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THREE SPECIFIC UDP-GLUCURONATE: FLAVONE-GLUCURONOSYLTRANSFERASES FROM PRIMARY LEAVES OF *SECALE CEREALE*

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Key Word Index—*Secale cereale*, Poaceae, luteolin mono-, di-, tri- glucuronides, UDP-glucuronate: flavone-glucuronosyltransferases

Abstract—A threefold glucuronidation of luteolin terminates the biosynthesis of luteolin 7-*O*-[β-D-glucuronosyl(1→2)β-D-glucuronide] 4′-*O*-β-D-glucuronide, the major flavone of the mesophyll of 5-day-old rye primary leaves. The strictly sequential transfer of glucuronate is catalysed by three specific enzymes: UDP-glucuronate: luteolin 7-*O*-glucuronosyltransferase (LGT), UDP-glucuronate: luteolin 7-*O*-glucuronide-glucuronosyltransferase (LMT) and UDP-glucuronate: luteolin 7-*O*-diglucuronide-glucuronosyltransferase (LDT). The three enzymes were separated on Ultrogel AcA-44 and DEAE-cellulose. The LGT was purified 170-fold and the LDT 23-fold, while the LMT was further chromatographed on hydroxylapatite, yielding a 99-fold purification The M_rs were 34 000 (LGT), 37 000 (LMT) and 29 000 (LDT). The isoelectric points of LGT and LDT were identical at a pH of 4 75, and an IEP of 4 80 was found for LMT The pH optima were at 6.5 (LGT), 6.5 and 8 5 (LMT), and at pH 7 (LDT) Temperature optima were 50° for LGT, 52° for LMT and 40° for LDT; the energies of activation were 50, 23 and 38 kJ/Mol, respectively. Each of the enzymes is highly specific for its natural substrate. Luteolin and UDP both had a strong inhibitory effect. For UDP, an uncompetitive inhibition was found, when the concentration of the glucuronate acceptor was varied. The LDT was not significantly influenced by the divalent cations Ca^{2+} and Mg^{2+}, whereas LGT and LMT were stimulated by Mg^{2+} ions (LGT: *ca* 20% up to 1 mM; LMT: up to 50% at 0.75 to 1 mM) and by Ca^{2+}-ions (LGT: *ca* 25% at 0 25 mM; LMT: *ca* 20% at 0 5 mM) The reactions are irreversible under the standard assay conditions

INTRODUCTION

Primary leaves of rye show a close correlation between organ development and metabolism of several flavonoids [1–4]. The age-dependent metabolism of the major leaf flavonoid, luteolin 7-*O*-[β-D-glucuronosyl(1→2)β-D-glucuronide]-4′-*O*-β-D-glucuronide (R^1), is characterized by significant accumulation up to the fifth day followed by a rapid decline during further leaf development [1]. This loss is due to a turnover which is initiated by a highly specific β-glucuronidase [5] which catalyses the removal of glucuronic acid in the 4′-position as the first catabolic step, producing luteolin 7-*O*-[β-D-glucuronosyl(1→2)β-D-glucuronide] (R^2)

Glycosidation reactions are known to be the last steps in the specific biosynthesis of several flavonoid glycosides [6–11]. Therefore, luteolin diglucuronide (R^2) may not only be the product of R^1-degradation but also an intermediate in R1-biosynthesis

A number of flavonoid-specific UDP-sugar transferases extracted from different plants have been described [6–11]. However, data on UDP-glucuronosyltransferases from plants are limited, the enzymes only being studied in crude extracts [12, 13]. Furthermore, there are only few reports on the sequential glycosylation of flavonoids [14, 15]. We now report on the partial purification and characterization from rye primary leaves of three UDP-glucuronosyltransferases involved in the sequential biosynthesis of luteolin triglucuronide (R^1)

RESULTS

Partial purification of glucuronosyltransferases

Four-day-old rye primary leaves are suitable for the extraction and purification of glucuronosyltransferases because of their high yield of catalytic activity for all three glucuronidation steps During this phase of development, from day three to day five, the luteolin triglucuronide is accumulated almost linearly in the leaves [1] When crude extracts were incubated with luteolin and UDP-glucuronic acid as substrates (cf. legend of Fig. 1), the three products were synthesized and identified by HPLC cochromatography as luteolin 7-*O*-glucuronide

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Abbreviations PEG Polyethyleneglycol, EGME: ethyleneglycol monomethyl ether, 2-ME 2-mercaptoethanol, -glur glucuronide, LGT UDP-glucuronate luteolin 7-*O*-glucuronosyltransferase, LMT UDP-glucuronate luteolin 7-*O*-glucuronide-glucuronosyltransferase, LDT. UDP-glucuronate luteolin 7-*O*-diglucuronide-4′-*O*-glucuronosyltransferase, L luteolin, N luteolin 7-*O*-β-D-glucuronide, R^2 luteolin 7-*O*-[β-D-glucuronosyl(1→2)β-D-glucuronide], R^1 luteolin 7-*O*-[β-D-glucuronosyl(1→2)β-D-glucuronide] 4′-*O*-β-D-glucuronide.

(N), luteolin 7-*O*-diglucuronide (R^2), and luteolin 7-*O*-diglucuronide-4′-*O*-glucuronide (R^1)

Figure 1 presents their time-dependent accumulation and it is suggested that N is the first intermediate as this does not accumulate further after the first 30 min and remains at a steady state concentration for up to 100 min. It is immediately converted to R^2, the second intermediate and this in turn is converted to R^1 the terminal product R^1 finally becomes the major product after a longer period of incubation (*ca* 3 hr, not shown)

Chromatography of a concentrated crude extract on Ultrogel AcA 44 did not separate the three glucuronosyltransferase activities Nevertheless, they were eluted after a major part of the applied protein had passed the column Separation of the three activities was successfully achieved by subsequent chromatography on DEAE-cellulose (Fig. 2). Using a K-Pi gradient, enzyme activities eluted at 0 06 to 0 07 M for LMT, 0 09 to 0.11 M for LGT and 0.13 to 0.15 M for LDT There was slight cross contamination of LMT and LGT (see below). After this second chromatographic step, the LGT showed a 170-fold purification whereas LMT and LDT were enriched by 47- and 23-fold, respectively (Table 1). In contrast to the very labile LGT and LDT, the relatively stable LMT could be further purified on hydroxylapatite resulting in a 99-fold purification

Ultrafiltration was chosen to concentrate them since the glucuronosyltransferases were sensitive to PEG and ammonium sulphate Sensitivity to the latter has also been described for a flavonoid-specific UDP-glucosyltransferase isolated from cell suspension cultures of parsley [7] The reason for the remarkable increase of the LMT-activity as well as the high loss of the LGT-activity after the first ultrafiltration step is unclear. There was no hint of a LMT-inhibitor of low molecular weight, capable of passing the ultrafiltration membrane during the concentration

The purified enzymes could be stored in 50% glycerol at −20° for more than three months with losses of activity of 30 to 50% Freezing (without glycerol) resulted in a complete deactivation Native electrophoresis showed that none of the glucuronosyltransferases was purified to homogeneity Nevertheless, they represented the major bands in gels after staining with Coomassie Blue (data not shown) Gel slices were assayed for enzyme activity using the substrates luteolin, N and R^2 Only the major bands showed the respective glucuronosyltransferase activity and no additional transferase could be detected in the gels

General properties of the partially purified glucuronosyltransferases

The transfer reactions were linear with time up to 30 min for LMT, and up to 40 min for the two other glucuronosyltransferases. Protein concentrations up to 3 μg per assay resulted in a linear increase in activity for all three enzymes. In general, high ionic strength had an inhibitory effect The LGT showed highest activity at pH 6.5 in 0.01 M citrate buffer (50% activity at pH 5 6, citrate buffer and pH 9, Bicine buffer) The pH optimum of LDT was at pH 7 in 0.05 M K-Pi buffer (50% activity at pH 6 in citrate buffer and pH 9 in Bicine buffer) The LMT exhibited two pH optima one at pH 6 5 in 0 01 M K-Pi buffer and a second at pH 8 5 in 0 01 M Bicine buffer (50% activity at pH 6, K-Pi buffer and pH 9,

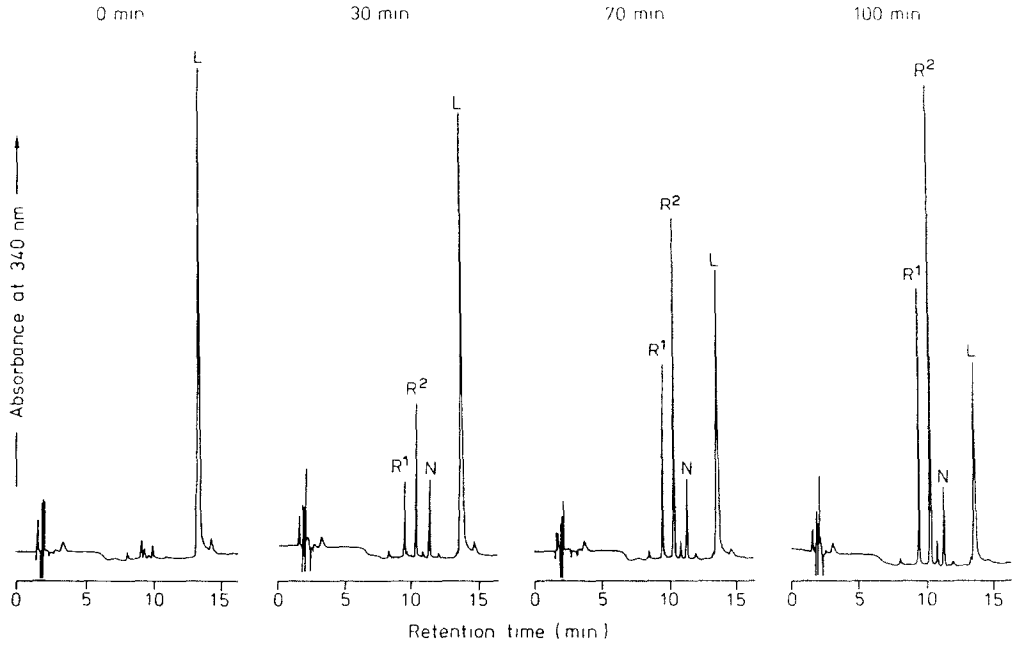

Fig 1 HPLC-chromatograms of crude extract incubations with luteolin (20 μM in EGME) and UDP-glucuronic acid (500 μM) as substrates in 10 mM citrate buffer, pH 5 5, 10 mM 2-ME time dependent sequential synthesis of luteolin 7-*O*-glucuronide (N), luteolin 7-*O*-diglucuronide (R^2), and luteolin 7-*O*-diglucuronosyl-4′-*O*-glucuronide (R^1) The reaction was started by the addition of 15 μl crude extract (total assay volume = 100 μl) and stopped with 100 μl MeOH The elution of the products and of luteolin (L) was performed with a linear gradient from 100% water (1% H_3PO_4) to 100% acetonitrile in 20 min, detection wavelength = 340 nm

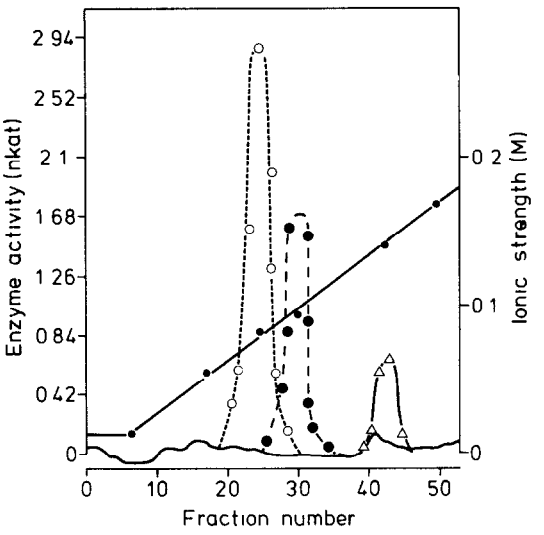

Fig 2. Chromatographic separation of the three UDP-glucuronate:flavone-glucuronosyltransferases on DEAE-cellulose The linear gradient ranges from 0 01 to 0 2 M K-Pi, flow rate· 60 ml/hr, 4.5 ml-fractions were collected and assayed for transferase activities, substrates as in Fig 1 —protein (A_{280} nm), ○——○ LMT, ●——● LGT, △——△ LDT, — — phosphate gradient

Bicine buffer, 30–40% activity at pH 7.5, K-Pi buffer) In crude extracts, a second pH-optimum at 8.5 was absent, and it is possible that the properties of the LMT might change during purification. For LGT and LMT there were almost identical temperature optima of 50°, whereas the optimum was 40° for LDT. Figure 3(a–c) shows the Arrhenius plots for each enzyme used to calculate the energies of activation. These differed between the enzymes with values of 50 kJ/mol for LGT, 23 kJ/mol for LMT, and 38 kJ/mol for LDT. M_rs of 37 000 for LMT, 34 000 for LGT and 29 000 for LDT were recorded after chromatography on Ultrogel AcA-44 These results were supported by SDS electrophoresis and therefore the three enzymes are monomeric. The apparent IEP's, estimated by chromatofocussing, were nearly identical with pH 4 75 (LGT, LMT) and pH 4 8 (LDT)

Substrate specificity and kinetics of glucuronosyltransferase

Several flavonoids and (hydroxy)cinnamic acid(s) were tested as possible glucuronate acceptors. The results are listed in Table 2, and apparent K_m and V_{max} values are given in Table 3. The LGT showed the highest activity with luteolin as substrate (100%), while apigenin was accepted at 38%. There was low activity with the two flavone 7-*O*-glucuronides, which might be due to the slight contamination of LGT with LMT (cf Fig 2) For LGT none of the other compounds tested was a suitable substrate. Luteolin 7-*O*-glucuronide was the best substrate for LMT. This enzyme also catalysed the transfer of glucuronate to the two other flavone 7-*O*-glucuronides and to apigenin 7-*O*-glucoside with high efficiency. The affinity to the flavone aglycones was very low and no product could be detected with the flavonols, flavone-*C*-glycosides, a flavanone, and cinnamic acids. The LDT did not accept flavone aglycones, and the best substrate was luteolin 7-*O*-diglucuronide. However, there was some transfer to the flavone 7-*O*-glucuronides and in the case of luteolin 7-*O*-glucuronide, the diglucuronide was synthesized The other compounds were not suitable substrates

Each of the glucuronosyltransferases showed highest activity to its natural acceptor substrate with high and similar affinities (low K_m-values) but different apparent V_{max}values (Table 3) None of the three enzymes accepted apigenin 7,4′-*O*-diglucuronide as a substrate This fact as well as the absence of luteolin 7,4′-*O*-diglucuronide as a

Table 1 Partial purification of the glucuronosyltransferases from rye primary leaves

Purification step	Enzyme	Total activity (pkat)	Yield (%)	Specific activity (μkat/kg)	Protein (mg)	Purification (-fold)
Crude extract	OA-activity*	9255	100	114 2		1
	LMT	13 100	100	162	81	1
	LDT	8209	100	101.6		1
Ultrafiltration	OA-activity*	7149	77	109	65 7	0 95
	LMT	44 242	337	737 4		4 5
	LDT	2453	30	37 4		0 4
Ultrogel AcA 44	OA-activity*	4883	53	241 5	14 7	2
Ultrafiltration	LMT	27 913	213	1889		12
Sephadex G-25	LDT	2100	25.5	143		1 4
DEAE-Cellulose	LGT	3883	42	19 415	0 2	170
Ultrafiltration	LMT	10 671	81	7622	1 4	47
	LDT	1373	16.7	2288	0 6	23
Hydroxylapatite Ultrafiltration	LMT	1173	8 5	16 048	0.07	99

*Over-all activity (OA) of LGT, LMT, LDT as assayed under standard assay conditions for LGT, with luteolin as flavone substrate

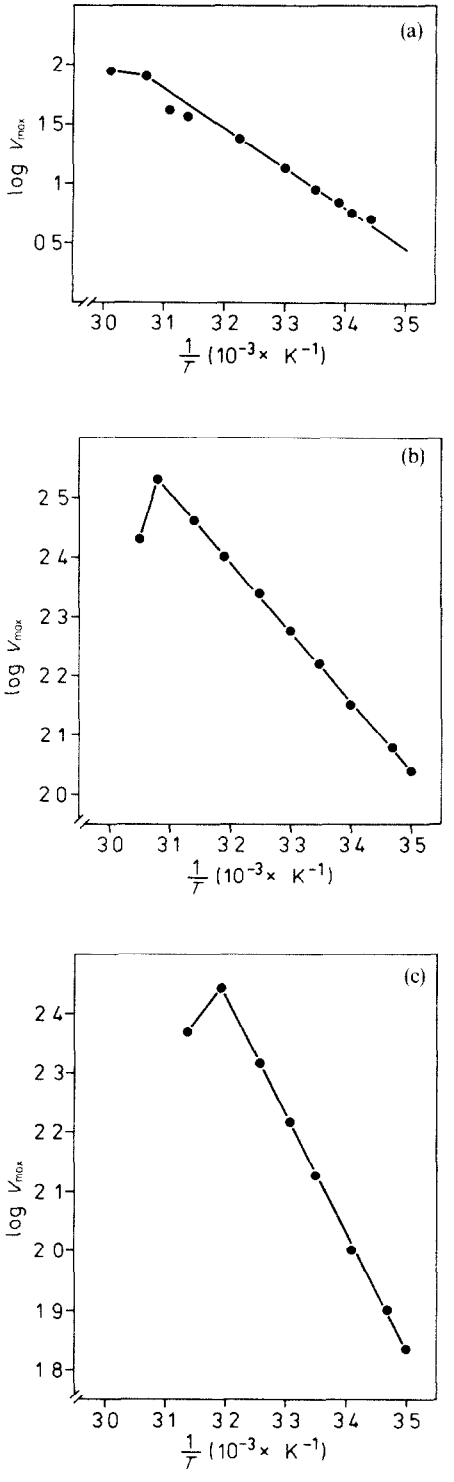

Fig 3 Arrhenius plots for the determination of the apparent energy of activation of purified (a) LGT, (b) LMT and (c) LDT

reaction product or intermediate, reflects the strict positional specificity of the threefold sequential glucuronidation of luteolin to R^1 via N and R^2 by three enzymes

To investigate the donor specificity of the three glucuronosyltransferases, several UDP-sugars were tested (Table 2). All three enzymes showed highest activity with UDP-glucuronate. The LGT and LDT showed a 10% or lower acceptance of the other UDP-sugars tested, and the LMT showed only a 3–4% acceptance. Apparent K_m and V_{max} values varied with the donor molecule The LGT showed the lowest K_m and V_{max} values ($K_m = 12\ \mu M$; $V_{max} = 777\ \mu Kat/kg$ protein), while LMT had an apparent K_m more than three fold higher (40 μM) and also a higher V_{max} (7692 $\mu Kat/kg$ protein) The LDT showed a lower affinity to the donor molecule ($K_m = 90\ \mu M$, $V_{max} = 3000\ \mu Kat/kg$). Similar K_m-values have been described for most of the flavonoid-specific glycosyltransferases studied In many cases the K_m-value for the sugar donor was much higher than that found for the phenolic reactant [6, 7, 15, 16]

Influence of cations, UDP, ADP, and luteolin on glucuronosyltransferase activities

The LDT was not affected by Mg^{2+} and Ca^{2+} ions up to 0 5 (0 75) mM whereas LMT showed a 20% stimulation with 0.5 mM Ca^{2+} and LGT a 20% stimulation with 0 25 mM Ca^{2+} Further increases in Ca^{2+} concentration caused a decline of activities For LGT, a similar rise in activity was obtained by the addition of Mg^{2+} ions up to 1 mM, whereas LMT activity showed a 50% increase with the addition of 0 8 mM Mg^{2+} The latter enzyme was however inhibited by 40% with the addition of heavy metal ions (Cu^{2+}, Pb^{2+}, and Ag^{+}) at a concentration of 0.75 mM The LGT and LDT were less sensitive to the heavy metal ions with no significant response to concentrations up to 1 mM

Luteolin reduced the activities of all three glucuronosyltransferases For LGT, a substrate inhibition of 60% was observed with 140 μM luteolin, while LMT was inhibited by 50% with 100 μM luteolin and LDT was inhibited by 50% with 20 μM luteolin. In addition to the inhibition of flavonoid-specific glycosyltransferases by aglycones the inhibitory effect of the reaction product UDP has been reported by other workers [7, 15, 17, 18] For the glucuronosyltransferases UDP was a more effective inhibitor than luteolin Kinetic studies indicated an uncompetitive inhibition by UDP when the phenolic substrate was varied The K_{ii}-values were determined by secondary plots of the intercepts (Lineweaver–Burk diagrams) on the v^{-1} axis vs the concentration of UDP A similar K_{ii}-values of 120 μM was found for both LMT and LDT, but LGT had a higher K_{ii} of 260 μM UDP In contrast to UDP, ADP had no influence on the enzyme activities Free reversibility of an UDP-glucose flavonol glucosyltransferase reaction has been described, with UDP-glucose appeared as a product [16] and the same result was found by Sutter *et al* for a similar enzyme [18]. However, the three glucuronosyltransferases from rye were not observed to catalyse the reverse reactions in the presence of UDP and the equivalent glucuronylated luteolin derivative (R^1, R^2, N) as substrates (standard assays)

DISCUSSION

The three glucuronosyltransferases involved in the sequential biosynthesis of luteolin triglucuronide (R^1) are similar to other flavonoid-specific glycosyltransferases studied so far [6, 7, 15, 17–19] They have low M_rs and there was no evidence of subunits capable of catalysing all three glucuronidation steps *in vitro* Nevertheless,

Table 2 Substrate specificity of the glucuronosyltransferases*

Substrate	LGT		LMT		LDT	
	pkat/assay	Activity (%)	pkat/assay	Activity (%)	pkat/assay	Activity (%)
Luteolin	1 32	100	0.4	6	—	—
Luteolin 7-*O*-glur (N)	0 07	6	6 45	100	0 32	34
Luteolin 7-*O*-diglur(R^2)	—	—	—	—	1 1	100
Apigenin	0 5	38	0 39	6	—	—
Apigenin 7-*O*-glur	0.2	17	5 7	88	0 22	20
Apigenin 7-*O*-glc	—	—	3 32	51.5	—	—
Apigenin 7,4'-*O*-diglur	—	—	—	—	—	—
Chrysoeriol 7-*O*-glur	0.3	23	4 2	65	0 07	12
UDP-Glur	1 18	100	6 23	100	1 08	100
UDP-Xyl	0 12	10 5	0 25	4	0 13	10
UDP-Gal	0 11	9 3	0 18	3	0 08	7 5
UDP-Glc	0.12	10	0.27	4 3	0.11	10 6

*Standard enzyme assays were used with the partially purified enzymes as described in the Experimental Identity of reaction products was verified by co-chromatography (HPLC) with reference compounds The reaction products with apigenin 7-*O*-glucuronide, apigenin 7-*O*-glucoside and chrysoeriol 7-*O*-glucuronide as substrates were not further identified Vitexin, saponarin, lutonarin, quercetin 3-*O*-glur, quercetin, kaempferol, naringenin, and *p*-coumaric acid, ferulic acid, cinnamic acid were not accepted as substrates (results not listed)

Table 3 Kinetic properties of the glucuronosyltransferases*

Substrate	LGT		LMT		LDT	
	K_m (μM)	V_{max} (μKat/kg)	K_m (μM)	V_{max} (μKat/kg)	K_m (μM)	V_{max} (μKat/kg)
UDP-glucuronic acid	12	777	40	7692	90	3000
Luteolin	8	1600				
Luteolin 7-*O*-glucuronide			12	9231		
Luteolin 7-*O*-diglucuronide					9	741

*Apparent K_m- and V_{max} values for the substrates of the three UDP-glucuronosyltransferases were obtained with a constant glucuronate donor concentration of 500 μM UDP-glucuronic acid per assay. In order to determine K_m- and V_{max}values for UDP-glucuronic acid measurements were done with the following constant acceptor concentration 20 μM luteolin/assay (LGT), 60 μM N/assay (LMT), 30 μM R^2 assay (LDT) The protein concentration per assay was 3 μg (LGT), (LDT) and 1 5 μg (LMT), respectively

under *in vivo* conditions the three glucuronosyltransferases may be weakly associated. Dissociation of a transferase-complex by the destruction of inner cell structures cannot be excluded and the properties of the separated enzymes may be different The low K_m-values of LGT and LMT may explain the absence of luteolin and luteolin 7-*O*-glucuronide accumulation in rye primary leaves In intact leaves, luteolin diglucuronide (R^2), a product of the LMT-catalysed reaction, never becomes a major flavonoid [1] However, the accumulation of the luteolin glucuronides R^1 and R^2 in rye primary leaves does not reflect the results of *in vitro* measurements. We therefore suggest a channelling of R^1 synthesis that does not allow the accumulation of R^2 in higher amounts in the anabolic pathway The amount of R^2 observed [1] may be primarily due to the degradation of R^1 by a R^1-specific β-glucuronidase [5]. Flavonoid glycosidation has been postulated to be weakly associated with membranes of the tonoplast or the ER [20, 21]. The presence of the three glucuronosyltransferase activities in the soluble cellular fraction together with their pH optima (6 5, 7, 8 5) supports a cytosolic localization and seems to exclude a tight membrane association.

EXPERIMENTAL

Plant material Caryopses of *Secale cereale* L. var Kustro were purchased from F. von Lochow-Petkus, Bergen (F.R G), and seedlings were grown in a phytotron as previously described [1]

Substrates and other chemicals Luteolin, apigenin, apigenin 7-*O*-glucoside, vitexin, kaempferol, naringenin, *p*-coumaric acid, ferulic acid, and cinnamic acid were obtained from Roth (Karlsruhe) Further substrates were isolated from suitable plants and purified cf [5]. All phenolic substrates were 95–98% pure as analysed by HPLC [5] Quercetin 3-*O*-glucuronide was a gift from Prof E. Wollenweber (Darmstadt) UDP-glucuronic acid, UDP-galactose, UDP-xylose, and UDP-glucose were purchased from Boehringer, Mannheim DEAE-cellulose 52 was from Whatman, Sephadex G 25 and kits for chromatofocussing were from Pharmacia Ultrogel AcA 44 was purchased from LKB, Freiburg, Hydroxylapatite Bio-gel HTP powder and stan-

dard proteins for SDS—gel electrophoresis from Bio-Rad (München) BSA, fraction V powder was from Sigma, and Dowex AG 1 × 2(200–400, Cl^--form), Polyclar AT as well as standard proteins for M_r determination from Serva (Heidelberg) All other chemicals were of analytical grade

Enzyme extraction and purification All operations were carried out at 0–4°, buffers contained 10 mM 2-mercaptoethanol,(4-day-old primary leaves (10–15 g fr wt) were frozen in liquid N_2 and ground in a mortar The powder was stirred for 40 min in 0 1 M K-Pi buffer, pH 7 0, containing 50% (w/w) Dowex AG 1 × 2 (Cl form) and 15% (w/w) Polyclar AT The crude extract was filtered through Miracloth (Calbiochem), and concd by ultrafiltration to 10 ml (ultrafiltration cell model 8050, ultrafiltration membrane 10 PM 10, from Amicon) The concentrate was applied to an Ultrogel AcA 44 column (1 5 × 85 cm) The column was previously equilibrated with elution buffer, 0 05 M K-Pi buffer pH 7 The flow rate was 10 ml/hr and 3 ml fractions were collected Fractions with glucuronosyltransferase activities were combined, concentrated by ultrafiltration, and desalted by chromatography on Sephadex G 25 with 0 01 M K-Pi buffer at pH 7 The eluate was applied to a DEAE-cellulose 52 column (1 × 25 cm), equilibrated with 0 01 M K-Pi buffer at pH 7 After washing with 50 ml of equilibration buffer, transferases were eluted with a linear gradient of 160 ml 0 01–0 2 M K-Pi buffer at pH 7 and a flow rate of 60 ml/hr Fractions of 4 5 ml were collected, those with highest activity of the respective transferase were either directly used for measurements or stored in 50% glycerol at −20°

For further purification of the LMT, the DEAE-cellulose fractions of highest activity were combined and concd by ultrafiltration After 2 × 30 min dialysis against 0 01 M K-Pi buffer pH 7, the protein solution was applied to a hydroxylapatite column (1 × 5 cm, prewashed with 0 01 M K-Pi buffer pH 7) After washing the column with starting buffer (20 ml), the enzyme was eluted with a linear gradient of 100 ml 0 01–0 2 M K-Pi buffer pH 7 with a flow rate of 20 ml/hr 3 5 ml fractions were collected, those with LMT activity combined and concentrated by ultrafiltration to 2 ml The enzyme solution was stored as indicated above Protein was determined according to Bradford [22], using BSA as standard

Enzyme assays (i) UDP-glucuronate luteolin 7-*O*-glucuronosyltransferase (LGT) The standard assay consisted of 20 μM luteolin (dissolved in EGME) up to 20 μl of the partially purified enzyme (2 7 μg protein), and 0 01 M citrate buffer, pH 6 5, in a total volume of 100 μl The reaction was started by the addition of 500 μM UDP-glucuronic acid (dissolved in citrate buffer), the incubation temperature was 37° After 30 min, the incubation was terminated by the addition of 100 μl MeOH

(ii) UDP-glucuronate luteolin 7-*O*-glucuronide-glucuronosyltransferase (LMT) The standard assay consisted of 60 μM luteolin 7-*O*-glucuronide (N) (dissolved in H_2O), 500 μM UDP-glucuronic acid (dissolved in citrate buffer), 8 μl of the partially purified enzyme (1 5 μg protein), and 0 01 M citrate buffer pH 6 5 in a total volume of 100 μl Start and incubation conditions were as in (i) Reactions were stopped after 25 min, with MeOH

(iii) UDP-glucuronate luteolin 7-*O*-diglucuronide-4′-*O*-glucuronosyltransferase (LDT) The standard assay consisted of 30 μM luteolin 7-*O*-diglucuronide (dissolved in H_2O), 500 μM UDP-glucuronic acid (dissolved in K-Pi buffer), up to 20 μl of the partially purified enzyme (3 μg protein) and 0 05 M K-Pi buffer pH 7 a total volume of 100 μl Start and incubation conditions were as in (i), the reaction time was 30 min

Apparent K_m and V_{max} values for the flavonoid substrates of the three UDP-glucuronosyltransferases (Table 3) were obtained with a constant concentration of 500 μM UDP-glucuronic acid per assay K_m and V_{max} values for UDP-glucuronic acid were estimated with the following acceptor concentrations, 20 μM luteolin per assay for LGT, 60 μM N for LMT and 30 μM R^2 for LDT Protein concentrations were 3 μg for the LGT and LDT assays, and 1 5 μg for the LMT assay

Determination of enzyme activities by HPLC Glucuronosyltransferase activities were measured by HPLC The HPLC system and chromatographic procedures for the separation of flavonoid aglycones, mono-, di-, and triglucuronides and glycosides, respectively, were the same as described in [5] Evaluations were performed with the SP 4270 Integrator (Spectraphysics), using R^1 and luteolin as external standards

M_r *determinations* M_rs were estimated by gel filtration, the Ultrogel AcA 44 column was calibrated with standard proteins The partially purified glucuronosyltransferases were eluted with 0 05 M K-Pi buffer, at pH 7 M_rs were also determined by SDS electrophoresis according to [23] For LMT, the concd enzyme was used after chromatography on hydroxylapatite For LGT and LDT, the appropriate bands of activity in gels after native electrophoresis [24] were eluted and prepared for SDS electrophoresis Native electrophoresis was performed with enzyme solutions after chromatography on DEAE-cellulose 52

Determinations of isoelectric points The apparent isoelectric points of the three glucuronosyltransferases were determined by chromatofocussing, using a pH gradient from pH 7 to pH 4 The gradient was controlled by pH measurements of each fraction, 20 μl aliquots of the fractions were incubated with luteolin, N, and R^2 as acceptor substrates

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