ISOELECTRIC FOCUSING OF INDOLEACETIC ACID DEGRADING ENZYMES FROM PEA ROOTS.

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SUMMARY

In homogenates of pea roots only one protein complex is responsible for the degradation of IAA. It consists at least of two enzymes which are probably intimately associated. This protein complex possesses an isoelectric point of 9.5 and has only a small net charge at neutral pH. At least 3 other peroxidases are present with a similar range of molecular weight as commonly displayed by the IAA degrading protein. Probably these enzymes are also bound to membranes.

1. INTRODUCTION

Several studies on IAA-oxidase from roots of plants have been performed with gel chromatography. SEQUEIRA & MINEO (1966) reported the presence of two distinct fractions capable of degrading IAA in homogenates of tobacco roots. CRONENBERGER *et al.* (1966) found only one fraction when working with pea roots. JANSSEN (1969) found two enzyme fractions in roots of peas in which one fraction could be converted to the other, possibly indicating the presence of the same enzyme in both fractions.

Most authors ascribe one enzyme to one peak of activity. But gel chromatography can only offer group separation because the resolution of compounds with small differences in molecular weight is inadequate. Use of other techniques is necessary before the degradation of IAA can be assigned to a specific enzyme. The present author (1969) already showed that another column support, i.e. polyvinylpyrrolidone, gives results indicating the presence of two different types of enzyme molecules capable of degrading IAA in homogenates of pea roots. One type is a peroxidative enzyme while the other type is non-peroxidative.

Another difficulty is the ubiquitous presence of peroxidases. Identification of IAA-oxidase as a peroxidase is commonly founded on the fact that a peak of peroxidase activity is coincidental with a peak of IAA degradation. But a causal relationship is difficult to prove with gel chromatography and proof is impossible when crude homogenates are assayed.

In the present study use has been made of isoelectric focusing, a form of electrophoresis (VESTERBERG *et al.* 1967). Under influence of the applied current a pH gradient is established in which proteins move until their isoelectric point is reached. The various peroxidases are easily resolved by this

technique when performed in polyacrylamide gel. Fractions obtained from a column of Sephadex G-100, containing IAA degrading enzymes have been analyzed by this method.

2. MATERIAL AND METHODS

Seeds of *Pisum sativum* cv. "Vlijmsche Gele Krombek" were grown as described earlier (VAN DER MAST 1969). Homogenation of the roots was carried out in Mc Ilvaine's buffer, pH 5.0, or in Tris-HCl buffer, pH 7.4, which contained 10 mM $MgCl_2$. Liquid isoelectric focusing was performed according to the instructions from the manufacturer (LKB-produkter, Stockholm-Bromma, Sweden) in a column with a volume of 110 ml. The concentration of the Ampholine carrier was 1%. Usually 4.5 ml of the 27000 g supernatant was added to the vessel containing the lighter solution. The temperature was maintained at 2°C. The duration of the focusing was 20–24 hours at all pH-ranges used, *i.e.* pH 3–10 and 7–10. The voltage in the former case was kept at 300 V, in the latter stepped up to 700 V; the current decreased steadily to 1 mA. In these experiments the kathode was taken as the upper electrode to avoid disturbances from coagulating compounds in the acid region of the gradient. The column was drained with a peristaltic pump. Fractions of 2–2 1/4 ml were obtained. The degradation of IAA was tested as described earlier in Mc Ilvaine's buffer. As the buffering capacity of the carrier solution is low, the addition of this buffer virtually eliminated variations of pH in the different fractions.

Polyacrylamide isoelectric focusing was performed according to the method of WRIGLEY (1968) and FAWCETT (1968). The polymerisation of the gel was carried out with ammonium persulphate. In the first experiments the excess of this compound was removed by applying current during 30 minutes as recommended by MITCHELL (1967). This step was omitted later as no influence of the persulphate was detectable. The concentration of acrylamide was 3 or 7.5%. Eight gels with a length of 15 cm and a diameter of 0.5 cm were used simultaneously. Usually 3/4 ml containing the Ampholine carrier, 40% sucrose and the enzymes was layered on the gels and separated from the electrode solution by 3/4 ml of distilled water containing the carrier and 20% sucrose. The duration of focusing was 16–17 hours in which the potential was maintained at 250 V, resulting in 12 V/cm and 2.6 mA per gel. Drifting of the pH gradient was negligible at this potential, but higher at 25 V/cm. The cathode was taken as the lower electrode. After a run the resulting pH's were measured in the gels at intervals of 1 cm with an Ingold microelectrode, nr 406 M 5, in which the salt bridge, through an extension, is level with the glass membrane.

In some instances the gels were cut into pieces of 1 cm and immersed in 1 ml of Mc Ilvaine's buffer, pH 5.0, containing 50 ppm IAA and 2 ppm p-coumaric acid. The incubation was carried out overnight in a cold room. The pieces were then removed and the residual IAA was determined by the addition of 4 ml Salkowski's reagent.

Gel chromatography on Sephadex G-100 was performed in columns with

dimensions of 35 × 1.9 cm. Fractions of 5 ml were obtained. The first 8 fractions after the passage of the void volume were lyophilized, taken up in 1 ml of destilled water containing the Ampholine carrier and 40% sucrose and focused on polyacrylamide gels as mentioned.

Ion exchange was carried out on CM-Sephadex C-25, a weakly acidic cation exchanger. The column dimensions were 35 × 1.9 cm. The elution was performed with NaH_2PO_4-NaOH buffer, pH 6.5 and ionic strength 0.06. The removal of the retained proteins was effected with the same buffer by raising the ionic strength to 0.3. Fractions of 5 ml were obtained.

The isolation of membrane-bound IAA degrading enzymes was carried out in a Spinco model L 50 centrifuge at 48000 RPM during 1 hour in rotor 50. The pellet was resuspended with the aid of a glass cylinder and a tightly fitting teflon piston.

Peroxidases were located with benzidine as reducing agent.

Total phosphate was determined according to the method of AMES & DUBIN (1960).

3. RESULTS

Liquid isoelectric focusing of pea root supernatant using an Ampholine carrier with a range of pH 3–10 gives the results represented in *fig. 1.*

One peak of IAA degrading activity is apparent with an isoelectric point

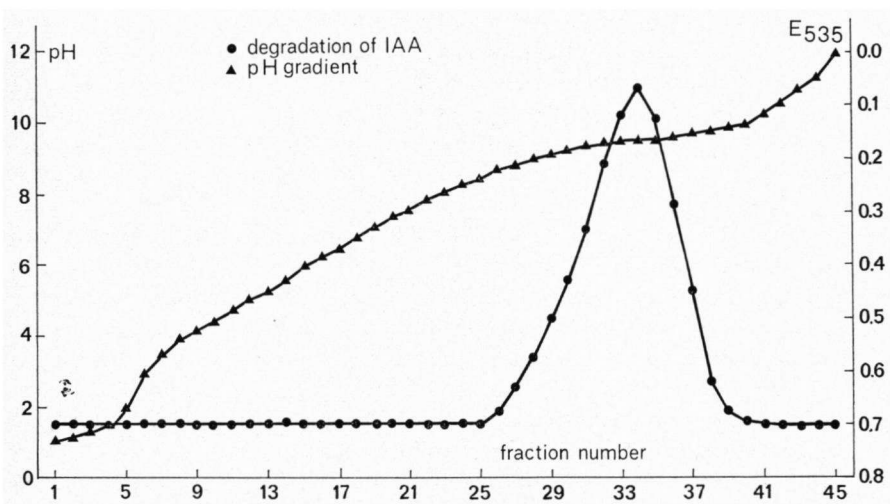

Fig. 1. Liquid isoelectric focusing of pea root supernatant during 20 hours. An Ampholine carrier establishing a gradient of pH 3–10 was used. The first 6 fractions contain the anode solution. The last 6 fractions consisting of the cathode solution are not shown in the figure. Peroxidases are found in all the fractions between pH 3 and pH 11. The degradation of IAA is localised around an I.E.P. of 9.5.

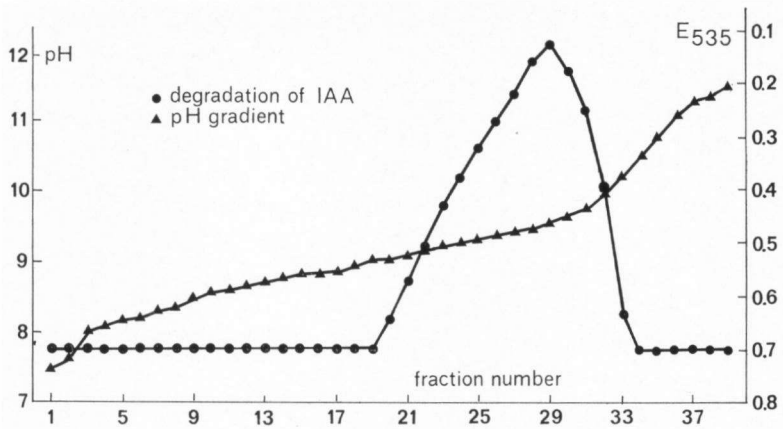

Fig. 2. Liquid isoelectric focusing of pea root supernatant in a gradient of pH 7–10 during 20 hours. Several fractions have been omitted at the acid side of the gradient. The fraction size is the same as in the foregoing figure. The slope of the gradient is even more gradual than the one depicted in *fig. 1*. The degradation of IAA is still localised around an I.E.P. of 9.5, but the broadness of the peak does not increase indicating the presence of only one protein responsible for IAA conversion.

(I.E.P.) of 9.5. Due to the gradual slope of the pH gradient this peak is rather broad. This could mask the presence of other IAA degrading enzymes with slightly different I.E.P.'s. If, however, focusing was performed in a pH gradient of 7–10 (*fig. 2*) this peak did not become broader, although the slope of such a gradient is even more gradual than the one given in *fig. 1*. If pea root supernatant was first focused in a gradient of pH 3–10 and the fractions with a pH of 8.4 to 9.7 were pooled and refocused in a gradient of pH 7–10, only the same peak of activity could be found. This all indicates that only one protein complex is responsible for the degradation of IAA. Peroxidase activity, however, was apparent in all fractions outside the ones containing the electrode solutions. If the duration of focusing in a gradient of pH 3–10 was reduced to 10 hours instead of 20 hours, a second peak of IAA degrading activity was found with an I.E.P. of 5.6. In this region coagulated compounds are present. Nearly all phosphate was situated between pH 1.2 and 6.5. When IAA degrading enzymes were found at pH 5.6 all the fractions between this pH and pH 9.5 showed IAA degrading activity. This is probably due to a continuous release of these enzymes from the membranes and the concomitant migration towards the kathode. Removal of membranes and ribosomes by ultracentrifugation totally abolished this activity and only the peak at pH 9.5 was then apparent.

When pea root supernatant was focused in a gradient of pH 3–10 during 20 hours and all the fractions with a pH of 8.4. to 9.7 were pooled, concentrated with Sephadex G-25 (VAN DER MAST 1970) and subsequently gel-chromatographed on Sephadex G-100, the distribution of enzyme molecules was the same

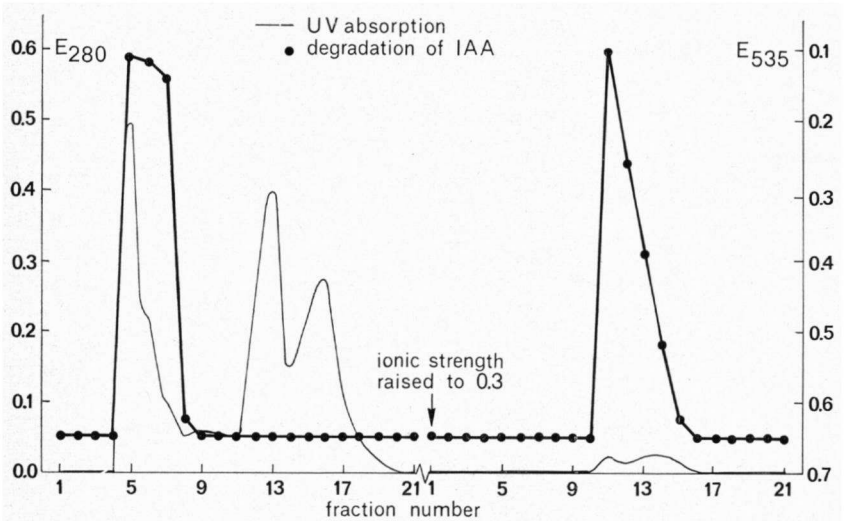

Fig. 3. Ion exchange study of IAA degrading enzymes at pH 6.5 on CM-Sephadex C-25 at ionic strength 0.06 with sodium as counterions. The first peak of activity is largely due to membrane-bound enzymes, while the retained peak consists of free IAA degrading protein molecules. The elution of this retained peak can be effected by raising the ionic strength. No influence of the flow rate on the retention was detected although this parameter in the various experiments varied between 8–25 ml/cm²/hour.

as found after addition of KCl or urea (VAN DER MAST 1969), *i.e.* no enzyme is present in the high molecular weight peak but only its lower molecular weight form can then be detected.

Ion exchange on CM-Sephadex C-25 with sodium as counterions gives rise to two peaks of activity as shown in *fig. 3*. The first peak elutes after the passage of the void volume and consists mostly of membranes and ribosomes. These organelles have a negative charge at pH 6.5, the pH of the eluant buffer, because of their lower I.E.P., and are therefore not retained by the column. The presence of ribosomes was confirmed with the orcinol test. Most of the IAA degrading activity in this peak is due to membrane-bound enzymes as after removal of these cell structures most of this activity disappeared. The IAA degrading activity not removed in this way is possibly due to the relatively weak charge of these molecules at this pH. Lowering the ionic strength of the elution buffer from 0.06 to 0.005 resulted in total removal of the free enzyme molecules from this first peak. Further lowering the ionic strength by carrying out the homogenation and the elution with destilled water resulted in total retention of all IAA degrading enzymes, even membrane-bound molecules, by the column. Ion exchange chromatography of isolated membranes with an elution buffer of ionic strength 0.06 did not give rise to a retained peak of IAA degrading activity.

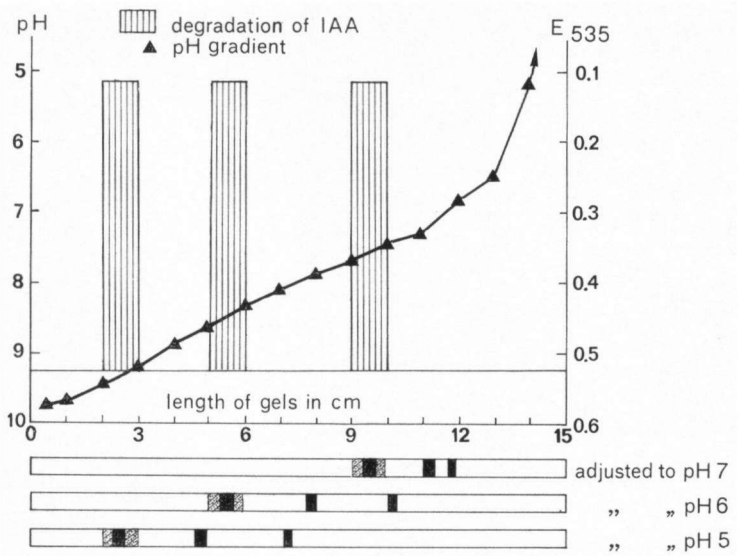

Fig. 4. Polyacrylamide isoelectric focusing of liquid focused IAA degrading enzymes. The fraction with a pH of 9.3 (see *fig. 1*) was isolated and divided into 3 portions. The portions were adjusted, after the addition of the carrier, to pH 7, 6 and 5, respectively. In this fraction 3 peroxidases are present. The degradation of IAA is confined to the faster moving protein in each gel. Due to the varying net charges of this protein in the various portions only the IAA degrading enzymes adjusted to pH 5 have reached their I.E.P. Separation of the two types of enzyme molecules does not occur. The acrylamide concentration employed was 7.5%. The stippled areas around the enzyme band indicate the small amount of diffusion while the hatching denotes that the blue colour achieved with benzidine quickly turns to red.

The retained IAA degrading enzymes elute from the column after raising the ionic strength of the elution buffer to 0.3.

The association of the two enzyme types can be inferred from the following experiments: Pea root supernatant was first focused in liquid in a gradient of pH 3–10. The fraction with a pH of 9.3 was isolated and divided into 3 portions. These were adjusted with citric acid after addition of the carrier to pH 7, 6 and 5, respectively. Refocusing on 3% or 7.5% polyacrylamide took place in a gradient of pH 7–10. The results are given in *fig. 4*.

Due to the varying net charge of the enzyme molecules in the 3 portions after adjustment of the pH, the enzymes in several gels have not yet reached their I.E.P. after 16 hours of focusing although the pH gradient has established itself in this time. It can be seen from *fig. 4* that the degradation of IAA is always associated with the faster moving protein. Two other peroxidases are present in the initial fraction of pH 9.3. Repeating the experiment with the fraction of pH 9.0 similar results were found, but in the fractions with pH 9.5

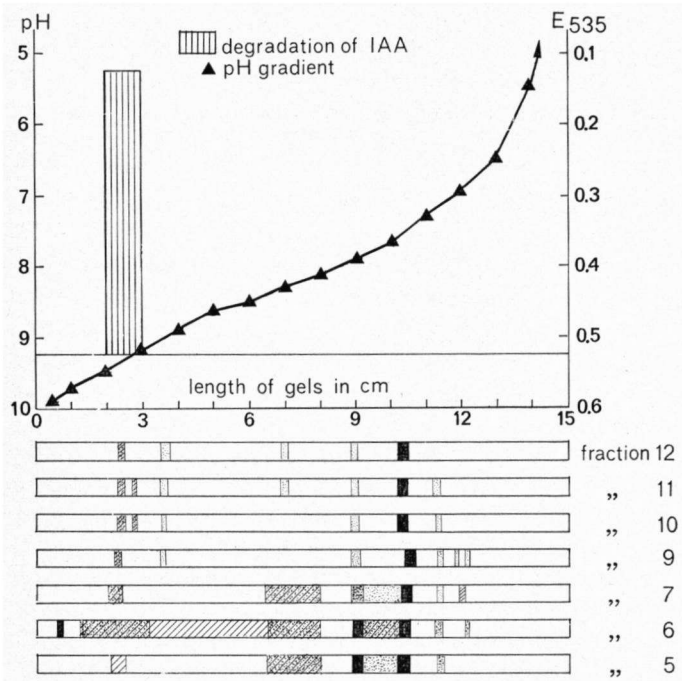

Fig. 5. Polyacrylamide isoelectric focusing of the first 8 fractions of pea root supernatant gelchromatographed on Sephadex G-100. The pH of the elution buffer was 5.0. The addition of the carrier, range pH 7–10, raises this pH to 6.5, implying, that only peroxidases possessing a positive charge at this pH are shown in the figure. The acrylamide concentration was 7.5%. The gel corresponding to fraction 8 was used for measuring the pH gradient. The variation of the pH at the same point in the various gels was ± 0.1. All the degradation of IAA is localised in the 3th cm of the gels around an I.E.P. of 9.4 except in the gel corresponding with fraction 6 due to the large amount of membranes present here. Three other peroxidases are present in the same range of molecular weight as the IAA degrading protein. The relative concentration of the same protein in the various fractions is indicated by corresponding differences in the shading. Hatching indicates areas where the blue colour achieved with benzidine turns to red within a few minutes.

and 9.7 the slowest moving protein was absent. In gels with a concentration of 3% acrylamide the migration of the IAA degrading protein was twice as fast.

In the next experiments pea root supernatant was gelchromatographed on Sephadex G-100. The first 8 fractions after the passage of the void volume were focused on polyacrylamide after freeze-drying. The results are shown in *fig.* 5.

Only peroxidases with a positive charge at pH 6.5 are shown, the negative ones being displaced from the gel towards the anode. All the fractions display a peroxidase with an I.E.P. of 9.4. Only this protein showed degradation of IAA when the gels were cut into pieces and incubated with IAA. However, the gel corresponding to fraction 6 showed IAA degradation throughout the gel till

the 13th cm. This is probably due to the presence of the membranes in this fraction. These structures will move towards the anode until they reach the transition zône between the carrier solution and the electrode solution. The subsequent release of bound IAA degrading enzymes may then account for the deviating result in this gel.

4. DISCUSSION

From the results given in *figs. 1* and *2* it could be apparent that only one complex of proteins is responsible for the degradation of IAA. But in *fig. 4* is shown that even around the I.E.P. of this protein two more peroxidases are present. But as these do not degrade IAA this conclusion seems to remain valid. The two other peroxidases are probably identical with the ones possessing I.E.P.'s of 9.0 and 8.3 depicted in *fig. 5*.

This protein complex which promotes the degradation of IAA apparently consists of at least two enzyme molecules, i.e. a peroxidative and a non-peroxidative one, as chromatography on polyvinylpyrrolidone showed the presence of the latter (VAN DER MAST 1969). In that paper another enzyme fraction was mentioned that did not adsorb to this compound. It was assumed that this was a peroxidase, but all the other peroxidases were also located in the non-adsorbed peak. This assumption is now proved because after removal of all other peroxidases there still remains a peroxidase capable of degrading IAA, as shown in *fig. 4*.

The conclusion that both types of enzyme molecules are intimately associated to one protein seems not far-fetched. When both enzyme types move separately to an I.E.P. of 9.5 they should at least have the same ratio of positive and negative charges. This indicates that separation on an ion exchanger should then be possible. This was not the case, even when all the IAA degrading enzymes were bound to the exchanger at zero ionic strength and elution was performed with a very shallow gradient of increasing concentrations of sodium ions. It still remains possible, of course, that both types of enzymes have an equal amount of positive and of negative charges distributed over each molecule in the same way. Also separation did not occur in the polyacrylamide gels, which should happen when only a small difference in size is present. At a concentration of 7.5% acrylamide the mean pore diameter of this gel is 50 nm and at 3% 150 nm (MARGOLIS & KENRICK 1967). At the latter concentration the enzyme band travelled twice as fast as in 7.5% polyacrylamide, indicating interference by the gel at a concentration of 7.5% acrylamide. But separation of the two types did not occur as degradation of IAA was never found outside the peroxidase positive enzyme band, indicating the presence of non-peroxidative IAA degrading enzyme molecules in this band.

From *fig. 5* it can be seen that in all the fractions displaying degradation of IAA a protein is present with an I.E.P. of 9.4. In a previous paper (VAN DER MAST 1970) evidence was presented indicating that both the membrane-bound and the free IAA degrading enzyme molecules consist of the two enzyme types

mentioned. The large range of molecular weight of the free IAA degrading enzymes found after gel chromatography on Sephadex is probably due to a continuous release of membrane-bound molecules to the free state during chromatography. Several indications for this phenomenon are found in the electro-focusing experiments. Further evidence will be communicated later.

It can also be seen from *fig. 5* that three other, basic, peroxidases show the same phenomenon as the IAA degrading protein, i.e. these molecules cover a range of molecular weights. Possibly these peroxidases are also attached to larger structures. Membrane-bound or ribosome-bound peroxidases not capable of degrading IAA nevertheless participate in this process as they stimulate this degradation of IAA by the specific protein with a factor 2–3 (to be communicated). This overlap of several identical peroxidases shows the impossibility of discrimination between these groups of enzymes by gel chromatography.

Due to the fact that the IAA degrading enzymes are present in two phases, electrophoresis experiments of short duration, such as when performed on cellulose acetate strips or in starch, can introduce faulty indications. SIEGEL & GALSTON (1967) already noted that acetocarmine positive material was present among anodic peroxidases from roots of *Pisum sativum*. They presumed that this was due to intact cellular organelles. It is possible that the anodic peroxidases found in this material are in fact cathodic but attached to membranes or to other anodic cell structures. This is also the case in electro-focusing experiments of short duration for the IAA degrading protein from peas.

A comparison between *fig. 1* and *fig. 3* shows the better resolution of peroxidases achieved on polyacrylamide gel. This is due to the restricted diffusion of the protein imposed by the gel structure.

As the two types of enzymes composing the IAA degrading protein seem to be inseparable by ion exchange and electrophoresis, the separation achieved on polyvinylpyrrolidone (VAN DER MAST 1969) probably can not be accounted for by differences in the charge of these enzymes. The assumption that adsorption of the non-peroxidative enzyme molecules to this compound is due to polyphenols, although open to question, still seems valid.

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REFERENCES

AMES, B. N. & D. T. DUBIN (1960): The role of polyamines in the neutralization of bacteriophage deoxyribonucleic acid. *J. Biol. Chem.* **235**: 769–775.

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

FAWCETT, J. S. (1968): Isoelectric fractionation of proteins on polyacrylamide gels. *FEBS letters* **1**: 81–82.

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

MARGOLIS, J. & K. G. KENRICK (1967): Polyacrylamide gel-electrophoresis across a molecular sieve gradient. *Nature (London)* **214**: 1334–1336.

MAST, C. A. VAN DER (1969): Separation of IAA degrading enzymes from pea roots on columns of polyvinylpyrrolidone. *Acta Bot. Neerl.* **18**: 620–626.

— (1970): Separation of IAA degrading enzymes from pea roots on columns of polyvinylpyrrolidone. II. *Acta Bot. Neerl.* **19**: 141–146.

MITCHELL, W. M. (1967): A potential source of electrophoretic artifacts in polyacrylamide gels. *Biochim. Biophys. Acta* **147**: 171–174.

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): The isoperoxidases of Pisum sativum. *Plant Physiol.* **42**: 221–226.

VESTERBERG, O., T. WADSTRÖM, K. VESTERBERG, H. SVENSSON & B. MALMGREN (1967): Studies on extracellular proteins from Staphylococcus aureus. I. Separation and characterization of enzymes and toxins by isoelectric focusing. *Biochem. Biophys. Acta* **133**: 435–445.

WRIGLEY, C. (1968): Gel electrofocusing – A technique for analyzing multiple protein samples by isoelectric focusing. *Science Tools* **15**: 17–23.