J Appl Biol Chem (2020) 63(3), 197–201
https://doi.org/10.3839/jabc.2020.027

Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Food Science

Viscozyme L aided flavonoid extraction and identification of quercetin from *Saururus chinensis* (Lour.) Baill

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Received: 24 June 2020 / Accepted: 25 July 2020 / Published Online: 30 September 2020

Abstract In order to enhance the extraction efficiency of flavonoid from *Saururus chinensis*, carbohydrate-hydrolyzing enzyme Viscozyme L aided extraction techniques have been studied. Then flavonoid composition, as well as quercetin, were also identified using UV/Vis, HPLC/MS, and ^{1}H-NMR. The results showed that favorable extraction conditions were Viscozyme L concentration of 0.25 mg/g, pH 4.2, reaction at 45 °C for 12 h. Under the favorable extraction condition, total flavonoid yield (37.9 mg/g) and quercetin yield (0.86 mg/g) increased by about 2.0 and 9.6 times, respectively, compared to control group. Interestingly, as a significant flavonoid of *S. chinensis*, flavonoid glycones rutin was hydrolyzed to aglycones quercetin by Viscozyme L. These findings provide scientific and theoretical support for the development quercetin-rich products, which was quickly absorbed by the human body than rutin.

Keywords Flavonoid · Identify · Quercetin · *Saururus chinensis* (Lour.) Baill · Viscozyme L

Introduction

Saururus Chinensis (Lour.) Baill is a perennial herb that mainly grows in eastern Asia, including Korea, China, and Japan [1-2]. Which has been reported to exert a variety of biological activities such as anti-allergic activity, anti-arteriosclerosis activity and anti-inflammatory activity due to the abundant polyphenols, such as flavan-3-ols, hydroxycinnamic acids, dihydrochalcones, and flavonoid [3-5]. Flavonoids are a large family of secondary plant metabolites, which present in plant tissues be covalently linked to polysaccharides via sugar residues esterified to polysaccharides by α-1,4 or β-1,4 linkage [6]. Quercetin, one of the most important flavonoid, which was most frequently found in fruits, vegetables, and other plant foods as conjugates in glycosylated forms as rutin [7-8]. According to a previous study on the metabolism flavonoid, quercetin was quickly absorbed by the human small intestine than rutin [9-10].

In recent years, to improve the extraction efficiency of flavonoids in plants, carbohydrate hydrolases are often introduced to release complex flavonoids from cell walls [11]. For instance, it is used to extract flavonoid aglycone from persimmon peel [12] and unripe apple [13].

In the present study, the carbohydrate-hydrolyzing enzyme, Viscozyme L aided extraction of flavonoids from *S. chinensis*, was examined. Besides, flavonoid composition, as well as quercetin, were also identified using UV/Vis, HPLC/MS, and ^{1}H-NMR.

Materials and Methods

Materials and reagent

S. chinensis (Lour.) Baill pieces were dried and powdered. Viscozyme L was obtained from Novozymes Co. (from Aspergillus aculeatus, 100 fungal β-glucanase units/mL, Bagsvaerd, Denmark), the Silica gel (40 μm), Octadesylsilane (50 μm), rutin, isoquercetrin, quercetrin, and quercetin were obtained from Sigma Co. (St. Louis, MO, USA). The other reagents were obtained from Duksan Co. (Seoul, Korea). HPLC/UVD (SPD-10A, Shimadzu, Co., Kyoto, Japan), Moderate pressure liquid chromatography (MPLC, Yamazen 540, Japan), HPLC/MS (Agilent Technologies, Palo Alto, CA, USA), NMR (Varian Inc., Palo Alto, CA, USA) were used in this study.

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Flavonoid analysis

The determination of flavonoid content was according to described by Jin et al. [14] and expressed in mg rutin equivalents per gram of sample (mg RE/g). The investigation of flavonoid composition was performed by a modification of the method of Zheng et al. [11].

Viscozyme L aided flavonoid extraction

To select an appropriate Viscozyme L aided quercetin extraction conditions, enzyme concentration, solution pH, reaction temperature, and time were studied. Briefly, for enzyme concentration, 1 g of *S. chinensis* powder was added at different concentrations of enzymes from 0.05 to 1 mg/g, with the enzyme hydrolyzed for 12 h in 45 °C at pH 4.0. For solution pH, the sample was added with 0.25 mg/g enzyme and hydrolyzed for 12 h at 45 °C at different enzyme hydrolysis buffer pH ranging from 3.5 to 5.8, which adjusted with 0.2 mol/L NaOH and 0.2 mol/L KH2PO4. For the preferred reaction temperature, the sample was added with 0.25 mg/g enzyme and hydrolyzed for 12 h, pH 4.0 at different temperatures ranging from 25 to 65 °C. For reaction time, sample was added with 0.25 mg/g enzyme and hydrolyzed at a different time from 6 to 48 h in 45 °C, pH 4.0. After the reaction, all the solution was extracted with 95% ethanol for 12 h at 80 °C and used to determine the quercetin, respectively.

Isolation and purification of quercetin

One hundred grams of dried *S. chinensis* powder with enzyme aided extracted at Viscozyme L concentration of 0.25 mg/g, reaction temperature 45 °C for 12 h, pH 4.2, and the crude solution was extracted with 95% ethanol for 12 h in 80 °C. Then, the crude solution was collected for flavonoid analysis.The crude solution was concentrated by rotary evaporator and isolated by partitioning extract first between ethyl acetate and aqueous phase according to its polarity for purification of quercetin. The ethyl acetate portion was further treated chromatographically, alternating between the Silica gel column (30×300 mm, i.d.) and ODS column (26×300 mm, i.d.). Then, the column was washed sequentially using the solvent systems and obtained sub-fractions 1-5. After checked with UV/Vis and HPLC, compound 2 was later rotary-evaporated, freeze-dried, and stored at −15 °C for structure analysis (Fig. 1).

Instrumental identification

The HPLC/MS analysis was carried out to identify the molecular weight of the isolated compound. An ODS column (Thermo Hypersil Gold, i.d.; 5 μM, 250×4.6 mm), mobile phase solvent A (acetic acid/H_2O), solvent B (acetic acid/acetonitrile/H_2O) were adopted. Mass spectra were simultaneously acquired using electrospray ionization in the negative ionization (NI) modes at fragmentation voltages (80 eV) for the mass range of 100-2000 amu (350 °C, 30 psi). ^{1}H NMR spectra were measured on a Varian model AMX-500 Spectrometer at 500 MHz. The isolated compound was soluted in CD3OD, which was previously contained the TMS as an internal standard. Chemical shifts were expressed in ppm relative to TMS, and coupling constants (J) are reported in Hertz (Hz).

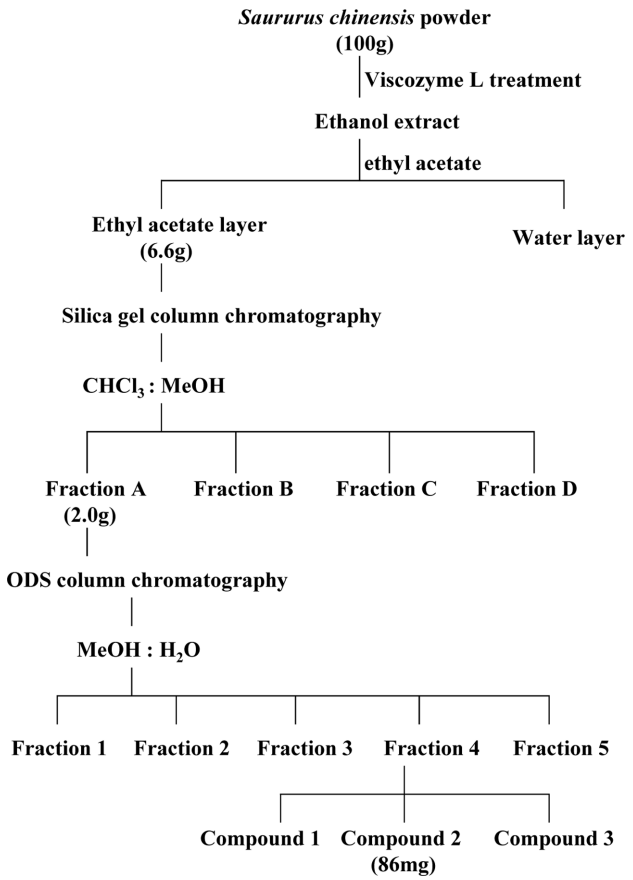

Fig. 1 Extraction and isolation schemes of flavonoid from *S. chinensis*

Results and Discussion

Effects of variables on flavonoid extraction

In general, the efficiency of the enzyme aided extraction of flavonoids from the plant materials was influenced by enzyme concentration, reaction temperature, time, and solution pH, etc. [13]. So, the effects of key variables were investigated for determining the best flavonoid extraction condition from *S. chinensis*. Fig. 2A showed the effects of the Viscozyme L concentration on flavonoid extraction. Flavonoid content was sharply increased at a low concentration of Viscozyme L treatments but maintained invariant at a high concentration of 0.25 mg/g. The reaction solution's pH affects enzyme activity, so it plays a vital role in cell wall hydrolysis and flavonoid extraction in plants [11]. Figure 2B showed that flavonoid contents sharply increased, peaked at the reaction solution pH of 4.2, and then decreased. The effects of the reaction temperature on the flavonoid extraction showed in Fig.

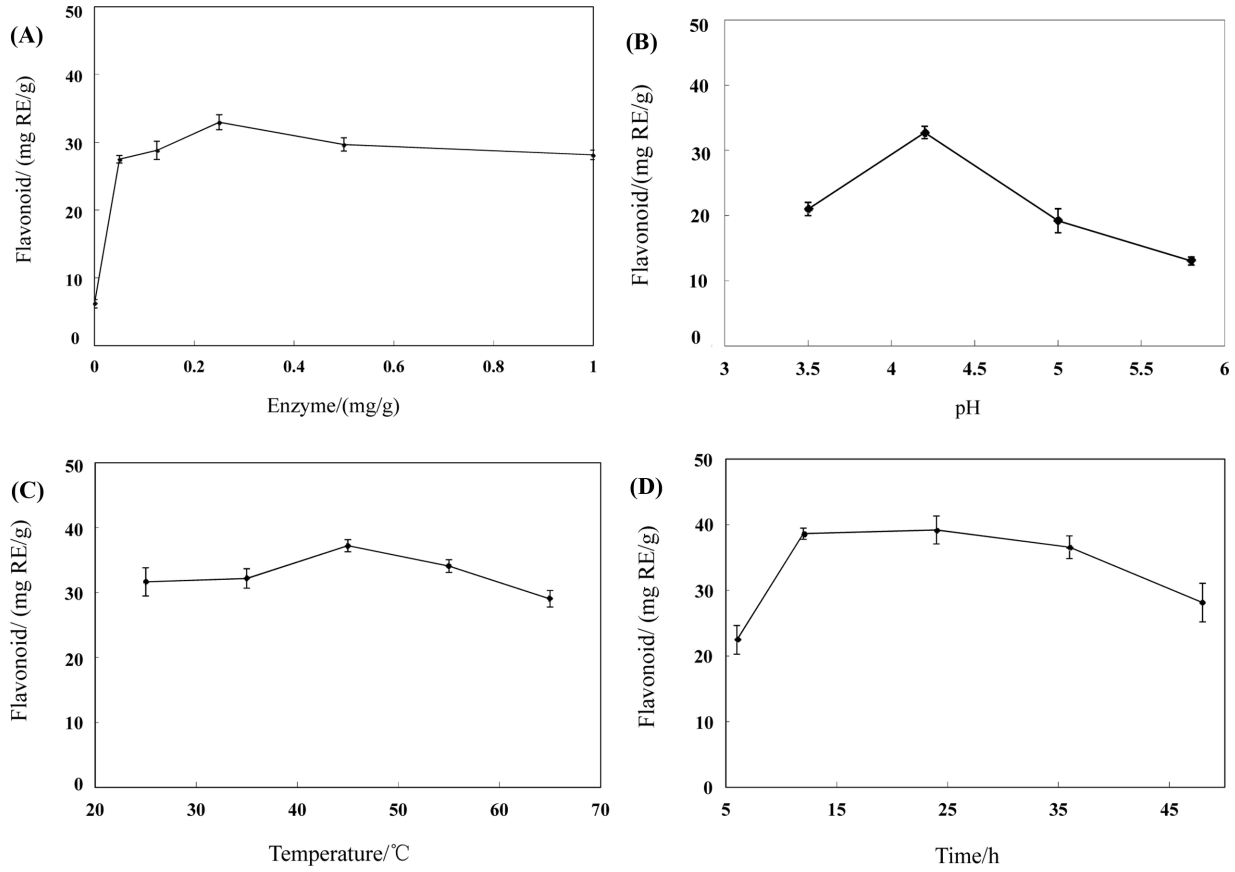

Fig. 2 Effects of (A) the concentration of Viscozyme L; (B) reaction solvent pH; (C) reaction temperature and (D) reaction time on the flavonoid extraction from *S. chinensis*.

2C. Flavonoid contents of enzyme treatments increased smoothly but maintained invariant or gradually decreased over-temperature around 45 °C. These results indicate that within a certain temperature range, with the increase of enzyme reaction temperature, it may be beneficial to increase in the enzyme activity, which could lead to an increase the decomposition activity. In addition, according to the previous research results, an excess decrease or increase of reaction temperature partly inhibited enzyme activity, thereby decreasing flavonoid extraction efficiency. Similar results were reported by Pinelo et al. [15]. They suggested that appropriately temperature may promote possible decomposition of flavonoid from the cell wall, while too high or low temperature had a significantly negative effect on the flavonoid extraction. The effects of the reaction time on flavonoid extraction are shown in Fig. 2D. Flavonoid contents increased sharply along with the reaction time. They reached a peak at 12 h of reaction and gradually decreased, which may be due to the further decomposition of flavonoids in excess reaction time, destroying the molecular structure of flavonoids. According to experiment results, Viscozyme L concentration of 0.25 mg/g, pH 4.2, reaction at 45 °C for 12 h were selected as the most favorable extraction condition for future purification and identification of flavonoid.

The extraction efficiency of flavonoid with Viscozyme L treatment

The composition of *S. chinensis* with Viscozyme L aided HPLC analyzed extraction. The four kinds of flavonoids, including rutin, isoquercitrin, queritrin, and quercetin were separated and their contents were detected. As the main flavonoids of *S. chinensis*, rutin and isoquercetin accounted for about 80% of total flavonoids [14,16]. However, the flavonoid composition of the *S. chinensis* in Viscozyme L treatment was significantly different compared to without enzyme treated control, which reflects the reduced rutin content associated with induced quercetin content. Quercetin content induced 9.6 times, whereas the rutin content reduced to 1/9 (Table 1 and Fig. 3). Interestingly, isoquercitrin and queritrin contents showed no significant change between with and without enzyme-treated samples. Rutin (3-*O*-rhamnosylglucoside) was the derivative compound of quercetin (Fig. 4), which is frequently detected in plants [6]. In this study, an increase of quercetin could be observed by Viscozyme L treatment. Means rutin, a major flavonoid of *S. chinensis* was hydrolyzed to quercetin by carbohydrate-hydrolyzing enzyme Viscozyme L. The similar results were reported by Zheng et al. [13]. They announced that chlorogenic acid was transformed to caffeic acid from unripe apple during

Table 1 Extraction efficiency of flavonoid with Viscozyme L treatment from *S. chinensis*

Flavonoid	Control	Viscozyme L
Total flavonoid Content (mg RE/g)	18.8±0.84	37.9±1.02
Rutin (mg/g)	0.95±0.07	0.10±0.03
Isoquercitrin (mg/g)	0.48±0.05	0.51±0.06
Queritrin (mg/g)	0.38±0.08	0.35±0.05
Quercetin (mg/g)	0.09±0.01	0.86±0.03

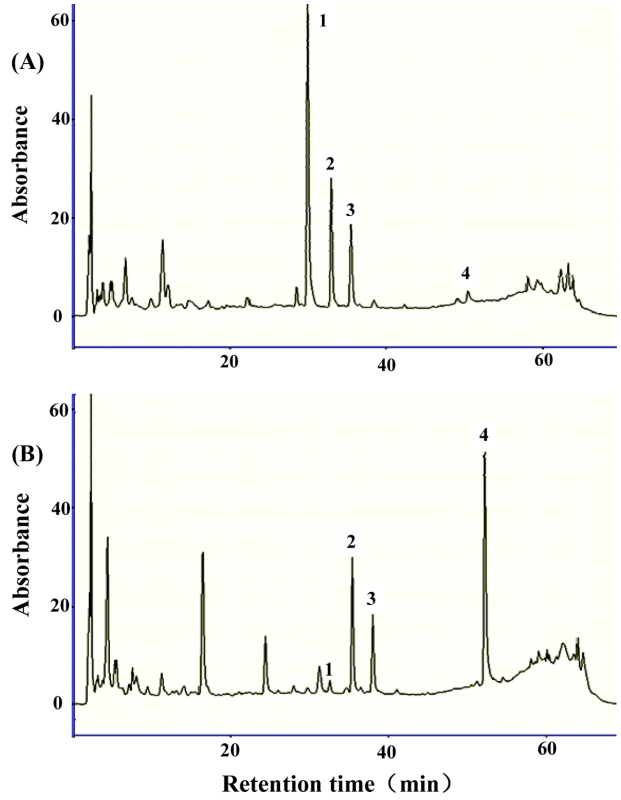

Fig. 3 HPLC chromatograms of *S. chinensis* extracted without (A) and with (B) Viscozyme L aided condition. 1:Rutin, 2: Isoquercitrin, 3: Queritrin, 4: Quercetin

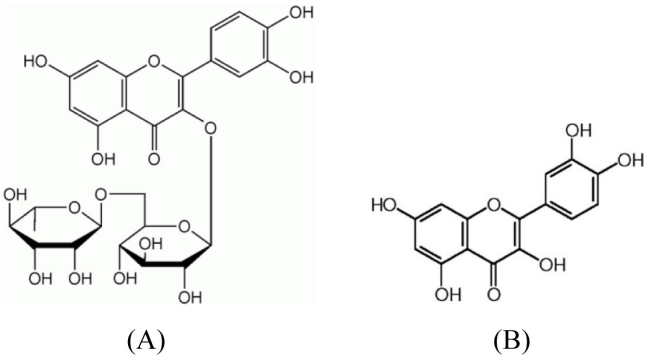

Fig. 4 The structure of rutin (A) and quercetin (B)

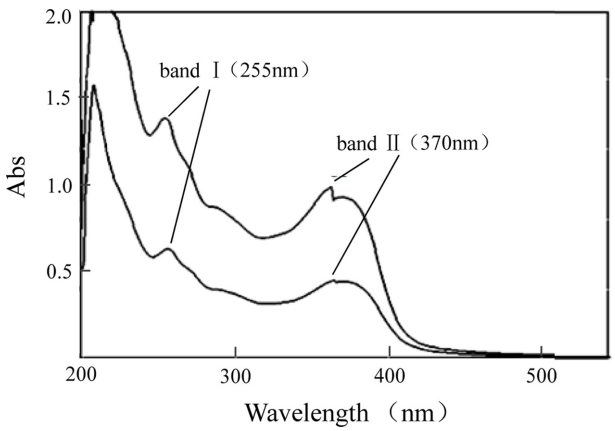

Fig. 5 UV-Vis absorption spectra of quercetin standard (up) and compound 2 (down)

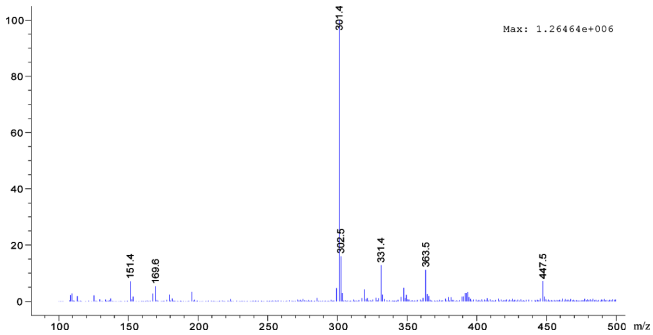

Fig. 6 LC/MS analysis of compound 2 isolated from *S. chinensis* extracted with Viscozyme L aided condition

carbohydrate-hydrolyzing enzyme extraction. Since then£¨caffeic acid has been widely recognized as an important indicator of lignin decomposition during enzymatic hydrolysis. Hence, quercetin also has been suggested as a marker to detect whether cleavage of rutin by carbohydrate-hydrolyzing enzymes has occurred in the plant cell wall.

Isolation and purification of quercetin

Isolation and purification of quercetin were carried out using MPLC, UV-Visible spectrophotometer. The Silica gel column fraction provides four fractions (Fraction A-D) according to UV absorbance, and then estimated fractions Fr.A was evaporated to dryness under vacuum for further fractionated by an ODS column chromatography using step-gradient elution of MeOH:H_2O (0:100-100:0), to yield five sub-fraction (Fraction 1-5), respectively. After removed the solvent by vacuum evaporation, compound 2 (Fig. 5) was identified by HPLC/MS, and ^{1}H-NMR analysis.

Identification of an isolated compound

To identify the chemical structure of compound 2, HPLC/MS, and

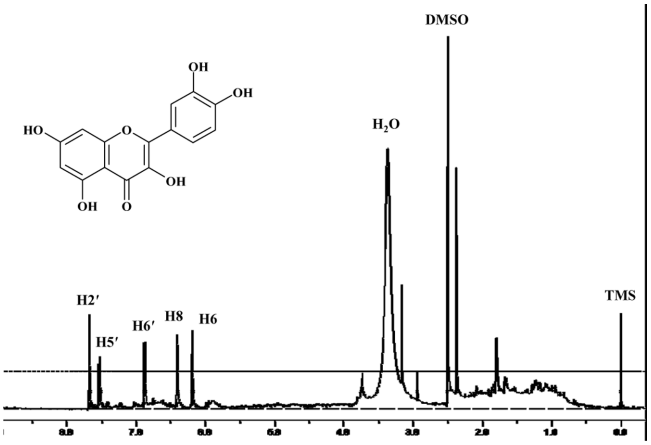

Fig 7 ^{1}H-NMR spectrum of compound 2 isolated from *S. chinensis* extracted with Viscozyme L aided condition

^{1}H NMR analyses were conducted. The MS spectrum mainly showed the ions corresponding to the deprotonated molecule $[M-H]^-$, which can provide the molecular weight of the compound. The MS spectra presented the ions corresponding to the sugar unit's cleavage or other easily broken units, which can provide the neutral loss information [1]. Compound 2 exhibited a fragment m/z 301 (Fig. 6). Considering this result and previous research [17-18], the molecular mass of compound 2 could be assumed as quercetin.

^{1}H-NMR spectrum of compound 2 shows the presence of meta-coupled aromatic protons at δ 6.187 (1H, d, J=2.1 Hz, H-6), δ 6.409 (1H, d, J=1.52 Hz, H-8), and ortho-coupled ABX type protons at signal δ 7.677 (1H, d, J=2.0 Hz, H-2'), δ 7.571 (1H, dd, J=2.0, 8.4 Hz, H-6'), δ 6.885 (1H, d, J=8.4 Hz, H-5') on the A-ring and B-ring of the skeleton, respectively (Fig. 7). Based on the considering chemical shift of proton and coupling constant, compared with the literature data [19-20], compound 2 was identified as quercetin.

Author's contributions HZZ and SKC conceived and designed the experiments. And both of them performed most of the experiments and wrote the manuscript. SKC revised and edited the manuscript and supervised the work. Both authors have read and approved the final manuscript.

Competing interests The authors declare that they have no competing interests.

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