# The Separation of Two Different Enzymes Catalyzing the Formation of Hydroxycinnamic Acid Glucosides and Esters

Paul Alfred Bäumker, Michael Jütte, and Rolf Wiermann

Botanisches Institut der Universität Münster, Schloßgarten 3, D-4400 Münster, Bundesrepublik Deutschland

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Protein extracts from anthers of *Tulipa* cv. Apeldoorn catalyze the formation of glucosides and glucose esters of hydroxycinnamic acid with UDP-glucose as the glucosyl donor. By chromatofocusing and by HPLC (anion exchange chromatography) it could be demonstrated for the first time that two different enzymes are involved in these reactions. By using the molecular sieving (HPLC) a molecular weight of about 45000 D was determined for the GT (E) (= catalyzing the formation of esters) and of about 25000 D for the GT (G) (= catalyzing the formation of glucosides). Both enzymes exhibit a high spezificity for free hydroxycinnamic acids.

It is assumed that these transferases are involved in the formation of feruloylglucose and ferulic acid glucoside, the latter of which can be isolated from anthers at early developmental stages. Furthermore, it is hypothesized that feruloylglucose functions as a starter molecule for the transacylation reaction by which di- and triferuloylsucrose are formed.

## Introduction

Hydroxycinnamic acids are ubiquitously distributed in the plant kingdom. They are important as intermediates and as accumulation products. Conjugates with a sugar compound in the form of esters or glycosides are widespread [1], the ester compound is said to have the greater significance [2]. Besides the wider distribution of the esters this assumption is indicated by the metabolic activity of these compounds in transacylating reactions as shown recently [3–6]. Analogous reactions have been reported from aromatic acid glucose esters [7–9] and from the indole acetic acid glucose ester [10].

In contrast to the enzymatic formation of esters [11–14] and their metabolism, very little is known about the turn over and the significance of hydroxycinnamic acid glucosides [1, 2, 15]. The substrates for the synthesis of the glucose esters as well as the glucosides of hydroxycinnamic acids are the same: the free acids and UDPG [16]. The enzymatic synthesis of both products has been shown by cell-free extracts of tomato fruits [12]. These authors suggest that only one single enzyme is involved in the synthesis of these conjugates.

In tulip anthers of an early developmental stage the ferulic acid glucoside but not the ferulic acid ester (feruloylglucose) is accumulated (Bäumker, Arendt and Wiermann, in preparation). However, both conjugates were demonstrated to be formed by cell-free extracts from these anthers. In this paper the chromatographic separation of two different glycosyltransferases involved in the formation of glucosides and esters of hydroxycinnamic acids is reported for the first time.

These studies are part of our investigations concerning the metabolism of ferulic acid sucrose esters in anthers of *Tulipa* and other Liliaceae [17–19]. It is hypothesized that feruloylglucose functions as a starter molecule for transacylation reactions by which di- and triferuloylsucrose are formed; the main compounds in the loculus of young anthers.

*Abbreviations:* PC, paper chromatography; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; GT-I, UDP-glucose: flavonol 3-0-glucosyltransferase; GT-II, UDP-rhamnose: flavonol 3-0-glucosid rhamnosyltransferase; GT-III, UDP-xylose: flavonol 3-glycoside xylosyltransferase; GT (E), glycosyltransferase forming esters; GT (G), glycosyltransferase forming glucosides; TCA, trichlor acetic acid.

Reprint requests should be sent to Prof. Dr. R. Wiermann.

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## Materials and Methods

### *Plant material*

Tulip bulbs (cv. Apeldoorn) purchased form Nebelung (Münster, FRG) were grown in the Botanical Garden of the University of Münster. For the isolation and purification of enzymes anthers with early stages of pollen differentiation were used.

*Chemicals*

UDP-D-Glucose was purchased from Boehringer (Mannheim, FRG) and UDP-D-[U-$^{14}$C]glucose from Amersham Buchler (Braunschweig, FRG). The hydroxycinnamic acids *p*-coumaric, caffeic, ferulic and sinapic acid and [$^{14}$C]ferulic acid were synthesized chemically. Reference compounds: ferulic acid glucosid was a kind gift of Prof. Dr. Barz (University of Münster). Feruloylglucose was extracted from cell cultures of *Chenopodium rubrum*. Reference proteins were obtained from Bio-Rad (München, FRG).

*Buffer solutions*

Buffer A: 0.1 M TRIS/HCl, pH 8.0, containing 0.25 M sucrose, 0.1 mM DTE and 0.1 mM EDTA. Buffer B: 0.1 M TRIS/HCl, pH 7.5. Buffer C: 25 mM imidazole/HCl, pH 7.4. Buffer D: Polybuffer (PB 74, 1:8 dilution), pH 4.0. Buffer E: 50 mM TRIS/HCl, pH 7.4.

*High performance liquid chromatography (HPLC)*

HPLC analyses were carried out with a chromatograph from Bio-Rad (München, FRG). It was used for the protein fractionation, the analyses of the reaction products and determination of enzyme activity. Solvents for reversed-phase HPLC: Solvent A: acetic acid (1% in distilled water), solvent B: acetonitrile (85% in distilled water).

*Enzyme preparation*

Anthers were ground in a pre-cooled (0–4 °C) mortar in the presence of Polyclar AT (2 g added to 100 ml) and buffer A. The homogenate was centrifuged at 10,000 × *g* for 10 min. Solid $(NH_4)_2\,SO_4$ was added to the supernatant to obtain 80% saturation. The precipitated protein was collected by centrifugation (27,000 × *g*, 20 min) and finally dissolved in buffer B for molecular sieving, or in buffer C for chromatofocusing, or buffer E for ion exchange chromatography.

Molecular sieving (Sephadex G-200): 1 ml of the protein solution was applied to a Sephadex G-200 column (0.9 × 50 cm; Pharmacia). The fractionation was carried out at a constant flow rate of 3.5 ml/h; fractions of 1.5 ml were collected.

High performance molecular sieving (Bio-Gel TSK): The protein solution (250 µl were applied to the column (7.5 × 0.75 cm; Bio-Rad) by injection without any pretreatment. The fractionation was accomplished by isokratic elution (60 ml/h); fractions of 0.7 ml were collected.

High performance ion exchange chromatography (Bio-Gel TSK): 2 ml of the protein solution were loaded onto the ion exchange column (7.5 × 0.75 cm, Bio-Rad) equilibrated with buffer E. Unbound protein was removed by washing with buffer E. The elution of bound protein was accomplished by linear gradient elution (60 ml/h) from 0–500 mM NaCl in 20 min; fractions of 1 ml were collected.

Chromatofocusing (PBE 94): The crude extract obtained after ammonium sulfate precipitation was desalted by Sephadex G-25 equilibrated with buffer C. The protein fraction was applied to a chromatofocusing column (0.9 × 20 cm, Pharmacia, Uppsala, Sweden) equilibrated with buffer C. Elution was carried out with buffer D (flow rate: 11 ml/h), fractions of 3 ml were collected. The pH of the fraction was adjusted to 7.5 by tritation with 0.1 M TRIS/HCl, pH 9.0.

*Enzyme assay*

The reaction mixture contained the following components: 50 µl protein solution, 100 nmol HCA and 100 nmol UDPG, each in 10 µl of buffer B. The reaction was started by the introduction of the enzyme preparation and was stopped after incubation at 35 °C for 60 min by addition of 5 µl TCA to induce protein precipitation. After centrifugation (8000 × *g*; 1 min) the complete supernatant was applied to HPLC or stored at −20 °C.

Enzyme activity was determined by HPLC analyses measuring peak areas.

PC: After removing the protein by TCA precipitation and centrifugation the incubation mixtures were chromatographed on Whatman 3 MM chromatography paper in 1-butanol-methanol-water (4:1:5 /v/v/v/; organic phase).

TLC: Hydroxycinnamic acid derivatives extracted by ethylacetate were chromatographed in acetic acid (3%) on microcristalline cellulose (Merck, Darmstadt, FRG).

HPLC: The analyses were carried out by reversed phase chromatography. The chromatographic col-

umn (120 × 8 mm) was packed with Lichrosorb RP-18 (5 μm; Knauer, Bad Homburg, FRG). Separations were accomplished by gradient elution from 10% solvent B in A to 50% solvent B in A in 15 min. Flow rate: 2 ml/min; detection at 300 nm (UV-detector: Model 1305 A, Bio-Rad, München). The different enzyme products were collected separately and analyzed in the range of 200–500 nm (UV 810, Kontron, Düsseldorf) to determine spectroscopic data.

*Hydrolyses*

The reaction products were treated with 1 N HCl for 30 min or 1 N NaOH for 5 min at 100 °C. Enzymic hydrolysis was carried out by β-glucosidase from almonds (Sigma, München, FRG) and esterase from porcine liver (Sigma).

**Results**

The incubation of a crude protein extract of young anthers desalted by Sephadex G 25 with UDPG and free hydroxycinnamic acids led to the formation of two different reaction products (see Fig. 1 (1), A and B). Fig. 1 (1) shows the results of a HPLC-analysis of an assay with ferulic acid and UDPG as substrates. The products A and B were purified by TLC, PC and HPLC and were examined spectroscopically and by means of enzymatic and chemical hydrolyses (data see Table I). They were identified as 1-feruloylglucose and ferulic acid β-glucosid, respectively. All data obtained correspond exactly to those from reference substances.

By using radioactive labelled substrates ([$^{14}$C]ferulic acid and/or UDP-[U-$^{14}$C]glucose) it was

Table I. The characterization of the reaction products A and B synthesized by two different glycosyltransferase activities (see also Fig. 1; 1–3). In every case, all data of A are completely identical with those of the reference substance ferulic acid glucosid and of B with those of feruloylglucose. (1): Mixture of acetonitrile and acetic acid according to the gradient elution at the retention time ($R_t$) of the products A and B. (2): see Materials and Methods.

| Reaction products references characteristics | A (ferulic acid glucosid) | | B (feruloylglucose) | |
|---|---|---|---|---|
| spectral data $\lambda_{max}$ (nm) [sh: shoulder] | | | | |
| Acetonitril acetic acid[1]) | 290 | sh 315 | 324 | sh 294 |
| + NaOH | 279 | sh 300 | 371 | |
| + HCl | 290 | sh 315 | 324 | sh 294 |
| behaviour in UV-light (UV 350 nm) | | | | |
| without $NH_3$ vapour | blue | | blue | |
| with $NH_3$ vapour | blue | | green | |
| Hydrolyses | | | | |
| NaOH | − | | + | |
| HCl | + | | + | |
| esterase[2]) | − | | + | |
| β-glucosidase[2]) | + | | + | |
| Chromatographic properties (for solvents see Materials and Methods) | | | | |
| HPLC $R_t$ [min] | 10.2 | | 11.4 | |
| TLC [$R_f \times 100$] | 55 | | 64 | |
| PC [$R_f \times 100$] | 32–12 | | 80–54 | |

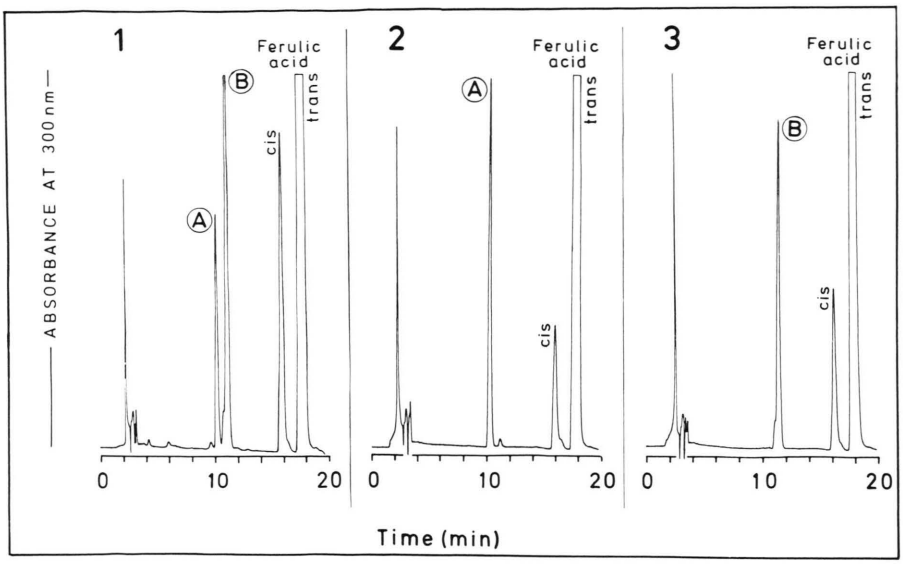


Fig. 1. HPLC-analyses of glycosyltransferase assays incubated with UDPG and ferulic acid as substrates. UDPG is not detectable under this HPLC conditions.

Protein source in 1: desalted crude extract from anthers of young developmental stages; in 2: protein of fraction 39 after chromatofocusing; in 3: protein of fraction 48 after chromatofocusing (see Fig. 4).

A: identified as ferulic acid glucosid (see Table I); B: identified as feruloylglucose (see Table I).

shown that both reaction products are composed of ferulic acid and glucose (Fig. 2).

A filtration of the crude extract through Sephadex G-200 hinted that two different glycosyltransferases could be involved in synthesis of feruloylglucose and ferulic acid glucosid (Fig. 3; GT (E), GT (G)). By using chromatofocusing a distinct separation of two different enzyme activities catalyzing ester and glucosid syntheses could be obtained (Fig. 4). Furthermore, it was shown by this procedure that two different enzyme spezies form the glucoseester of ferulic acid.

Ferulic acid glucosid is the main product in assays with protein from fraction 39 (Fig. 4) apart from a very small amount of feruloylglucose (see Fig. 1.2); on the other hand, fraction 48 (Fig. 4) is absolutely

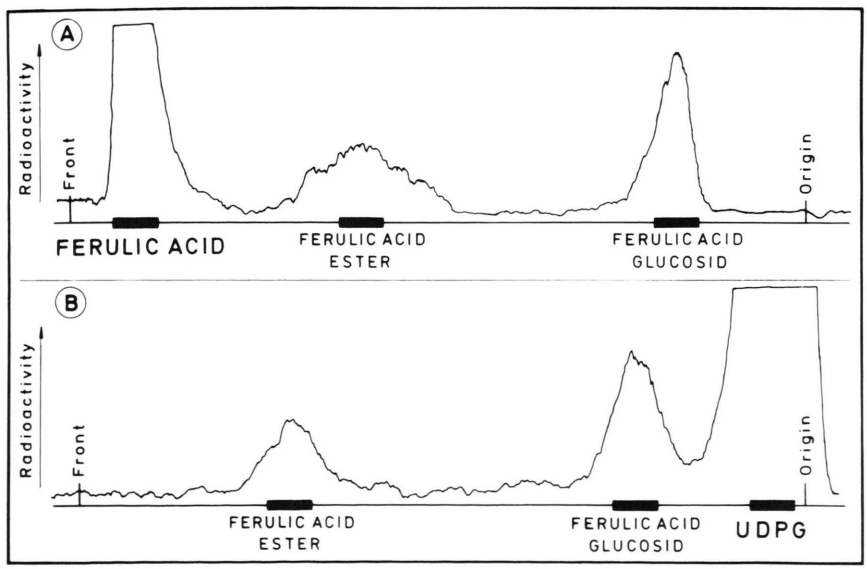


Fig. 2. Radioscannograms of PCs of complete transferase assay indicating the formation of ferulic acid ester and glucosid. Substrates in A: [$^{14}$C]ferulic acid, in B: UDP-D [U-$^{14}$C]glucose.

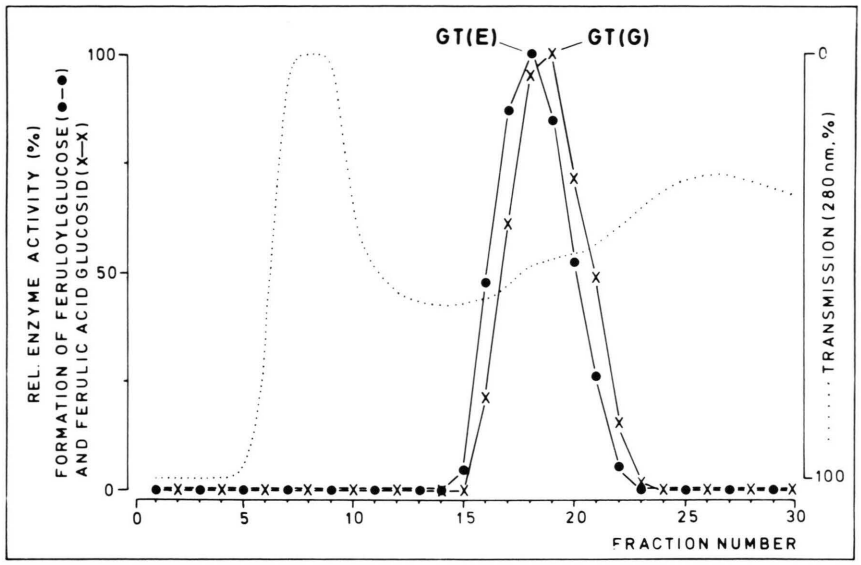


Fig. 3. The elution of glycosyltransferases after Sephadex G-200 fractionation.

free of enzyme activity forming ferulic acid glucosid (Fig. 1.3).

For further studies the protein of the fractions 30–39 and 40–49, were combined and separately subjected to an anion exchange chromatography. The fractionation resulted in the elution of GT (G) at 0.32 M NaCl and of GT (E) at 0.39 M NaCl (Fig. 5). Thus, in contrast to the results of Fleuriet *et al.* [12] a distinct separation of the two glycosyltransferases could be achieved by anion exchange chromatography.

When applying protein from the fractions with the highest enzyme activity of GT (E) and GT (G) (see Fig. 5) to molecular sieving by HPLC the molecular weights could be determined. A comparison with reference proteins shows that the main activity of GT (E) has a molecular weight of about 45,000 D and the GT (G) of about 25,000 D (Fig. 6).

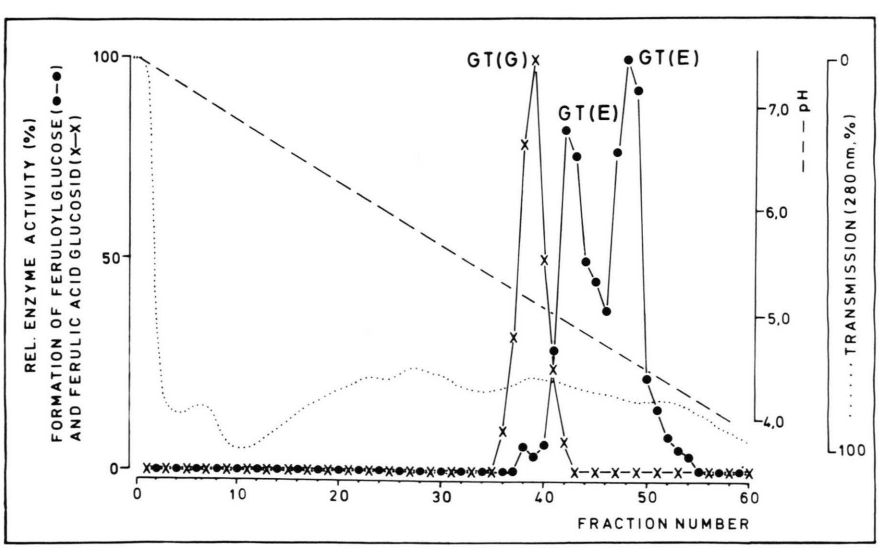


Fig. 4. The fractionation of different glycosyltransferases by chromatofocusing.

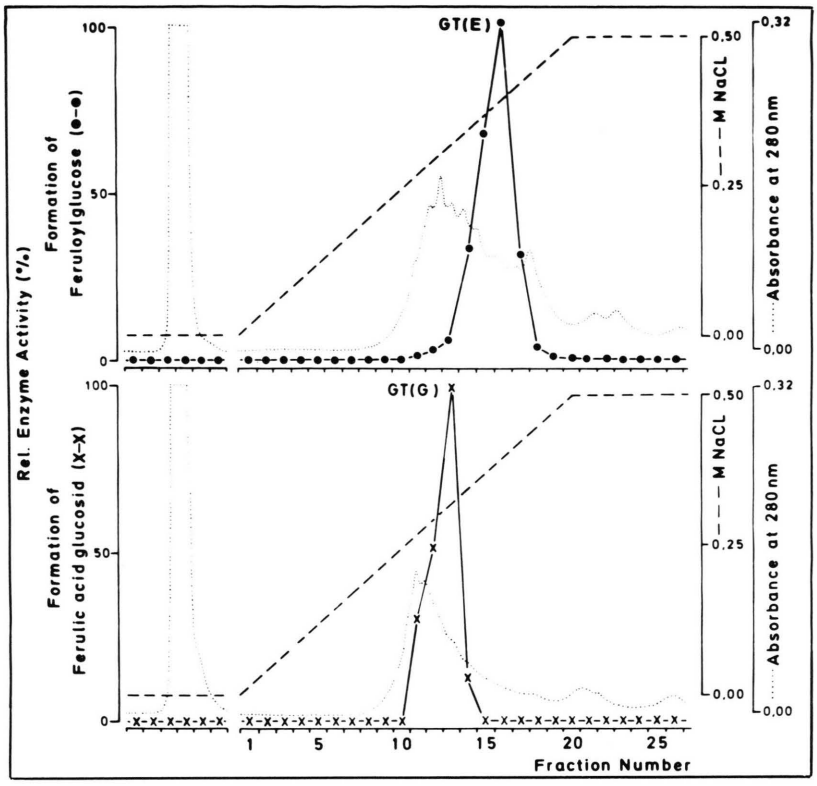


Fig. 5. The separation of the glycosyltransferases (GT (E) and GT (G)) by high performance ion exchange chromatography.

## Discussion

It is well-known that enzymatic synthesis of cinnamic acid derivatives like glucose esters and glucosides needs both the free acids and UDPG as substrates [16]. But there is a lack of information concerning the question whether these products are formed by one or two different enzyme(s). Fleuriet *et al.* [12] suggested that the formation of feruloylglucose and ferulic acid glucoside are catalyzed by the same enzyme, but the possibility that a second activity might be involved in the glycosylation of hydroxycinnamic acids was not completely excluded.

This is the first report about the evidence and separation of two different glycosyltransferases catalyzing the formation of either the glucose ester or the glucosid of hydroxycinnamic acids (Fig. 7). Both enzymes exhibit a high specificity for free hydroxycinnamic acids. Besides ferulic acid, other hydroxycinnamic acids like *p*-coumaric, caffeic or sinapic acid also act as substrates for the transferases.

At present it is assumed that the formation of feruloylglucose is to be seen in the context of the synthesis of di- and triferuloylsaccharose, which are accumulated in high amounts in the loculus of anthers of young developmental stages [17, 18]. In this sense, feruloylglucose as an activated intermediate may play an important role as starter molecule in transacylation reactions [4, 6, 8, 9, 19, 20].

The glucosyltransferase forming the glucoside may be involved in reactions, which lead to the accumulation of ferulic acid glucoside; the occurrence of this substance in the loculus material has been shown, but the significance of this compound in the phenylpropanoid metabolism of tulip anthers is not clear as yet.

At present there is evidence that both enzymes GT (E) and GT (G) prefer particularly hydroxycinnamic acids as acceptor substrates for the glycosyl moiety in comparison for example with flavonoid compounds (data unpublished). This indicates that the glycosyltransferases described in this paper differ from those involved in the formation of di- and triglycosides of flavonoids in the later stages of pollen development [21].

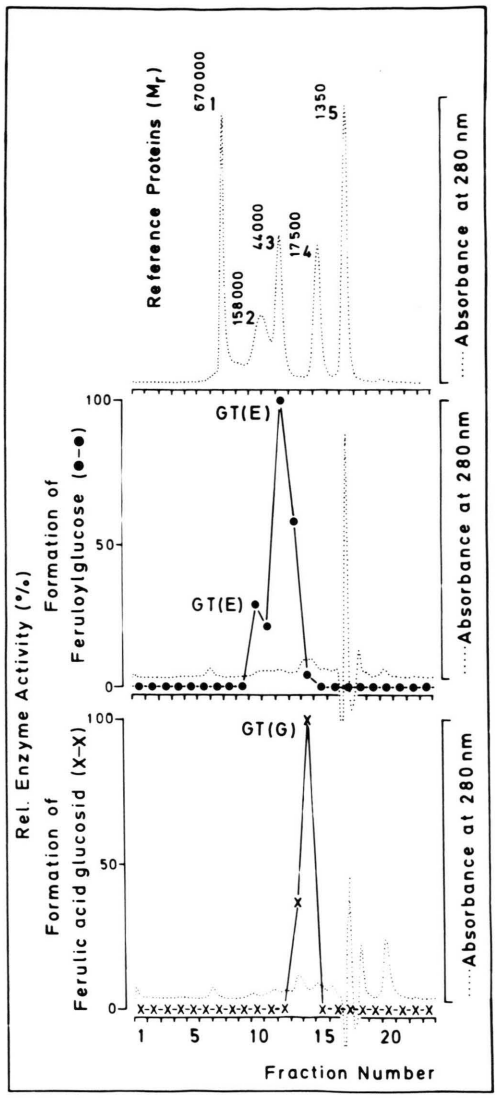


Fig. 6. The separation of the two glycosyltransferases (GT (E) and GT (G) and the determination of the molecular weight by high performance molecular sieving (1: tryoglobulin, 2: gammaglobulin, 3: ovalbumin, 4: myoglobin, 5: vitamin B 12).

Both glycosyltransferases, GT III [21] and GT (G) show a lower molecular weight in comparison with GT I, GT II and GT (E). GT (G) and GT III exhibit nearly identical IEPs of about 5.0 and GT III-activity was also found in early stages of pollen development. But in contrast to GT (G), the GT III strictly requires $Ca^{2+}$ and/or $NH_4^+$ for activity, whereas the formation of the glucosid by GT (G) is independent of these ions.

In further studies more details of the separated GT (E) and GT (G) have to be characterized and their development during pollen differentiation and ripening has to be analyzed.

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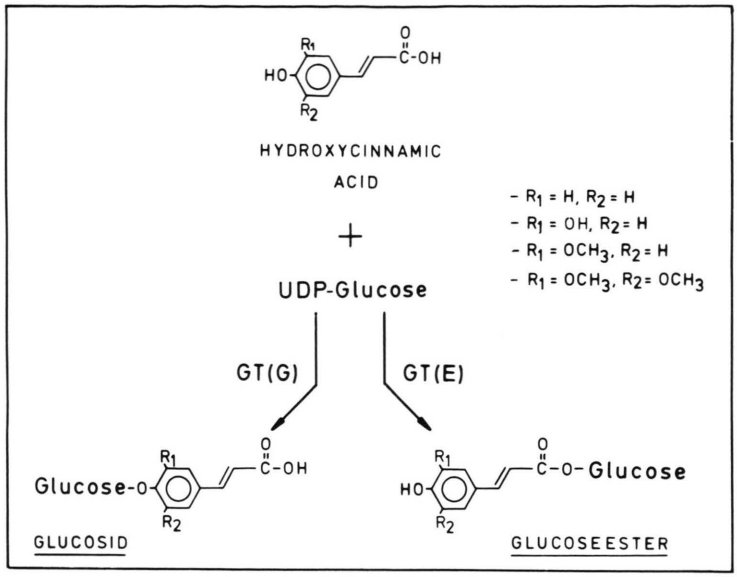


Fig. 7. Scheme deduced from the results concerning the formation of hydroxycinnamic acid glucose ester and glucosid by the GT (E) and GT (G).

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