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Preparation and properties of rutin-hydrolyzing enzyme from tartary buckwheat seeds

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ABSTRACT

A rutin hydrolyzing enzyme (RHE) was isolated from *Fagopyrum tataricum* Moench seeds by using ammonium sulphate fractionation, anion exchange and size exclusion chromatography. The purified RHE has an apparent molecular weight of about 70 kDa determined by SDS–PAGE, with an isoelectric point (pI) (determined by isoelectric focusing) of 6.7. RHE has a specific catalytic activity toward rutin when incubated together with rutin at 37 °C for 30 min in the presence of 20% ethanol, and its K_m value for rutin is 1.04×10^{-3} M. The RHE catalytic product analyzed by HPLC displayed high similarity with quercetin and this is confirmed by ^{1}H NMR spectroscopy and LC-ESI-MS/MS, suggesting that the RHE hydrolysis product is quercetin. These results suggest that the RHE from tartary buckwheat seeds is a specific rutin-hydrolyzing enzyme, providing a new enzymatic preparation method for quercetin.

1. Introduction

Flavonoids are a group of polyphenolic compounds, containing a basic skeleton of diphenylpropane (C_6–C_3–C_6). More than 4000 different flavonoids exist and are classified into the following subclasses: flavones, flavonols, flavan-3-ols, isoflavones, flavanones, anthocyanins and chalcones (Murakami, Ashida, & Terao, 2008). Flavonoids, widely found in fruits and vegetables, have attracted much attention as potential anti-carcinogens. Quercetin (3,3′,4′,5,7-pentahydroxylflavone) is a typical flavonoid present in the plant kingdom as a secondary metabolite. Compared to other flavonoids, quercetin has been demonstrated to be very effective in anti-tumour, antioxidant and anti-inflammatory activities in vitro systems (Hidalgo, Sánchez-Moreno, & Pascual-Teresa, 2010; Hodek, Trefil, & Stiborova, 2002). These activities of quercetin are mainly due to the *o*-hydroxy structure in the B ring, the 2, 3 double bond in conjugation with the 4-oxo function in the C ring and the 3- and 5-OH groups with the 4-oxo function in the A and C rings (Lien, Ren, Bui, & Wang, 1999).

In plants, quercetin exists as glycosylated forms such as glucoside, galactoside, rhamnoside, arabinoside and rutinoside (Herrera & Luque de Castro, 2004). Rutin is a flavonol glycoside synthesized by higher plants to defend against ultraviolet radiation and diseases (Gaberscik, Voncina, Trost, & Germ, 2002). Rutin and other rutin derivatives are used for the treatment and curing of increased frangibility and permeability of blood capillaries caused by various diseases, for example, vascular-based pathological hemophilia, lesion in the retina in diabetes. Quercetin has a higher antioxidant activity than rutin, exhibiting antiallergenic activities, anti-carcinoma activity, blood glucose reducing activity, and antibacterial activity (Gaberscik et al., 2002). High concentrations of quercetin are found in onions (300 mg/kg), kale (450 mg/kg), broccoli (100 mg/kg), beans (50 mg/kg), apples (50 mg/kg), blackcurrants (40 mg/kg), and tea (30 mg/kg) (Hollman & Arts, 2000). In 1999, the International Agency for Research on Cancer (IARC) concluded that quercetin is a potential anti-cancer agent for humans. In the United States and Europe, supplements of quercetin are commercially available (Okamota, 2005).

Buckwheat, belonging to *Polygnaceae Fagopyrum* Mill, is a very important cereal grain that is loaded with nutritional material. Buckwheat, especially tartary buckwheat, contains biologically important rutin, quercetin and fagopyrins not found in other cereal crops, and the content of flavonoids in tartary buckwheat flour is 10 to 100 times higher than that in common buckwheat (Fabjan, Rode, Kosir, Wang, & Zhang, 2003). Therefore tartary buckwheat products are regarded as a source of medicine with some unique components, and much attention has recently been paid to quercetin considering its important role in treating and preventing various diseases (Gaberscik et al., 2002). The molecular structures of rutin and quercetin are similar except rutinoside in C-ring is replaced with –OH in quercetin (Fig. 1), indicating that they have isogenous relationship. In order to convert rutin into quercetin, in this study, we purified a specific rutin hydrolyzing enzyme (RHE) from tartary buckwheat, analyzed its enzymatic properties,

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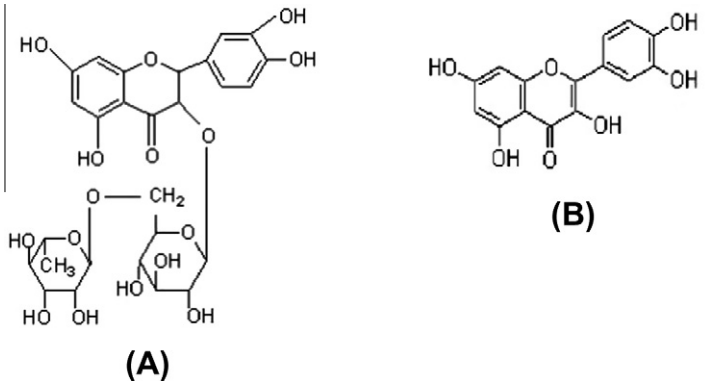

Fig. 1. Molecular structure of rutin and quercetin. A is rutin and B is quercetin.

and eventually developed an affordable technique for preparation of quercetin on a large scale.

2. Materials and methods

2.1. Materials

Tartary buckwheat seeds were harvested from ShouYang County, China, in October 2009. Resource™ Q (6.4 × 30 mm) anion exchange column and Superdex™ 75 10/300 GL gel filtration column were purchased from GE Healthcare (Uppsala, Sweden). Quercetin and rutin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solvents for HPLC were of chromatography grade. All other reagents were of the highest purity available.

2.2. Extraction and purification of RHE

Air-dried tartary buckwheat seeds were ground into powder in a mini grinder (Huaxin, China), and the powder could pass through 40 mesh sieve. All operations were carried out at 4 °C. Fifty grams of the powder were mixed in 500 ml of 20 mM acetate buffer (ACE buffer, pH 5.0) for 24 h at 4 °C. After the insoluble material was removed by centrifugation at 8000*g* for 20 min, solid $(NH_4)_2SO_4$ was slowly added to the supernatant to 80% saturation and continuously stirred for 4 h. The precipitate was collected by centrifugation at 12,000*g* for 30 min at 4 °C, and was then redissolved in 20 mM Tris–HCl buffer (pH 7.0). Then the crude proteins were dialyzed in deionized water at 4 °C for 4 h. The dialyzate was finally centrifuged and lyophilized to obtain crude proteins for subsequent purification.

Crude proteins (10 mg) obtained above was dissolved in 10 ml of 20 mM Tris–HCl buffer (pH 7.0) and centrifuged at 10,000*g* for 20 min. The supernatant (1 ml) obtained was loaded onto a Resource™ Q column equilibrated with 20 mM Tris–HCl buffer (pH 7.0) and then eluted with the same buffer containing 1 M NaCl. Unbound protein and eluted proteins were collected in Eppendorf tubes. The proteins in different tubes were measured for RHE activity according to the method described in the section below. The fraction containing RHE activity was further separated on a Superdex™ 75 10/300 GL gel filtration column (GE Healthcare, Uppsala, Sweden). The purified RHE was lyophilized for subsequent research. Protein concentration was determined according to Bradford (1976) with bovine serum albumin as a standard.

2.3. SDS–PAGE and IEF analysis

SDS–PAGE was performed to estimate the molecular weight of RHE using the method of Laemmli (1970). Samples were dissolved in 6× loading buffer, heated at 100 °C for 3 min, and centrifuged at 10,000*g* for 1 min. Electrophoresis was conducted under a constant current of 20 mA for 3 h. The gels were stained with Coomassie Brilliant Blue R-250 (Pharmacia, Uppsala, Sweden). The molecular weight of individual protein bands was determined using a GeneGenius densitometric image analysis system (Syngene, Cambridge, UK) with protein standards. In addition, the isoelectric points (pI) of the proteins were analyzed by isoelectric focusing under reducing conditions. RHE (0.5 mg) was solubilized in 500 μl of a rehydration buffer containing urea (7 M), thiourea (2 M), CHAPS (2%, w/v), IPG buffer pH 3–10 (2%, w/v), and a trace of Bromophenol Blue dye. The mixture was kept at room temperature for 1 h, and then centrifuged at 12,000*g* for 5 min. The aliquots of the sample solution were loaded onto IPG strips (pH 3–10, 13 cm; GE Healthcare, Uppsala, Sweden). The strips were allowed to rehydrate overnight in a reswelling tray. The IEF was carried out in a Multiphor II unit (GE Healthcare, Piscataway, NJ). Focusing was performed at 20 °C under the following conditions: 100 V (gradient over 1 min); 100 V (fixed for 120 min); 500 V (gradient over 1 min); 3500 V (gradient over 90 min); 3500 V (fixed for 6 h). After IEF, the IPG strips were stained with Coomassie Brilliant Blue R-250. Completely bleaching IPG strips, the position of band was measured with a ruler. The pI was determined according to the relative position of protein bands in the gel.

2.4. Catalytic activity of RHE

Rutin, at a final concentration of 1 mg/ml, was dissolved in ethanol and 100 μl of which was then mixed with 350 μl of deionized water and 50 μl of purified RHE (50 μg/ml). The mixture was incubated for 15 min at 37 °C, and the enzyme reaction was terminated by addition of 500 μl of methanol. After centrifugation, the mixture was subjected to HPLC using a C_{18} column (250 × 4.6 mm, i.d. 5 μm, Waters, Milford, MA, USA). Specific activity of RHE was calculated from the amount of quercetin released from the rutin as a substrate. In order to simply monitor the activity of RHE, enzyme reactions were kept at room temperature for 30 min until yellow precipitation completely took place. After 12,000*g* centrifugation, supernatant was removed and precipitation was dissolved in ethanol. The supernatant and precipitation were identified with HPLC.

2.5. Product analysis by HPLC

HPLC analysis was carried out at room temperature on a Waters 1525 system using a Waters 2487 photodiode array detector set at 360 nm. The products which were prepared as described above were dissolved in methanol. After centrifugation, 20 μl of the supernatant were injected onto a 250 × 4.6 mm Hypersil ODS-2 column, 5 μm particle size (Thermo Fisher Scientific, Walthan,

MA, USA). Mobile phase was methanol–0.4% phosphoric acid (50:50, v/v), flow rate 1 ml/min. At the same time, standards rutin and quercetin were used as controls. Prepared products were identified on the basis of their retention times in HPLC by direct chromatographic comparison with quercetin. Purity of the product was calculated according to the peak areas.

After centrifugation, the supernatant was subjected to HPLC. Mobile phase was methanol–0.4% phosphoric acid (50:50, v/v), flow rate 1 ml/min, injection volume 20 μl. At the same time, rutin and quercetin standards were used as positive controls. Products were identified on the basis of their retention times in HPLC by direct chromatographic comparison with standards. Purity of the product was estimated according to the peak areas.

2.6. Nuclear Magnetic Resonance spectroscopy (NMR)

Prepared product compounds were analyzed by ^{1}H NMR on a DRX–300 instrument (Bruker, Fallanden, Switzerland). Sample (10.0 mg) and quercetin standard (10.0 mg) were dissolved in 0.5 ml deuterated methanol in dimethyl sulphoxide (DMSO) for NMR analysis, and residual signals (2.30 ppm) used for internal standard. Chemical shifts and coupling constants were expressed in δ (ppm) and Hz, respectively.

2.7. LC-ESI-MS/MS analyses

Liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) is often used to identify phenolic compounds (Seeram, Lee, Scheuller, & Heber, 2006). The analysis was carried out using a Waters ACQUITY™ UPLC system. An ACQUITY UPLC™ BEH C_{18} column (50 × 2.1 mm, 1.7 mm, Waters Corp., Milford, MA, USA) was employed for separation, with the column temperature maintained at 35 °C. The gradient elution for UPLC analysis consisted of two solvent compositions: Solvent A: 1% acetonitrile in water (pH 3.0) and solvent B: 0.1% formic acid in acetonitrile. The constant gradient began with 20.0% eluent A. After establishing the final conditions for the chromatographic analysis of samples, the detector interface and mass spectrometer were systematically optimized to maximize the response for $[M–H]^-$ ion with detection in the multiple reaction monitoring (MRM) mode. Throughout the UPLC process, the flow rate was set at 0.3 ml/min and the run time was 5 min while the volume of injection was 10 μl.

A Waters TQDTM tandem quadropole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) interface was used for analytical detection. The ESI source was set in negative ionization mode. Quantification was performed using MRM of the transitions of *m/z* 301.187 → 151.109 for quercetin and the samples, with scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary voltage 3.5 kV, cone voltage 30 V, source temperature 120 °C and desolvation temperature 350 °C. Nitrogen was used as the desolvation gas with a flow rate of 60 L/h. All data collected in multi-channel analysis (MCA) mode were acquired and processed using MassLynx™ V 4.1 software with QuanLynx™ V 4.1 program (Waters Corp., Milford, MA, USA).

3. Results

3.1. Isolation of rutin hydrolysis enzyme (RHE)

Seed storage proteins are a set of proteins that accumulate to high levels in seeds during the late stages of seed development. They are usually classified into four groups: globulins, albumins, prolamins, and glutelins. We used different solutions to extract RHE, and found that RHE has the highest solubility in ACE buffer (without NaCl) when pH is 5.0. Based on this property, we speculate that RHE belongs to albumins.

The crude proteins, following dialysis in 20 mM Tris–HCl buffer (pH 7.0), were loaded onto a Resource™ Q anion exchange column and eluted as described in the Section 2. As shown in Fig. 2A, peak II, corresponding to RHE, was eluted between 34.0 and 43.0 ml by 1 M NaCl. Peak II has two major protein bands on SDS–PAGE with approximate molecular weights of 70 and 20 kDa (data not shown). To obtain pure protein and analyze its properties and function, peak II, collected from the anion exchange columns was further separated by size exclusion chromatography (SEC) on a Superdex™ 75 10/300 GL column with 20 mM Tris–HCl buffer at pH 7.0 (Fig. 2B). The protein eluted in the first peak (elution volume 11.0 ml) displayed catalytic activity to hydrolyze rutin, and SDS–PAGE revealed a highly purified single protein band (Fig. 3, lane 3) with an approximate molecular weight of 70 kDa. RHE was successfully purified 5.25 folds from the powder prepared from dried tartary buckwheat seeds, with a total recovery of 60% (Table 1). Isoelectric focusing was used to determine the pI of RHE. According to relative mobility of RHE in IPG strip, the pI of RHE was calculated to be 6.7. This is different than glycosidases from common buckwheat seeds (Bourbouze, Pratviel-Sosa, & Percheron, 1974), which indicates that these enzymes are not identical to RHE described here.

3.2. Activity of RHE

Rutin was used as a substrate for the activity analysis of RHE. Since the solubility of rutin and quercetin in water were very

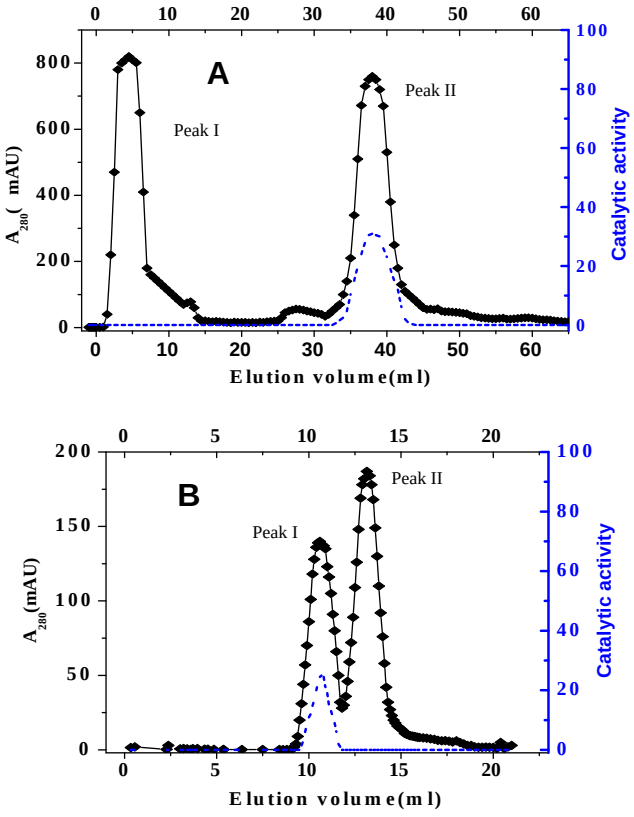

Fig. 2. Chromatographic characterization of RHE. (A) Anion exchange chromatography. Crude extracts were loaded onto a Resource Q anion exchange column (6.4 × 30 mm) pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.0) and were eluted using 1 M NaCl in the same buffer. Rutin as substrate is used to monitor RHE activity. (B) Size exclusion chromatography. Peak II recovered from the anion exchange column, which contained RHE activity, was loaded onto a Superdex™ 75 10/300 GL gel filtration column equilibrated with 20 mM Tris–HCl buffer (pH 7.0). The column was eluted with the same buffer at a constant flow rate of 0.5 ml/min.

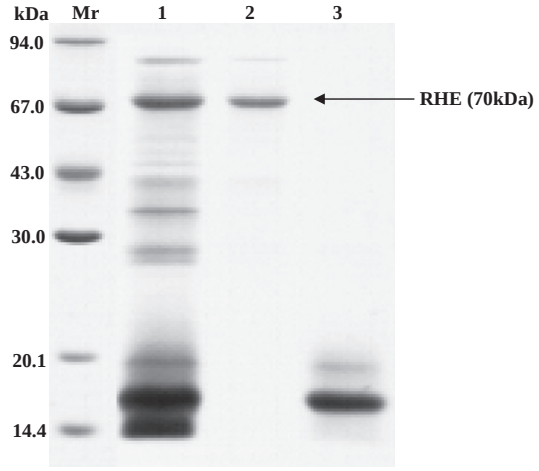

Fig. 3. SDS–PAGE analysis of RHE purified by gel filtration. A 12.5% solution of polyacrylamide gel (containing 1% sodium dodecyl sulphate) was used to separate the samples. Lane1 is molecular weight marker; lane 2 is peak I protein fraction purified by gel filtration (target protein); lane 3 is peak II from gel filtration.

different, we adopted a low-alcohol solution as a reaction system. In this system, we held the ethanol concentration at 20% because high concentrations of ethanol (>30%) would inhibit the activity of RHE. K_m value of RHE for rutin was calculated to be 1.04×10^{-3} M according to the peak areas obtained from HPLC.

Following the incubation of substrate rutin and purified RHE at 37 °C for 15 min, yellow precipitation was observed in the reaction system. The supernatant was removed by centrifugation at 12,000*g* for 10 min at 4 °C. The precipitate was dissolved in methanol and evaluated by HPLC (Fig. 4). Each sample of the supernatant and the precipitation was injected into HPLC system. The precipitate eluted at 21.8 min (Fig. 4D), similar to the quercetin standard (Fig. 4B), thereby confirming our hypothesis. On the other hand, the supernatant was eluted at 7.9 and 21.8 min, respectively, with very low absorbance (Fig. 4C); and both retention times in the supernatant were consistent with rutin and quercetin, as shown in Fig. 4A and B, respectively. This phenomenon suggests that RHE may effectively convert rutin to quercetin. The elution peak at 5.7 min may be the absorption peak of RHE. As shown in Fig. 4D, the RHE reaction product is of high purity. According to the HPLC peak areas (data not shown), the purity of the product is about 98%.

3.3. ^{1}H NMR spectroscopy and LC-ESI-MS/MS analysis

We employed ^{1}H NMR spectroscopy to elucidate the structure of the hydrolysis product obtained from RHE reaction. Quercetin has a simple ^{1}H NMR spectrum. ^{1}H NMR showed five phenolic hydroxyl groups (δ 12.5, 10.8, 9.58, 9.35, 9.29, 1H, s). ^{1}H NMR analysis of the hydrolysis product indicated chemical shifts in the region of δ 6.00 to 8.00 ppm, characteristics of flavone nucleus: 7.64 (1H, d, H-2), 7.53 (1H, dd, J = 8.6 Hz, H-6), 6.87 (1H, d, J = 8.6 Hz, H-5), 6.40 (1H, J = 2 Hz, H-8), 6.16 (1H, d, J = 2 Hz, H-6). These data coincide with those of the standard quercetin.

In order to further determine the structure of hydrolysates, LC-ESI-MS/MS was employed to determine the molecular structure of the RHE-hydrolyzed products. UPLC–MS/MS chromatograms of standard quercetin and the hydrolysis product are shown in Fig. 5A and B. The retention times for standard quercetin and the hydrolysis product were similar, approximately 3.70 and 3.82 min, respectively. Fig. 5C and D are characteristic ion scan spectra for quercetin and the hydrolysate. We found that some characteristic groups are present in both quercetin and the hydrolysate, such as 78.56 *m/z*, 97.29 *m/z*, 194.45 *m/z*. Combining these results with the ^{1}H NMR and HPLC data, it can be conclude that the hydrolysate by RHE is indeed quercetin.

4. Discussion

The importance of endogenous enzymes on the stability of active ingredients has rarely been investigated in the past. Endogenous enzymes may influence the quality and the nutritional value of foods. In the food industry, the appropriate processing technology is essential for food preservation.

We started our investigations by using different methods to analyze the contents of flavonoids in tartary buckwheat seeds and found a dramatic difference in the proportion of rutin and quercetin. Assuming that quercetin may be a hydrolyzing product of rutin, we aimed at the isolation of specific rutin hydrolysis enzyme (RHE). Using a 2-step purification method described here, we succeeded in isolating a RHE with a molecular weight of 70 kDa, which is responsible for rutin hydrolysis in 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 to RHE described here. On the other hand, isoelectric point determined here for RHE is in the range of those reported for other plant β-glucosidases (Schliemann, 1987).

2Rutin glucosidase activities have been reported in plant and microorganisms (Hay, Westlake, & Simpson, 1961; Kurusawa, Ikeda, & Egami, 1973; Narikawa, Hirofumi, & Takaaki, 2000; Yasuda & Nakagawa, 1994); different enzymes may be involved in the degradation or modification of genuine flavonol glycosides. In 1959, pioneer papers dealing with microbial aerobic degradation of flavonoids were published (Hattori & Noguchi 1959; Westlake, Talbot, Blakley, & Simpson, 1959). However, the enzymes which could lead to rutinose plus quercetin upon action on rutin are only Aspergillus enzyme and Penicillium enzyme (Hay et al., 1961; Narikawa et al., 2000). The purified Aspergillus enzyme was shown to be stable with an optimum pH of 5.6 .The substrate specificity of the enzyme was shown to be quite restricted. The K_m value for rutin substrate was found to be 7.3×10^{-5} M. During rutin hydrolysis a 1:1 ratio was found between the two products (quercetin and rutinose). Furthermore, the Penicillium enzyme, a β-rutinosidase in microorganisms, was purified from *Penicillium rugulosum* IFO 7242 (Narikawa et al., 2000), which can release the disaccharide rutinose from the flavonoid glycoside rutin. This enzyme had a molecular weight of 245 kDa, a very low optimum pH of 2.2, and had 3-glucosidase activity for catalyze rutin and isoquercitrin into quercitrin. The two kinds of enzymes were called rutinosidase. It is noteworthy

Table 1
Purification of RHE from tartary buckwheat seeds.

Stage	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg protein)	Recovery (%)	Purification (fold)
Crude extract	15.19	1035	68	100	1
Resource Q	5.03	828	164	80	2.41
Superdex G75	1.83	654	357	60	5.25

nkat is defined as the amount of enzyme required to raise the rate of reaction by 1 nmol/s under defined assay conditions.

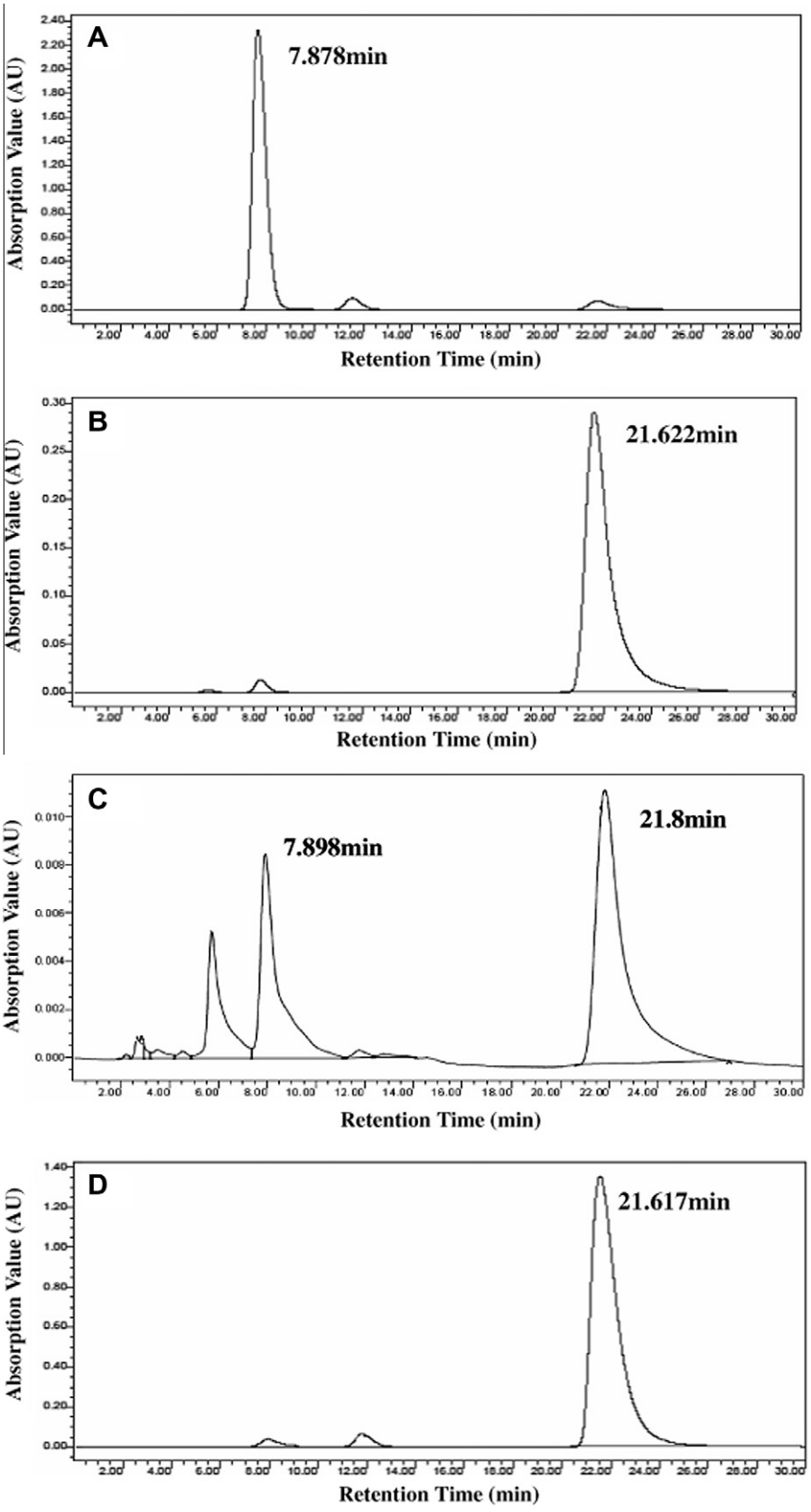

Fig. 4. HPLC chromatograms for quercetin and the hydrolyzate by RHE. (A) HPLC for rutin; (B) HPLC for quercetin; (C) HPLC for supernatant from reaction system after centrifugation; (D) HPLC for the hydrolyzate by RHE.

that rutinosidase is a quite specific enzyme discriminating both the sugar part and the flavonol aglycone part of its substrate (Sylvain, Pierre, & Gilles, 2010).

Rutin glucosidase in plant has been purified only from buckwheat seeds (Yasuda & Nakagawa, 1994; Baumgertel, Grimm, Eisenbeiß, & Kreis, 2003; Narikawa et al., 2000). By comparison,

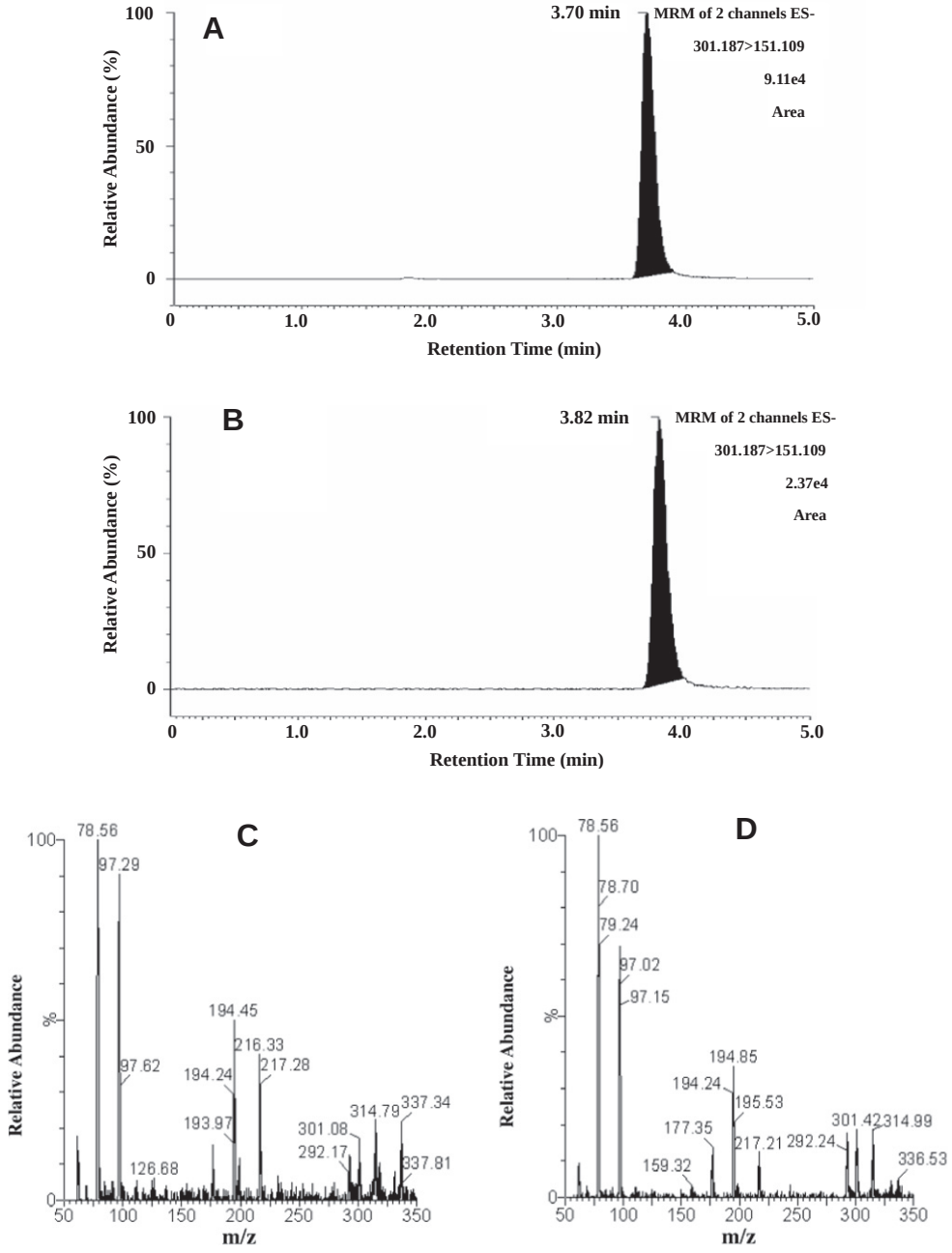

Fig. 5. LC-ESI-MS/MS chromatograms analysis of quercetin and the hydrolyzate by RHE in negative mode (301.187 > 151.109 *m/z*). (A) MRM chromatograms of quercetin. (B) MRM chromatograms of hydrolyzate by RHE. (C) Characteristic ions scan spectra for quercetin. (D) Characteristic ions scan spectra for hydrolysate by RHE.

we found that these glycosides described so far including rutin-degrading enzymes (RDE I and RDE II), flavonol-3-O-β-heterodisaccharide glycosidase (FHG I) and RHE reported here from tartary buckwheat share some characteristics. They have similar masses (about 70 kDa), and FHG I and RHE have similar isoelectric points (about 6.5) (Baumgertel et al., 2003; Yasuda & Nakagawa, 1994), which indicates that these enzymes are not identical to glycosidases from common buckwheat seeds (Bourbouze et al., 1974).

The cleavage of rutin into quercetin and rutinose is the first step in enzymatic rutin degradation (Surholt & Hosel, 1978). The aglycone released by this reaction may further be degraded by the so-called 'flavonol oxidases' (Barz & Koster, 1981) yielding products such as 3,4-dihydroxybenzoic acid and 2,4,5-trihydroxybenzoic acid. This process is harmful in the foods, because quercetin was further degraded and yielded phloroglucinol carboxylic acid and protocatechuic acid at various stages of a food processing process. However glycosidases responsible for the hydrolysis of secondary plant products have been shown to possess a high degree of specificity. Our research indicated that there are no other compounds present after RHE incubated with quercetin for a long time. This phenomenon suggests that RHE is a specific enzyme which hydrolyzes rutin to quercetin. RHE could be used in the preparation of quercetin from rutin, a process that was first explored by us and reported here.

To the best of our knowledge, so for, use of rutin hydrolysis enzyme in plants to prepare quercetin has not been reported. Here we explored the possibility of using rutin hydrolase in the preparation process of quercetin. Therefore, it can be concluded that this

enzymatic preparation method for quercetin is very useful to enhance the biological effect of rutin or quercetin and their incorporation into food products.

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