INDOLEACETIC ACID OXIDASE, PEROXIDASE AND POLYPHENOLOXIDASE OF PEA ROOTS

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SUMMARY

The elution pattern of IAA-oxidase, peroxidase and polyphenoloxidase after filtration of crude pea root extract through Sephadex G 100 columns is affected by the pretreatment of the extract and also by the effluent. The changes are the same for all three enzymes. The IAA-oxidase activity and the polyphenoloxidase activity are strongly inhibited by DIECA, but the peroxidase activity is unaffected by it.

1. INTRODUCTION

The nature of the enzymes that catalyse the destruction of indoleacetic acid (IAA) has been the subject of many investigations. According to TANG & BONNER (1947) the IAA-oxidase of pea epicotyls is an iron protein. WAGENKNECHT & BURRIS (1950) found that the destruction of IAA by bean root extracts was inhibited by sodium-diethyldithiocarbamate (DIECA) and concluded that a copper enzyme was involved. BRIGGS *c.s.* (1955), however, found no influence of DIECA on the IAA-oxidase activity of extracts from *Osmunda cinnamomea*, and they stated that this IAA-oxidase is an iron enzyme. Moreover DINANT & GASPAR (1967) have pointed to the fact that DIECA does not chelate copper exclusively.

GOLDACRE (1951) suggested the involvement of a peroxidase in the destruction of IAA by extracts from pea epicotyls. This idea is supported by many authors. STUTZ (1957) failed to separate the IAA-oxidase activity from the peroxidase activity in extracts from lupine by means of electrophoresis. He supposed the IAA-oxidase activity and the peroxidase activity to be associated with different centers of the same enzyme. RAY (1960) considered IAA-oxidase and peroxidase activity of *Omphalia flavida* to be due to a single enzyme, for the activities run parallel both during thermal inactivation and in the course of the purification of the enzyme. Also the optimum pH value was at 3.5–3.7 for both activities.

The IAA-oxidase activity, however, also has been ascribed to other enzyme systems. According to FÅHRAEUS & TULLANDER (1956) a laccase was involved in the destruction of IAA by *Polyporus versicolor*. This also has been found by LEGRAND (1957) for IAA-oxidase from *Lactaria*. BRIGGS & RAY (1956) demonstrated that IAA could be destroyed by tyrosinase from mushrooms and from the leaves of *Osmunda*. Purified phenoloxidase from *Prunus* was found capable of destroying IAA (TOMASZEWSKI 1959). KONINGS (1964) suggested that IAA-oxidase from pea roots was more likely to be a polyphenoloxidase than a per-

oxidase on account of a different sensitivity to KCN. Most of the evidence, however, indicated that generally a peroxidase was involved in the destruction of IAA by plant extracts.

The results obtained by SEQUEIRA & MINEO (1966) show that IAA-oxidase activity sometimes may be due to a more specific enzyme. After purification of extracts from tobacco roots by means of column chromatography they found two distinct fractions with high IAA-oxidase activity. Only one of the IAA oxidizing fractions exhibited peroxidase activity. MACE (1966) found a complete loss of IAA-oxidase activity in banana root extract after it had been frozen and thawed twice, but the oxidation of guaiacol by peroxidase was unaffected by this procedure. SIEGEL & GALSTON (1967) showed that the peroxidase activity of horse-radish peroxidase was abolished after removal of the heme prosthetic group. The apoenzyme, however, retained its IAA-oxidase activity. After purification of pea root extracts on columns of polyvinylpyrrolidone one of the IAA oxidizing fractions showed no peroxidase activity (VAN DER MAST 1969). PILET & LAVANCHY (1969) purified extracts from *Lens* roots on Sephadex columns but could not separate IAA-oxidase and peroxidase activity. After completion of the present study, however, PILET & SEVHONKIAN (1969) reported that the fractions which exhibited IAA-oxidase and peroxidase activity also showed polyphenoloxidase activity. At almost the same time GASPAR *c.s.* (1969) published the results of experiments with extracts from roots of *Lens* and *Pisum* similar to those of Pilet & Sevhonkian. They concluded that the IAA-oxidase activity in both cases was due to a peroxidase.

It has been demonstrated that the elution pattern of IAA-oxidase after filtration of an extract from pea roots through a Sephadex G 100 column depends on the pretreatment of the extract (JANSSEN 1969b) and on the effluent (unpublished results). We have now investigated whether and how the elution pattern of peroxidase and polyphenoloxidase is affected by the pretreatment of the extract and by the effluent.

The results in the literature obtained with DIECA are not uniform. Therefore, the effect of DIECA on the IAA-oxidase, the peroxidase and the polyphenoloxidase activity of pea root extracts was investigated too.

The results from the experiments with columns indicate that a close correlation exists between IAA-oxidase, peroxidase and polyphenoloxidase. The results from the experiments with DIECA, however, point to differences.

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 distilled water. To every 60 tips 1 ml was added. The tips were ground with sand and centrifuged at 27000 g for 30 minutes. The supernatant (the crude enzyme preparation) was used immediately or after storage at -20 °C. Before an extract was frozen NaCl was added to a final concentration of 5%. An extract kept at -20 °C, was centrifuged for a second time before use.

The purification procedure by means of Sephadex G 100 columns has been described before (JANSSEN 1969b). The columns were washed with effluent for 17 hours before use.

The IAA-oxidase activity of the individual fractions was determined in a reaction mixture consisting of 3.3 ml phosphate-citrate buffer solution according to Mc.Ilvaine of pH 5.0, 0.5 ml p-coumaric acid 10^{-5} g/ml, 0.2 ml IAA 10^{-3} g/ml and 1.0 ml of a fraction. After an incubation time of 15 minutes the remaining IAA was determined with the Salkowski reagent according to TANG & BONNER (1947).

The peroxidase activity was determined in a reaction mixture consisting of 8.3 ml phosphate-citrate buffer of pH 7.0, 75 ml 0.3% H_2O_2, 0.75 ml 0.1 M pyrogallol and 0.2 ml of a fraction. After an incubation time of 1 minute the intensity of the brown colour of the purpurogallin formed was measured with a Cenco colorimeter, using the blue filter.

The determination of the polyphenoloxidase activity was performed in a reaction mixture containing 0.5 ml of a 5% catechol solution, 3.0 ml phosphate-citrate buffer pH 6.5 and 0.5 ml of a fraction. After 30 minutes the intensity of the brown colour was measured with a Cenco colorimeter, using the blue filter.

The protein content of the various fractions was determined with the Folin phenol reagent according to LOWRY *c.s.* (1951).

The experiments were performed at 22°C.

3. RESULTS

3.1. The elution pattern of IAA-oxidase, peroxidase and polyphenoloxidase activity after purification of the crude enzyme through Sephadex G 100 columns

In *fig. 1a* the results are presented of an experiment performed with a freshly prepared extract fractionated with a 0.075% NaCl solution as effluent. The extracts used in the experiments presented in *figs. 1b* and *1c* were stored at -20°C for some time before use. In the experiment of *fig. 1b* 10x diluted phosphate-citrate buffer solution of pH 5 was used as effluent and in the experiment of *fig. 1c* distilled water was used. It is evident that the fractions that exhibited IAA-oxidase activity also contained both polyphenoloxidase activity and peroxidase activity in all cases.

3.2. The influence of sodiumdiethyldithiocarbamate on the IAA-oxidase, the peroxidase and the polyphenoloxidase activity

The experiments described in this section were performed with extracts that were partially purified through Sephadex G 25 columns.

From *figures 2* and *4* it is clear that the IAA-oxidase and the polyphenoloxidase activities were inhibited by DIECA. *Fig. 3* shows that the peroxidase activity was not influenced by DIECA.

As the tests were performed at different pH values, the different results might be caused by a pH dependent effect of DIECA. Therefore, the effect of DIECA

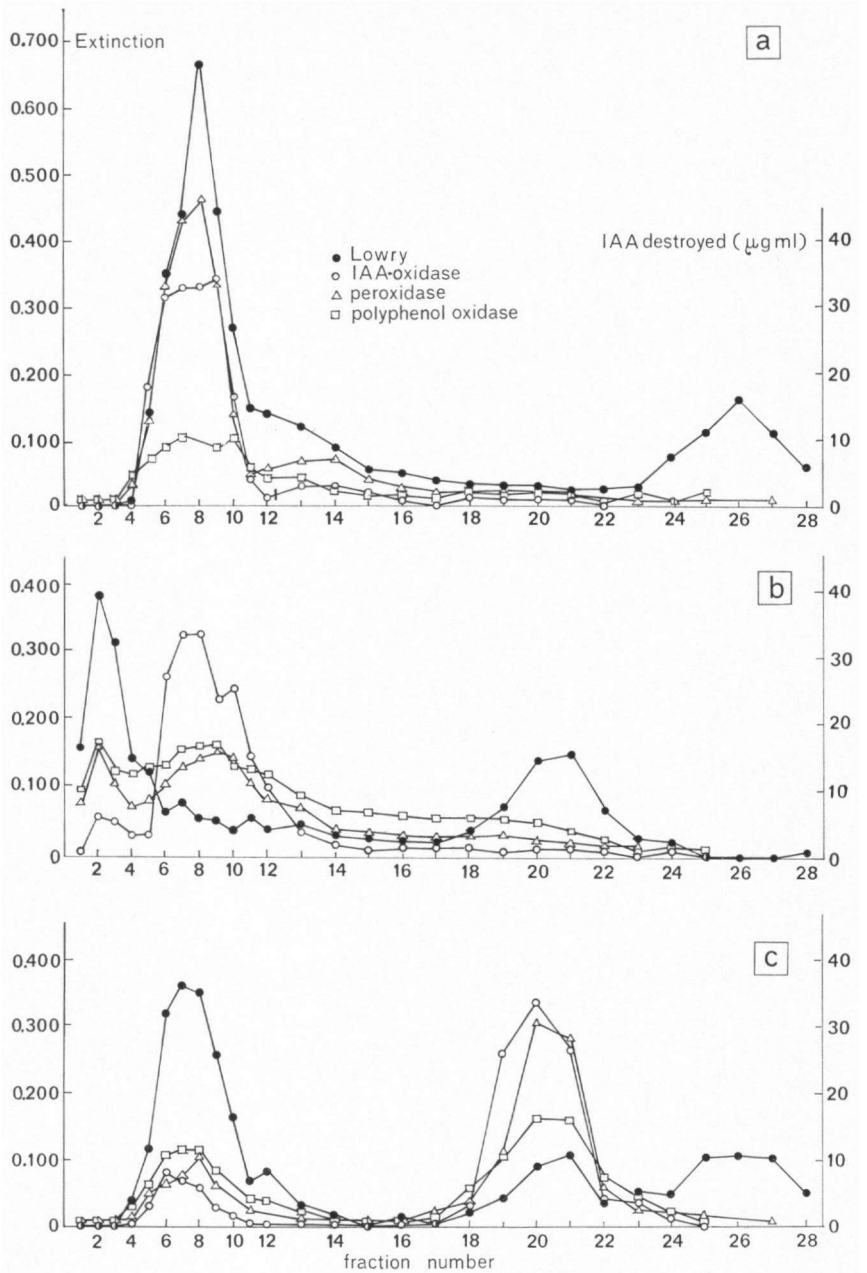
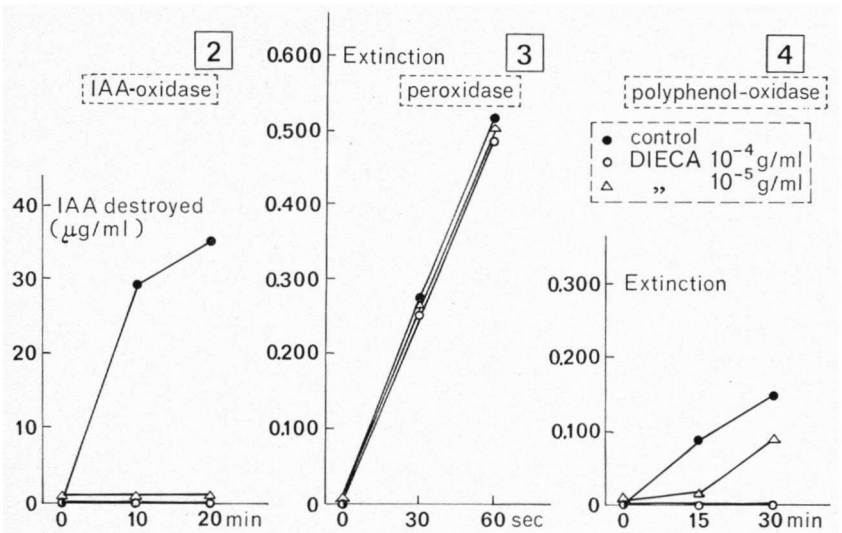


Fig. 1. The IAA-oxidase activity, the peroxidase activity, the polyphenoloxidase activity and the Lowry measurements of the various fractions after filtration of 3 ml pea root extract through a Sephadex G 100 column. After a void volume of 25 ml (b) or 30 ml (a and c) had passed, 12 fractions of 2.5 ml each and 16 fractions of 5.0 ml each were collected. a. Extract: freshly prepared. Effluent: 0.075% NaCl. b. Extract: stored at –20 °C before use. Effluent: 10× diluted phosphate-citrate buffer pH 5. c. Extract: stored at –20 °C before use. Effluent: distilled water.



Figs. 2–4. The influence of sodiumdiethyldithiocarbamate on the IAA-oxidase activity (fig. 2), the peroxidase activity (fig. 3) and the polyphenoloxidase activity (fig. 4) of extracts from pea roots.

on the peroxidase activity was investigated at pH 5 (the pH value of the IAA-oxidase test).

Fig. 5 shows that the peroxidase activity was much less at pH 5. It is also clear that the effect of DIECA on it was similar to the effect at pH 7.

Only in the peroxidase test H_2O_2 was added to the reaction mixture. The lack of effect of DIECA on the peroxidase activity might be caused by a reaction of H_2O_2 with DIECA. Therefore, we investigated whether the inhibition of the IAA-oxidase activity by DIECA was influenced by H_2O_2. This was complicated by the fact that H_2O_2 in the concentration used influenced the colour development of the Salkowski reaction. The colour development occurs much faster in the presence of H_2O_2, but the decolourization already starts after about 10 minutes. The intensity of the colour from the Salkowski reaction was measured therefore 8 minutes instead of 45 minutes after addition of the sample to the reagent. The results of an experiment are presented in *fig. 6*. At this concen-

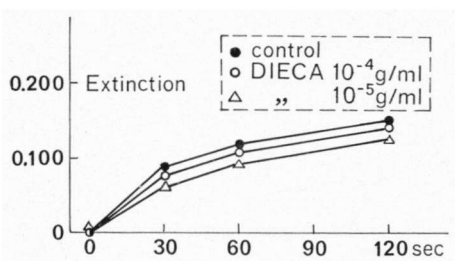


Fig. 5. The influence of sodiumdiethyldithiocarbamate on the peroxidase activity at pH 5 of extracts from pea roots.

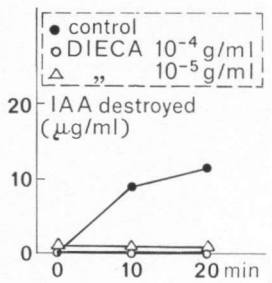


Fig. 6. The effect of H_2O_2 on the influence of sodiumdiethyldithiocarbamate on the IAA-oxidase acitivity of extracts from pea roots.

tration of H_2O_2 the IAA-oxidase activity was inhibited. The inhibition of the IAA-oxidase activity by DIECA, however, remained unaffected.

It is clear that both the IAA-oxidase reaction and the polyphenoloxidase reaction are much more sensitive to DIECA than the peroxidase reaction is.

4. DISCUSSION

From the results presented in this paper it is clear that the elution pattern of IAA-oxidase, peroxidase and polyphenoloxidase activities from pea roots after filtration through Sephadex G 100 columns are the same in all cases; possibly they are due to one enzyme. The sensitivity to DIECA of IAA-oxidase and polyphenoloxidase and the insensitivity of peroxidase may be an indication that the activities are located at different active centers of the enzyme.

According to OAKS & SHAW (1960) the peroxidase from *Melampsora lini* was mildly inhibited by DIECA, whereas the IAA-oxidase was strongly inhibited. Nevertheless they concluded that this IAA-oxidase was a peroxidase. The oxidation of pyrogallol was stimulated by copper, which was suggested to be necessary as a cofactor. DIECA might act by removing the copper from the solution rather than by reacting with the copper component of the enzyme.

PILET & SEVHONKIAN (1969) suggested that IAA-oxidase and polyphenoloxidase of *Lens* roots are enzymes of the same molecular weight. This does not seem very likely here. The changes in the elution pattern caused by the treatment of the extract and by the variations in the effluent should then be the same for all three enzymes.

It is not clear why our results are different from those of GASPAR *c.s.* (1969). Judging from the results presented in this paper and those presented by KONINGS (1964) IAA-oxidase from pea roots is a polyphenoloxidase rather than a peroxidase.

However, it is evident that not every IAA-oxidase can be a polyphenoloxidase. For instance RAY (1960) with *Osmunda*, BASTIN (1964) with *Impatiens* and DINANT & GASPAR (1967) with *Phaseolus* found both IAA-oxidase and peroxidase activity, but no polyphenoloxidase. A crude extract of *Coleus blumei* only showed both polyphenoloxidase activity and peroxidase activity; after purification, however, peroxidase and IAA-oxidase activity was found (KAMINSKI 1966).

From the available results it is clear that IAA-oxidase is not always either a peroxidase or a polyphenoloxidase. It seems possible that IAA-oxidase activity in some cases is due to a more specific enzyme.

ACKNOWLEDGEMENTS

The author is much indebted to miss W. van Hiele for her skillful technical assistance.

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