THE INHIBITING EFFECT OF CAFFEIC ACID ON THE ENZYMATIC DEGRADATION OF IAA IS EXERTED VIA THE NON-IAA DEGRADING PEROXIDASES.

C. A. VAN DER MAST

Botanisch Laboratorium, Utrecht

SUMMARY

Caffeic acid inhibits the enzymatic degradation of IAA, probably by formation of semiquinones. These radicals then occupy the sites on the non-IAA degrading peroxidases which are normally used for the transfer of the IAA free radicals, thereby closing pathway I (see *fig.1*). H_2O_2 cancels this inhibiting effect by directing the flow of the IAA free radicals via pathway II to the cofactor.

1. INTRODUCTION

The degradation of IAA in homogenates of pea roots is performed by protein complexes (VAN DER MAST 1970a) each of which consists of at least two enzyme molecules able to convert this plant hormone but having different physicochemical properties. The two enzyme types can be separated by chromatography on polyvinylpyrrolidone (VAN DER MAST 1969, 1970b). Part of these complexes are bound to the membranes but can be removed from them by KCl (VAN DER MAST 1970c). Both the free and the bound complexes are active in the conversion of IAA.

It was also found that part of the peroxidases which are incapable of degrading IAA are bound to the membranes, although the manner of attachment is different from the mode found for the IAA degrading complexes (VAN DER MAST 1970a, 1970d). By using these membrane-bound peroxidases as a tool for varying their amount in the homogenates it was possible to increase or decrease the lag-time of the IAA degradative reaction by the IAA degrading enzymes. This implies that the aforementioned peroxidases, although not capable of degrading IAA, nevertheless participate in this reaction (VAN DER MAST 1970d). FOX & PURVES (1968) proposed a reaction scheme for the inactivation of the IAA free radicals which originate from the action of horse radish peroxidase on IAA. It seems very likely that the non-IAA degrading peroxidases play a part in the transfer of these radicals. The peroxidases are inactivated during this process but reactivation takes place when a cofactor is present (VAN DER MAST 1970d).

The addition of H_2O_2 to an incubation mixture with a low peroxidase content completely cancelled lag-time phenomena, implying that another pathway for the IAA free radicals is present which is independent of the amount of peroxidases (VAN DER MAST 1970d).

The reaction schemes proposed in the previous paper (VAN DER MAST 1970d) are summarized in *fig. 1.*

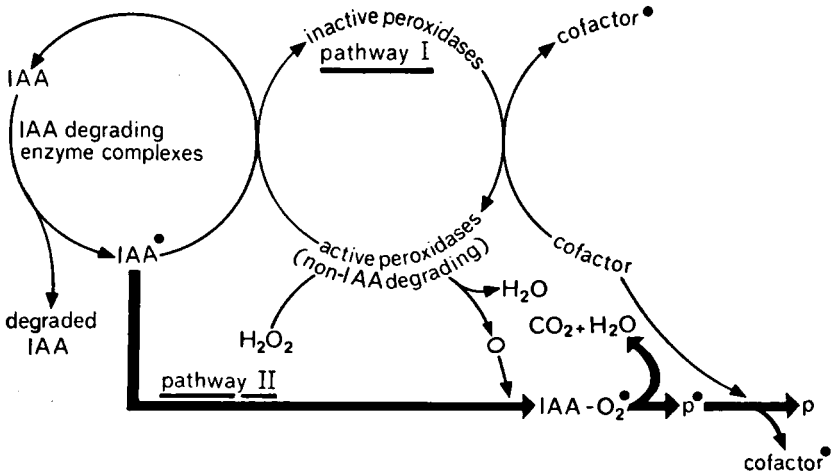

Fig. 1. Pathways of IAA free radicals generated by the enzymatic breakdown of IAA. Pathway I operates in the absence of H_2O_2 and is dependent on the amount of non-IAA degrading peroxidases. These proteins transfer the radicals to the cofactors which are thus inactivated.
Pathway II only operates in the presence of H_2O_2 and is independent of the amount of peroxidases present in the preparation, thus abolishing lag-time phenomena.
The radicals are indicated by a dot.

The inhibitors of the IAA degradative reaction are usually polyphenolic compounds which have at least one free hydroxyl group on an aromatic ring in an ortho or para position to another hydroxyl group (HARE 1964). A compilation of these substances is given by JANSSEN (1970). They differ greatly in their chemical structure which makes it quite unlikely that they act directly on the IAA degrading enzymes via an inhibitor site. A less specific interaction with the radical transferring groups on the non-IAA degrading peroxidases seems to be indicated.

2. MATERIAL AND METHODS

Seeds of *Pisum sativum* L. cv. 'Vlijmsche Gele Krombek' were grown as described earlier (VAN DER MAST 1969). Roots with a length of 2–3 cm were homogenized in Tris-HCl buffer (35 mM Tris, 0.5 mM $CaCl_2$ and 10 mM $MgCl_2$), pH 7.4. Sometimes KCl was added to the buffer in a concentration of 50 mM. The abbreviations TCM and TCMK buffer, respectively, will be used.

For every gram fresh weight of roots 2 ml of a homogenation buffer was added. Cell debris was removed by centrifuging the homogenate during 20 minutes at 27000 g. The membranes and the ribosomes were removed by a further

centrifuging period of 1 hour at 180000 g in rotor 50 of a Spinco model L50 ultracentrifuge.

The degradation of IAA was performed in Mc Ilvaine's buffer, pH 5.0. This buffer contained IAA and sometimes p-coumairic acid (pCA), cafferic acid (CA) or H_2O_2. To 8 ml of this mixture 2 ml of the enzyme preparations were added. The end concentration of IAA was 50×10^{-6}g, of pCA 2×10^{-6}g, of CA 4×10^{-6} g and of H_2O_2 4×10^{-3}M. The incubation was carried out at 25 °C. To 1 ml samples from the reaction mixture 4 ml of Salkowski's reagent were added.

When H_2O_2 was present in the incubate the amount of colour due to residual IAA was read after 3 minutes at 535 nm, in other cases after 30 minutes.

3. RESULTS

The influences of the various compounds on the degradation of IAA by a 27000 g supernatant are shown in *fig. 2.*

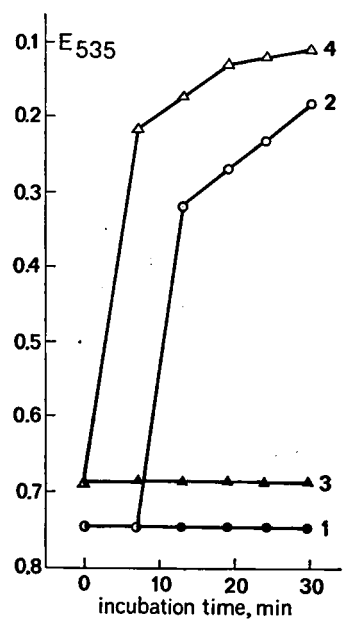

Fig. 2. Influence of pCA, CA and H_2O_2 on the IAA degradation by a 27000 g TCM supernatant.
The amount of CA added guarantees a fully inhibited reaction as indicated by line 1 and 3. When only pCA is added to the homogenate the results found are expressed by line 2. The addition of H_2O_2 to a supernatant containing CA and pCA cancels the inhibiting effect of CA and overcomes the lag-time (line 4).
●——● (line 1) IAA degradation, pCA and CA added.
○——○ (line 2) IAA degradation, pCA added.
▲——▲ (Line 3) IAA degradation, CA and H_2O_2 added.
△——△ (line 4) IAA degradation, pCA, CA and H_2O_2 added.

When pCA and CA are added together the inhibiting effect of the latter compound on the IAA conversion is still fully exercised as indicated by line 1. When only pCA is present the results found are expressed by line 2. In this case the reaction goes to completion after a certain lag-time as observed earlier (VAN DER MAST 1970d). When both CA and H_2O_2 are added, but pCA is omitted, the inhibition is still complete (line 3) but after the addition of pCA the reaction pro-

ceeds normally (line 4). Due to the presence of H_2O_2 the reaction does not show a lag-time.

As shown by *fig. 3* the removal of the membranous fraction does not alter the results mentioned above.

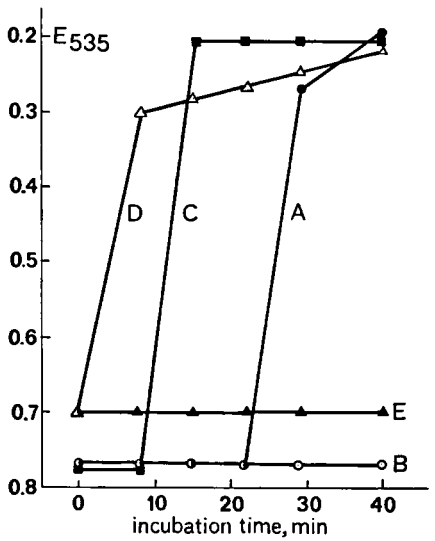

Fig. 3. Influence of pCA, CA and H_2O_2 on the IAA degradation by ultracentrifuged samples.
The addition of pCA to a TCM supernatant shows a normal conversion of IAA (Line A) with a lag-time due to partial blockage of pathway I (fig.1). A further addition of CA totally inhibits this degradation by TCM or TCMK supernatants or of TCM supernatants with added TCMK membranes (line B) although the amounts of peroxidases vary in these cases as shown by line C (only pCA added) which exhibits a shorter lag-time for TCMK homogenates and TCM + TCMK membrane preparations. The addition of pCA, CA and H_2O_2 to various preparations gives rise to a normal conversion of IAA without lag-times (line D), whereas omission of pCA effectuates the inhibition by CA (line E).

●——● (line A) IAA degradation by a TCM supernatant, pCA added.
■——■ (line C) IAA degradation by a TCM supernatant + TCMK membranes (ratio 1:1) or of a TCMK supernatant, pCA added.
▲——▲ (line E) IAA degradation by the aforementioned preparations, CA and H_2O_2 added.
○——○ (line B) IAA degradation by a TCM or TCMK supernatant or of a TCM supernatant + TCMK membranes (ratio 1:1), pCA and CA added.
△——△ (line D) IAA degradation by a TCM or TCMK supernatant or of a TCM supernatant + TCMK membranes, pCA, CA and H_2O_2 added.

The degradation of IAA by a 180000 g TCM supernatant in the presence of pCA proceeds normally after a considerable lag-time (line A) as described earlier.

The addition of both pCA and CA to either a 180000 g TCM supernatant or a 180000 g TCMK supernatant or a 180000 g TCM supernatant in which TCMK membranes had been resuspended causes a complete inhibition of the IAA degradative reaction (line B). When, however, pCA, CA and H_2O_2 were added to the enzyme preparations mentioned above, a normal conversion of IAA was achieved without a lag-time (line D). The omission of pCA from this mixture gave the results expressed by line E.

The degradation of IAA in the presence of only pCA by a 180000 g TCMK supernatant or a 180000 g TCM supernatant which contained TCMK membranes in a ratio of 1:1 is expressed by line C as described earlier.

4. DISCUSSION

The lag-times of the IAA degradative reaction displayed by the various preparations are all in accord with the results described earlier (VAN DER MAST 1970d). In this paper the abolishment of the lag-time, in the presence of H_2O_2, of a 180000 g TCM supernatant to which TCMK membranes had been added was mentioned. Line D in *fig. 3* shows that even a 180000 g TCM supernatant, which displays the longest lag-time due to the least amount of non-IAA degrading peroxidase molecules, can be made to start the reaction with IAA immediately when H_2O_2 is added. This means that in the presence of H_2O_2 the degradation of IAA becomes independent of the amount of these peroxidases, except for a small amount needed to convert H_2O_2.

The inhibition of the reaction by CA or other phenolic compounds is usually ascribed to the formation of quinones from them (JANSSEN 1970). According to FOMIN *et al.* (1968) semiquinone free radicals form in solutions of quinones at physiological pH values in the presence of proteins. The appearance of the radicals is apparently associated with the reaction of the quinones with water (OH^- ions). Proteins accelerate this reaction through the high mobility of the OH^- ion over the layer of water structured on the surface of these biopolymers and stabilize the semiquinone radicals formed on their surface.

It seems very likely that the semiquinone free radicals of the inhibitors react with the sites used for the transfer of the IAA free radicals on the surface of the non-IAA degrading peroxidases, thereby closing pathway I, as shown in *fig. 4*.

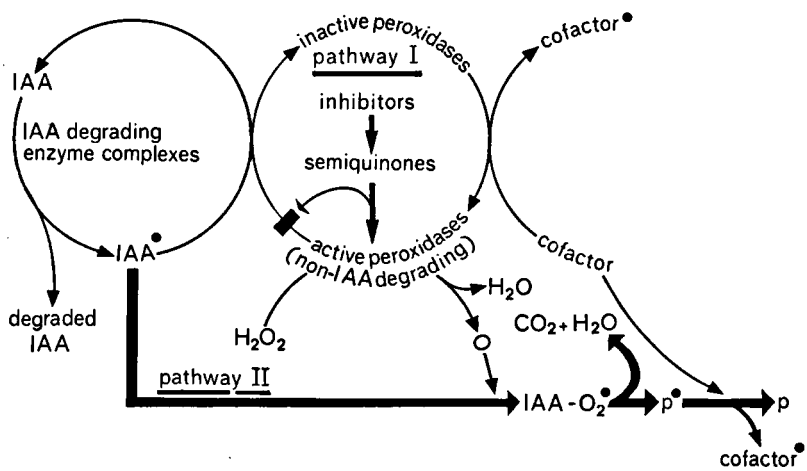

Fig. 4. Reaction scheme explaining the inhibiting effect of caffeic acid. This compound and probably all phenolic inhibitors act via the formation of semiquinones which radicals then occupy the sites present on the non-IAA degrading peroxidases for the transfer of the IAA free radicals.
Pathway II bypasses these peroxidases and is thus not inhibited by the phenolic compounds.
The presence of a cofactor as a final radical trap is obligatory in both pathways.

The addition of H_2O_2 opens pathway II which is independent of the amount of peroxidases present and thus insensitive to phenolic inhibitors. It is highly unlikely that H_2O_2 reacts directly with CA and thus inactivates this compound, as in the absence of pCA full inhibition is achieved.

Both pathways are dependent on the presence of a cofactor, like pCA, because such a compound acts as a final radical trap.

KONINGS (1964) noted that an amount of 10^{-6} g CA in the presence of 10^{-5} g 2,4-dichlorophenol gave full inhibition of the IAA degrading reaction. This is consistent with the scheme presented in *fig. 4.* in which the amount of the cofactor added has no influence on the inhibition achieved with the inhibitor as the two compounds do not compete for the so-called active sites on the IAA degrading enzymes but work at different levels. Konings also found that a reaction started in the presence of a cofactor needed a higher concentration of an inhibitor to achieve the same amount of inhibition as found when the cofactor and the inhibitor were added simultaneously to the reaction medium. This is probably due to the high concentration of IAA free radicals on the sites of the non-IAA degrading peroxidases in the former case, which are not displaced from these sites by a normal inhibiting concentration of the inhibitor.

It seems very likely that all phenolic inhibitors of the enzymatic conversion of IAA act in a similar way via these peroxidases.

REFERENCES

FOMIN, G. V., N. N. KATMAZOVSKII, L. G. IGNAT'EVA & L. A. BLYUMENFEL'D (1968): Interaction of the hydroxyl ion with quinones in protein solutions. *Biophysics* **13**: 899–906.

FOX, R. & W. K. PURVES (1968): Mechanism of enhancement of IAA oxidation by 2,4-dichlorophenol. *Plant Physiol.* **43**: 454–456.

HARE, R. C. (1964): Indoleacetic acid oxidase. *Bot. Rev.* **30**: 129–165.

JANSSEN, M. G. H. (1970): Inhibitors of pea root indoleacetic acid oxidase. *Acta Bot. Neerl.* **19**: 109–111.

KONINGS, H. (1964): On the indoleacetic acid converting enzyme of pea roots and its relation to geotropism, straight growth and cell wall properties. *Acta Bot. Neerl.* **13**: 566–622.

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

— (1970a): Isoelectric focusing of indoleacetic acid degrading enzymes from pea roots. *Acta Bot. Neerl.* **19**: 363–372.

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

— (1970c): The presence of membrane-bound IAA degrading protein complexes in homogenates of pea roots and the manner of attachment to these membranes. *Acta Bot. Neerl.* **19**: 546–559.

— (1970d): The influence of membrane-bound peroxidases on the degradation of IAA by the specific IAA degrading complexes in homogenates of pea roots. *Acta Bot. Neerl.* **19**: 727–736.