Partial Purification and Some Properties of a Hydroxycinnamoyl Glucosyltransferase from Tomato Fruits

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A glucosyltransferase was isolated from immature “cherry” tomatoes and was partially purified (200-fold) by ammonium sulphate precipitation and successive chromatography on Sephadex G-100 and DEAE-cellulose columns.

The enzyme utilised the free hydroxycinnamic acids and UDP-glucose in the formation of their respective glucosides (pH 8.0) and glucose esters (pH 7.0); but did not accept the CoA thiolesters of HCAs in the presence of glucose-1-phosphate. The constant glucoside/glucose ester ratio observed during purification suggests that both reactions are catalysed by the same enzyme.

The $K_m$ values for $p$-coumaric, caffeic, ferulic and sinapic acids were 0.8, 1.5, 1.4 and 2.5 $\mu$m, respectively. With ferulic acid as substrate, the $K_m$ value for UDPG was 10 $\mu$m. The enzyme required an $-\text{SH}$ group for activity and the reaction was strongly inhibited by EDTA, divalent metal ions and UDP.

Introduction

Hydroxycinnamic acid (HCA) derivatives occur naturally in plants [1], including many fruit tissues [2–4], both as glucosides and esters of glucose or quinic acid. Whereas HCA glucosides were believed to be of more restricted distribution in Nature than their glucosyl or quinyl esters [1], however, tomato fruits are known to accumulate both the glucosides and smaller amounts of the glucose esters of a number of HCAs [5].

A number of glucosyltransferases (GTs) has been shown to catalyse the transfer of glucose from UDPG to simple hydroxyphenols [6–10], phenolic acids [10, 11], lignin precursors [12, 13], dihydroxy-coumarins [14] and flavonoids [15–20], with the formation of their respective glucosides. Whereas earlier work [21, 22] gave evidence for the in vitro formation of glucose esters in intact tissues which were administered free HCAs and UDPG, it was only recently that Macheix [23] and Strack [24] demonstrated the formation of feruloyl glucose and sinapoyl glucose by cell-free extracts of apple fruit and radish seedlings, respectively.

It may be envisaged that the formation of both glucosides and glucose esters of HCAs is catalysed either by one GT that utilises the free acids and UDPG as the sugar donor, or by two distinct enzymes/isoenzymes (Fig. 1). However, this does not exclude the possibility of the involvement of the hydroxycinnamoyl CoA esters and glucose-1-phosphate in the formation of their glucose ester derivatives (Fig. 1), in a manner similar to the hydroxycinnamoyl quinate esters [25]. The present report examines these possibilities and describes the par-

Abbreviations: HCAs, hydroxycinnamic acids; GT, glucosyltransferase; UDPG, uridine diphosphoglucose.

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Fig. 1. Possible routes for the glucosylation of hydroxycinnamic acids.
tial purification and some properties of a GT from tomato fruits which utilises the free HCAs and UDPG in the formation of both their glucosides and glucose esters. This enzyme does not accept the hydroxycinnamoyl CoA thiolesters in presence of glucose-1-phosphate in catalysing this reaction.

**Experimental**

**Plant material**

Tomato (*Lycopersicum esculentum* var. *cerasiforme*) plants were grown under greenhouse conditions and the fruits were collected in the green stage (ca. 12–15 mm dia.). Previous work [5] indicated that, at this growth stage, the fruit contain detectable amounts of p-coumaroyl glucose and feruloyl glucose in addition to high amounts of the glucosides of HCAs.

**Enzyme extraction and purification**

Fresh or frozen (−20 °C) fruits were homogenised with cold 0.1 M phosphate buffer, pH 8.0 in the presence of 20 mM β-mercaptoethanol, Polyclar AT, polyethylene glycol (MW 6000) and fine sand. The homogenate was filtered through miracloth and the filtrate was centrifuged at 20000 × g for 20 min. The supernatant was adjusted with KOH to pH 7.0, stirred for 20 min with Dowex 1 X 2 which had been equilibrated with the same buffer, then filtered. The filtrate was fractionated with solid (NH₄)₂SO₄ and the protein fraction which precipitated between 45–65% salt saturation was collected by centrifugation and resuspended in the minimum amount of 25 mM phosphate buffer, pH 7.5. The latter was desalted on Sephadex G-25 column using the same buffer. The protein extract, after being concentrated by ultrafiltration (Amicon), was applied to Sephadex G-100 column which had previously been equilibrated with phosphate buffer, pH 7.5 and 3-ml fractions were collected and assayed for GT activity against p-coumaric, caffeic, ferulic and sinapic acids. The active fractions were combined and were further chromatographed on a DEAE-cellulose (Cellex-D, 0.76 meq.) column. The latter was washed with two volumes of 25 mM phosphate buffer, pH 7.5, where approximately 30% of the applied protein fell through. The remaining protein was eluted with 4–5 volumes of a salt gradient between 0–500 mM KCl in the same buffer and the fractions collected were assayed for enzyme activity. The protein content of the enzyme preparation was determined by the method of Lowry *et al.* [26].

**Glucosyltransferase assay**

The standard assay mixture consisted of 25–50 nmol of the HCA, 0.17 nmol UDP-[U⁴C]-glucose containing 0.05 μCi, 1.4 μmol β-mercaptoethanol, 1 μmol MnCl₂ and 50–100 μl enzyme protein in a total volume of 140 μl. The assay mixture was incubated at 30 °C for 60 min and the reaction was terminated by the addition of 10 μl of 6 N HCl. The reaction products were extracted by shaking with 150 μl of 40% (NH₄)₂SO₄ and 400 μl ethyl acetate. After centrifugation, 100 μl of the organic layer was transferred to a scintillation vial and counted for radioactivity in a toluene-based liquid.

**Identification of reaction products**

The ethyl acetate extracts containing the reaction products were concentrated and chromatographed in butyl acetate-acetic acid-water (4 : 1 : 5, v/v, organic layer) on Whatman no. 3 paper for 24 h. The glucosides and glucose esters of HCAs were identified by co-chromatography with reference compounds, fluorescence in UV-light, UV-absorption maxima and hydrolysis with KOH and β-glucosidase as previously described [5]. The spots corresponding to the glucosides and glucose esters of HCAs were cut off the paper chromatograms and their radioactivity was determined by liquid scintillation.

**Results**

**Enzyme purification**

The purification procedure of tomato fruit GT, against ferulic acid as substrate, is outlined in Table I and Figs. 2 and 3. The combination of gel filtration and ion-exchange chromatography resulted in the elimination of about 98% of the contaminating protein and 200-fold increase in the specific activity of the enzyme preparation as compared with the original extract. The final fraction was eluted at about 0.33 M salt concentration and had specific activity of 18.8 pkat/mg protein. Similar purification data was obtained with p-coumaric, caffeic and sinapic acids as substrates (not shown).
Table I. Purification data of tomato fruit glucosyltransferase.

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<tbody>
<tr>
<td>Crude extract (Dowex 1X2)</td>
<td>120</td>
<td>11.28</td>
<td>0.09</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (45–65% satn)[^c]</td>
<td>29</td>
<td>15.70</td>
<td>0.54</td>
<td>3.36</td>
<td>8</td>
</tr>
<tr>
<td>Sephadex G-100</td>
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<td>21.17</td>
<td>1.89</td>
<td>2.83</td>
<td>20</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>1.5</td>
<td>28.20</td>
<td>18.80</td>
<td>2.75</td>
<td>200</td>
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[^a]: The standard assay was used as described in the Experimental section with ferulic acid as substrate.

[^b]: Determined after chromatography of reaction products and counting radioactivity of the corresponding compounds.

[^c]: After desalting on Sephadex G-25 column.

Fig. 2. Elution profile of glucosyltransferase activity on Sephadex G-100 column.

Fig. 3. Elution profile of glucosyltransferase activity on DEAE-cellulose column.
The products of the enzyme reaction against the different substrates were identified by the standard procedures [5] as the glucosides and glucose esters of p-coumaric, ferulic and sinapic acids. The enzyme reaction with caffeic acid gave the 4-O-glucoside, but no corresponding glucose ester was formed. The enzymatic synthesis of the glucosides of the former substrates was consistently higher than that of their respective glucose esters, as shown by the glucoside/glucose ester ratio for ferulic acid (Table I). Similar ratios were obtained with p-coumaric and sinapic acids, though exhibited slight variations during the various steps of purification.

**Enzyme properties**

**Stability of the enzyme:** The purified enzyme preparation lost approximately 40% and 70% of its activity after storage at 0–4 °C for 24 h and 48 h, respectively. Storage at −20 °C resulted in almost complete loss of enzyme activity within 12–24 h. The loss of activity could not be prevented by the addition of bovine serum albumin or ethylene glycol. Most of the property studies were conducted, therefore, with the Sephadex G-100 fraction which had a half-life of 24–30 h.

**Enzyme kinetics:** The glucosylation of HCAs was proportional with protein concentration up to 75 μg protein and with time up to 30 min. The rate of reaction was not inhibited by substrate concentration up to 1 mM. The apparent \( K_m \) values, as determined from Lineweaver-Burk plots, were 0.8, 1.4, 1.5 and 2.5 μM for p-coumaric, ferulic, caffeic and sinapic acids, respectively. With ferulic acid as substrate, the apparent \( K_m \) value for UDPG was 10 μM.

**pH optimum:** The pH optima for glucosylation and glucose ester formation were determined using the HCAs and non-labelled UDPG in 3-ml reaction media. The products were isolated by chromatography and quantitated by a spectrophotometric technique. The pH optima for glucose ester and glucoside formation of p-coumaric acid were observed at pH 7.0 and 8.0, respectively (Fig. 4) and were similar to those of the other HCAs.

**Substrate specificity:** During all purification steps, the enzyme exhibited an expressed glucose acceptor ability for the free HCAs and UDPG as the glucosyl donor. At no time did any of the enzyme fractions (Table I) accept the CoA thiolester derivatives of HCAs (p-coumaroyl CoA, caffeoyl CoA or feruloyl CoA) when supplied as substrates in presence of glucose-1-phosphate. The enzyme exhibited somewhat broad specificity, since it reacted with other phenolic substrates such as kaempferol, quercetin, aesculetin, daphnetin and scopoletin.

**Effect of divalent cations and other reagents:** Whereas Ca\(^{2+}\) or Mg\(^{2+}\) inhibited the enzyme activity by 20–50% at a concentration range between \(10^{-4}\) and \(10^{-2}\) M, the inhibition by Mn\(^{2+}\) amounted to 20–80% at the same concentration range. However, glucosylation of HCAs was stimulated by about 10% in the presence of 6–10 μM MnCl\(_2\). EDTA inhibited the reaction by 30% at a concentration of \(10^{-6}\) M and the enzyme was completely inactivated at \(10^{-5}\) M, indicating a metal ion requirement for activity. Iodoacetate or p-chloromercuri-
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Benzoate resulted in 50–70% inhibition of enzyme activity at a concentration range between $10^{-6}$ and $10^{-3}$ M. Almost 80% of the activity with pCMB ($10^{-6}$ M) was restored after the addition of 10 mM β-mercaptoethanol, indicating that an —SH group is essential for enzyme activity.

**Inhibition by UDP:** Glucosylation of either p-coumaric or ferulic acids was inhibited by one of the reaction products, UDP. It inhibited the enzyme reaction by 10–40% when added at a concentration range between $10^{-6}$ and $10^{-3}$ M. When 1 mM UDP was added after 30 min of enzyme reaction, the activity was inhibited by 40% and 70% after 60 min and 120 min reaction, respectively (Fig. 5).

**Discussion**

This is the first report of a glucosyltransferase which catalyses the formation of both the glucosides and glucose esters (except caffeoyl glucose) of HCA's in the presence of UDPG as the glucosyl donor. The enzyme does not accept any of the CoA thiolester derivatives tested, in the presence of glucose-1-phosphate, in order to catalyse this reaction. This seems to indicate that the formation of glucose esters of HCA's, in tomato fruits, does not involve their CoA esters as intermediates. Furthermore, the constant ratio of glucosides/glucose esters observed during enzyme purification (Table I) suggests that the formation of both glucosylated derivatives may be catalysed by the same enzyme. However, the fact that this enzyme could not be further purified due to its stability does not exclude the possibility of other enzyme/isoenzyme which may be involved in the glucosylation of HCA's in tomato fruits.

Tomato fruits GT appears different from those reported in apple fruits [23] and radish seedlings [24] which catalysed the formation of feruloyl glucose and sinapoyl glucose, respectively; but none of their corresponding glucosides. An earlier survey [1] demonstrated that plants which predominantly contained glucose esters of HCA's did not form the corresponding glucosides when administered the free acids. This finding is of considerable biochemical interest in view of the fact that immature tomato fruits accumulate both the glucosides and smaller amounts of the glucose esters of the four HCA's, except caffeoyl glucose. Furthermore, the enzymatic synthesis of the latter compound in tomato could not be demonstrated in vitro; which might be due to its instability or enzymatic breakdown by an active esterase, as was previously suggested [5]. The latter possibility was tested by incubating the enzyme with p-coumaroyl glucose as substrate in the presence of UDP-[14C]-glucose and the recovery of labelled glucosylated products (70% of control) and free p-coumaric acid. This indicates the presence of an esterase activity associated with the enzyme preparation, which may account for the predominance of glucosides to their corresponding glucose esters in tomato fruits [5].

The pH optima observed for both reactions (Fig. 4) are similar to those reported for other glucosides [8, 12–14] and glucose esters [23, 24]. However, both pH optima seem to influence the chemical reactivity of the —OH and —COOH groups and hence, the stereospecificity of the enzyme towards the substrates. It is possible to assume, therefore, that both reactions — glucosylation and glucose ester formation — are catalysed by one GT with two active sites, which may be regulated by the pH of the reaction medium. The fact that these experiments were catalysed at pH 7.5 seemed to favor glucosylation over glucose ester formation. Furthermore, the ratio reported for glucosides/glucose esters (Table I) is comparable with those reported in the intact fruit [5].

In contrast with the radish enzyme [24], tomato fruit GT exhibited similar affinities towards the four HCA's and was strongly inhibited by EDTA and divalent metal ions. With p-coumaric acid as substrate, glucosylation was inhibited by UDP concentrations between $10^{-6}$ and $10^{-3}$ M; the lower limit of which was within the concentration range of UDPG present in the reaction medium. So far, we have no sufficient experimental evidence to suggest the reversion of enzyme activity by UDP, as was reported with the radish enzyme [24], due to the association of an esterase activity with the tomato enzyme.

**Acknowledgements**

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