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EFFECTS OF CHLOROGENIC AND CAFFEIC ACIDS ON IAA OXIDASE PREPARATIONS FROM SWEET POTATO ROOTS

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Key Word Index—*Ipomoea batatas;* Convolvulaceae; indolyl-3-acetic acid oxidase; chlorogenic acid; caffeic acid.

Abstract—IAA oxidase preparations from sweet potato (*Ipomoea batatas*) roots oxidised IAA in the absence of added phenolics. Activity was optimal around pH 6·8 and a minor pH optimum occurred around pH 4·3. Both chlorogenic and caffeic acids inhibited IAA oxidase activity at high concentrations (0·6–5·7 nmol/ml) but stimulated enzyme activity at low concentrations (0·10–0·55 nmol/ml); these effects were dependent on IAA and enzyme concentration and on pH. The activities of both substances are compared with those of other phenolics known to stimulate and inhibit plant IAA oxidases.

INTRODUCTION

CHLOROGENIC and caffeic acids occur widely in higher plants,[1–4] including sweet potato.[2,5] Free quinic acid also occurs in several plant species.[6] Both chlorogenic acid and caffeic acids are well known inhibitors of IAA oxidase,[7–9] while quinic acid has been found to have no effect on this enzyme.[7] In this paper, caffeic and chlorogenic acids are shown both to inhibit and to stimulate sweet potato IAA oxidase, depending on the concentration of substrate and phenolics used and on the pH of the reaction mixtures.

RESULTS

Characteristics of Enzyme Preparations

IAA oxidase preparations from sweet potato roots catalysed the destruction of IAA in the absence of added phenolics. The enzyme used in this study was previously described as 7·0 enzyme[10] with a typical major pH optimum at approx. pH 6·8 and a minor optimum at approx. 4·4. All preparations showed both peroxidase and phenolase activities.[10] The phenolase activity of preparations was specific for *ortho*dihydroxyphenols and preparations oxidized chlorogenic and caffeic acids rapidly with optimal activity at pH 3·8–4·2, which was close to the minor pH optimum for IAA oxidase activity (pH 4·4). The pH optimum for catechol oxidation was, however, around pH 6·8, similar to that of the major pH optimum for IAA oxidase activity.[10]

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IAA oxidase activity was determined routinely by estimating residual IAA by the Salkowski method, since this procedure allowed for simultaneous assay of a large number of reaction mixtures. Simultaneous assay of reaction mixtures was desirable in the experiments described because of the impure nature of preparations and the resulting difficulty in interpreting results of reaction rates based on the appearance of intermediate compounds. Stimulation and inhibition of enzyme activity were, however, demonstrated spectrophotometrically at 247, 261 and 292 nm. Disappearance of IAA measured by the Salkowski method was found to correspond with spectrophotometric assays of enzyme activity at the above wavelengths, over 150 min at 28°.

Inhibitory and Stimulatory Effects of Chlorogenic and Caffeic Acids

Typical curves of the inhibition and stimulation of IAA oxidase activity at pH 6·8 by chlorogenic and caffeic acids in the concentration range 0·24–2·40 nmol/ml are shown in Fig. 1.

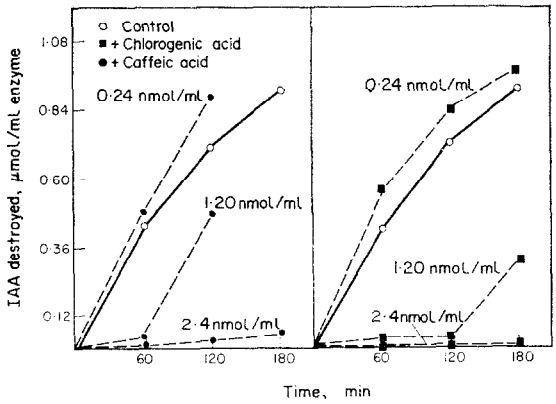

FIG. 1. STIMULATION AND INHIBITION OF SWEET POTATO ROOT IAA OXIDASE ACTIVITY AS A FUNCTION OF CHLOROGENIC AND CAFFEIC ACID CONCENTRATIONS.
Reaction mixtures were incubated at 33° and contained 0·6 μmol IAA, 200 μmol phosphate-citrate buffer pH 6·0, 0·5 ml enzyme in total vol. of 4·1 ml.

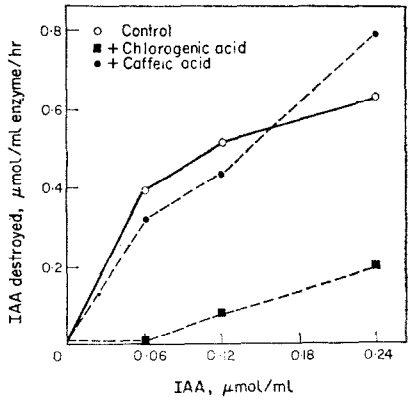

FIG. 2. EFFECT OF IAA CONCENTRATION ON SWEET POTATO ROOT IAA OXIDASE ACTIVITY IN THE PRESENCE OF CHLOROGENIC AND CAFFEIC ACIDS.
Reaction mixtures were incubated at 33° and contained IAA as shown, 250 μmol phosphate-citrate buffer pH 7·0, 0·02 μmol chlorogenic or caffeic acids in total vol. of 4·2 ml.

The enzyme was inhibited by 2·4 nmol of either phenol in the presence of 0·14 nmol IAA/ml, for 60 min. Chlorogenic acid inhibition was maintained up to 180 min, but the inhibition caused by caffeic acid slowly decreased with time to 95% inhibition at 180 min.

Chlorogenic acid at a concentration of 1·2 nmol/ml (Fig. 1) also resulted in total inhibition of IAA oxidation for 120 min but inhibition decreased with time thereafter. Oxidation of IAA in the presence of 1·2 nmol of caffeic acid increased after 60 min to 56% that of the control at 120 min. Although the sensitivities of various preparations to chlorogenic and caffeic acids differed somewhat, chlorogenic acid was always the more effective inhibitor. At phenol concentrations of 0·24 nmol/ml, enzyme activity was stimulated 17 and 24% by chlorogenic and caffeic acids respectively.

The Effect of Substrate Concentration

Increasing substrate concentration in the presence of a constant concentration of phenols decreased inhibition by both chlorogenic and caffeic acids (Fig. 2). At an IAA concentration of 0·24 nmol/ml, the inhibitory effect of caffeic acid was converted to a 25% stimulation of IAA oxidase activity, and chlorogenic acid inhibition was reduced to 70%. Increasing IAA concentration had a greater effect on the reversal of caffeic acid inhibition.

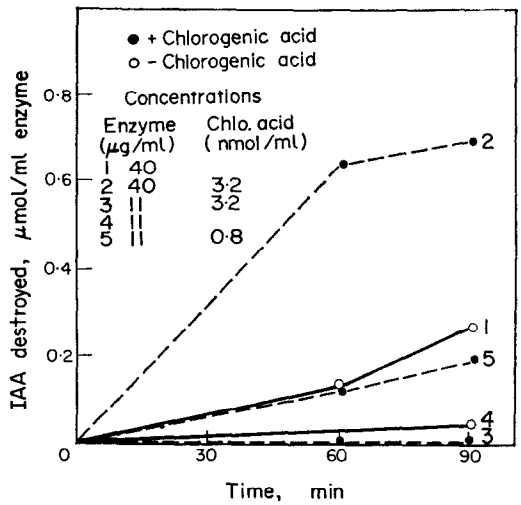

FIG. 3. STIMULATION AND INHIBITION OF SWEET POTATO ROOT IAA OXIDASE ACTIVITY AS A FUNCTION OF RELATIVE CONCENTRATIONS OF ENZYME AND CHLOROGENIC ACID.
All reaction mixtures were incubated at 33° and contained 1 μmol IAA, 300 μmol phosphate-citrate buffer, pH 4·3 in total vol. of 8·0 ml μg/ml.

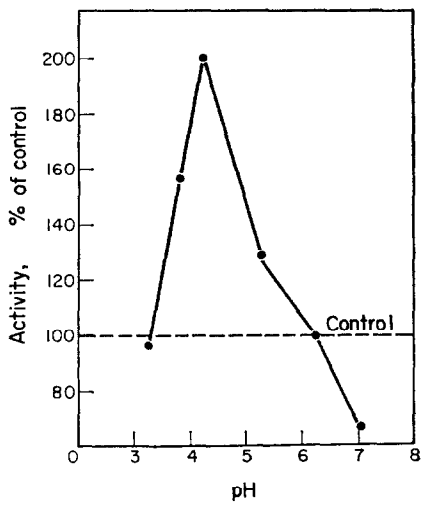

FIG. 4. STIMULATION AND INHIBITION OF SWEET POTATO ROOT IAA OXIDASE ACTIVITY BY A FIXED CONCENTRATION OF CHLOROGENIC ACID, AS A FUNCTION OF pH.
Reaction mixtures were incubated at 33° and contained 0·05 μmol IAA, 100 μmol phosphate citrate buffer, 1 nmole chlorogenic acid, 0·75 ml enzyme in a total vol. of 2·85 ml and with pH values adjusted as shown above.

The Effect of Enzyme Concentration

When enzyme protein concentration was decreased by dilution with phosphate–citrate buffer from 40 to 11 μg/ml (Fig. 3) a 150% stimulation of IAA oxidase activity by chlorogenic acid was reversed to total inhibition. However, decreasing the chlorogenic acid concentration from 3·2 to 0·8 nmol/ml in the presence of 11 μg/ml of enzyme protein restored the stimulation.

The Effect of pH

IAA oxidase activities at the two pH optima of the sweet potato root enzyme preparation, in the presence of levels of chlorogenic and caffeic acids (5·7 nmol/ml) previously found to be inhibitory at the major pH optimum are shown in Table 1.

Enzyme activity in the absence of phenols at the major pH optimum (6·8) was approx. six times that at the minor optimum (pH 4·4). At pH 6·8, there was total and 48% inhibition of IAA oxidase activity by chlorogenic and caffeic acids respectively. The same concentration of phenols resulted, however, in 8-fold stimulation of enzyme activity by chlorogenic

acid and 2-fold stimulation by caffeic acid at pH 4·4. The level of enzyme activity recorded at pH 4·4 in the presence of chlorogenic acid was also 41 % higher than IAA oxidase activity at pH 6·8 in the absence of these phenols.

Data (Fig. 4) indicated that the pH optimum for sweet potato root IAA oxidase activity in the presence of chlorogenic acid was near pH 4·2. Stimulation of activity by chlorogenic acid was limited to the pH range 3·2–6·3; outside this range, the phenol was inhibitory.

TABLE 1. THE EFFECT OF pH ON SWEET POTATO ROOT IAA OXIDASE ACTIVITY IN THE PRESENCE OF CHLOROGENIC CAFFEIC ACIDS

Reaction mixture	IAA oxidase activity (μmol IAA destroyed/ml enzyme/hr) pH 4·4	pH 6·8
Control	0·07	0·39
+ chlorogenic acid	0·55	0·00
+ caffeic acid	0·14	0·20

Reaction mixture contained IAA (2 μmol), buffer (500 μmol), chlorogenic or caffeic acids (0·03 μmol) and enzyme (1·0 ml) in a total vol. of 8·3 ml.

Comparison of the Action of Chlorogenic Acid with Scopoletin

Data comparing the stimulation of sweet potato IAA oxidase by equimolar amounts of chlorogenic acid and scopoletin at three selected pH values are given in Table 2. Results indicated that at pH 4·2 stimulation was maximal and at this pH, the percentage stimulation of enzyme activity by scopoletin (161 %) and chlorogenic acid (130 %) was similar. IAA oxidase activity in the presence of chlorogenic acid was, however, much more sensitive to changes in pH than in the presence of scopoletin.

TABLE 2. COMPARISON OF THE ACTION OF CHLOROGENIC ACID AND SCOPOLETIN ON SWEET POTATO IAA OXIDASE AT SELECTED pH VALUES

Reaction mixture	IAA oxidase activity (μmol IAA destroyed/ml enzyme/hr) pH 3·4	pH 4·2	pH 7·0
Control	0·19	0·23	0·45
+ chlorogenic acid (3·6 nmoles/ml)	0·33	0·53	0·00
+ scopoletin (3·6 nmoles/ml)	0·57	0·60	0·38

DISCUSSION

Both mono and *ortho*dihydroxy benzoic acids have been found to stimulate tobacco callus[11] and pea root IAA oxidases.[12] Monohydroxy cinnamic acids, *p*-coumaric acid[13]

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and ferulic acid[14] as well as lactone derivatives of cinnamic acids, scopoletin[15] and 4-methylumbelliferone[16] have also been shown to stimulate IAA oxidase activity. Recently, Imbert and Wilson[15] demonstrated that scopoletin both stimulated and inhibited sweet potato IAA oxidase, depending on concentration. It has now been found that the *ortho*dihydroxy cinnamic acid, caffeic acid, like its benzoic acid analogue, 3,4-dihydroxybenzoic acid, both stimulated and inhibited sweet potato IAA oxidase. Similar stimulatory activity here reported for chlorogenic acid indicated that esterification of caffeic acid with quinic acid, like lactonization of cinnamic acids, did not destroy the stimulatory effect of these molecules on sweet potato root IAA oxidase activity.

Chlorogenic acid was reported to be a competitive inhibitor and caffeic acid a non-competitive inhibitor of pea root IAA oxidase by Rabin and Kline.[7] Since quinic acid had no effect on enzyme activity, it was postulated that caffeic acid might be the reactive moiety of the chlorogenic acid molecule, but that esterification with quinic acid was necessary for true competitive inhibition. Both compounds are now shown to be competitive inhibitors of sweet potato root IAA oxidase, as indicated by relief of inhibition at high concentrations of IAA (Fig. 2). Inhibition produced by both chlorogenic acid and caffeic acids decreased with time (Fig. 1) indicating these phenolics might have been used up during the reaction. Steric factors introduced by the modification of the caffeic acid molecule through esterification with quinic acid might be responsible for the slower decrease in inhibition with time, in the presence of chlorogenic acid. In contrast, stimulation of IAA oxidase activity by 3,4-dihydroxy-benzoic acid was reversed to inhibition in the course of enzyme reaction[11] indicating either that this phenolic was not used up with time or was not a substrate for enzyme(s) in the IAA oxidase preparation used.

Stimulation or inhibition of sweet potato root IAA oxidase by chlorogenic and caffeic acids was shown to be determined by phenolic concentration (Fig. 1), substrate concentration (Fig. 2), enzyme concentration (Fig. 3) and pH (Fig. 4). Effects of phenolic concentration on stimulation/inhibition were similar to those obtained by Lee and Skoog[11] for 3,4-dihydroxy-benzoic acid and by Imbert and Wilson[15] for scopoletin. The former authors explained these effects on the basis of the relative affinities of 'co-factor' for two sites on the enzyme molecule, one for activation and another for inhibition. This explanation is not incompatible with the 'co-operative effect' proposed by Imbert and Wilson to explain the stimulatory activity of low concentrations of scopoletin, also a competitive inhibitor of sweet potato root IAA oxidase. The fact that inhibitory levels of both chlorogenic and caffeic acids could be made stimulatory by altering either substrate or enzyme concentration indicated that there was a critical substrate-enzyme-phenolic concentration relationship for stimulation of enzyme activity. Sweet potato IAA oxidase preparations used in our experiments, however, showed peroxidase, catecholase and caffeic acid oxidase as well as chlorogenic acid oxidase activities. Interpretations of intermediate reactions involving a single enzyme species therefore, can only be tentative.

The present results might also be explained by assuming that the first step in the promotion of the phenol-induced IAA oxidase reaction is the oxidation of phenols,[17] in such a way that the formation of an enzyme intermediate is favoured, followed by the oxidation of IAA by phenolic radicals so produced.[18] Such an explanation is compatible with the role

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of phenolics in autocatalytic, cyclical mechanisms for IAA oxidation.[17] On this basis, the reversal of inhibition to stimulation at pH 4·4 might be due to the activity of the highly reactive *o*-quinones and semi-quinones with high redox potentials, formed by the oxidation of both chlorogenic and caffeic acids. Activity of quinones and semi-quinones, which could act as oxidants and reductants respectively, would be greatly influenced by pH. The greater stimulatory power of chlorogenic acid might be due to differences in redox potentials of quinones produced from the two phenols. It is interesting to note that the pH optimum for chlorogenic and caffeic acid—stimulated IAA oxidase was similar to that for chlorogenic and caffeic acid oxidase activity of the enzyme preparation, at around pH 4·0.[10] Rapid removal of these phenols by oxidation at low pH might also reduce their concentrations to stimulatory levels. Although it has been reported[19] that the quinones of chlorogenic and caffeic acids could result in the non-enzymic removal of IAA from solution at low pH, the low concentrations of both phenols used could not account for the disappearance of IAA observed in our experiments.

EXPERIMENTAL

IAA oxidase preparations from sweet potato roots were prepared as previously described.[10,15] IAA oxidase activity was assayed by the modified 'Salkowski method',[20] and by spectrophotometric determinations at 247, 261 and 292 nm, but only results obtained with the former method are here reported.

As it has been stated that proteins[21] and phenolic co-factors[22,23] can affect IAA oxidase determinations by the Salkowski method, special care was taken to eliminate such interference. No precipitation of protein occurred on adding the Salkowski reagent to the reaction mixtures. In preliminary experiments using high concentrations of chlorogenic and caffeic acids (0·1 μmol/ml) the reported inhibition[23] of colour development at 530 nm in the presence of polyphenols was confirmed. Neither colour intensity nor rate of colour development was affected by the low concentrations of co-factors subsequently used (5·7 nmol/ml). Polyphenolase and peroxidase activities were assayed spectrophotometrically as previously described.[10] The pH of reaction mixtures was determined before and after each enzyme assay and protein estimated by the Folin reagent.[24]

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