AN INVESTIGATION OF THE POLYPHENOLOXIDASE TEST WITH CATECHOL AND PROLINE

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SUMMARY

It will be demonstrated that the formation of the purple pigment in a mixture of catechol, proline and a crude enzyme preparation cannot be accepted as a qualitative test for polyphenoloxidase activity as proposed by DRAWERT & GEBBING (1963).

1. INTRODUCTION

The oxidation of catechol (or homocatechol) to o-benzoquinone is catalysed by polyphenoloxidase. With proline (or hydroxyproline) this quinone forms non-enzymatically a purple pigment (JACKSON & KENDAL 1949). They identified the pigment formed from homocatechol and hydroxyprolineethylester as 4-(4'-hydroxy-2'-carbethoxy-1'-pyrrolidyl)-5-methyl-o-benzoquinone.

DRAWERT & GEBBING (1963) reported a rapid qualitative polyphenoloxidase test with catechol and proline which is based on this principle. This method was used by KONINGS (1964) to demonstrate the presence of polyphenoloxidase in pea roots.

We investigated whether the formation of the purple pigment could be used as a quantitative colorimetric test for polyphenoloxidase activity. We found that a polyphenoloxidase preparation of pea roots clearly accelerated the browning (oxidation) of catechol, but that the formation of the purple colour in solutions containing proline was almost equal in the presence and in the absence of the enzyme preparation.

Drawert & Gebbing found no colour formation after addition of KCN, sodiumdiethyldithiocarbamate (DIECA), sodiumthiosulfate or cystein and after boiling the enzyme preparation, which they accepted as proof for the enzymatic formation of the purple pigment. It will be demonstrated, however, that their conclusions are based on insufficient information and an alternative explanation of their results will be presented.

2. MATERIAL AND METHODS

Seeds of *Pisum sativum* L. cv. 'Vlijmsche Gele Krombek' were sterilised during 10 minutes in a 0.2% $HgCl_2$ solution, rinsed with tap water, soaked for 4 hours, then put in petri dishes on moist filter paper and grown in darkness during 65 hours at 22 °C. Root tips, 5 mm long, were cut off and collected in an ice-cold phosphate-citrate buffer solution pH = 6.5, 30 tips per ml, ground with sand

and centrifuged at 27.000 g during 30 minutes. The supernatant was used as the crude enzyme preparation.

A purified enzyme preparation was obtained by filtration of the crude enzyme through a Sephadex G 25 column.

The composition of the various reaction mixtures will be described in the pertinent sections.

The experiments were carried out in daylight at room temperature.

3. RESULTS

3.1. The nonenzymatic formation of the purple pigment

The formation of the purple pigment was found in a mixture of 0.5 ml of 2% proline, 0.5 ml of 10% catechol solution and 1 ml buffer solution of pH = 6.5.

If no buffer solution was added, no colour developed within 30 min. The pH of the mixture in this case was about 5. We therefore investigated the pigment formation at different pH values.

At pH = 7 some purple colour was formed almost immediately, but at pH = 5 it took about two hours before it became visible.

DRAWERT & GEBBING (1963) found no pigment formation when DIECA, sodiumthiosulfate or cystein were added to a mixture of proline, catechol and crude enzyme preparation. We therefore investigated the effect of these compounds on the non-enzymatic pigment formation. Besides these substances we also tested ethylenediaminetetraacetic acid (EDTA) and ascorbic acid. As DRAWERT & GEBBING (1963) probably carried out their experiments at pH = 6.5 (see DRAWERT & GEBBING 1967) all reaction mixtures were adjusted to this pH value. Each of these compounds prevented the colour formation under these conditions.

Thus the inhibition by DIECA, sodiumthiosulfate or cystein does not support the role of polyphenoloxidase in the production of the purple pigment, as stated by Drawert & Gebbing.

3.2. Different effects of crude and purified enzyme preparations on the pigment formation

A boiled crude enzyme preparation added to a reaction mixture prevented the formation of the purple pigment, as found by Drawert & Gebbing. A boiled purified enzyme preparation, however, did not influence the non-enzymatic colour formation (see *table 1*).

Comparison of the effects of reaction mixtures 4 and 5 (*table 1*) shows that the failure of colour formation after addition of the boiled crude enzyme preparation is no consequence of the inactiviaton of the polyphenoloxidase, but must be caused by substances present in the crude extract and removed by purifying the crude enzyme preparation through a Sephadex G 25 column. These substances could be amino acids, which according to SUZUKI (1957) cause bleaching of the pigment. In our experiments especially glycine caused a rapid decolourization.

Table 1. The effect of boiling on crude and purified enzyme preparations. Each reaction mixture contained 1 ml buffer solution pH = 6.5, 0.5 ml 2% proline and 0.5 ml 10% catechol.

Mixture no.	addition	Intensity of purple colour after 20 min.
1.	0.5 ml destilled water	++
2.	0.5 ml crude enzyme	++
3.	0.5 ml purified enzyme	+++
4.	0.5 ml boiled crude enzyme	±
5.	0.5 ml boiled purified enzyme	++

The reactions influencing the pigment formation can accordingly be summarised as follows:

$$\text{catechol} + \text{proline} \xrightarrow[\text{enzymatically (II)}]{\text{non-enzymatically (I)}} \text{purple pigment}$$

$$\text{purple pigment} + \text{amino acids} \xrightarrow{\text{non-enzymatically (III)}} \text{decolouring.}$$

From the effects of mixtures 2 and 3 we see that the purified enzyme causes more colourization than the crude enzyme does. In both cases the purple pigment is formed by reactions I and II. In the reaction mixture with crude enzyme some of the pigment is bleached by reaction III. In mixture 4 we see almost no colourization. By boiling the enzyme preparation reaction II is stopped. Reaction III causes an almost complete decolourization of the pigment formed by reaction I. The colour intensity in mixture 5 is the same as in mixture 1. When only catechol and proline are present only reaction I can occur. The results with mixtures 3 and 5 suggest that the enzyme preparation of pea roots contained phenoloxidase.

The reaction scheme is further supported by the following results.

3.3. The effect of bovine albumine

By hydrolysis of an albumine solution with diluted HCl, free amino acids will be formed. These amino acids will cause a decolourization (reaction III), provided that our reaction scheme is correct. Whereas a bovine albumine solution did not influence the colour formation, a hydrolysed albumine solution indeed inhibited the development of the purple colour.

3.4. The effect of KCN on the crude and the purified enzyme preparation

Drawert & Gebbing found that the pigment formation was prevented by KCN. Finally therefore KCN was added to a purified enzyme preparation. In this case we supposed that reaction II would be blocked by KCN and reaction III would not take place in the abscence of free amino acids so that only reaction I would occur. The results, presented in *table 2* support this view.

Table 2. The effect of KCN on crude and purified enzyme preparations. Each reaction mixture contained 1 ml buffer solution pH = 6.5, 0.5 ml 2% proline and 0.5 ml 10% catechol.

Mixture no.	addition	colour intensity after 20 min.
1.	1 ml destilled water	++
2.	0.5 ml destilled water + 0.5 ml KCN (2 mg/ml)	++
3.	0.5 ml crude enzyme + 0.5 ml KCN (2 mg/ml)	——
4.	0.5 ml purified enzyme + 0.5 ml KCN (2 mg/ml)	++

From the results with reaction mixtures 1 and 2 we see that KCN in the concentration used has no effect on the non-enzymatic pigment formation. From mixture 4 it is clear that KCN stops reaction II. In mixture 3 the pigment formed by reaction I is bleached by reaction III.

4. DISCUSSION

The results presented in the previous sections show clearly that in a mixture of catechol and proline at a proper pH the purple pigment is formed in the absense of an enzyme preparation. The reaction scheme given in section 3.2 can fully explain our results and can also explain the results of DRAWERT & GEBBING (1963). Their conclusion that the pigment formation in a mixture of catechol, proline and a crude enzyme preparation is a qualitative test for polyphenoloxidase, was based on insufficient information about the factors which influence the pigment formation and must therefore be rejected.

ACKNOWLEDGEMENT

The author is much indebted to Prof. R. van der Veen and to Prof. J. van Die for their stimulating interest and to miss Wera van Hiele for her skilful technical assistance.

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