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High-performance liquid chromatographic assay of glycosyltransferases using flavonoids as substrate

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Abstract

An HPLC method for the determination of glycosyltransferase activity, alternative to the radioactive assay, is proposed. The method is suitable for following the kinetics of consecutive enzymes that yield monoglucosides, diglucosides and triglucosides, as demonstrated with a pea seedling extract containing a mixture of three glucosyltransferases using flavonoids as substrate and UDP-glucose as carbohydrate donor. In this instance the HPLC determination of the three glucosides could be accomplished after separation of the aglycones by solid extraction on a Sep-Pak C_{18} microcolumn. After isolation of the enzyme catalysing the production of the monoglucoside of quercetin (isoquercitrin) or kaempferol (astragalin), the kinetics of the reaction were determined by HPLC, following both the increase of the product and the disappearance of the substrate. The increasing amounts of isoquercitrin and astragalin were consistent with the decrease in the amount of aglycone measured after direct injection of the reaction mixture into the HPLC system and its elution with a less polar solvent.

1. Introduction

Flavonoids are pigments widely found in plant tissues which are involved in a protection mechanism against predatory insects but can work also as a lure for pollinating agents. The technological uses of flavonoids concern mainly their antioxidant activity as radical scavengers, which inhibits the peroxidation of lipids [1–3], and the therapeutic properties of many glycosides derived from this kind of aglycone [4–7]. The glycosylation of flavonoids is very important not only for the latter purpose but mainly because the glycosides are much more soluble than the aglycones and therefore can be easily conveyed through physiological fluids. The enzymes that catalyse the glycosylation of flavonoids (O-glycosyltransferases) use uridine diphosphate as carbohydrate donor and are often specific for the sugar and the position of binding.

The most common assay of the enzyme activity involves the use of radioactive sugars and the determination of the products by TLC or paper chromatography [8–13], although a few methods using an HPLC linear gradient have been mentioned [14,15]. We have developed an improved HPLC procedure, employing isocratic elution, able to determine the activity of O-glycosyltrans-

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ferases even when various enzymes of this kind are present in the same sample. After isolation of three 3-O-glucosyltransferases from pea seedling, according to the method described by Jourdan and Mansell [16], their activity was determined by isocratic HPLC elution, measuring the products formed and/or the substrate left over. The enzymes present in pea seedlings work in sequence, yielding a monoglucoside, a diglucoside and a triglucoside of flavonoids, and therefore a prior separation of the glucosides from the aglycones by means of solid-phase extraction might sometimes be needed.

2. Experimental

2.1. Materials

Kaempferol (K), quercetin (Q) and uridine-5′-diphosphoglucose (UDP-G), used as enzyme substrates, were obtained from Sigma (St. Louis, MO, USA). Kaempferol, quercetin, kaempferol-3-O-glucoside (K-G) and quercetin-3-O-glucoside (Q-G) used as standards were purchased from Extrasynthese (Genay, France). Methanol, tetrahydrofuran (THF), 1-propanol and 2-propanol were of HPLC grade (Baker, Deventer, Netherlands) and water was distilled twice before use. All other reagents were of analytical-reagent grade.

O-Glucosyltransferases were extracted from pea seedlings grown for 1 week under continuous light at 25°C. After harvesting, the seedlings were stored at −80°C until used. The enzymes were purified according to the procedure of Jourdan and Mansell [16]. Frozen seedlings were ground in a Waring blender together with dry-ice and extracted with borate buffer (pH 7.7) containing 20 m*M* 2-mercaptoethanol at 4°C. After double precipitation with 40% and 70% ammonium sulfate, the salts in excess were removed by gel filtration on Sephadex G-25 and the solution containing the O-glucosyltransferases was used for the simultaneous assay of the three enzymes by HPLC. The 3-O-glucosyltransferase which catalyses the production of the monoglucoside was purified from the mixture of the three enzymes by ion-exchange chromatography on cellulose DE-52 equilibrated with 0.05 *M* phosphate buffer (pH 7.3) containing 20 m*M* 2-mercaptoethanol and eluted with a linear gradient (0–0.1 *M*) of KCl.

2.2. Assay of enzyme activity

The reaction mixture used for the assay of glucosyltransferase activity consisted of 10 μl of substrate (3 m*M* kaempferol or quercetin, dissolved in ethylene glycol monomethyl ether) added to 900 μl of 25 m*M* phosphate buffer (pH 7.3) containing 60 μ*M* acceptor (UDP-G) incubated at 25°C. The reaction was started by addition of 100 μl of enzyme solution. At fixed times an aliquot of the reaction mixture was assayed by HPLC for the amounts of substrate and product(s). When purified O-glucosyltransferase catalysing the monoglucosylation was used, 20 μl of reaction mixture were injected in the HPLC system equilibrated with the aglycones eluent as described in Section 2.3. Otherwise, 200 μl of sample containing a mixture of the three glucosyltransferases were loaded on a Sep-Pak C_{18} microcolumn (Waters, Milford, MA, USA) previously washed with methanol (5 ml) and water (10 ml), and successively eluted with 5 ml of water, 5 ml of 50% methanol and 5 ml of pure methanol. The fraction in 50% methanol was dried under reduced pressure, dissolved in 200 μl of methanol and 20 μl of this solution were injected in the HPLC system equilibrated with the glucosides eluent (see Section 2.3).

2.3. Chromatographic conditions

HPLC analyses of the reaction mixture were performed with a Gilson apparatus (Gilson Medical Electronics, Middleton, WI, USA) equipped with Model 712 HPLC system controller or with a Waters apparatus supplied with a

Model 1040 A photodiode-array detector (Hewlett-Packard, Waldbronn, Germany). Separations were carried out on a C_8 (7-μm) Aquapore RP 300 column (220 × 2.1 mm I.D.) or a C_8 (5-μm) Hypersil MOS column (200 × 4.6 mm I.D.) with a precolumn of the same type, using a flow-rate of 1.5 ml/min at a pressure of 20.68 MPa. The aglycones eluent, used for the determination of the activity of O-monoglucosyl transferase, consisted of THF–1-propanol–0.6% citric acid (7.5:12.5:80) and the glycosides eluent consisted of THF–2-propanol–water (5:10:85) and was used for the assay of the mixture of the three enzymes when the three glucosides produced were previously separated from the aglycones after solid-phase extraction with Sep-Pak C_{18}. Calibration graphs for aglycones (kaempferol and quercetin) and their glucosides (kaempferol 3-O-glucoside and quercetin 3-O-glucoside) were established in presence and absence of rutin (quercetin-3-rutinoside) as an internal standard.

3. Results and discussion

Glucosyltransferases from pea seedlings were chosen because their purification is simple and, at the same time, three different enzymes could be obtained with a minimum time consumption [16]. These enzymes (OGT-1, OGT-2 and OGT-3) catalyse the consecutive glucosylation of flavonoids (F) according to the following reactions:

$$F \xrightarrow{\text{OGT-1+UDP-G}} F\text{-}G \xrightarrow{\text{OGT-2+UDP-G}}$$

$$F\text{-}GG \xrightarrow{\text{OGT-3+UDP-G}} F\text{-}GGG$$

where OGT = 3-O-glucosyltransferase (OGT-1 uses an aglycone as substrate; OGT-2 uses a monoglucoside as substrate and OGT-3 uses a diglucoside as substrate), and -G, -GG and -GGC represent mono-, di- and triglucoside, respectively.

By means of HPLC it was possible to de-

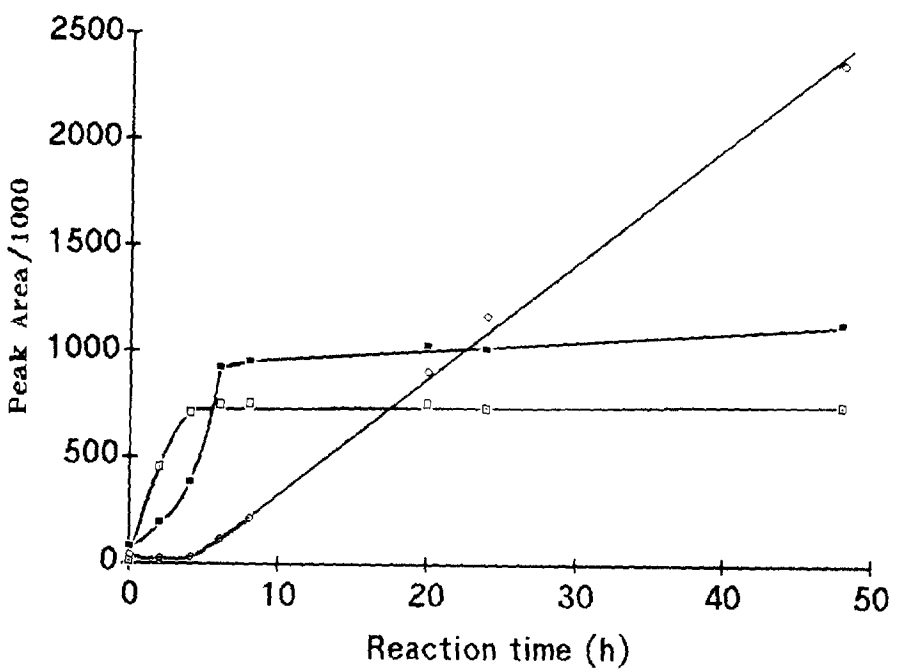

Fig. 1. Kinetics of consecutive glucosylation of kaempferol by the mixture of three O-glucosyltransferases from pea seedlings. □ = Kinetics of monoglucoside-producing enzyme (OGT-1); ■ = OGT-2; ○ = OGT-3.

termine simultaneously the products of the consecutive reactions in the mixture of the three enzymes. The kinetics of the consecutive reactions are reported in Fig. 1, where kaempferol was used as substrate.

As the aglycones and the glucosides have distinct polar properties, the three glucosides could be characterized after solid-phase extraction with Sep-Pak C_{18} and HPLC elution with the glucosides eluent, THF–2-propanol–water (5:10:85). This eluent required too long a time for the complete elution of the mixture of glucosides and aglycones, whereas the aglycones eluent, THF–1-propanol–0.6% citric acid (7.5:12.5:80), although faster, caused overlapping of the peaks of the glucosides, which therefore could not be individually identified.

Among the products of the three O-glucosyl transferases, only the monoglucosides (K-G, astragalin; Q-G, isoquercitrin) were available as standards, therefore the diglucosides and the triglucosides of kaempferol and quercetin were identified by the typical flavonoid spectrum obtained with the diode-array detector from the HPLC peaks (Fig. 2). The correlation between the peak areas of the substrate (aglycone) or product (monoglucoside) and their amounts (nanomoles or micrograms) was assessed by means of a calibration graph and used for the determination of the activity of the O-glucosyltransferase catalysing the first reaction (OGT-1). The relationship between peak area and amount was linear both for aglycones (quercetin, $R = 0.989$; kaempferol, $R = 0.999$) and glucosides (isoquercitrin, $R = 0.976$; astragalin, $R = 0.996$).

The assay of the isolated enzyme catalysing the monoglucosylation of kaempferol and quercetin (OGT-1) was simply carried out with the aglycones eluent without solid-phase extraction, as the only product obtained could be easily identified from the substrate (Fig. 3). In this way it was possible to obtain the enzyme kinetics through the disappearance of the substrate and the corresponding increase in the product. Fig. 4 shows typical kinetics where the decrease in the substrate (kaempferol) is in good agreement with the increase in the product (kaempferol-3-O-glucoside). In this case a consumption of 7.3 nmol of substrate per hour ($R = 0.989$) corresponds to the production of 7.6 nmol per hour of kaempferol-3-O-glucoside ($R = 0.955$).

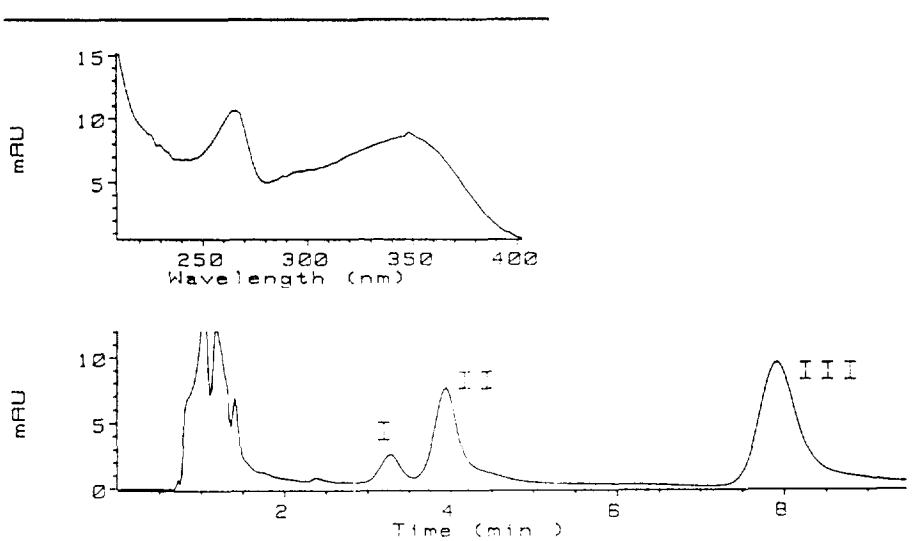

Fig. 2. Bottom: HPLC profile of the three glucosides of kaempferol after 8 h of reaction with the mixture of the three enzymes. Peaks I, II and III correspond to triglucoside, diglucoside and monoglucoside, respectively. Top: spectrum of peak II in the bottom trace. The elution was accomplished with the glucosides eluent, THF–2-propanol–water (5:10:85) after solid-phase extraction with Sep-Pak C_{18} as described in the text.

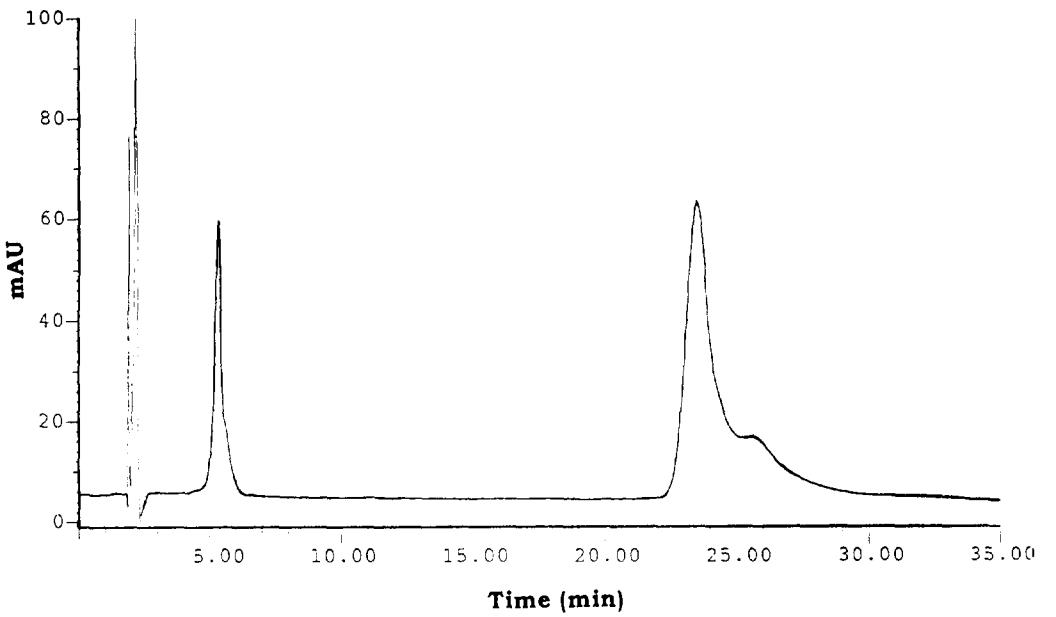

Fig. 3. Elution pattern of kaempferol (I, right peak) and astragalin (kaempferol-3-O-glucoside) (II, left peak) with the aglycones eluent, THF–1-propanol–0.6% citric acid (7.5:12.5:80) as described in the text.

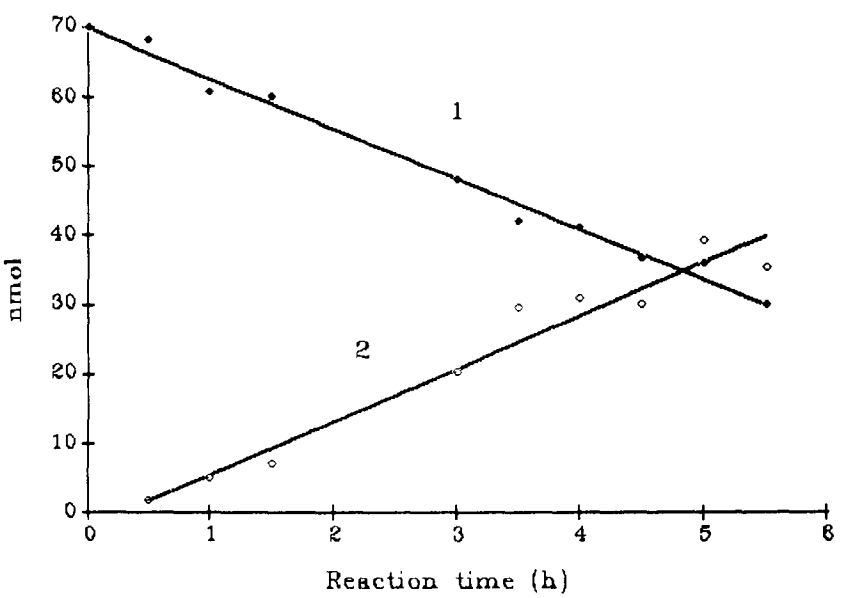

Fig. 4. Assay of 3-O-glucosyl transferase with kaempferol as a substrate. Simultaneous determination of substrate (◆) and product (◇). The kinetics of the disappearance of kaempferol are in good agreement with that of the increase in astragalin.

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