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SCREENING FOR FLAVONOL 3-GLYCOSIDE SPECIFIC β-GLYCOSIDASES IN PLANTS USING A SPECTROPHOTOMETRIC ENZYMATIC ASSAY

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Abstract—A simple and rapid spectrophotometric assay for following the hydrolysis of flavonol 3-glycosides has been developed. The assay profits from the fact that peroxidase converts flavonol aglycones to their corresponding 2,3-dihydroxyflavanones, producing a large shift in UV absorption, whereas flavonol 3-glycosides are not attacked. The amount of liberated aglycone can therefore be calculated from the decrease of flavonol absorption at 350–380 nm. A horseradish peroxidase–H_2O_2 test system can be used to investigate the hydrolysis of most flavonol 3-glycosides, whereas quercetin 3-glycosides can be tested using a peroxidase preparation from *Mentha* sp. which uses O_2 as cofactor rather than H_2O_2. Flavonol 3-glycoside synthesis, e.g. with UDP-sugars as cofactors, may also be tested by this particular system. Various plants and plant cell cultures were screened for kaempferol and quercetin 3-glycoside specific β-glycosidases. However, in no case could any specific activity be detected.

INTRODUCTION

Flavonols, the most important group among flavonoids, occur mainly as 3-glycosides [1]. Several reports have shown flavonol 3-glycosides to undergo turnover in plants [2–7] and in a few cases total degradation could be demonstrated [8, 9]. Cleavage of the sugar–aglycone linkage catalysed by specific β-glycosidases might be expected to be the first catabolic step according to the degradation pathway found in microorganisms [10–12]. To our knowledge flavonol 3-glycoside specific β-glycosidases have not yet been unequivocally demonstrated in plants. Several plant β-glycosidases are reported to be able to split flavonol 3-glycosides [13–15], but hydrolysis has only been demonstrated qualitatively using chromatographic methods, and velocities have not been measured. The incubation conditions which have been used, e.g. large amounts of substrate and β-glycosidase, and long incubation periods (cf. [13, 14]), indicate that the specificity of those β-glycosidases for flavonol 3-glycosides was low or absent. In no case has specificity, such as for a β-glycosidase of *Aspergillus* sp. [16], been demonstrated.

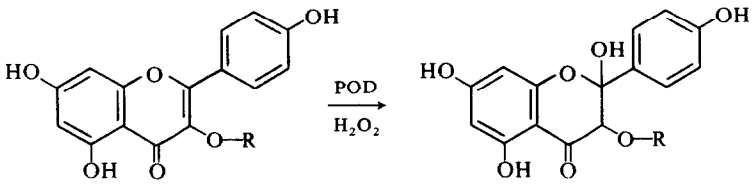
There is no doubt that the lack of a specific, rapid test system is the reason for the lack of quantitative data on enzymatic flavonol 3-glycoside hydrolysis. Convenient methods to measure glycosidase activity are not practicable for these substrates. For instance, the use of alkali, as in the case of nitrophenyl glycosides [17] or isoflavone 7-glycosides [18], is not possible mainly due to the instability of flavonols in alkali. Determination of glucose using the glucose oxidase–peroxidase assay is also not possible due to the peroxidase-catalysed conversion of flavonols [19, 20]. Test systems using NAD(P) reduction cannot be used because of the high flavonol absorption at 340 nm. Finally, chromatographic methods are only applicable for exact quantitative

On the other hand, the conversion of flavonols by peroxidase would allow quantitative measurement of the enzymatic hydrolysis of flavonol 3-glycosides in a coupled assay. In this paper we describe this new test system and its application to the screening of flavonol 3-glycoside specific β-glycosidase activities in plants and plant cell cultures.

RESULTS

Method of assay

Flavonols are converted quantitatively by peroxidase, in the presence of hydrogen peroxide, to their corresponding 2,3-dihydroxyflavanones [19, 20] (Fig. 1). This reaction occurs only if the hydroxyl group at the C-3 of the flavonol nucleus is free; flavonol 3-glycosides do not react [20, 21]. The UV absorption spectra of flavonols and 2,3-dihydroxyflavanones are very different (Fig. 1). Since flavonol 3-glycosides do not react in this manner, it is possible to selectively convert the liberated flavonol aglycone by peroxidase in the presence of the flavonol 3-glycoside. The flavonol conversion is best followed by measuring the absorption decrease in the flavonol maximum at 350–380 nm (cf. Fig. 1). The amount of liberated aglycone can be calculated from the absorption decrease at these wavelengths. Further possible conversions of 2,3-dihydroxyflavanones by peroxidase [20, 23] do not upset measurements in any way since these reactions are very slow compared to the first step, and the reaction



R = H; kaempferol: reaction possible.
R = sugar; kaempferol 3-glycoside: reaction not possible.

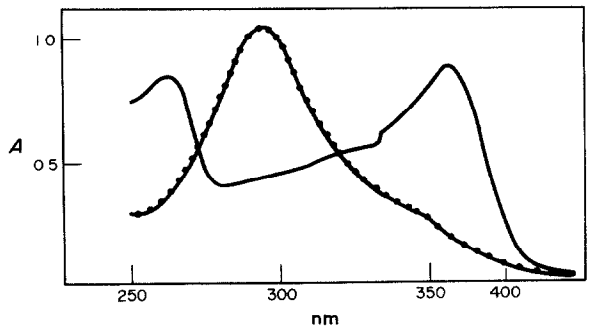


Fig. 1 Absorption change in the course of the peroxidase catalysed reaction of flavonols to 2,3-dihydroxyflavanones. ——Kaempferol 3-glycoside absorption in buffer at pH 5 (the kaempferol aglycone absorption is only slightly different), -•-•-• 2,3,5,7,4'-Pentahydroxyflavanone absorption in buffer at pH 5

products do not show absorption in the wavelength range 350–380 nm [20, 22, 23]. The determination of flavonol 3-glycoside hydrolysis can be handled in two different ways. (1) Flavonol 3-glycosides are incubated together with the glycosidase preparation and the reaction is stopped by heating. Peroxidase and H_2O_2 are then added and the absorption decrease is measured after another period of incubation (method *a*). (2) Peroxidase is administered together with the glycosidase preparation and the absorption decrease is directly followed spectrophotometrically (method *b*).

Hydrolysis of kaempferol 3-glucoside using pure β-glucosidase from almonds in the presence of peroxidase and H_2O_2, and of quercetin 3-glucuronide with β-glucuronidase from *Helix pomatia* in the presence of *Mentha* peroxidase according to method *b*, are illustrated in Fig. 2.

Most flavonol 3-glycosides can be investigated using the horseradish peroxidase (HRP)–H_2O_2 system. However, the widely occurring quercetin 3-glycosides (e.g. rutin) are an exception in so far as they are already attacked by small amounts of peroxidase and H_2O_2 (cf. [20]). This is mainly due to the catechol nucleus in ring B of the quercetin skeleton; hence the HRP–H_2O_2 system cannot be used to investigate their hydrolysis. This disadvantage can be overcome by using a peroxidase preparation from *Mentha* sp., which metabolizes quercetin as shown in Fig. 1, but does not attack quercetin 3-glycosides [24]. This particular peroxidase uses molecular oxygen rather than H_2O_2 as cofactor. Flavonols without a catechol-based B-ring (e.g. kaempferol) are not substrates for this fairly unusual peroxidases [24]. Peppermint plants or *Mentha arvensis* cell cultures show high activities for this peroxidase, hence crude protein extracts can be used for the assay.

Effects of pH and substrate concentration

Incubation for glycosidase activity according to method *a* can be done at all pHs where the substrate is stable,

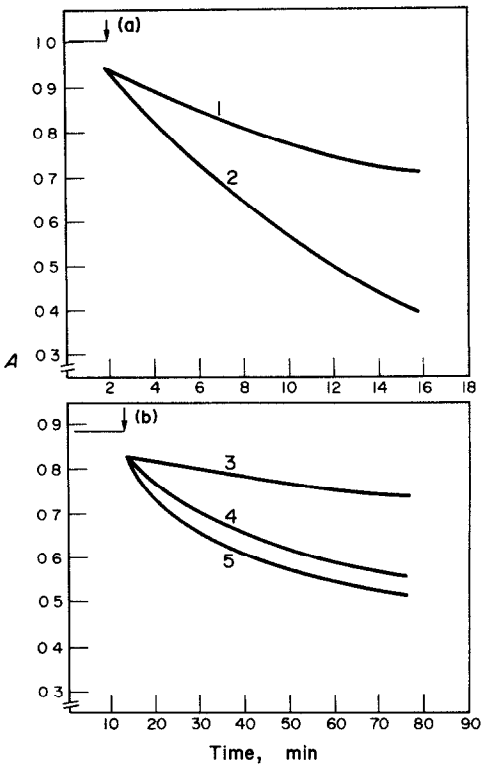


Fig. 2 Direct spectrophotometric determination of quercetin 3-glucuronide hydrolysis by β-glucuronidase from *Helix pomatia* (a) and of kaempferol 3-glucoside hydrolysis by β-glucosidase from almonds (b). The arrows indicate addition of β-glucuronidase and β-glucosidase (respectively) and the sudden decrease in absorption results from dilution of the incubation mixtures. Assays were: 0.11 U β-glucuronidase at pH 7.5 (1) and pH 5 (2); 10 U β-glucosidase at pH 5 (4), 20 U β-glucosidase at pH 5 (5) respectively pH 7.5 (3). Further conditions are as described in Experimental.

and the following peroxidase incubation is best done at pH 5. Substrate concentration may be varied over a wide range because appropriate aliquots can be applied for the peroxidase incubation. The flavonol converting activity of HRP is not very different at pH values from 4–8.5 and of *Mentha* peroxidase from 5–7.5. Hence it is possible to investigate hydrolysis of flavonol 3-glycosides according to method *b* over these pH ranges. Since flavonol 3-glycoside hydrolysis is followed by absorption decrease, the substrate concentrations which may be investigated are limited by this method; generally it would not be possible to exceed 0.1 mM. It is further necessary, for quantitative measurements with method *b*, to ensure that the glycosidase reaction is the rate limiting step in the test system. Exact conditions for measuring kaempferol and quercetin 3-glycosides are given in the Experimental.

Quantitative calculation of flavonol 3-glycoside hydrolysis

The influence of pH on the absorption maxima and ε values in the case of kaempferol and quercetin 3-glycosides, as well as for the corresponding 2,3-dihydroxyflavanones, are given in Table 1. While wavelengths of maximal flavonol absorption are shifted bathochromically by increasing pH, the ε-value of maximal flavonol absorption remains unchanged. The same holds true for the 2,3-dihydroxyflavanones when the maximal absorption shifts from 295 to 335 nm, together with a slight increase of the ε-value by increasing pH.

The 2,3-dihydroxyflavanone absorption at the wavelength under investigation has to be taken into account for quantitative calculations. It can be determined by total conversion of the flavonol aglycone with peroxidase, so that the original ε-value corresponds to total flavonol conversion. Table 1 shows examples for calculations concerning kaempferol and quercetin at different pH values, measured at a flavonol concentration of 0.05 mM.

Precautions in application of the assay

One has to ensure that the flavonol 3-glycosides under investigation are not attacked by peroxidase. This is important because flavonol 3-glycosides are known to be converted by large amounts of peroxidase and H_2O_2 in an as yet unknown manner (cf. [20]), differently from that depicted in Fig. 1. It is therefore necessary to determine the right amount of peroxidase which will completely oxidize the aglycone without affecting the 3-glycoside. Examples of suitable amounts of peroxidase for measuring kaempferol and quercetin 3-glycosides are given below. Endogenous peroxidase in crude plant protein extracts can be neglected if H_2O_2 is absent. This holds true for Sephadex G 25-filtered or $(NH_4)_2SO_4$-precipitated protein extracts, or can easily be achieved by addition of catalase. Using method *a*, endogenous peroxidase is destroyed in the heat denaturation step, and is therefore without influence in the second incubation. For direct measurement (method *b*) the endogenous peroxidase content has to be determined and the peroxidase added must be lowered by this particular amount. In some instances, the endogenous plant peroxidase activity is so high (roots of *Cucurbita maxima*) that the addition of HRP had to be omitted.

Heat treatment over 3 min in boiling water does not influence the substrates at pH values lower than 7. However, some flavonol 3-glycosides may be unstable during the heat step at pH values above 7.

Calculation of suitable amounts of peroxidase

Horseradish peroxidase (60 EU) should be used in a concentration of 0.05 μg/ml test volume to analyse kaempferol 3-glycosides. This value corresponds to a kaempferol-converting activity of 6 μmol/min at pHs 5 and 7.5 (30°) in the presence of 0.25 mM H_2O_2 under standard conditions (see Experimental). If endogenous peroxidase is present, the total activity should not exceed 30 μmol/min at pH 5 and 10 μmol/min at pH 7.5 in the assay. Quercetin 3-glycosides can be tested by a crude protein preparation of *Mentha* sp.; this peroxidase activity (without addition of H_2O_2) converts 20 μmol quercetin/ml test volume at pH 5 respectively 7.5 (30°). If peroxidase concentrations are kept within the above limits, quantitative conversion of flavonol aglycones is assured, whereas flavonol 3-glycosides are not attacked.

Analysis of transferase activity

The above test can also be used to analyse transferase activity resulting in the synthesis of flavonol 3-glycosides, e.g. flavonol: UDP-sugar transferase. This is best done according to method *a*. After incubation and heat denaturation, excess nonglycosylated aglycone is destroyed by peroxidase. Absorption increase against blanks can be used in this assay to calculate transferase activity.

Table 1. Data for the quantitative calculation of kaempferol and quercetin 3-glycoside hydrolysis at different pH values

	Kaempferol 3-glycosides				Quercetin 3-glycosides			
pH	λ_{max} (nm)	$\varepsilon(M^{-1}.cm^{-1})$ at λ_{max}	μmol corresponding to $\Delta E = 0.1$ at λ_{max}	λ_{max} of 2,3,5,7,4′-pentahydroxyflavanone	λ_{max} (nm)	$\varepsilon(M^{-1}.cm^{-1})$ at λ_{max}	μmol corresponding to $\Delta E = 0.1$ at λ_{max}	λ_{max} of 2,3,5,7,3′,4′-hexahydroxyflavanone
4.0	345	16000	8.3	295	348	22400	*Mentha* peroxidase	295
4.5	345	16000	8.3	295	348	22400	activity to low	295
5.0	345	16000	8.3	295	348	22400	6.1	295
5.5	345	16000	8.3	295	348	22400	6.1	295
6.0	347	16000	8.3	295	348	22400	6.1	298, 335 *sh*
6.5	348	16000	11.4	297, 335 *sh*	350	22400	7.15	330, 295
7.0	349	16000	11.4	299, 333 *sh*	356	22400	7.15	295 *sh*, 330
7.5	360	16000	11.4	297 *sh*, 333	368	22400	7.15	330
8.0	370	16000	11.4	333	372	22400	calculation not possible due to	
8.5	380	16000	11.4	333	380	22400	instability of quercetin in alkali	

sh = shoulder.

Table 2. Plants and plant tissue cultures which were shown to lack specific β-glycosidase activity for flavonol 3-glycosides

Plant	Plant organ	Age (days)
Cicer arietinum L.*	leaves	14
	roots	14
	tissue cultures	10
Phaseolus vulgaris	leaves	12
Pisum sativum	leaves	10
*Cucurbita maxima**	cotyledones	14
	roots	13
*Fagopyrum esculentum**	cotyledones	14
	hypocotyls	4–10
Glycine max	cotyledones	20
Trifolium repens	aerial parts	17
Phaseolus aureus	leaves	9
	tissue cultures	10
Brassica napus	tissue cultures	8
Lupinus angustifolius	leaves	11
Mentha arvensis	tissue cultures	12
Sinapis alba	cotyledones	7
Tulipa cv Apeldoorn	anthers	—

Rutin and quercetin 3-rhamnoside were used as substrates throughout; * indicates that kaempferol 3-glucoside and kaempferol 3-apiosylglucoside were also tested.

Screening of plants and plant cell cultures for flavonol 3-glycoside specific β-glycosidases

Various plants which are known to contain flavonol 3-glycosides or to show turnover [4, 6] or catabolism [8] of these compounds were checked for specific β-glycosidases. In each case crude protein preparations as well as Sephadex G 25-filtered protein were used as a source for β-glycosidase activity and incubations were carried out at both pH 5 and 7.5. Glycosidase activity was followed predominantly by method *b* at 30° for at least ten min. Glycosidase preparation (0.1 ml) was used in the standard assay (see Experimental). Flavonol 3-glycosides investigated were rutin (quercetin 3-rhamnosylglucoside), quercitrin (quercetin 3-rhamnoside), kaempferol 3-glucoside, kaempferol 3-rhamnosylglucoside and kaempferol 3-apiosylglucoside at substrate concentrations of 0.05–0.1 mM throughout. As can be seen from Table 2, all plants and plant cell cultures tested failed to split these flavonol 3-glycosides.

It should be noted that all plant extracts contained β-glycosidase activity, sometimes in high amounts, on incubation with 4-nitrophenyl-β-glucoside.

Cicer arietinum, *Cucurbita maxima* and *Fagopyrum esculentum* were examined for flavonol 3-glycoside specific β-glycosidase activity. All β-glycosidases separated so far from *Cicer arietinum* plants [18, 25] or cell suspension cultures [26], as well as from crude cell walls isolated from cell suspension cultures (cf. [8, 26]), did not catalyse flavonol 3-glycoside hydrolysis. Six β-glycosidases from *Cucurbita maxima* seedlings, separated by CM chromatography also did not catalyse hydrolysis. *Fagopyrum esculentum* seedlings were described by Bourbouze *et al* [14] to contain a rhamnodiastase-like activity which hydrolyses rutin. Protein extracts from this plant under exactly the same conditions as described [14] failed to hydrolyse quercetin 3-rhamnoside and rutin under the test conditions described above, either at pH 5 or 7.5. Only after long incubation periods (e.g. 18 hr) could slight hydrolysis be observed with rutin at pH 5, but this is no proof for a flavonol 3-glycoside specific activity. Furthermore, we followed β-glycosidase activity in the course of seedling development from day 4 to day 10 where the rutin content in hypocotyls increases rapidly. There was again no rutin hydrolysis detectable during this whole period, whereas β-glycosidase activity was present with 4-nitrophenyl-β-glucoside as substrate throughout.

DISCUSSION

Our new assay allows the specific and rapid determination of flavonol 3-glycoside hydrolysis. It can therefore be used to look for specific β-glycosidases for these substrates. With slight modification, it can also be employed for measuring synthesis of flavonol 3-glycosides by transferases. In contrast to a similar test system described earlier using quercetinase from fungi [16], the peroxidase system shows two advantages. First, the peroxidases used are easily available. Secondly, the wide specificity of horseradish peroxidase for flavonol aglycones allows investigations of very different flavonol 3-glycosides. The use of *Mentha* peroxidase, which shows a rather narrow specificity for quercetin, fills the gap where this type of 3-glycosides cannot be tested with the HRP–H_2O_2 system. Substrates like flavonol 3,7-diglycosides can be analysed, too, because the remaining flavonol 7-glycosides are converted by peroxidase according to Fig. 1 [20]. It is obvious that only hydrolysis of the sugar–aglycone bond at the C-3 of the flavonol can be detected by this assay. Hydrolysis between sugars or at other positions has to be investigated independently.

In spite of the necessity of measuring absorption decreases to follow glycosidase activity, the test allows large variation of the substrate concentration due to the possibility of making endpoint determinations as well as direct spectrophotometric measurements.

β-Glycosidases specific for flavonol 3-glycosides could not yet be demonstrated in our experiments, although a number of plants have been investigated. Furthermore, experiments with cell cultures which are able to hydrolyse exogenously applied flavonol 3-glucosides [8] revealed no specific glycosidases, either in the soluble or in the insoluble fractions of the extracts. We would suggest from our experiments that such plant β-glycosidases, which have been described to hydrolyse flavonol 3-glycosides [13, 14], do not possess specificity for this type of substrate. This suggestion is based on the very low hydrolysis velocity shown by these β-glycosidase preparations in the assay. We are not able to definitely rule out that very low activities of glycosidases specific for flavonol 3-glycosides may occur in plants, or that activities might have been destroyed in the course of the investigations. But taking into consideration that specific β-glycosidases for other plant phenolics are easily detectable (cf. [18, 25, 27, 28]) and are often very stable, we would suggest that specific β-glycosidases for flavonol 3-glycosides are lacking in the plant material under investigation. On the other hand, it seems unlikely that turnover of those substances occurs without removal of the sugar moiety. It might therefore be possible that removal of the sugars from position 3 of flavonols is catalysed by a transferase-like reaction, due to the fact enough to be transferred to a suitable acceptor [29].

EXPERIMENTAL

Substrates and enzymes. Kaempferol, quercetin, kaempferol 3-rhamnosylglucoside, quercetin 3-rhamnoside, rutin and horseradish peroxidase (60 EU) were purchased from Roth, Karlsruhe. β-Glucosidase from almonds (40 U/mg; 25°, salicin for substrate) and β-glucuronidase from *Helix pomatia* (4.5 U/ml; 25°, 4-nitrophenyl-β-glucuronide) came from Boehringer, Mannheim. Kaempferol 3-glucoside and 3-apiosylglucoside were isolated from leaves of chick pea plants as described [30].

Plant material. All seeds used were provided by the botanical garden of the University of Münster. They were soaked under flowing H_2O for 24 hr and grown at 26° in vermiculite under a white light (15 hr)–dark (9 hr) program. Conditions of cell culture cultivation have previously been described [23].

Preparation of protein extracts for glycosidase activity tests. Plant material, quartz sand p.a. and citrate–Na-phosphate buffer pH 5, 0.2 M (1:1:2 w/w) were ground for 10 min in a mortar, then centrifuged at 20000 *g* for 20 min. The supernatant was filtered through glass wool. Part of the protein extract was chromatographed on Sephadex G 25 equilibrated with the extraction buffer. Purification and separation of β-glycosidases from *Cicer arietinum* seedlings and cell suspension cultures were carried out as described [18, 25, 26]. β-Glycosidases from *Cucurbita maxima* were purified using the same methods.

Preparation of Mentha *peroxidase.* Plants of *Mentha piperita* or cells of suspension cultures of *Mentha arvensis* were used for the prepn of the peroxidase. Cell suspension cultures were filtered off, washed and freeze-dried before use [24]. 50 mg Me_2CO powder of whole plants (or 50 mg freeze-dried cells), 50 mg quartz sand p.a. and 3 ml phosphate buffer 0.1 M, pH 7.5, were ground in a mortar for 3 min and centrifuged at 20000 *g* for 5 min. The supernatant was used for the tests.

Glycosidase measurements with kaempferol- and quercetin 3-glycosides. Method a. 25 μl substrate soln (in ethyleneglycol monomethyl ether; concn of substrate 1–40 mM), *x* μl glycosidase prepn and (975 − *x*) μl buffer, 0.05 M, are incubated under appropriate conditions. Catalase (i.g. 1 μg/ml) can be added to prevent peroxidase reaction if necessary. Incubation is stopped by treatment in boiling H_2O (3 min) and denaturated protein is removed by centrifugation if necessary. 0.5 ml of the test vol. (or lower aliquots, if substrate concn exceeds 0.1 mM) are then incubated together with 25 μl H_2O_2 10 mM, 25 μl HRP soln, and 0.45 ml McIlvaine buffer, 0.2 M, pH 5, for 10 min at 30°. The pH of the second incubation should be 5. Absorption is measured after the incubation against a blank without substrates in the maximum of flavonol glycoside absorption. In the case of quercetin 3-glycosides, 50 μl of crude protein prepn of *Mentha* sp. is used instead of HRP–H_2O_2. Amount of hydrolysed substrate can be calculated as outlined under Results. *Method* b. 25 μl substrate soln (in ethyleneglycol monomethyl ether; concn of substrate 0.5–4 mM), *x* μl glycosidase prepn, 25 μl H_2O_2 10 mM, 25 μl HRP (or 50 μl *Mentha* protein prepn instead of HRP and H_2O_2) and (925 − *x*) μl buffer are incubated in a cuvette at 30° and the absorption decrease is recorded spectrophotometrically at an appropriate wavelength. Reaction is started by addition of the glycosidase prepn. Correct wavelength and quantitative calculation depend on pH used and can be seen from Table 1. Before using method *b*, one has to look for interfering components as pointed out under Results. Incubations with heat denaturated plant protein extracts were carried out as controls throughout.

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