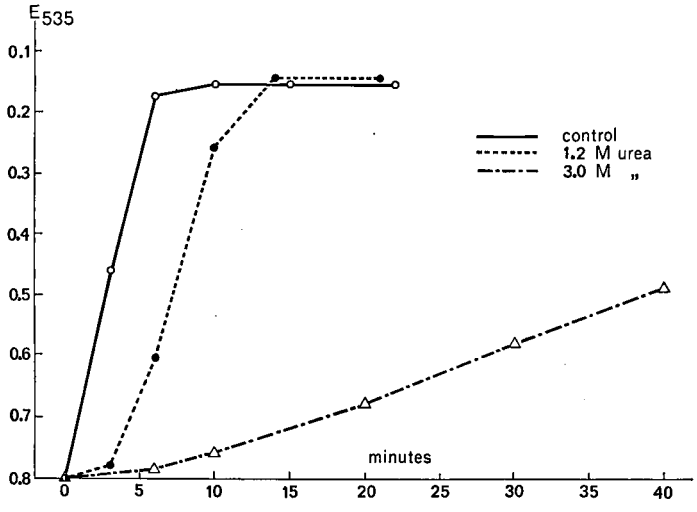

Fig. 3. The effect of urea on the rate of IAA degradation.

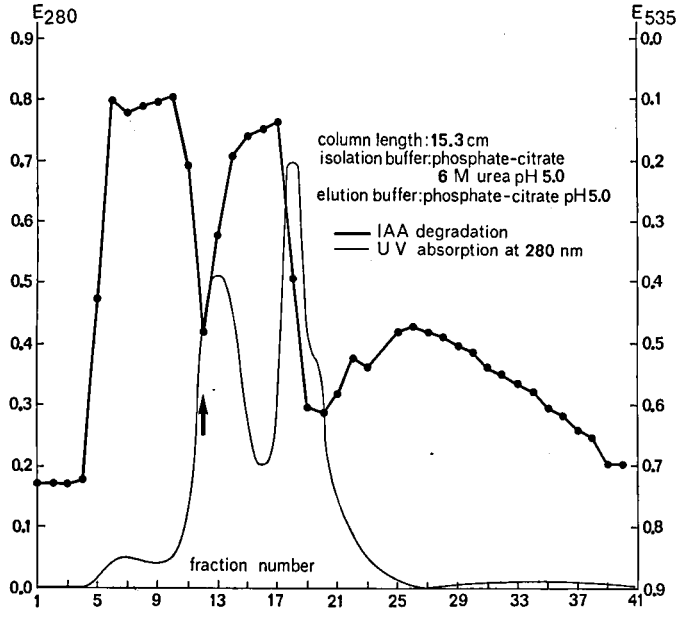

Fig. 4. The effect of urea on the adsorption of IAA degrading enzymes.

4. DISCUSSION

The experiments show that PVP also possesses gelchromatographic qualities outside the already reported adsorptionchromatographic properties (LERNER *c.s.* 1968).

From the results presented in this paper it is clear that there are two types of IAA-degrading enzymes present in homogenates of pea roots.

Adsorption of enzyme molecules proceeds better at a relatively low pH, as shown in *figs. 1* and *2*. But even at pH 7.4 adsorption probably takes place. From experiments of CRONENBERGER *c.s.* (1966) and JANSSEN (1969) with pea root homogenates it is clear that this type of enzyme activity is never found in the low molecular weight peak as obtained with gel chromatography over Sephadex G-100. As the present work makes probable that 35000 is the lowest molecular weight for IAA-degrading enzymes from pea roots and that these molecules elute when chromatographed over PVP just after the void volume has passed, it seems plausible that enzymes found in the second and the third peak in *fig. 1* are adsorbed. This extension of IAA-degrading enzymes into the second peak persists at pH 5.0. Probably enzyme molecules present here adsorb in a different manner than those present in the third and the fourth peak (*fig. 2*).

According to WAUGH (1954) urea in the concentrations used in these experiments disrupts hydrophobic bonds by formation of hexagonal hydrogen-bonded cagelike structures into which fit the apolar side chains of the constituent amino-acids of a protein.

As hydrophobic bonds form a major stabilizing factor in maintaining the configuration of a protein (BIGELOW 1967), the denaturation of the adsorbed enzyme reported in the present paper could be tentatively interpreted by assuming a larger ratio of apolar to polar side chains in its molecules than in those of the enzyme fraction which maintains its activity in urea. Also the action of deoxycholate, which possesses a large hydrophobic side in its molecule and as a result has a detergent action (SMALL *c.s.* 1969), points in that direction. Deoxycholate destroys the same enzyme fractions which are made inactive by urea.

JANSSEN (1969) reported that isolation of pea root enzymes in Mc Ilvaine's buffer resulted in IAA-degradative activity being present in the high molecular weight peak, i.e. having a molecular weight larger than 100000. He also found that various treatments could split these larger molecules to smaller ones with a molecular weight around the value found by the present author after urea had been added.

Preliminary experiments demonstrated that following the addition of 400 mg dry PVP per ml of root supernatant these high molecular weight enzyme molecules were quantitatively removed. This could mean that the enzyme molecules with high hydrophobicity and those with a lower one are associated as this high molecular weight peak exhibits a high peroxidase activity which is normally not retained by PVP. Furthermore denaturation of the enzyme molecules with the assumed high degree of hydrophobicity results in a lower rate of reaction by the ones with a more hydrophilic nature as shown in *fig. 3*. After purification over

PVP these enzymes attain a much higher reaction rate in the presence of the same amount of urea.

This could mean that purification releases the enzyme molecules with a relatively high hydrophobicity, from the urea-resistant fraction. According to BIGELOW (1967) proteins with a more hydrophobic character are often of the associating type. However, possible association of the enzyme molecules can not be entirely accounted for by a fraction of proteins with a large ratio of apolar to polar side chains. Addition of 50 mM KCl or $CaCl_2$ destroys the high molecular weight complex just as well as urea, while ions tend to stabilize hydrophobic associations (KAUZMANN 1959).

It seems possible from these experiments that proteins with a large ratio of apolar to polar side chains form complexes with the apolar parts of phenolic compounds, so that the free charged groups form a hydrophilic outer shell around the molecule. These groups could then participate in the formation of larger complexes with other proteins in which they are very active (LOOMIS & BATAILLE 1966).

ACKNOWLEDGEMENT

The author is indebted to Miss M. A. T. Gebbink for her help with the experiments.

REFERENCES

ANDERSEN, R. A. & J. A. SOWERS (1968): Optimum conditions for bonding plant phenols to insoluble polyvinylpyrrolidone. *Phytochem.* **7**: 293–301.

BIGELOW, C. C. (1967): On the average hydrophobicity of proteins and the relation between it and protein structure. *J. Theoret. Biol.* **16**: 187–211.

COLOWICK, S. P. & N. O. KAPLAN (1957): *Methods in enzymology.* Academic Press, vol. **3**: 88.

CRONENBERGER, L., R. VILLE & H. PACHECO (1966): Purification partielle des "auxine-oxydases" des racines de Pisum sativum. *Bull. Soc. Chim. Biol.* **48**: 833–836.

DETERMANN, H. (1967): *Gelchromatographie.* Springer Verlag, p. 118.

JANSSEN, M. G. H. (1969): The form of indoleacetic acid oxidase of pea roots. *Acta Bot. Neerl.* **18**: 429–433.

KAUZMANN, W. (1959): Some factors in the interpretation of protein denaturation. *Adv. protein chemistry.* **14**: 1–63.

LERNER, J., T. M. DOUGHERTY & A. I. SCHEPARTZ (1968): Selective properties of polyvinyl-pyrrolidone in column chromatography of nucleotides, their derivatives and related compounds. *J. Chrom.* **37**: 453–457.

LOOMIS, W. D. & J. BATAILLE (1966): Plant phenolic compounds and the isolation of plant enzymes. *Phytochem.* **5**: 423–438.

PILET, P. E. & P. LAVANCHY (1969): Purification d'extraits peroxydasiques (racine de Lens) à activité "auxines-oxydasique". *Physiol. Vég.* **7**: 19–29.

SEQUEIRA, L. & L. MINEO (1966): Partial purification and kinetics of indoleacetic acid oxidase from tobacco roots. *Plant Physiol.* **41**: 1200–1208.

SIEGEL, B. Z. & A. W. GALSTON (1967): Indoleacetic acid oxidase activity of apoperoxidase. *Science* **157**: 1557–1559.

SMALL, D. M., S. A. PENKETT & D. CHAPMAN (1969): Studies on simple and mixed bile salt micelles by nuclear magnetic resonance spectroscopy. *Biochim. Biophys. Acta* **176**: 178–189.

WAUGH, D. F. (1954): Protein-protein interactions. *Adv. Protein chemistry* **9**: 325–437.