In Vivo Pharmacokinetics of Hesperidin Are Affected by Treatment with Glucosidase-like BglA Protein Isolated from Yeasts

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Hesperidin is an abundant flavanone glycoside in citrus fruits and has been reported to possess a wide range of biological activities. However, hesperidin has poor bioavailability. Here, we tested the hypothesis that hesperetin found in chenpi will have a better bioavailability than hesperidin and that treatment of hesperidin with the glucosidase-like yeast Bg1A protein will increase its bioavailability. The results indicate that hesperidin in pure or extract form is hydrolyzed by BglA protein extracted from *Sporobolomyces singularis* or expressed in *Escherichia coli* BL21 (DE3). This biotransformation affected the plasma pharmacokinetics of total hesperetin in rats, in that the plasma T_{max} was significantly shorter after administration of BglA protein-treated hesperidin than after administration of hesperidin extract. In addition, the area under the curve values for total hesperetin after administration of Bg1A-treated hesperidin were ~4-fold higher by oral administration and 3-fold higher by intravenous administration, respectively. In contrast, the plasma clearance value and volume of distribution after administration of Bg1A-treated hesperidin extract or pure hesperetin were significantly smaller than after administration of untreated hesperidin extract or pure hesperetin. This is the first study that systemically determines the absolute bioavailability of hesperidin and hesperetin simultaneously, shows clearly that hesperetin is more bioavailable than hesperidin regardless of the route of administration, and shows that prior transformation of hesperidin to hesperetin via fermentation should significantly increase its bioavailability because of the action of the yeast glycosidase-like protein BglA.

KEYWORDS: Hesperidin; chenpi; biotransformation; glycosidase; pharmacokinetics; bioavailability

INTRODUCTION

Hesperidin is an abundant flavanone glycoside found in citrus fruits and is often consumed in the form of orange juice (*1*). Hesperidin has been reported to possess anti-inflammatory, antioxidant, and regulation activities of hepatic cholesterol synthesis (*2–4*). Other reported activities of these flavonoids include lowering cancer risk and inhibiting bone resorption (*5–7*). However, hesperidin, like many other flavonoids, has a poor bioavailability (*8–12*). The reasons for this poor bioavailability include the low water solubility of hesperidin and disposition via phase II enzymes (*13, 14*). Additionally, glycosides of hesperetin have been shown to be effluxed in Caco-2 cells (*15*), consistent with observations that many flavonoid glycosides are effluxed by enterocytes (*16–18*). The bioavailability is also highly variable, perhaps because of interindividual differences

in human intestinal microflora that are capable of hydrolyzing hesperidin (*19, 20*).

In Western countries, hesperidin represents a major flavonoid consumed daily, and some estimates suggest that it could even be responsible for up to 50% of the daily dietary intake of flavonoids in a few developed countries (*5*). Hesperidin is a major active ingredient in the Chinese traditional medicinal herb called chenpi, which is made from tangerine peel and has been used for more than 10 centuries. Chenpi also is used in the traditional medicines of other Asian countries such as Japan, where it is called chinpi (*13*). Chenpi has expectorant activities and a broncho-dilative effect and is usually used to treat upper respiratory tract infections using traditional Chinese medicinal formulae. Chenpi is prescribed as an herbal medicine for inflammation, allergies, and hepatopathy in Japan (*13*). Chenpi is prepared using a well-documented and ancient nine-step fermentation procedure because chenpi so produced appears to have a greater bioactivity than air- or sun-dried tangerine peel. In preliminary studies, we isolated a yeast strain from chenpi

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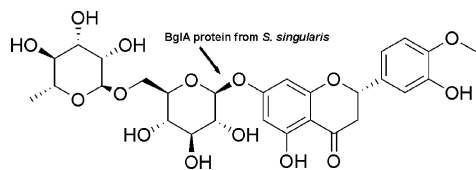‡ University of Houston.

Figure 1. Structure of hesperidin and proposed site of hydrolysis by the BglA protein.

and identified it to be a yeast strain closely related to *Sporobolomyces singularis*. This strain has glycosidase activities that are similar to other yeast strains identified previously to produce a glycosidase-like BglA protein (*21, 22*).

We were interested in determining the presence of BglA in *S. singularis* because this glucosidase-like protein, an enzyme isoform belonging to family 1 glycosyl hydrolases, can hydrolyze hesperidin abundant in citrus peels to hesperetin. Since hesperetin is absorbed much more quickly than hesperidin (*15*), we hypothesized that ancient Chinese fermentation methods increase the bioavailability of chenpi by removing the glycosides and thereby increasing the bioavailability of hesperetin (**Figure 1**). Testing this hypothesis allowed us to provide a mechanistic explanation as to why fermentation-prepared chenpi may have higher activities, which until now has been unclear. To test this hypothesis, we biotransformed hesperidin extract with BglA protein extracted from *S. singularis* or expressed in *Escherichia coli* BL21 (DE3) in vitro. We also determined how the BglA protein influenced the plasma pharmacokinetics of total hesperetin in rats after administration of BglA protein-treated or untreated hesperidin extracts. For comparison purposes, pharmacokinetic studies also were conducted using pure hesperetin and its glycoside hesperidin.

MATERIALS AND METHODS

Materials. Chenpi was obtained from a local drugstore. Standard hesperidin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Standard hesperetin, β-glucuronidase type VII-A, *H*5,β-glucosidase, and *p*-nitrophenyl-β-D-glucopyranoside (PNP-Glc) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical or HPLC grade. *S. singularis* (ATCC 24193) was obtained from ATCC (Manassas, VA) and cultured in YM medium containing 3 g of yeast extract, 3 g of malt extract, 5 g of peptone, 10 g of dextrose, and 20 g of agar in 1.0 L of distilled water (pH 5.0) and was maintained aerobically at 24 °C.

Extraction of Hesperidin from Chenpi. The hesperidin was extracted from chenpi with an ultrasonic method adapted from a published report (*23*). Briefly, 4 g of chenpi was ground to 0.5 to ∼1 mm, blended with 200 mL of methanol, and placed in an ultrasonic machine (KQ-100A, Kunming, China) running at 60 mHz for 60 min at 40 °C. After initial extraction, the solvent was recovered and then replaced with fresh methanol, and the process was then repeated. At the end of the second extraction procedure, methanol was then separated from the chenpi and then combined with the first methanol extract solution. The combined extract solution was filtered, and the filtrate was evaporated to dryness. The residues were again dissolved in medium at a maximum of 5% (v/v) propylene glycol with a concentration of 10 mg/mL. Then, the solutions were acidified with 0.1 mL of 0.01 M oxalic acid and quantified by HPLC.

Extraction and Purification of BglA Protein from *S. singularis*. The *S. singularis* yeast cells in midexponential phase were harvested and centrifuged (2000*g*, 5 min). The pellet was washed twice with 50 mM sodium phosphate buffer (pH 7.0), and cells were resuspended in 50 mM phosphate citrate buffer (pH 4.0) and treated with 1% (w/v) Usukizyme (Kyowa, Japan) for 2 h at 37 °C. The soluble fraction of lysate was mixed with the supernatant prepared by homogenization to give a crude extract. The purification steps were carried out according to previous reports (*24*). Briefly, ammonium sulfate was added to the

cell extracts and then centrifuged (10 000*g*, 30 min). The pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.0) and loaded onto a DEAE sepharose CL-6B column (50 mm × 200 mm, Pharmacia). Further purification was performed by gel filtration on a sephacryl S-300 HR column (15 mm × 900 mm, Pharmacia). The eluent was 50 mM sodium phosphate buffer containing 0.1 M NaCl (pH 7.2). The β-glucosidase activity of the eluted fractions was measured as described later.

Cloning, Expression, and Purification of BglA Gene in *E. coli* BL21 (DE3). *S. singularis* was harvested, and the total RNA was extracted with TRIzol reagent (Invitrogen). PCR was performed using the following procedure: initial denaturation, 94 °C for 2 min; 30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min; and final extension at 72 °C for 5 min. PCR products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV light. A pair of primers (5′-CGC GGATCC ATGATGCTGCATGCGGCAC-3′ (forward primer) and 5′-CCC AAGCTT TCAGAGGTGGTTGCGACC-3′′ (backward primer)) were designed and synthesized with BamH I and Hind III restriction sites added to the primers (underlined). The PCR product was digested with BamH I and Hind III. The digested fragments were purified by gel extraction and then ligated to the pQE30-Xa vector (Qiagen) to generate the recombinant expression vector, pQE30-Xa-*BglA*. The ligated sequence was then confirmed by sequencing (not shown).

Subsequently, pQE30-Xa-*BglA* was used to transfect the *E. coli* BL21 (DE3) strain. Briefly, a single transfected colony was picked from a selective plate and inoculated into LB medium supplemented with ampicillin (200 μg/mL). When the OD$_{600}$ of the cultured media reached 0.6, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the media at a final media concentration of 1 mM. After an additional 4 h of incubation, a portion of the bacteria was suspended in 200 μL of the sample buffer and boiled for 5 min at 90 °C. The expression of BglA was detected by SDS-PAGE, carried out according to the method of Laemmli (*25*). Proteins were stained with the Bio-Safe Coomassie G250 stain (Amresco) according to manufacturer's instructions. The remaining bacteria were harvested and washed twice with 50 mM potassium phosphate buffer (pH 7.0) to pellet the bacteria using centrifugation (4000*g*, 20 min).

To purify the BglA protein form the bacteria, each gram of cell pellet was resuspended in 3 mL of TE buffer. Following the addition of lysozyme to a final concentration of 0.2 mg/mL, the cell suspension was incubated for 20 min at 37 °C with gentle shaking. Then cell extracts were obtained by centrifugation (12 000*g*, 30 min), and the β-glucosidase activity of the crude BglA protein was measured as described next. An additional purification step, as described previously using various columns, was needed to further purify the BglA protein from bacteria to higher specific activities.

Assay for β-Glucosidase Activity of BglA Protein. The β-glucosidase activity was measured using PNP-Glc as the substrate (*24*). The incubation mixture (total volume of 1 mL) contained 1.5 mM PNP-Glc, 50 mM phosphate buffer (pH 6.0), and a 0.1 mL solution of crude or purified enzyme preparation. The reaction was carried out at 37 °C for 4 h and then terminated by adding 4 mL of 0.25 M Na$_2$CO$_3$. The absorbance at 420 nm, subtracting the blank, was used as a measure of the amount of *p*-nitrophenol (PNP) released. One unit of β-glucosidase activity was defined as the amount of enzyme that hydrolyzes 1 μmol of PNP-Glc per minute under the assay conditions.

In Vitro Biotransformation of Hesperidin by BglA Protein. Each reaction mixture (total of 1 mL) containing 0.5 μM hesperidin, 50 mM phosphate citrate buffer (pH 6.0), and 0.01 mg of enzyme equivalent of BglA protein was either extracted from *S. singularis* or expressed in *E. coli* BL21 (DE3). The mixture without enzyme was preincubated at 40 °C for 5 min, and the reaction was started by the addition of enzyme. After incubation at 40 °C for 30 min, the reaction was stopped by boiling for 5 min. Another two mixtures were incubated under the same conditions, which were the positive control of β-glucosidase and the mock control. Hhesperidin and hesperetin in the reaction mixture were analyzed by HPLC. Samples removed from the reaction mixture for HPLC application were centrifuged (3000*g*, 20 min) and extracted with 500 μL of methanol from the supernatant. The organic layer was transferred to a microcentrifuge tube and dried under vacuum. The
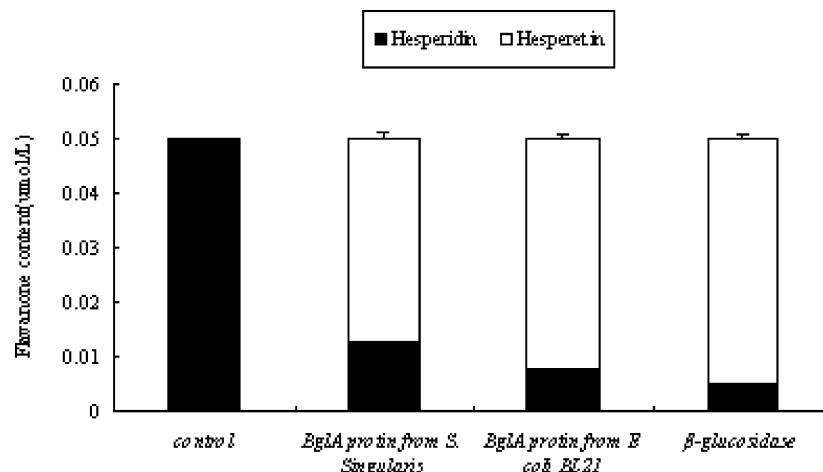
Figure 2. Metabolism of hesperidin by BglA protein and β-glucosidase. The experiments were performed using 0.5 μmol/L of hesperidin and 0.01 mg of enzyme equivalent prepared from yeast or expressed in *E. coli* (DE3) using procedures described in the Materials and Methods.
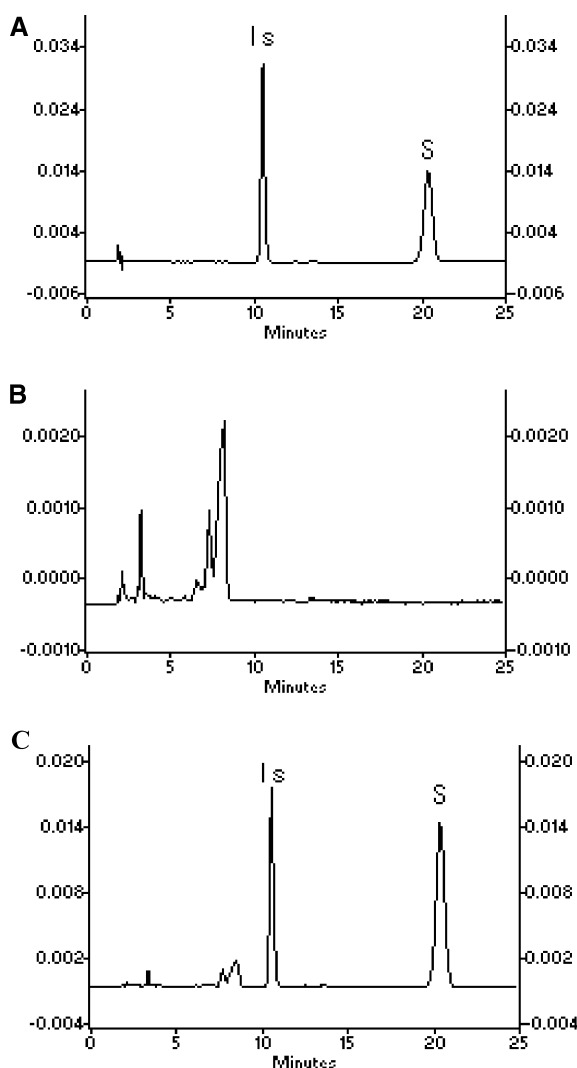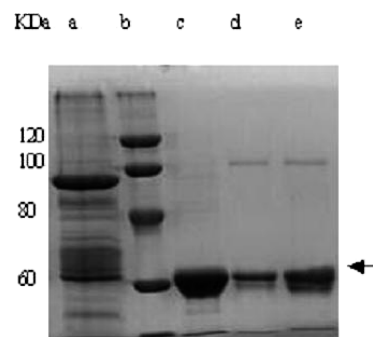

Figure 4. SDS-PAGE of BglA protein extracted from *E. coli* BL21 (DE3) and undergoing various steps of purification. Lane a: bacteria cell extract; lane b: molecular mass standards; lane c: (NH$_4$)$_2$SO$_4$ precipitate (55−60% saturation); lane d: pooled DEAE sepharose fractions; and lane e: pooled sephacryl fractions. The narrow band indicates the BglA protein.

Table 1. Purification of BglA Protein Expressed in *E. coli* BL21 (DE3)

step	total activity (U)	total protein (mg)	specific activity (U/mg protein)[a]	purification (-fold)	recovery (%)
crude extract	216.0	772.0	0.3	1.0	100.0
ammonium sulfate	49.2	19.6	2.5	8.4	22.8
DEAE sepharose	25.6	8.5	3.0	10.0	11.9
sephacryl	16.2	2.8	5.8	19.3	7.5

[a] One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1 μmol of PNP-Glc per min under assay conditions.

Figure 3. Representative HPLC chromatograms of (**A**) standards of hesperetin and hesperidin, (**B**) blank rat plasma, and (**C**) 0.02 μmol/L of hesperetin and 0.02 μmol/L of hesperidin in rat plasma.

residue was reconstituted with 100 μL of methanol and centrifuged (3000g, 10 min). Then, the supernatant was transferred for HPLC analysis. Experiments were carried out in triplicate for each experiment.

HPLC Assays of Hesperidin and Hesperetin. The concentrations of hesperidin and hesperetin were analyzed by HPLC as follows: system, Shimadzu LC-6 with variable wavelength detector; column, YWG-C$_{18}$ (4.6 mm × 150 mm, 5 μm, Shimadzu); column temperature, 30 °C; injection volume, 20 μL; detector wavelength, 283 nm; flow rate, 1 mL/min; and mobile phase, 40:60 (v/v) mixture of methanol and 0.04 M KH$_2$PO$_4$ (pH adjusted to 4.0 using H$_3$PO$_4$). Quantification of hesperidin and hesperetin peaks from all matrices was based on the standard curves. For the analysis of hesperetin and hesperidin, standard solutions containing 0.00, 0.002, 0.005, 0.02, 0.05, 0.2, 0.5, and 1 μmol of added hesperetin/liter or hesperidin/liter were prepared, respectively. The standard curve was obtained by plotting the peak height of standards versus hesperetin and hesperidin concentrations. Day-to-day variation (CV %) for hesperetin and hesperidin from all matrices was 11%, and within-day variation for them was 7%. The limits of detection for hesperetin and hesperidin from plasma were 1.5 and 3.5 nmol/L, respectively. The linear response ranges of the method for hesperetin and hesperidin were 1.5 to ~1000 and 3.5 to ~1000 nmol/mL, respectively. The equations for linear responses, described by the relationship between peak height (y) and concentration (x), were y

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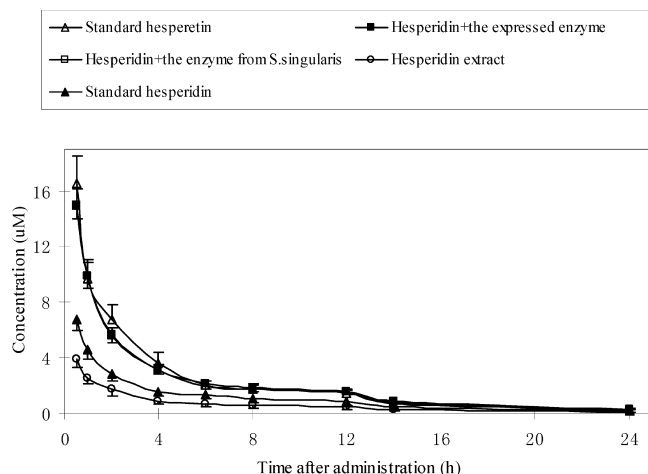*J. Agric. Food Chem.,* Vol. 56, No. 14, 2008   **5553**

Figure 5. Changes in plasma concentration of total hesperetin in rats following IV administration. The rats were divided into five groups, and each rat was administered intravenously a 31 μmol/kg equivalent amount of hesperidin. Plasma samples were treated with β-glucuronidase, and therefore, the plasma levels represent the total hesperetin equivalent. Values were presented as mean $\pm$ SEM ($n = 5$).

$= 0.1676x - 0.0092$ ($r^2 = 0.998$) for hesperidin and $y = 0.1083x - 0.0065$ ($r^2 = 0.998$) for hesperetin, respectively. Recoveries of these compounds by this method were <95%.

Animals and Study Design. Male Wistar rats (6 weeks old, 180 to ~220 g, Beijing Administration Office of Laboratory Animal, Beijing, China; certification number SCXK-Military-2002) were housed in an air-conditioned room (23 $\pm$ 2 °C) under a 12 h light−dark cycle for a 1 week period of acclimatization. Rats fasted overnight before the experiments. The animals were handled according to the Guidelines for the Care and Use of Laboratory Animals, and the experiments were conducted at Tianjin Medical University.

Before the experiment started, rats were weighed and divided by weight in 10 groups as follows: in test group 1 (test 1), rats were gavaged with purified hesperidin; in test 2, rats were gavaged with purified hesperetin; in test 3, rats were gavaged with the untreated hesperidin extracts; in test 4, rats were gavaged with hesperidin extract treated with expressed BglA protein; and in test 5, rats were gavaged with hesperidin treated with BglA protein isolated from *S. singularis*. The other five tests (5 rats per test) were carried out using hesperidin or hesperetin administered intravenously with the chemicals in the same order as stated previously. For both oral and IV administration, 18.9 mg/kg of hesperidin or 9.4 mg/kg of hesperetin was administered as an aqueous isotonic solution at a maximum of 5% (v/v) propylene glycol. The dose of flavonoids given was based on recommended amounts of chenpi given to people by the Chinese Pharmacopoeia. In cases of oral administration, blood samples were withdrawn from the jugular artery cannula before and at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, and 24 h after flavonoid administration. Before the flavonoid solution can be used intravenously, the solution is processed to minimize potential hazards to the rats. First, the flavonoid solution was carefully chilled (4 °C), filtered, and centrifuged (10 000*g*, 2 min) to remove any particulate matter. After the solution was shown to be nonhemolytic, it was autoclaved at 121 °C, cooled, and then used for intravenous administration. Similar to oral administration, the plasma samples were collected before intravenous administration and at 0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h after IV administration. Plasma samples were prepared by centrifugation (10 000*g*, 2 min), and the plasma was removed and stored at −20 °C within 30 min after a blood sample was taken.

The plasma samples after administration were used for the quantification of total hesperetin, after the metabolites were released by hydrolysis with β-glucuronidase. Plasma samples (50 μL) were incubated with appropriate amounts of β-glucuronidase type H-5 (500 units/mL) in 0.1 mM sodium acetate buffer (pH 5.0) at 37 °C for 90 min. To stop the reaction of enzyme, 0.01 M oxalic acid (100 μL) was added to the reaction mixture. Then, the mixture was extracted with 500 μL of methanol by vortexing the mixture for 2 min and

centrifugation (3000*g*, 10 min). The supernatant layer was transferred to a microcentrifuge tube and vacuum-dried. The residue was reconstituted with 100 μL of methanol, vortexed, and then centrifuged (3000*g*, 10 min) to produce samples for HPLC analysis.

Calculation of Pharmacokinetic Indexes and Statistical Analysis. Pharmacokinetic analysis of the data was performed using WinNonlin (Pharsight) (version 5.2 Build 200701231637). The pharmacokinetic parameters were derived using noncompartmental analysis and a linear trapezoidal (Linear Interpolation) method, and other pharmacokinetic parameters were determined using standard equalizers. Each rat plasma concentration versus time course was analyzed individually. The statistical analysis of the pharmacokinetic parameters was achieved using one-way ANOVA with post hoc analysis. A statistically significant difference was set at 5% or $p > 0.05$.

RESULTS

Hydrolysis Hesperidin by BglA Protein in Vitro. BglA protein, whether isolated from *S. singularis* or expressed in *E. coli* BL21 (DE3), was capable of hydrolyzing hesperidin exclusively to hesperetin (**Figure 2**), as hesperetin was not detected in a mock mixture free of it. In BglA protein-treated hesperidin extracts, the hesperetin concentration was nearly similar to that treated with commercial β-glucosidase ($p > 0.05$) (not shown). In addition, the source of the BglA protein did not affect their activities as two different sources of BglA protein had nearly identical activities (not shown). Lastly, the identity of hesperidin was verified by being compared to an authentic standard using HPLC (as shown in **Figure 3**) since HPLC clearly separated hesperetin ($t_R = 10.77$ min) from hesperidin ($t_R = 20.38$ min).

Identification of BglA expressed in *E. coli*. SDS-PAGE analysis of the cell extract of *E. coli* BL21 (DE3) transfected with the BglA gene revealed a predominant protein band of ~66 kDa (**Figure 4**), which was in close agreement to the expected molecular mass of 65.67 kDa deduced from the amino acid sequence of the *BglA* gene. This predominant protein band was not detected in the cell extract from *E. coli* BL21 (DE3) not transfected with BglA plasmid. Lastly, the BglA protein activity in transfected *E. coli* BL21 (DE3) was approximately 100-fold higher than that in cell extracts from *S. singularis* (0.3 U mg of protein^{-1} vs 0.0027 U mg of protein^{-1}).

The BglA protein was enriched as described previously (in the Materials and Methods), and a summary of the purification procedure of the BglA protein from transfected *E. coli* BL21 (DE3) along with each step's yield is presented in **Table 1**. The heterologously expressed BglA protein was purified by ammonium sulfate fractionation and column chromatography with an (overall) 19.3-fold enrichment, and the recovery yield was 7.5%.

Effects of BglA Protein Treatment on in Vivo Pharmacokinetics of Total Hesperetin in Rats after IV Administration. We first conducted parallel IV pharmacokinetic studies of hesperetin and hesperidin because it had not been performed in the same laboratory. Previously, pharmacokinetic studies of hesperetin or hesperidin were conducted separately, and the major chemical species found in human plasma were glucuronide conjugates, while the sulfates or sulfate and glucuronide conjugates were only detected in minor quantities (*8, 11*). This was confirmed by another study in which free hesperetin or hesperidin was not detected in the rat plasma after administration of hesperidin not treated with β-glucuronidase (*13*). On the basis of this literature and the fact that hesperetin glucuronides are not commercially available, plasma samples from the present study also were treated with β-glucuronidase to release aglycone hesperetin. Our experiment could not quantify the hesperetin conjugates due to lack of standards; therefore, we termed

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Table 2. Pharmacokinetic Parameters of Total Hesperetin after Single IV Administration of Hesperidin or Hesperetin or Hesperidin Treated with Different BlgA Protein Treatments

parameters	pure hesperidin	pure hesperetin	hesperidin extract untreated	hesperidin extract + BlgA from *E. coli*	hesperidin extract + BlgA from yeast
AUC$_{obsd}$ av (nmol h/mL) SD	30.09	61.47[a]	18.01	59.40[b]	57.90[b]
	12.88	22.20	10.86	14.68	11.73
half-life av (h) SD	6.28	6.52	6.25	6.05	6.15
	0.28	0.61	0.81	1.01	0.32
V_z av (mL) SD	10.73	5.40[a]	19.27	4.70[b]	4.88[b]
	4.34	2.42	7.44	0.47	0.82
CL av (mL/h) SD	1.17	0.56[a]	2.10	0.54[b]	0.55[b]
	0.43	0.22	0.81	0.11	0.09

[a] There are statistically significant differences between AUC$_{obsd}$ for pure hesperidin and pure hesperetin ($p < 0.05$). [b] There are statistically significant differences between AUC$_{obsd}$, V_z, and CL for hesperidin with different BlgA protein treatment and untreated hesperidin extract ($p > 0.05$).
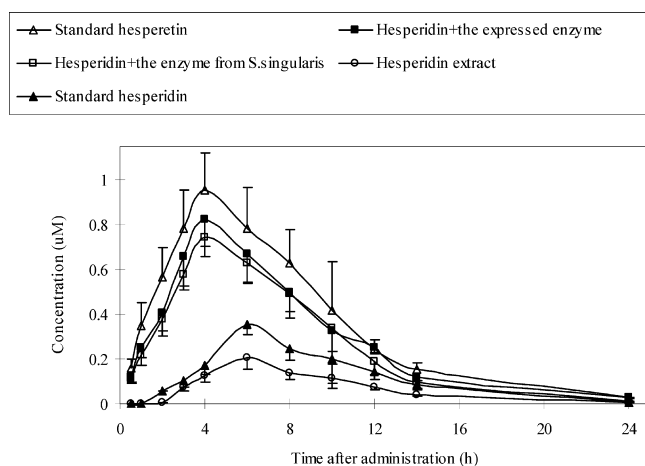

Figure 6. Changes in plasma concentration of total hesperetin in rats following oral administration. The rats were divided into five groups, and each rat was administered orally 31 μmol/kg equivalent amount of hesperidin. Plasma samples were treated with β-glucuronidase, and therefore, the plasma levels represent the total hesperetin equivalent. Values were presented as mean ± SEM ($n = 5$).

hesperetin released with β-glucuronidase from all species of hesperetin conjugates as total hesperetin. We quantified the plasma concentration of total hesperetin as well.

Analysis of plasma concentration−time curves of total hesperetin (shown in **Figure 5**) following IV administration indicated that pure hesperetin had a much larger area under curve (AUC) value than pure hesperidin and that the relative bioavailability of hesperetin doubled that of hesperidin (61.5 nmol h/mL vs 30.1 nmol h/mL) (**Table 2**). This large AUC value appears to be consistent with a slower clearance of hesperetin when compared to hesperidin (1.17 mL/h vs 0.56 mL/h). However, we also see that hesperetin has a much smaller volume of distribution, perhaps due to its tight binding to the plasma protein when compared to hesperidin. Several other important pharmacokinetic parameters of pure compounds are shown in **Table 2**.

In addition to pure compounds, we also injected purified hesperidin extracts with or without BlgA protein treatment, which hydrolyzed hesperidin to hesperetin. As expected, the hesperidin extract without BglA treatment behaved just like hesperidin shown earlier, whereas that treated with BglA protein behaved almost like pure hesperetin. The only exception was that the untreated hesperidin extract had an even smaller AUC value, faster clearance, and larger volume of distribution than pure hesperidin, suggesting that the herbal mixture contained additional phytochemicals that may interact with hesperidin to moderately change its pharmacokinetic parameters.

Effects of BglA Protein Treatment on in Vivo Pharmacokinetics of Total Hesperetin in Rats after Oral Administration. Hesperetin or hesperidin was absorbed by rats from all treatment groups. The corresponding times to reach maximum plasma concentration (T_{max}) in rats were significantly shorter after administration of BglA protein-treated hesperidin and the standard hesperetin than those in the other two groups (**Figure 6**). After dosing with the standard hesperidin and hesperidin extract, total hesperetin was detected at 2 h and reached a maximum (∼0.35 and 0.20 μM) at 6 h (**Table 3**). On the other hand, after the administration of BglA protein-treated hesperidin and standard hesperetin, total hesperetin was detected as early as 30 min after administration and reached a maximum concentration (∼0.82, 0.74, and 0.95 μM) at 4 h (**Table 3**). The results show that plasma C_{max} and T_{max} levels were similar in hesperetin and BglA-treated hesperidin groups since the latter rapidly hydrolyzed hesperidin to hesperetin.

Whereas untreated hesperidin not only was absorbed more slowly, it also had much smaller AUC values following IV administration (2-fold). In oral administration, the difference between pure hesperetin and hesperidin was more than 3-fold, whereas the difference between untreated hesperidin and BglA protein-treated hesperidin was even more at 4-fold or higher. Again, this is similar to IV administration where the extract had a larger difference since the extract may contain additional phytochemicals that may interact with the absorption and/or disposition process of hesperidin.
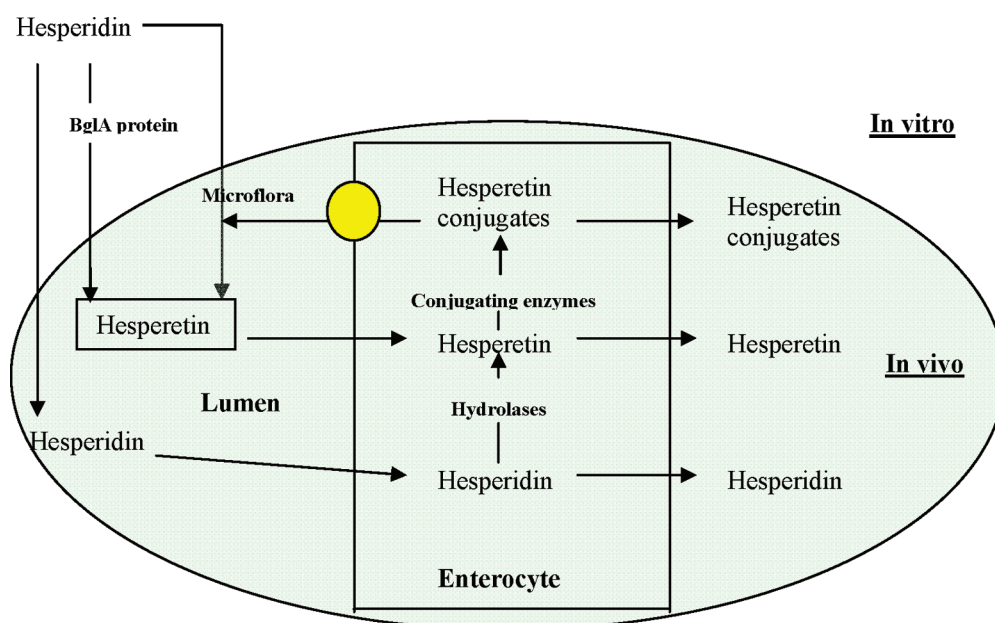
DISCUSSION

Hesperidin has been reported to have multiple biological activities but has a poor bioavailability. Chenpi is the common source of hesperidin, and it has been found to have a higher bioactivity after a nine-step fermentation process. Moreover, a yeast strain closely related to *S. singularis* and containing a β-glycosidase-like BglA protein was isolated from chenpi. Therefore, the present hypothesis is that better herbal materials may result from fermentation processes because they produce more bioavailable active species.

Our study provides strong evidence to show that the hydrolysis of hesperidin could be enabled in vitro by using yeast enzymes present in the chenpi fermentation process. For example, yeast BglA proteins extracted from *S. singularis* or expressed in *E. coli* BL21 (DE3) (**Figure 4**) are active against hesperidin (**Figure 2**). In the presence of this BglA protein (**Figure 2**), which hydrolyzed hesperidin to hesperetin, the total plasma hesperetin pharmacokinetics was significantly altered following IV and oral administration (**Figures 5** and **6**). For example, the plasma T_{max} value was significantly shorter, and the plasma concentrations of total hesperetin were significantly

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Table 3. Pharmacokinetic Parameters of Total Hesperetin after Single Oral Administration of Hesperidin or Hesperetin or Hesperidin Treated with Different BlgA Protein Treatments

parameters	pure hesperidin	pure hesperetin	hesperidin extract untreated	hesperidin extract + BlgA from *E. coli*	hesperidin extract + BlgA from yeast
AUC_{obs}d av (nmol h/mL) SD	2.63	7.91[a]	1.50	6.82[b]	5.93[b]
	1.48	4.15	0.79	2.53	2.26
C_{max} av (nmol/mL) SD	0.35	0.95	0.20	0.82	0.74
	0.10	0.37	0.10	0.27	0.19
T_{max} av (h) SD	6	4	6	4	4
	0	0	0	0	0
V_z av (mL) SD	9.86	5.65[a]	13.14	2.75[b]	4.29[b]
	5.05	5.28	1.73	1.55	2.09
CL av (mL/h) SD	56.49	19.15[a]	114.06	24.48[b]	26.95[b]
	27.42	6.34	32.24	5.99	10.32
F (%) av SD	8.53	13.26	10.36	11.39	10.03
	2.30	4.68	8.31	3.02	2.35

[a] There are statistically significant differences between AUC_{obsd} for pure hesperidin and pure hesperetin ($p > 0.05$). [b] There are statistically significant differences between AUC_{obsd}, V_z, and CL for hesperidin with different BlgA protein treatment and untreated hesperidin extract ($p > 0.05$).

Figure 7. Possible pathways for hesperidin and hesperidin disposition in the gut. The image shows hydrolysis of hesperidin by the BglA protein in vitro, by microflora in lumen in vivo, transport pathways of hesperidin and hesperetin, and disposition pathways of hesperidin and hesperetin.

higher after oral administration of BglA protein-treated hesperidin (**Figure 6**). Similarly, AUC values of hesperetin or treated hesperidin also were significantly higher than hesperidin or untreated hesperidin extracts (**Tables 2** and **3**). Therefore, the fermentation process utilizing yeasts capable of secreting the BglA protein likely will result in a higher bioavailability of the flavonoids present in chenpi.

The higher bioavailability is likely the result of increased uptake of hesperetin after administration of BglA protein-treated hesperidin (containing mostly hesperetin) as compared to untreated hesperidin extract (containing mostly hesperidin). Faster absorption of hesperetin as compared to hesperidin was shown previously in the Caco-2 cell culture model and various rat and human pharmacokinetic studies (*13, 26*). In our own studies, the data appear to suggest that efflux contributed to slower absorption for hesperidin (glycoside) when compared to hesperetin (aglycone) since T_{max} was longer and C_{max} was smaller for the glycoside (**Figure 6**).

Another important reason as to why hesperetin or BglA-treated hesperidin extract has a higher bioavailability is the faster clearance of the glycosides. In fact, clearance values were higher

following both oral and IV administration. Since we conducted the first side-by-side study of this kind, our data provide for the first time strong evidence that hesperidin (a glycoside) also was eliminated much more quickly than hesperetin (aglycone). This is an important finding since it complements the current theory that glycosides have a poor bioavailability because they are poorly permeable or effluxed by transporters such as MPR2 (*15, 17, 18, 27*). Another reason is the extensive metabolism of aglycone (hesperetin) after absorption (*8, 13, 26*).

Although hesperidin is poorly absorbed and rapidly eliminated, it has a reasonable half-life (6 h). This reasonable half-life came first from a prolonged absorption phase, as shown by a longer T_{max} (**Table 3**). The prolonged absorption phase came from its poor absorption, which forced a large portion of glycosides (e.g., hesperidin) to the ileum and the colon, where bacteria β-glucosidases capable of cleaving the flavonoid glycosides are abundantly present and impact bioavailability (*28*). Because of the action of intestinal

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microflora, significant absorption of hesperetin would occur after hesperidin gradually is hydrolyzed in the terminal ileum and colon.

Another reason for hesperetin to have a reasonable plasma half-life, as evidenced by the maintenance of the total hesperetin concentration at a reasonable level even at 24 h, is that hesperetin participates in enteric and enterohepatic dual recycling (*29*). This is because hesperetin has been shown to be extensively metabolized into phase II conjugates such as glucuronides and sulfates. These two metabolites are formed and eliminated by enterocytes and hepatocytes with a substantial portion of these metabolites excreted into the intestinal lumen (**Figure 7**). At the lower small intestine and colon, these metabolites may be hydrolyzed by bacteria β-glucuronidases and sulfatases to release the absorbable aglycones, which may be reabsorbed into the plasma (**Figure 7**).

In conclusion, this is the first comprehensive and comparative study of IV and oral pharmacokinetics of both hesperidin and hesperetin in the same laboratory. The results indicate for the first time that rapid plasma clearance is partially responsible for the poor bioavailability of flavonoid glycosides, adding to our knowledge that rapid clearance together with poor permeation, low solubility, and/or extensive intestinal efflux are responsible for the poor bioavailability. Because hesperetin, the aglycone form of hesperidin, is much better absorbed with a slower clearance and therefore higher bioavailability, a classical fermentation process utilizing BglA protein-containing yeasts should produce chenpi that have more bioavailable hesperetin than dried citrus peels, thereby increasing their biological activity.

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