THE INFLUENCE OF THE EFFLUENT AND OF THE TEMPERATURE ON THE SEPARATION OF INDOLEACETIC ACID OXIDASE OF PEA ROOTS ON SEPHADEX COLUMNS

M. G. H. JANSSEN

Botanisch Laboratorium, Utrecht

SUMMARY

The low molecular IAA-oxidase of pea roots, reported previously (Janssen 1969b), is formed from the larger units during the chromatographic procedure. This formation is enhanced both by increasing temperatures and by increasing ionic strength of the effluent.

It is also demonstrated that the low molecular IAA-oxidase can adsorb to the Sephadex. Once adsorbed the enzyme molecules can be eluted with a NaCl solution.

1. INTRODUCTION

It has been demonstrated (JANSSEN 1969b) that the elution pattern of indoleacetic acid oxidase (IAA-oxidase) from pea roots after filtration of a crude extract through Sephadex G 100 columns is influenced by the pretreatment of the extract. Extracts that had been prepared in distilled water showed only a high molecular IAA-oxidase peak, but after addition of NaCl (to a final concentration of 5%) to the extract which besides had been stored for some time at –20°C, also a low molecular enzyme was found (see *fig. 1*). The columns were eluted with a 10 × diluted phosphate-citrate buffer solution of pH 5 in these experiments.

Further experiments revealed that, if the buffer solution was replaced by distilled water, the low molecular enzyme was retarded and found in fractions which contained substances of still lower molecular weight. The results presented in this paper indicate that this is caused by adsorption of the low molecular enzyme molecules to the Sephadex. It will also be demonstrated, that the ionic strength of the effluent and the temperature at which the experiments are performed strongly influence the elution pattern.

2. MATERIAL AND METHODS

Seedlings of *Pisum sativum* cv. "Vlijmsche Gele Krombek" were grown in darkness as described before (JANSSEN 1969a). After 65 hours the root tips were collected in ice-cold distilled water. To every 60 tips 1 ml was added. The tips were ground with sand and centrifuged at 27000 g for 30 minutes (at 4°C). After addition of NaCl to a final concentration of 5%, the extract was stored at –20°C for some time and centrifuged at 27000 g for 10 minutes before use.

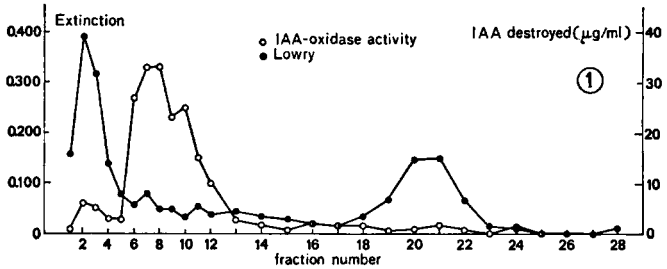


Fig. 1. The elution pattern of IAA oxidase activity of pea roots after filtration of a crude extract through a Sephadex G 100 column. Effluent: 10 × diluted phosphate-citrate buffer solution of pH 5. (From JANSSEN 1969b)

The purification procedure by means of Sephadex G 100 columns (dimensions 35 × 2 cm) has been described before (Janssen 1969b).

In the experiments with Sephadex G 25 the dimensions of the columns were 21 × 2 cm. After a void volume of 18 ml had passed 30 fractions of 3 ml each were collected.

The columns were equilibrated with the effluent for at least 16 hours. A sample of 3 ml was applied to a column.

The IAA-oxidase activity of the individual fractions was determined as described previously (JANSSEN 1969b).

The protein content of the various fractions was measured with the Folin phenol reagent according to LOWRY *et al.* (1951).

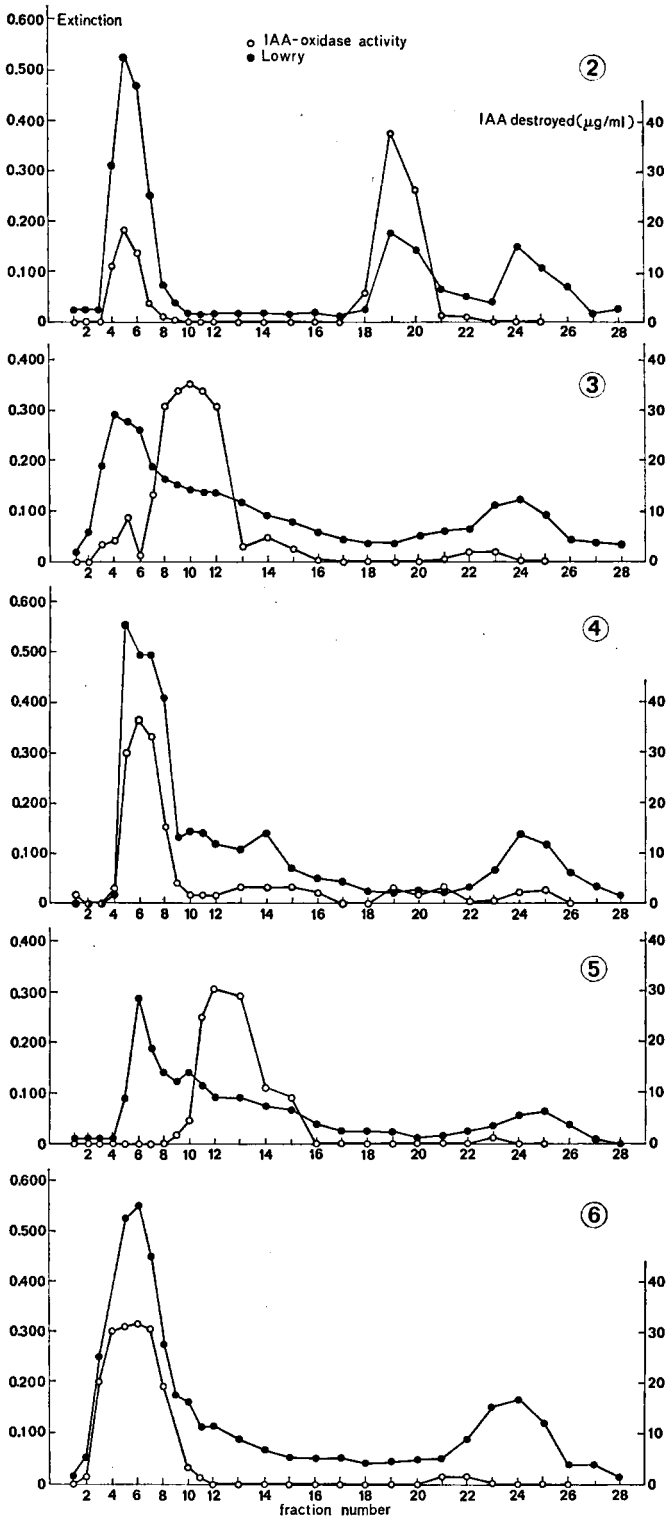
The experiments were performed at 22 °C, unless mentioned otherwise.

3. RESULTS AND CONCLUSIONS

3.1. The elution pattern on Sephadex G 100 with distilled water as the effluent

In *fig. 2* the results are presented of an experiment performed with an extract similar to that used in the experiment of *fig. 1*. The effluent was distilled water instead of 10 × diluted phosphate-citrate buffer solution. It is clear that the low molecular IAA-oxidase was found in fractions containing substances of much lower molecular weight. This could be caused by the different pH value during the chromatographic procedure or by the different ionic strength of the effluent.

Figs. 2–6. The elution pattern of IAA oxidase activity of pea roots after filtration of 3 ml of crude extract through a Sephadex G 100 column (dimensions 35 × 2 cm). After a void volume of 30 ml had passed 12 fractions of 2.5 ml each and 16 fractions of 5 ml each were collected. Incubation time: 15 minutes. Fig.2. Experiment at 22 °C with distilled water as the effluent. Fig. 3. Experiment at 22 °C with a 0.075 % NaCl solution as the effluent. Fig. 4. Experiment at 5 °C with a 0.075 % NaCl solution as the effluent. Fig. 5. Experiment at 5 °C with a 5 % NaCl solution as the effluent. Fig. 6. Experiment at 22 °C with a 0.01 % NaCl solution as the effluent.


Extinction
o IAA-oxidase activity
● Lowry
IAA destroyed(μg/ml)
②
③
④
⑤
⑥
fraction number

The precipitation method with tannic acid according to MEJBAUM-KATZENELLENBOGEN & DOBRYSZYCKA (1959) revealed that the positive reaction in the Lowry measurements of the fractions 19 and 20 was caused by proteins and of the fractions 24 and 25 by low molecular substances.

3.2. The elution pattern on Sephadex G 100 with a 0.075% NaCl solution as the effluent

Using a 0.075% NaCl solution as the effluent, the results were qualitatively similar to the results obtained with 10 × diluted phosphate-citrate buffer as the effluent (*cf. figs. 3* and *1*). The different results of the experiments presented in *figs. 1* and *2* must, therefore, be caused by a change in the ionic strength and not by a change in the pH value of the effluent.

3.3. The elution pattern on Sephadex G 100 at low temperature

The experiments described in the previous sections were performed at 22°C. In *fig. 4* the results are presented of an experiment performed at 5°C with a 0.075% NaCl solution as the effluent. The low molecular IAA-oxidase was not found while the high molecular fraction showed much more IAA-oxidase activity. Using distilled water as the effluent at 5°C, the results were similar to the results presented in *fig. 4*. This indicates that the low molecular IAA-oxidase must have been formed during the chromatographic procedure.

3.4. The elution pattern on Sephadex G 100 with a 5% or a 0.01% NaCl solution as the effluent

In *fig. 5* the results are presented of an experiment performed at 5°C, using a 5% NaCl solution as the effluent. At 22°C the results were almost similar. With a 5% NaCl solution as the effluent the low molecular IAA-oxidase is formed even at 5°C.

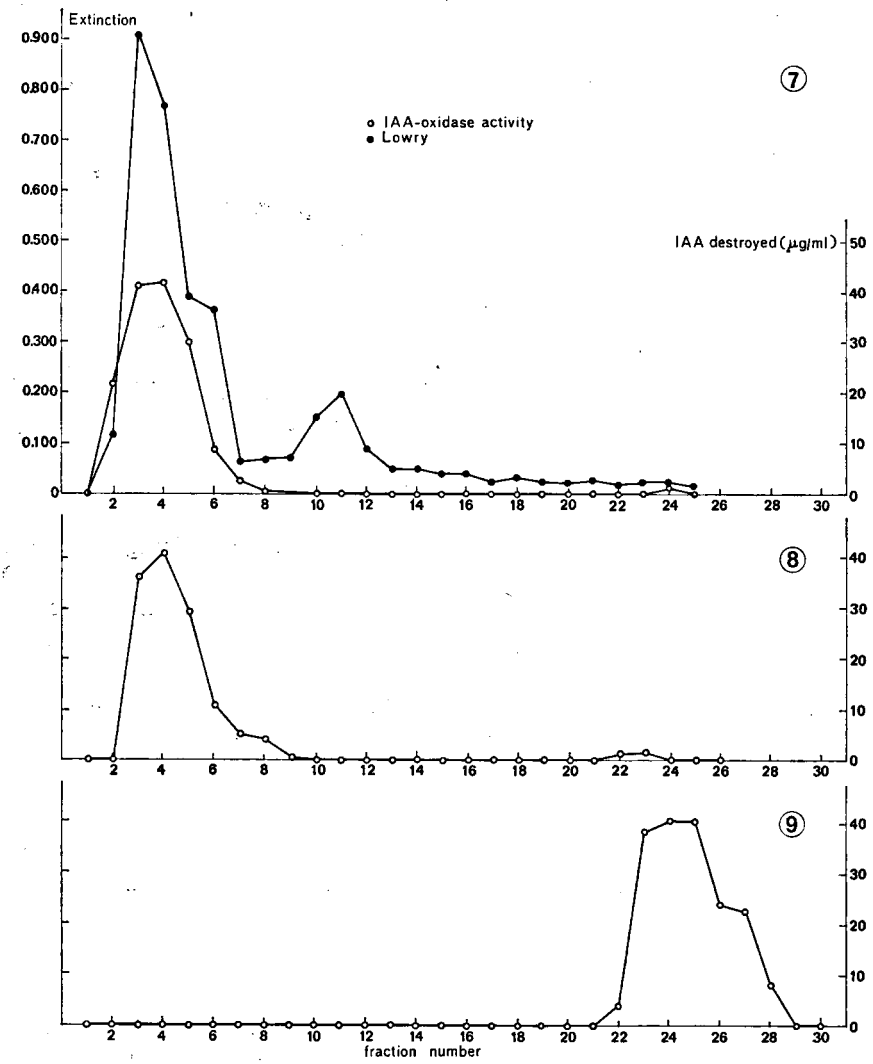
Using a 0.01% NaCl solution as the effluent even at 22°C only the high molecular IAA-oxidase was found (*fig. 6*). A low ionic strength of the effluent seems to have a stabilizing effect on the high molecular units.

3.5. Elution patterns on Sephadex G 25 columns

Using distilled water as the effluent, some proteins can be delayed during the chromatographic procedure by adsorption to the free carboxyl groups of the Sephadex (see DETERMANN 1967). This could have caused the shift of the low molecular IAA-oxidase mentioned in section 3.2.

The number of free carboxyl groups per gram Sephadex G 25 is equal to that per gram Sephadex G 100. To get equal gelbeds of Sephadex G 25 and G 100 about 3 × as much of Sephadex G 25 has to be used. If the low molecular enzyme molecules can be adsorbed to Sephadex G 100 this must also happen with Sephadex G 25.

The results of an experiment performed at 22°C with a Sephadex G 25 column, using a 0.075% NaCl solution as the effluent, are presented in *fig. 7*.



Figs. 7–9. The elution pattern of IAA-oxidase activity of pea roots after filtration of 3 ml enzyme preparation through a Sephadex G 25 column (dimensions 21 × 2 cm). The experiments were performed at 22 °C. Fig. 7. Enzyme: crude extract. Effluent: 0.075 % NaCl solution. Incubation time: 10 minutes. Fig. 8. Enzyme: the low molecular IAA-oxidase of fig. 2. Effluent: 0.075 % NaCl solution. Incubation time: 30 minutes. Fig. 9. Enzyme: the low molecular IAA-oxidase of fig. 2. Effluent: initially distilled water; after collecting 8 fractions 0.075 % NaCl. Incubation time: 90 minutes.

With distilled water an almost similar result was obtained. No adsorption of IAA-oxidase becomes evident.

Now the low molecular IAA-oxidase from *fig. 2* was passed through a Sephadex G 25 column with distilled water as the effluent. Even exhaustive washing failed to elute any IAA-oxidase.

Next this experiment was repeated with a 0.075% NaCl solution as the effluent during the second passage. From *fig. 8* appears that the IAA-oxidase activity was found immediately after the void volume had passed.

The failure to detect any IAA-oxidase activity with distilled water as the effluent could have been caused by a strong adsorption of the enzyme molecules to the Sephadex. Therefore, the low molecular IAA-oxidase of *fig. 2* was passed through a Sephadex G 25 column initially with distilled water as the effluent. After collecting 8 fractions, the experiment was continued with a 0.075% NaCl solution as the effluent. From *fig. 9* it is clear that now indeed IAA-oxidase activity was found. It may, therefore, be concluded that the low molecular IAA-oxidase can be adsorbed to the Sephadex in the absence of ions and that it can be removed from it by a NaCl solution.

The shift of the low molecular IAA-oxidase to fractions containing substances of much lower molecular weight in the experiments with Sephadex G 100 using distilled water as the effluent (see *fig. 2*), must be caused by adsorption of the enzyme molecules to the Sephadex as can be concluded from the results presented in *fig. 9*.

The adsorption of the low molecular enzyme molecules to the Sephadex may be an indication that the low molecular IAA-oxidase is a protein with basic properties.

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