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Purification and characterization of a flavonol 3-*O*-β-heterodisaccharidase from the dried herb of *Fagopyrum esculentum* Moench

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

A flavonol-3-*O*-β-heterodisaccharide glycosidase (FHG I) was isolated from dried aerial tissues of *Fagopyrum esculentum* Moench (Fagopyri herba). It has a specific enzyme activity of ca. 3.5 nkat mg^{-1} protein in buffered extracts when rutin (quercetin-3-*O*-rutinoside) was used as substrate and an optimal enzyme activity was seen at around pH 4.8 and 30 °C. FHG I was purified about 156-fold to apparent homogeneity by hydrophobic interaction, anion exchange and size exclusion chromatographic steps. The apparent molecular mass of FHG I was 74.5±2 kDa as determined by SDS-PAGE and it is a monomeric glycoprotein with a carbohydrate content of 23%. The isoelectric point as determined by isoelectric focusing was 5.7 and the energy of activation was 32 kJ mol^{-1}. FHG I exhibits a high substrate specificity, preferring flavonol 3-O-glycosides comprising the disaccharide rutinose. The K_m and V_{max} values for the natural substrate rutin were calculated to be 0.561 μM and 745 nkat mg $^{-1}$ protein, respectively. Two oligopeptide fragments obtained after enzymatic digestion of FHG I were sequenced and showed similarities to sequences of β-glucohydrolases from other plant species. Polyclonal antibodies were raised and their specificities determined. Another flavonol 3-*O*-β-heterodisaccharide glycosidase (FHG II) could also be detected in buckwheat herb, having a molecular mass of 85.3±2 kDa and an isoelectric point between pH 6.0 and 6.5.

Keywords: *Fagopyrum esculentum*; Polygonaceae; Rutinoside; Rutin; β-Glycosidase; Purification

1. Introduction

Rutin (quercetin 3-*O*-rutinoside) **1** is the dominant flavonol glycoside in the leaves and flowers of the common buckwheat, *Fagopyrum esculentum* Moench (Couch et al., 1946). Rutin **1** exhibits antioxidative, antihemorrhagic and blood vessel protecting properties (e.g., Gabor, 1972; Wojcicki et al., 1995a, 1995b; Rice-Evans et al., 1997). Therefore, the dried aerial parts of common buckwheat are used as tea preparations or in tablets for the treatment of vascular diseases and circulatory disorders (Ihme et al., 1996). Rutin **1** can be hydrolysed by microorganisms and enzymes isolated from common and tartary buckwheat seeds (Hay et al., 1961; Kurusawa et al., 1973; Bourbouze et al., 1974, 1976; MacDonald et al., 1984; Yasuda and Nakagawa, 1994). However, rutin-cleaving enzymes have not been investigated so far in leaves of *Fagopyrum esculentum* Moench or in Fagopyri herba, which is the commercially available dried buckwheat herb, although it is well known that the rutin **1** content of the drug may decrease after harvest (Nguyen-Hiep et al., 1964; Noguchi and Mori, 1969). From previous studies on the stability of rutin **1** in Fagopyri herba it may be deduced that the disappearance of the most important ingredient of Fagopyri herba, namely rutin **1**, and the transient appearance of quercetin **2** is a result of enzyme-catalyzed reactions, involving glycosidases and oxidases.

In the present paper we report for the first time the purification to apparent homogeneity and the molecular

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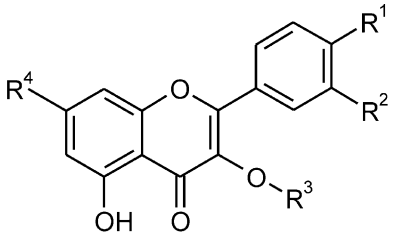


Flavonol glycoside (General structure)

1 Rutin: R^1 = OH, R^2 = OH, R^3 = rutinosyl (see **3**), R^4 = OH
2 Quercetin: R^1 = OH, R^2 = OH, R^3 = H, R^4 = OH
4 Isorhamnetin 3-*O*-rutinoside: R^1 = OH, R^2 = OCH_3, R^3 = rutinosyl (see **3**), R^4= OH
5 Kaempferol 3-*O*-rutinoside: R^1 = OH, R^2 = H, R^3 = rutinosyl (see **3**), R^4 = OH
6 Kaempferol 3-*O*-sophoroside-7-*O*-glucoside: R^1 = OH, R^2 = H, R^3 = sophorosyl (see **14**), R^4 = glucosyl
7 Kaempferol 3-*O*-glucoside: R^1 = OH, R^2 = H, R^3 = glucosyl, R^4 = OH
8 Kaempferol 3-*O*-glucoside-7-*O*-rhamnoside: R^1 = OH, R^2 = H, R^3 = glucosyl, R^4 = rhamnosyl (see **15**)
9 Quercitrin: R^1 = OH, R^2 = OH, R^3 = rhamnosyl (see **15**), R^4 = OH
10 Isorhamnetin: R^1 = OH, R^2 = OCH_3, R^3 = H, R^4 = OH
11 Kaempferol: R^1 = OH, R^2 = H, R^3 = H, R^4 = OH
12 Kaempferol 7-*O*-glucoside: R^1 = OH, R^2 = H, R^3 = H, R^4 = glucosyl
13 Kaempferol 7-*O*-rhamnoside: R^1 = OH, R^2 = H, R^3 = H, R^4 = rhamnosyl (see **15**)

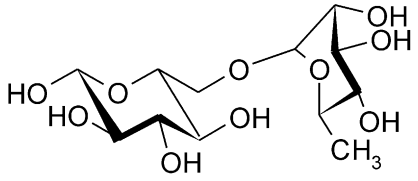
characterization of a flavonol-3-*O*-β-heterodisaccharide glycosidase isolated from dried buckwheat herb (Fagopyri herba)

2. Results

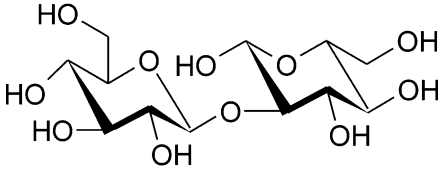
2.1. Enzyme purification

After incubating Fagopyri herba in water or different buffer solutions, rutin **1** was degraded fairly rapidly (Fig. 1), and quercetin **2** and rutinose **3** were identified as degradation products (data not shown). Rutin **1** was not degraded under the same conditions in drug samples heated prior to fermentation (Fig. 1) indicating that the degradation seen in the untreated samples can be attributed to an enzymatic process. The activity observed in crude protein extracts reached about 3.5 nkat/mg protein. Acetone powders prepared from Fagopyri herba were still quite active and served as the starting material for enzyme purification. Rutin **1**, a flavonol 3-*O*-heterodisaccharide, was the preferred substrate of the two enzymes characterized here, which were hence termed flavonol-3-*O*-heterodisaccharide glucohydrolases I and II (FHG I and II).

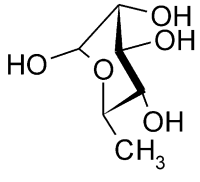
The major flavonol-3-*O*-heterodisaccharide glucohydrolase (FHG I) was successfully purified 156-fold from acetone powders prepared from dried buckwheat herb, with a total recovery of 16.4% (Table 1). Hydrophobic interaction chromatography on Phenylsepharose was the first chromatographic step used to separate FHG I from other proteins. The activity peaked at 0.34 M $(NH_4)_2SO_4$ (Fig. 2A). Fractions showing FHG activity were pooled, dialysed against water and subjected to anion exchange chromatography on Resource Q for



Rutinose, **3**



Sophorose, **14**



α-L-Rhamnose, **15**

further purification. A NaCl gradient between 0 and 0.7 M was then applied, and FHG I was released from the matrix at a NaCl concentration of 0.32 M (Fig. 2B). The active fractions were again combined, concentrated by membrane filtration and subjected to gel filtration, where the rutin-hydrolysing activity eluted as a single peak between 10 and 18 ml (Fig. 2C). The size of the holoenzyme was estimated to be approximately 80 kDa.

2.2. Molecular mass, isoelectric point, pH-optimum, temperature optimum and energy of activation

The purification steps were accompanied by SDS-PAGE in order to control the efficacy of the respective purification steps. The active fraction 14 obtained after gel filtration showed a single polypeptide band at 74.5±2 kDa (Fig. 3). SDS-PAGE under reducing conditions yielded similar results, suggesting that FHG I is a monomer (data not shown).

Purified FHG I was treated with trifluoromethane sulphonic acid to remove sugar residues covalently attached to the protein. The carbohydrate content of FHG I was then estimated by comparing the molecular masses of the native protein and its deglycosylated derivative seen in SDS-PAGE. The molecular mass of the deglycosylated protein was 58 kDa indicating a sugar content of 23% (Fig. 4).

The pI of the FHG I as determined by isoelectric focusing was 5.7 (Fig. 5).

Using rutin **1** as a substrate, pH and temperature dependencies of the purified enzyme were investigated. FHG I activity was optimal at pH 4.8 with half maximal values at pH 3.9 and 7.0 and proved to be stable over a wide temperature range, namely from 4 to 50 °C. The enzyme worked best at around 30 °C (42 nkat mg^{-1} protein), but surprisingly high enzyme activity was seen at 4 °C (37 nkat mg^{-1} protein) and was still active at 70 °C (11 nkat mg^{-1} protein). Enzyme activities determined

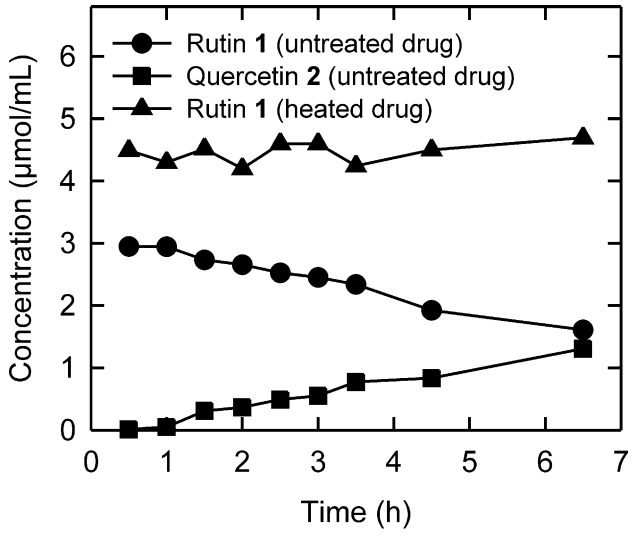


Fig. 1. Rutin **1** and quercetin **2** concentration after incubation of powdered crude drug in McIlvaine buffer pH 6.0 at 25 °C.

Table 1
Purification of flavonol 3-*O*-β-heterodisaccharidase I (FHG I) from the dried herb of *Fagopyrum esculentum* (buckwheat)

Stage	Protein [mg]	Total activity [nkat]	Specific activity [nkat/mg protein]	Recovery [%]	Purification (fold)
Crude extract	21.1	66	3.1	100	1
Phenylsepharose	1.4	33.1	23.6	50	7.6
Resource Q	0.98	28.9	29.5	44.7	9.5
Superdex 200	0.023	10.8	468	16.4	156

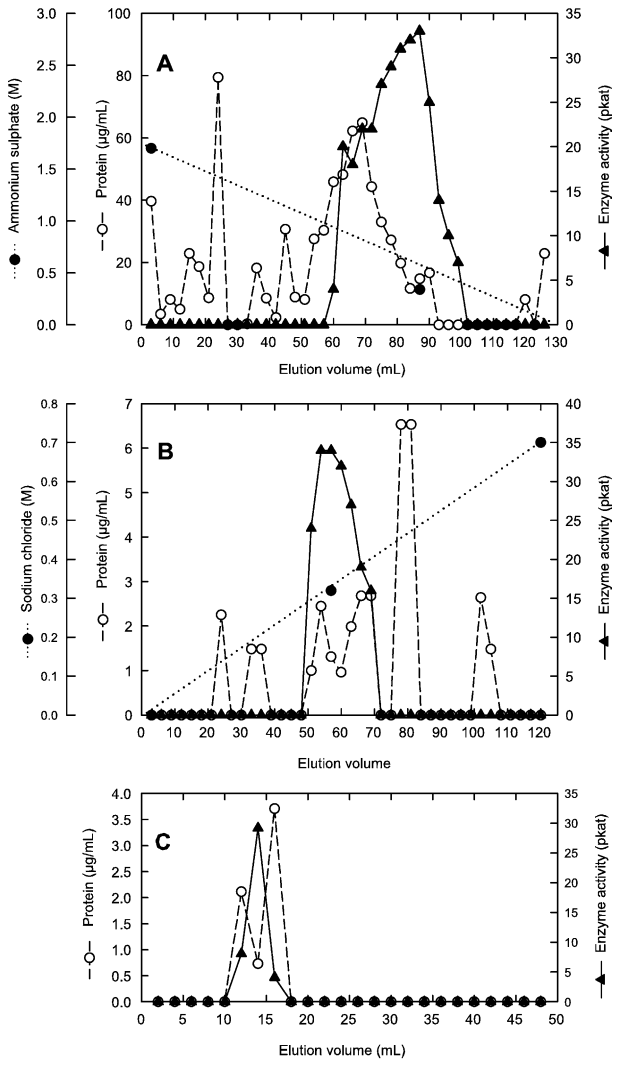


Fig. 2. Elution profiles of flavonol 3-*O*-β-heterodisaccharidase I (FHG I) purified from the dried herb of *Fagopyrum esculentum* (buckwheat). Acetone-precipitated protein was subjected to a three-step chromatographic procedure. (A) Hydrophobic interaction chromatography on Phenylsepharose, (B) anion exchange chromatography on Resource Q and (C) size exclusion chromatography on Superdex 200. Enzyme activity was checked using 1 mM rutin **1** as the substrate.

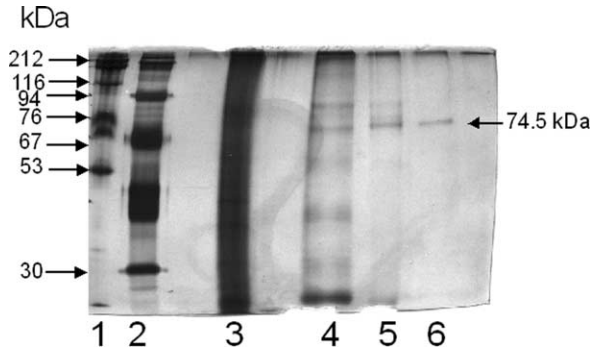


Fig. 3. SDS-PAGE analysis of the purification steps of FHG I. Track 1 and 2 show molecular weight markers. Track 3 shows the crude extract, track 4 active pooled fractions of hydrophobic interaction chromatography, track 5 the active pooled fractions of anion exchange chromatography, and track 6 the most active fraction (fraction 14) of size exclusion chromatography (see Fig. 2).

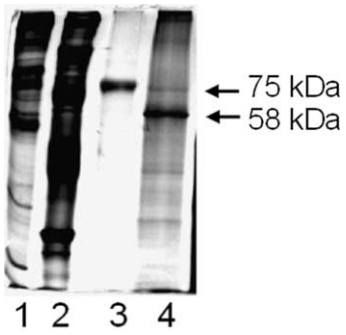


Fig. 4. SDS-PAGE analysis of native and deglycosylated FHG I. Tracks 1 and 2 show molecular weight markers; track 3 is FHG I before, track 4 FHG I after trifluoromethane sulphonic acid treatment.

between 4 and 30 °C were used to calculate the energy of activation, which was 32 kJ mol^{-1}.

2.3. Enzyme inhibition and substrate specifity

The substrate preferences of FHG I were studied with various other flavonol 3-*O*-glycosides **4**–**8**. Table 2 summarizes the kinetic parameters determined. Taking low K_m values as an indication of affinity, FHG I showed its highest affinity for kaempferol 3-*O*-monoglycosides, with K_m values being up to 19 times lower than those with kaempferol 3-*O*-disaccharides (**5**). Surprisingly, quercetin 3-*O*-monosaccharides and quercetin 3-*O*-sophoroside were not hydrolyzed at all by FHG I (data not shown), and sugars attached to the phenolic hydroxyl group at C7 of the flavonoid structure were not removed by FHG I. Cardiac glycosides, cyanogenic glycosides, oligosaccharides and *p*-nitrophenyl β-D-glucoside were not accepted as substrates either. Flavonol 3-*O*-rutinosides (**1**,**4**,**5**) were all cleaved by FHG I, however, the affinity of the enzyme seems to depend on the substitution pattern of the flavonol B-ring.

With regard to reaction velocities and relative activities, flavonol 3-*O*-disaccharides were better substrates for FHG I than the respective monosaccharides. The

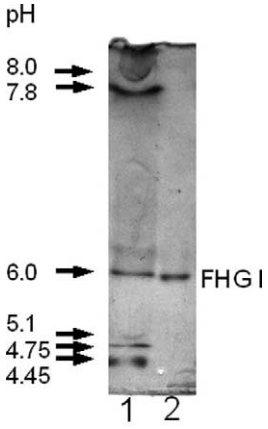


Fig. 5. Determination of the isoelectric point of FHG I using isoelectric focusing.

V_{max}/K_m ratios, which may be taken as a measure of the efficacy of the enzyme in the turn-over of a given substrate, were higher for disaccharides than for monosaccharides. Rutin **1**, probably the natural substrate of FHG I, was degraded with a V_{max}/K_m ratio of 1291. Using the relative velocity as a measure of specificity, kaempferol rutinoside **5** was hydrolysed best, among the other possible substrate tested.

Rutinose **1** was the only sugar released upon incubation of rutin **1** in the presence of FHG I; glucose or rhamnose could not be detected.

Table 2
Kinetic constants and specific activity of the FHG I from buckwheat herb. (Amounts calculated as kaempferol equivalents)

Substrate	Product	K_m [mM]	V_{max} [nkat/mg]	V_{max}/K_m [nkat/mg]	Specific activity [nkat/mg protein]	Specific activity [%]
Quercetin 3-*O*-rutinoside (Rutin) (**1**)	Quercetin (**2**)	0.561	724	1291	468	100
Isorhamnetin 3-*O*-rutinoside (**4**)	Isorhamnetin (**10**)	0.375	21	56	15	3
Kaempferol 3-*O*-rutinoside (**5**)	Kaempferol (**11**)	0.950	1211	1275	650	134
Kaempferol 3-*O*-sophoroside-7-glucoside (**6**)	Kaempferol 7-*O*-glucoside (**12**)	0.533	572	1073	374	80
Kaempferol 3-*O*-glucoside (**7**)	Kaempferol (**11**)	0.050	42	840	38	8
Kaempferol 3-*O*-glucoside-7-*O*-rhamnoside (**8**)	Kaempferol 7-*O*-rhamnoside (**13**)	0.060	50	833	47	10

The degradation of rutin **1** was only weakly inhibited by quercitrin (quercetin 3-O-rhamnoside **9**) or the semi-synthetic flavonol ether troxerutin (data not shown). The influence on FHG I of alcohols used as solvents for the preparation of herbal extracts was also investigated and methanol–H_2O at 1:2 (v/v) led to a loss of activity of about only 20%. However, the enzyme was completely inactivated by methanol–H_2O at $>$ 1:1 (v/v) and with ethanol–H_2O $>$ 1:2 (v/v).

2.4. FHG I Antibodies and protein sequence

FHG I purified from Fagopyri herba was used to raise polyclonal antibodies in mice. The antisera obtained contained antibodies recognizing FHG I in unreduced and reduced samples of purified FHG I and crude protein extracts (data not shown). Under the conditions used, immune globulins present in the pre-immune sera did not bind to buckwheat proteins. In addition to FHG I, a 87-kDa protein was recognized by the immune globulins present in the sera after immunization of the experimental animals with purified FHG I. This particular protein (appearing in fraction 18 together with FGH I) could be separated from FHG I in SDS-PAGE, and it was found that it also was capable of hydrolyzing rutin **1** (data not shown). Therefore, this enzyme was named FHG II. The isoelectric point of FHG II, as determined by isoelectric focusing, was at about pH 6.3. FHG II was not characterized further.

Finally, the purified FHG I was partially sequenced. The protein, which was blocked at the N-terminus, was digested using the endoproteinase LysC from *Lysobacter enzymogenes*. This digest yielded several oligopeptides, two of which were sequenced: a 15 amino acid fragment (fragment 1: LIVGDRLPTFTDEQT) and a 10 amino acid fragment (fragment 2: VSGGINQAGI). Comparing amino acid sequences of known glucosidases with the information obtained from our sequencing experiments revealed high homology of FHG I with prunasin hydrolase isoforms from *Prunus serotina* (*E*-values <0.1 for fragment 1) and various other (putative) β-glucohydrolases (*E*-values <1.0 for fragment 1). The properties described so far let us assume that the FHG I isolated here is a member of the Family 1 of glycoside hydrolases [EC 3.2.1.21.].

3. Discussion

The importance of endogenous enzymes on the stability of herbal drugs and their biological active ingredients has rarely been investigated in the past. After harvest and during drying and processing of a given drug the destruction of cell compartments may result in the degradation of important drug constituents, because enzymes still active in the drug may modify or even degrade the compounds in question (Nguyen-Hiep et al., 1964; Noguchi and Mori, 1969). Endogenous plant enzymes may in this way influence the quality and therapeutic value of drugs and drug preparations.

We started our investigations by fermenting heat-treated and untreated samples of Fagopyri herba in buffer solutions and found a dramatic decrease in rutin **1** in the untreated samples. This degradation was still seen in crude protein extracts containing 33% MeOH or in buffered protein extracts stored at 4 °C. This observation is of special pharmaceutical interest since it shows that stability of rutin **1** cannot be achieved under standard extraction and storage conditions. Assuming that the degradation of rutin **1** starts with the initial removal of the sugar moieties, we aimed at the isolation of a flavonol 3-*O*-glycosidase. Using the 3-step purification method described here, we succeeded in isolating two enzymes, termed FHG I and FHG II, which are responsible for rutin **1** hydrolysis in Fagopyri herba. Comparing the properties of FHG I with those of enzymes isolated from the seeds of common and tartary buckwheat (Bourbouze et al., 1974, 1976; Yasuda and Nakagawa, 1994), we found that the buckwheat glycosides described so far share some characteristics. The molecular masses of a rhamnodiastase and an α-L-rhamnosidase from *Fagopyrum esculentum* seeds, and the rutin-degrading enzymes RDE I and RDE II from *Fagopyrum tartaricum* are similar to the masses calculated for FHG I and FHG II in the present investigation. Other plant β-glucosidases of similar molecular masses are also known (see Schliemann, 1987, for a review). FHG I seems to be a monomer which corresponds to the previous findings of monomeric organisation of RDE I and II in tartary buckwheat seeds. RDE I and II contained 11 and 14% carbohydrates, respectively, as compared to 23% determined for FHG I in the present study. Isoelectric points have not been published for RDE I and II from tartary buckwheat seeds. The isoelectric points for glycosidases from common buckwheat seeds were around 3.7 (Bourbouze et al., 1974), which indicates that these enzymes are not identical with the FHGs described here. But, Bourbouze et al. (1974) did not use rutin **1** as the substrate to trace glycosidase activity (see below). On the other hand, isoelectric points determined here for the two FHGs are well in the range of those reported for other plant β-glucosidases (Schliemann, 1987; Esen, 1993). Somewhat striking was the observation that FHG I does not hydrolyse cyanogenic glycosides, although it shares considerable sequence homology with cyanogenic β-glucosidases (Oxtoby et al. 1991; Li et al., 1992; Zheng and Poulton, 1995; Zhou et al., 2002).

The substrate preferences of rutin-hydrolysing enzymes have been investigated in some detail. The glycosidases purified from common buckwheat by Bourbouze et al. (1974, 1976) were capable of hydrolysing

α-rhamnosides, but also the disaccharide rutinose, amygdalin, various *p*-nitrophenylglycosides and quercetin-3-sophoroside **9**. In contrast, only a small number of substrates tested were accepted by the FHG I described here. Cyanogenic glycosides, 7-*O*-glycosides, *p*-nitrophenyl β-D-glucoside, cardiac glycosides, and oligosaccharides were not hydrolysed by FHG I. The glycosidases described more recently (Yasuda and Nakagawa, 1994) were tested with rutin as the substrate, and both rutin-degrading enzymes showed a high specificity for this particular substrate.

As expected, the sugar residues attached to the 3-β-O-position have an influence on reaction velocity and affinity. Moreover, we found here that the structure of the aglycone, especially the group attached in the 3′ position of the B-ring, influences the catalytic activity of FHG I.

Different enzymes may be involved in the degradation or modification of genuine flavonol glycosides in Fagopyri herba. Glycosidases responsible for the hydrolysis of secondary plant products have been shown to possess a high degree of specificity. The cleavage of rutin **1** into quercetin **2** and rutinose **3** is the first step in enzymatic rutin **1** degradation (Surholt and Hösel, 1978). The aglycone released by this reaction may be further degraded by so-called "flavonol oxidases" (Barz and Köster, 1981) yielding products such as 3,4-dihydroxybenzoic acid and 2,4,5-trihydroxybenzoic acid. These compounds were not detected until after prolonged incubation of quercetin **2** by protein extracts prepared from buckwheat herb (data not shown). This may explain the observation that no increase in the quercetin **2** content of buckwheat herb is seen although rutin **1** eventually disappears. Enzymatic degradation of biologically active drug constituents may occur at various stages of a pharmaceutical process. As shown here, specific enzymes may even be active in ethanol extracts or at low temperatures. Therefore, we feel that a knowledge of enzymes active or becoming active in crude drugs is an important issue on our way to well-defined processes for the production of phytopharmaceuticals.

4. Experimental

4.1. Materials

Some rare flavonol glycosides, such as isorhamnetin 3-*O*-rutinoside **4**, kaempferol 3-*O*-rutinoside **5**, kaempferol 3-*O*-glucoside-7-*O*-rhamnoside **8**, kaempferol 3-sophoroside-7-*O*-glucoside **6** and quercetin 3-*O*-sophoroside were generous gifts from Dr. M. Veit, Universität Würzburg, Germany. Other glycosides and reference compounds used in the present study were purchased from Carl Roth GmbH & Co KG (Karlsruhe, Germany) or Boehringer Mannheim GmbH (Mannheim, Germany). Rutinose hepatacetate was obtained from Sigma (Steinheim, Germany). All other reagents used were obtained from Merck (Darmstadt, Germany) or Sigma (Steinheim, Germany).

The dried aerial tissues of *Fagopyrum esculentum* Moench (buckwheat herb, Fagopyri herba; Polygonaceae) were obtained from Fink GmbH (Glaxo SmithKline), Herrenberg, Germany.

4.2. Flavonoid and sugar analysis

Flavonoids were extracted with MeOH-H_2O (4:1) as described by Hagels et al. (1995) and the extracts then analyzed by HPLC. The flavonoids were separated on an RP 18 column (Lichrosphere 100, 5 μm, 125x 4, Merck) equilibrated with solution A (aq. phosphoric acid 0.85% (v/v) + 4% (v/v) acetonitrile). The flavonoids were eluted with solution B (acetonitrile containing 12.5% (v/v) phosphoric acid 0.85% (v/v)) using the following gradient: 0 min 6% B, 5 min 15% B, 11 min 15% B, 16.5 min 24% B, 22 min 41% B, 25 min 41% B, 33 min 6% B and detected by their UV-absorption at 340 nm. Flavonoids were identified on the basis of their retention times in HPLC by direct chromatographic comparison with authentic samples.

Sugars were analysed by TLC on silica gel 60 using toluene:acetone (4:1; v/v) as the mobile phase and thymol-H_2SO_4 for detection. Sugars were acetylated (Blakeney et al., 1983) prior to analysis since rutinose was commercially available only as its heptaacetate.

4.3. Enzyme assays

The standard assay for testing FHG I contained 1 mM substrate and about 15 nkat enzyme in a total volume of 105 μl buffer. 1.5-ml Eppendorf tubes containing this mixture were incubated for 15 min at 30 °C, and the enzyme reaction was terminated by the addition of 315 μl of methanol. After centrifugation the mixture was subjected to HPLC. Specific activities of FHG I were calculated from the amount of flavonol released from the corresponding flavonol-glycoside used as substrate.

Protein was determined according to Bradford (1976) with bovine serum albumin as the standard protein for establishing calibration curves.

4.4. Purification of FHG I

A High Load™ system (Pharmacia) and a FPLC™ system (Pharmacia) were utilized for the chromatographic purification steps. All operations were carried out at 4 °C. Acetone powders prepared from buckwheat herb were extracted with 10 volumes (w/v) of McIlvaine buffer, pH 6.0 and homogenized using an Ultra-Turrax. After centrifugation at 15,000 *g* for 20 min, the super-

natant was removed and filtered through Miracloth^{R} (Calbiochem). Proteins were precipitated by the addition of three volumes (w/v) cold acetone (−18 °C) and pelleted at 3000 *g* for 10 min. Precipitated protein was dissolved in buffer A (0.1 M potassium phosphate buffer pH 6.4 + 1.7 M $(NH_4)_2SO_4$). This solution was loaded on a Phenylsepharose 6 FF column (Amersham Biosciences) equilibrated with buffer A. The column was washed with the same buffer until the eluate was protein-free. Proteins were eluted with a linear gradient from 1.7 to 0 M $(NH_4)_2SO_4$ at a flow rate of 1.33 ml min^{-1}. The eluate was collected in 3-ml fractions that were then assayed for FHG I using rutin **1** as the substrate. The most active fractions were combined and dialysed against double-distilled water overnight.

The dialysed enzyme solution was adjusted to pH 6.5 and then loaded onto a Resource Q ion exchange column (Amersham Biosciences) previously equilibrated with buffer B (0.02 M sodium acetate buffer, pH 6.5). Proteins were eluted with a linear gradient from 0 to 0.7 M NaCl at a flow rate of 1.33 ml min^{-1} and collected in 3-ml fractions. The most active fractions were combined and concentrated by ultra-filtration using 30-kDa Macrosep membrane filters (Filtron).

The FHG I was further purified by size exclusion chromatography on Superdex 200 HR 10/30 (Amersham Biosciences). The column was equilibrated with McIlvaine buffer, pH 4.8. The proteins were eluted at a flow rate of 0.5 ml min^{-1}, and fractions of 1 ml each were collected. After having checked their activity and purity, fractions containing FHG I were pooled, concentrated, and used for the studies reported here.

4.5. Gel electrophoresis

Discontinuous SDS-PAGE was performed according to Laemmli (1970). Gels (10% polyacrylamide) were developed for 15 min at 80 V, then for 75 min at 120 V. Molecular weight standards were obtained from Biorad (Low and High Molecular Weight Marker Kit). Standards and protein samples were heated in sample buffer at 100 °C for 3 min prior to SDS-PAGE. Gels were silver-stained (Silver Staining Kit, Amersham Biosciences) according to the instructions of the manufacturer.

4.6. Enzyme properties

The isoelectric point of the purified FHG I was determined on PhastGel IEF 3-9 using the PhastSystem^{TM} (Amersham Biosciences). Proteins were silver-stained according to the instructions of the manufacturer. Proteins of the Amersham Biosciences pI Marker Kit served as the pI standards. The pH-optimum was determined in McIlvaine buffer in the presence of about 100 μg protein per ml and 1 mM rutin as the substrate. The incubation time was 15 min. The temperature optimum and the energy of activation were determined at pH 5.0; otherwise the above conditions were applied. The K_m and V_{max} values of the FHG I for the different substrates were determined using the hyperbolic regression method in the presence of about 1 μg protein mL^{-1} and an incubation time of 15 min. Relative reaction velocities were determined with 1 mM of the respective substrate.

The carbohydrate content of FHG I was determined after deglycosylation of the purified protein using the Glycofree Deglycosylation Kit K-500 (Oxford GlycoSystems) according to the manufacturer's instruction. The sequencing of oligopeptides obtained after LysC digestion was carried out by Toplab Martinsried, Germany.

4.7. Polyclonal antibodies

Polyclonal antibodies against purified FHG I were raised in white mice. Two weeks after the collection of pre-immune sera, three mice were immunized at different subcutaneous sites with FHG I homogenates containing 80 μg protein and 0.9% (w/v) NaCl. Three subsequent boost immunizations were administered after 16 (62 μg), 49 (10 μg) and 107 (50 μg). Antisera were collected 10 days after the final boost. IgG-enriched antisera were obtained from whole blood by centrifugation at 1000 *g* for 10 min and stored in small aliquots at −20 °C.

Antisera titers were determined by ELISA. Antisera specifities were analyzed by immunoblotting techniques (Western Blot). Crude protein extracts from buckwheat herb as well as homogeneous FHG I were subjected to SDS-PAGE. Proteins were electroblotted onto nitrocellulose membranes and probed using sera containing polyclonal antibodies raised against FHG I (see above).

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