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Distribution and in Vitro Availability of Selenium in Selenium-Containing Storage Protein from Selenium-Enriched Rice Utilizing Optimized Extraction

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Selenium (Se) distribution in Se-enriched rice and optimization of extraction for Se-containing protein were studied. Se availability in Se-containing protein product was simulated using an in vitro gastrointestinal digestion. The results showed that Se was predominately found as organic Se, whereas inorganic Se comprised only 2.85% of the total Se. The glutelin fraction contained the largest amount of Se, approximately 31.3% of the total Se in the rice gain. Utilizing orthogonal analysis, the optimum extraction conditions were selected at a volume to weight of 20:1, 0.08 M NaOH, an extraction time of 3 h, and at a temperature of 35 °C. A Se-containing rice protein product with 83.5% protein and 9.09 μg g^{-1} Se was sequestered using the optimal extraction method. This rice protein product with high molecular weight Se-containing protein can readily be digested to low molecular weight peptides and selenomethionine (52.3% of total Se in protein extract).

KEYWORDS: Selenium; rice protein; distribution; availability; extraction

INTRODUCTION

Selenium (Se), a crucial trace element, is required in small amounts in humans for the function of a number of Se-dependent enzymes, such as glutathione peroxidase (GPx) and thioredoxin reductase (*1*). The Recommended Dietary Allowance (RDA) of Se for men and woman is 55 μg day^{-1}, whereas the estimated safe and adequate daily dietary intakes (ESADDIs) for adults is between 50 and 200 μg day^{-1} (*2*). Unfortunately, low Se intake has become a problem for many people worldwide. This can be attributed to the low content and bioavailability of Se in soils and consequently low concentrations of Se in plant tissues (*3*, *4*). Se deficiency in developing countries is mainly derived from deficiencies of the element in staple food, such as rice or wheat in Asia and maize or sorghum in Africa (*5*). In China, cereals and cereal products contribute to approximately 70% of the total dietary intake of Se in the population living in the Se-deficient areas (*6*). For these reasons, we are trying to enhance the Se content of cereal products to increase the dietary intake of the element in these regions.

Rice, being one of the leading food crops for more than half of the world's population, is an important source of Se, especially for the people in China who depend on rice as a staple food. However, low levels and considerable differences of Se were found in global rice products, mainly depending on the Se concentration of the soil and rice genotype (*7*−*9*). Our previous studies have shown that foliar application of Se-enriched fertilizer can significantly increase the Se content of rice grains (*7*, *10*). Thus, it is feasible to improve Se nutrition of Se-deficient populations who depend on rice as a staple by providing Se-enriched rice in China. However, the beneficial nutritional value of Se is based on both the concentration ingested and its chemical form (*11*). Accordingly, information on the total Se content of Se-enriched rice alone is insufficient to ensure the biological availability of the products.

Analytical speciation studies allow for the elucidation of the different Se forms present within a sample. With selenized yeast, the most commonly used Se supplement, it has been shown that the primary Se species is selenomethionine (SeMet) (*12*). Other food-based products that have been extensively studied for Se speciation include Se-enriched garlic, onion, radish, carrot root, broccoli sprout, and florets (*11*, *13*−*17*). Major Se species reported in these selenized vegetables were Se-methyl-selenocysteine and γ-glutamyl-Se-methyl-selenocysteine, which are known to be more effective inhibitors of tumor formation compared to other Se species. Se bioaccessibility assessment in selenized yeast was carried out by Reyes et al. using an in vitro gastrointestinal digestion via two-dimensional chromatography approach, which showed that approximately 90% of the total Se was found in the soluble fraction and only 40% was determined to be SeMet (*18*). In our previous study, the major selenocompound identified in the Se-enriched rice by high-performance liquid chromatography (HPLC) coupled with inductively coupled

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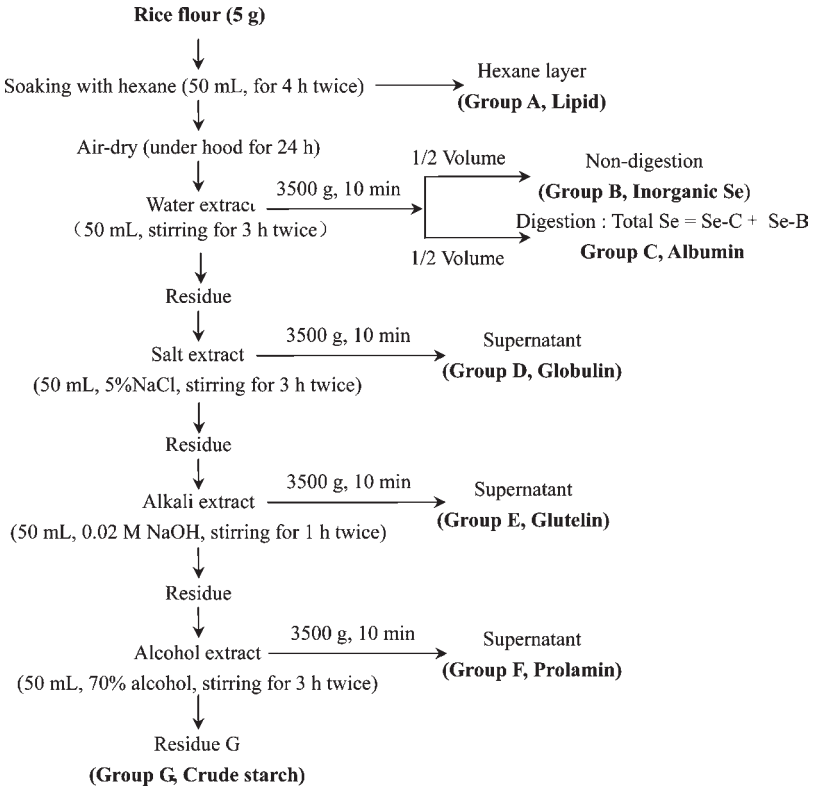

Figure 1. Scheme for separation procedure of selenium biochemical fractions in Se-enriched rice. The biochemical fractions A, B, C, D, E, F, and G are lipid, inorganic compound, albumin, globulin, glutelin, prolamin, and crude starch, respectively. The Se within the albumin fraction was calculated by the difference between total Se of water-soluble extract and inorganic Se.

plasma mass spectrometry (ICPMS) was SeMet, representing >86% of the total Se extracted (*19*). Because SeMet is a readily bioavailable Se species, Se-enriched rice has been considered for supplementation in Se-deficient geographical regions in China. Currently, Se-enriched rice has been widely produced by foliar application as an economical source of SeMet to enhance dietary Se intake levels in China, where Se-deficient soil is prevalent (*19*). However, byproducts of Se-enriched rice such as rice bran or broken rice, which are commonly fed to livestock, have not yet been fully utilized. Many studies worldwide are investigating various plant sources of proteins such as soybean protein (*20*), rice or rice bran protein (*21*), tea protein (*22*), and wheat protein (*23*), with the aim of improving the nutritional value of food products along with a low-cost assessment. Moreover, the increasing need to explore the functional properties of rice proteins and fully utilize the rice product as well as minimize the resource waste is of utmost importance. Therefore, it is of great interest to extract Se-containing protein from Se-enriched rice, which could be used as a novel Se supplement in Se-deficient areas.

The objectives of the current work are (a) to investigate the distribution of Se in biochemical fractions prepared by separation procedures of Se-enriched rice and (b) to conduct an orthogonal experiment to determine the optimal alkaline conditions of Se-containing protein extraction. In addition, (c) an in vitro gastrointestinal digestion is employed to evaluate the potential elemental accessibility of Se-containing protein products extracted from rice via size exclusion chromatography (SEC) coupled with ICPMS.

MATERIALS AND METHODS

Chemicals. All of the solutions were prepared in 18.2 MΩ cm^{-1} doubly deionized water (DDW) processed by Sybron/Barnstead (Boston, MA). Nitric acid (Suprapure, 68%), hydrochloric acid (Suprapure, 36%), and sodium hydroxide were purchased from Nanjing Chemical Industry (Nanjing, China). Calibration of the SEC column was performed using a standard mixture of cytochrome *c* (125 kDa), aprotinin (6.5 kDa), vitamin B_{12} (1.35 kDa), $(Gly)_6$ (0.36 kDa), and $(Gly)_3$ (0.189 kDa) (Sigma-Aldrich Co., St. Louis, MO). The following Sigma reagents and solutions were also used: seleno-DL-methionine (SeMet, 0.196 kDa, 99%), sodium dodecyl sulfate (SDS), protease inhibitor phenylmethanesulfonyl fluoride (PMSF), and porcine enzymes (pepsin, α-amylase, and pancreatin). Tris(hydroxymethyl)aminomethane (Tris), sodium hydroxide (NaOH), acetic acid, and acetone were also obtained from Fisher Scientific (Fair Lawn, NJ).

Se-Enriched Rice Sample. Se-enriched fertilizer was prepared from selenite, and the preparation method was detailed by Hu (*24*). The field experiment was conducted on September 9, 2008, at Nanjing (N 31° 56′, E 118° 47′), Jiangsu Province of the People's Republic of China. The soil pH of this region was 7.2, and the natural total Se content in the soil was 0.31 mg kg^{-1} of soil. The cultivar of test rice was Zhendao 10 (*Oryza sativa* L.). Foliar application of Se fertilizer to the test rice was detailed by Chen (*7*). Briefly, the Se fertilizer treatments as foliar application were diluted with water (about 740 L ha^{-1}) and sprayed once to the test paddy during the heading stage of growth. The rate of treatment with Se-enriched fertilizer was 60 g of Se ha^{-1}, and the control was sprayed with distilled water only. Paddy transplanting, irrigation, and other treatments were carried out acording to standard farming practices. When thoroughly tasseled, the grains were hand harvested on October 30, 2008. After harvesting, the rice grains were dried at 50 °C, then hulled, polished, and milled into powder for further analysis.

Separation of Biochemical Fraction in Se-Enriched Rice. The separation procedure for each biochemical fraction of Se-enriched rice was performed according to the modified method from Ju et al. (*25*). As illustrated in **Figure 1**, 5 g of dried rice powder was soaked in 50 mL of hexane. After air-drying, the defatted rice flour was extracted by stirring with 50 mL of DDW at 20 °C for 3 h, and then centrifuged at 3500*g* for 10 min. The supernatant was divided into halves, one for determination of total Se of water-soluble extract after acid digestion and the other for determination of inorganic Se after filtering through a 0.45 μm syringe filter without digestion. Accordingly, the Se within the albumin fraction was calculated by the difference between total Se of water extract and inorganic Se.

Following water extraction, the flour was extracted with 50 mL of 50% NaCl at 20 °C for 3 h. The sediment was then extracted for glutelin with 50 mL of 0.02 M NaOH at 20 °C for 1 h, followed by prolamin extraction with 50 mL of 70% ethanol at 20 °C for 3 h. To adequately separate each fraction from rice, extraction procedures were repeated twice. The supernatants were centrifuged at 3500*g* for 10 min and then combined in a crucible to dry via a boiling water bath before the digestion for Se analysis.

Extraction of Se-Containing Protein. The defatted rice flours were obtained from broken rice, then mixed with a NaOH solution, and stirred continuously for a set time (0.5−5 h) at a controlled temperature (20−50 °C) using a magnetic stirrer (85-2, Shanghai Shensheng Scientific Instrument, China). The mixture was then centrifuged at 3500*g* for 10 min, and the supernatant was carefully collected. The Se-containing protein from rice was precipitated from the supernatants by adding acetic acid to adjust the pH. The maximum extraction rates of protein and Se were obtained by subjecting a portion of each supernatant to a pH ranging from 3.0 to 6.5. The pH that produced the maximum extraction rates of protein and Se was selected as optimal pH for precipitation of Se-containing protein in rice. Subsequently, the precipitated proteins were centrifuged (3500*g* for 10 min), freeze-dried, weighed, and then stored at −4 °C until further analysis.

Determination of Total Se. The Se concentrations of each fraction isolated from Se-enriched rice was determined using the dual hydride generation atomic fluorescent spectrometry (AFS-3100, Beijing Kechuang Haiguang Instrument, China) (*5*). The sample was put into a Kjeldahl flask and digested with 10 mL of a mixture of concentrated HNO_3 and $HClO_4$ (v/v, 4:1) at 155−175 °C until the digest was completely mineralized. After cooling, 5 mL of 6 M HCl was added to the digest to reduce Se^{6+} to Se^{4+} at 120 °C until the solution became colorless and clear, and then the remainder was diluted with DDW. Blank digestions were also carried out in the same way.

The present procedure was validated against a certified reference material, GBW10010 (polished rice), purchased from the National Research Center (Beijing, China). The compared results are in good agreement with six replicates between the certified (0.061 ± 0.015 $\mu g\ g^{-1}$) and determined (0.059 ± 0.011 $\mu g\ g^{-1}$) results. A fresh 1000 ng mL^{-1} Se standard was prepared daily in 0.5% hydrochloric acid from the Se stock solution. The recovery and accuracy of the Se measurements for the procedure described above were determined to be 92−106%. Se extraction rates were calculated as a percentage of the summed amount of Se in the extracted protein to the total Se of rice flour.

Protein Content. Protein contents in rice flour and dried protein products were determined using the micro-Kjeldahl method (Foss 2300 Kjeltec Analyzer, Denmark), and the nitrogen content was multiplied by 5.95 (*26*). Three replicates were carried out for each sample. Protein extraction rates were calculated as an average percentage of the summed amount of proteins in the dried protein to the total protein of rice flour.

Amino Acid Composition. The protein samples were hydrolyzed under vacuum with 6 M HCl at 110 °C for 24 h in the presence of 1% phenol (v/v). The hydrolysates were analyzed with an amino acid analyzer (MLC-703; Atto Corp., Tokyo, Japan).

In Vitro Gastrointestinal Digestion Method. Approximately 2 mL of Se-containing protein extracted by the optimized extraction method was precipitated by adding 80% acetone and held at −4 °C for 12 h to precipitate the proteins. After this treatment, the mixture was centrifuged (3500*g*, 15 min) and the supernatant was discarded. Subsequently, the residue acetone remaining in the sediment was removed under a stream of pure N_2 gas, and then the dried proteins were resolubilized in 1 mL of Tris-HCl buffer (containing 1% SDS and 1 $\mu g\ mL^{-1}$ PMSF, pH 7.5). The final mixture was passed through a 0.45 μm syringe filter, and stored at −20 °C until further analysis.

The in vitro enzymolysis digestion was modified according to the procedures described by Crews et al. (*27*) and Cabanero et al. (*28*). The intent of the method was to simulate the conditions of digestion in the stomach and intestine. In brief, about 0.3 g of freeze-dried Se-containing protein was incubated in a shaking water bath at 37 °C for 2 h with 3 mL of gastric juice (1%, w/v, pepsin in 0.15 M NaCl acidified with HCl to pH 3.0). After gastric digestion, 2 M $NaHCO_3$ was added to raise the pH to 6.8. Then, 3 mL of intestinal juice containing 3% (w/v) pancreatin, 1.5% (w/v) amylase, and 1% (w/v) bile salts in 0.15 M NaCl was added. The sample was further incubated for 2 h at 37 °C, shaking periodically. Subsequently, the soluble gastrointestinal extract was centrifuged at 8945*g* for 15 min at 4 °C. The supernatant was decanted and stored at −20 °C. All of the mixtures were filtered through a 0.45 μm syringe filter to remove particulates before SEC-HPLC-ICPMS analysis.

The molecular weights of Se-containing proteins in resolubilized protein extract and the gastrointestinal extract were determined using SEC-HPLC-ICPMS. The proteins and standards were separated by Superdex Peptide 10-300 GL column (7−0.1 kDa, Tricorn, Amersham Biosciences). Calibration standards were detected with UV detection at the wavelength of 280 nm with an injection volume of 100 μL per sample. The mobile phase consisted of 30 mM Tris buffer (pH 7.5), which was pumped through the column isocratically at 0.5 mL min^{-1}. The coupling between the corresponding column outlet, and the sample introduction system of the ICPMS was achieved through a 300 mm long × 0.25 mm i.d. PEEK tubing. Signals at m/z 77, 78, 80, and 82 (Se isotopes) were monitored with a dwell time of 0.1 s. Possible polyatomic interferences were removed by the Agilent Octopole Reaction System operating in the hydrogen gas mode with a flow rate of 3.5 mL min^{-1}. Other ICPMS instrumental conditions were as follows: RF forward power, 1450 W; plasma Ar gas flow rate, 15.0 L min^{-1}; carrier Ar gas flow rate, 1.05 L min^{-1}.

Statistical Analysis. The data are presented as the mean ± standard deviation of determinations. The difference of Se content and protein content for each sample was analyzed by one-way analysis of variance (ANOVA). Multiple comparisons of means were separated at $P < 0.01$ by Duncan's multiple-range tests. All computations were made by employing statistical software (SAS, version 8.2).

RESULTS

Distribution of Se in Biochemical Fractions of Se-Enriched Rice. The overall Se contents found within different biochemical fractions of Se-enriched rice obtained resulting from various treatments are presented in **Table 1**. The majority of Se found was organic Se, whereas only 2.48% of total Se was determined to be inorganic Se; in addition, a minuscule amount was found in lipid fraction. Four types of proteins were extracted and separated from the Se-enriched rice via a combination of separation and centrifugal methods as described in **Figure 1**. Nearly 54% of the total Se was metabolized into extracted proteins albumin-Se, globulin-Se, glutelin-Se, and prolamin-Se. Comparison of the Se contents of the subsequent protein fractions revealed that the glutelin portion contained a significantly larger amount of Se with approximately 31.3% of the total Se in the rice ($P < 0.01$), which is in agreement with the study reported by Zhang et al. (*29*). The other three types of protein fractions are albumin, globulin, and prolamin ordered with decreasing Se concentration, respectively. As shown in the method outline, the dominant Se-containing protein from glutelin can be readily extracted by alkaline methods. However, even with the extensive methods applied, >33.8% of total Se was sequestered in the residue (crude starch) and contained nearly 1.5% (w/w) protein under further investigation (data not shown in **Table 1**). After the proposed analysis, it was concluded that a small portion of Se was still bound to unextracted protein and/or starch structures. According to the study by Ferri et al., it is suggested that starch is the main Se storage site of the Se-enriched potato (*30*). Present observations, however, reveal that Se was mostly stored in rice protein and a small Se portion was retained in starch complexes. Further studies should focus on the optimization of Se-containing protein extraction in starch matrices.

Selection of pH To Precipitate Extracted Proteins. Ju et al. reported that albumin and glutelin have two isoelectric points (Ips) at pH 4.1 and 4.8 determined by turbidity measurements, respectively, at which the maximum amounts of proteins can be precipitated by acid from the protein mixture (*25*). However, in this investigation, extraction rates of protein and Se were used to select the optimum pH for precipitating extracted proteins. As shown in **Figure 2**, when pH values were increased in an acidic pH range of 3.0−6.5, two peak values at 4.0 and 5.4 were observed from

the extraction data of the Se-containing protein, suggesting high extraction conditions. Due to higher yields of Se-containing protein, pH 5.4 was selected as the optimal pH for the continued study.

Table 1. Detailed Distribution of Selenium in Biochemical Fraction of Se-Enriched Rice

group[a]	mass of Se[b] (ng)	ratio to total Se of rice (%)
lipid	1.4 ± 0.5 e	0.03
inorganic	125.4 ± 4.4 d	2.48
albumin	490.7 ± 12.1 b	9.72
globulin	351.2 ± 10.2 bc	6.95
glutelin	1579.5 ± 87.9 a	31.28
prolamin	303.3 ± 21.7 c	6.01
crude starch	1710.9 ± 68.4 a	33.88
Se loss	488.0 ± 15.0 b	9.66
total Se	5050.4	100.00

[a] The detailed fractionation procedure can be traced in **Figure 1**. The Se loss means the difference of total Se of 5 g of Se-enriched rice and Se in lipid, inorganic, albumin, globulin, gultelin, prolamin, and crude starch group. [b] Data are expressed as the mean of three determinations ± standard deviation. Values followed by different letters are different ($P < 0.01$) from one another.

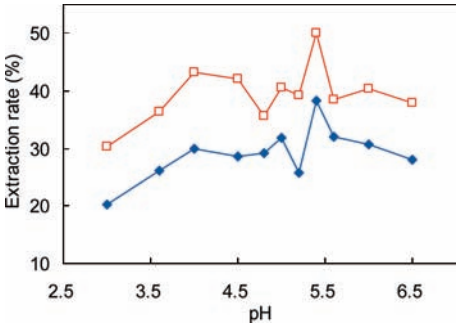

Figure 2. Extraction rate of Se-containing protein with pH values (3.0−6.5): (□) protein extraction rate; (◆), Se extraction rate.

Effect of Volume/Weight Ratio, Alkali Concentration, Extraction Temperature, and Time on the Se-Containing Protein Extraction Rate. Because most of the Se was incorporated into the alkali-soluble protein fraction, alkaline extraction was employed as the method for Se-containing rice protein extraction in the following experiments. Extraction efficiency depends highly on the extraction conditions such as agent concentration, temperature, time, and volume/weight ratio of solvent to raw material. Sodium hydroxide was used as the sole extraction agent due to the high alkaline concentration, which is able to break down hydrogen bonds as well as dissociate hydrogen from carbolic and sulfate groups, thus releasing protein from starch complexes (*22*). The aim of the present experiments was to investigate the effect of volume/weight, alkali concentration, temperature, and time on the extraction rates of protein and Se to obtain optimal conditions for extraction of Se-containing protein from the rice. **Figure 3** depicts the Se-containing protein extraction rate as a function of each main variable and various operation parameters tested. As expected, the extraction yield varied when different conditions were applied.

To study the effect of the volume/weight ratio, extraction experiments were conducted at a constant concentration (0.08 M NaOH), fixed time (1 h), and a constant temperature (20 °C). As shown in **Figure 3A**, both of the extraction rates of protein and Se are increased linearly with the volume/weight ratio up to 20:1, followed by a slight decrease with larger ratios. This can be attributed to the protein concentration difference between that in the solid phase and that in the solution. The higher solvent volumes produce lower protein concentrations in the solvent and, therefore, allow for increased mass diffusion during the extraction process. However, this beneficial effect is diminished gradually as larger volumes of solvent are added. From these data it was concluded that a volume/weight ratio of 20:1 is suitable for Se-containing protein extraction using the alkaline method. To determine the effect of the alkali concentration, extraction experiments were carried out at a fixed time (1 h), a constant temperature (20 °C), and a constant volume/weight ratio (10:1). It was found that the extraction rates increased sharply with the

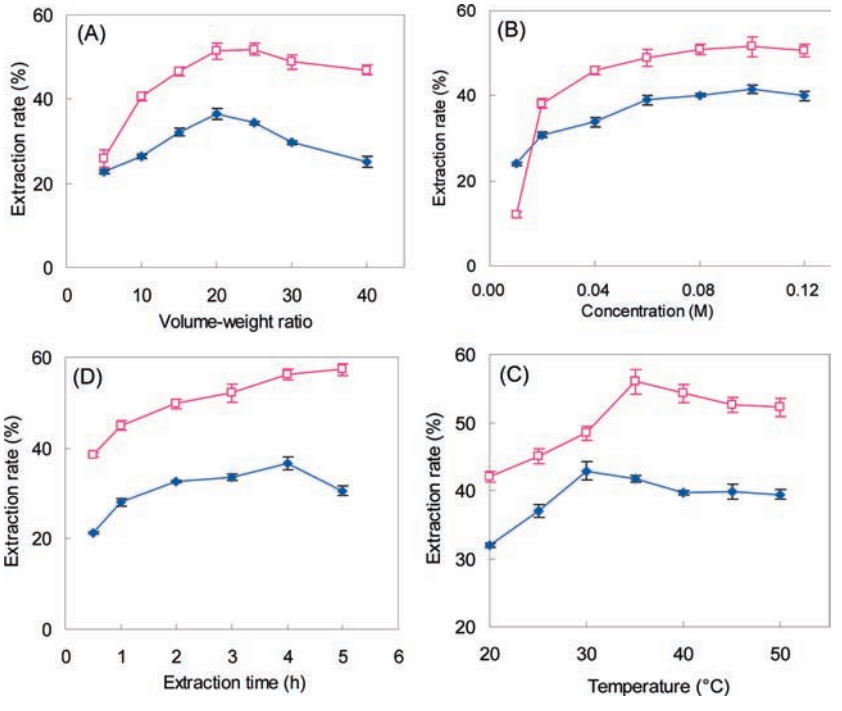

Figure 3. Effect of various factors on the Se-containing protein extraction rate using the alkaline method: (**A**) effect of volume/weight ratio of solvent and rice flour; (**B**) effect of alkali concentration; (**C**) effect of extraction temperature; (**D**) effect of extraction time; (□) protein extraction rate; (◆) Se extraction rate.

Table 2. Extraction Rates of Se-Containing Protein from Se-Enriched Rice Using the Alkaline Method in the Orthogonal Experiment L_9 (3^4)

	A^a	B	C	D	extraction rate (%)	
run	(v/w)	(NaOH, M)	(temperature, °C)	(time, h)	protein	Se
1	10	0.02	25	1	54.9 ± 1.6[b]	39.9 ± 1.9
2	10	0.05	35	2	66.8 ± 1.5	51.1 ± 0.3
3	10	0.08	45	3	67.9 ± 0.6	57.3 ± 0.8
4	15	0.02	25	3	74.7 ± 1.3	55.7 ± 0.5
5	15	0.05	45	1	71.2 ± 0.5	59.8 ± 0.1
6	15	0.08	35	2	64.5 ± 3.4	54.3 ± 0.9
7	20	0.02	35	2	70.8 ± 2.1	41.2 ± 1.5
8	20	0.05	25	3	71.3 ± 0.1	55.9 ± 1.1
9	20	0.08	35	1	68.7 ± 2.7	55.5 ± 2.0
			Protein			
k_1[c]	63.2	66.8	63.6	64.9		
k_2	70.1	69.8	70.1	67.4		
k_3	70.2	67.0	70.0	71.3		
R[d]	7.0	3.0	6.5	6.3		
Q[e]	A_3	B_2	C_2	D_3		
			Se			
k_1	49.4	45.6	50.0	51.8		
k_2	56.6	55.6	54.1	48.9		
k_3	50.9	55.7	52.8	56.3		
R	7.1	10.1	4.1	7.4		
Q	A_2	B_3	C_2	D_3		

[a] The letters *A*, *B*, *C*, and *D* indicate volume/weight ratio, NaOH concentration, extraction temperature, and extraction time, respectively. [b] Values are the mean of two determinations ± standard deviation. [c] *k* represents the average extraction rate of protein or Se at each level. [d] *R* value indicates the range between three average extraction rates of protein or Se of each level. [e] *Q* represents the optimal level of four factors.

increase of NaOH concentration from 0.01 to 0.04 M; however, little increase was observed when the NaOH concentration increased from 0.06 to 0.12 M (**Figure 3B**). The effect of extraction time for Se-containing protein yield is shown in **Figure 3C** (at fixed contraction conditions: 20 °C, 0.08 M NaOH, and volume/weight ratio 10:1). Overall, it was found that a longer extraction time provided higher extraction rates; however. the most dramatic effect of the extraction time was observed during the first 2 h, when the protein yield increased significantly. The time effect was less significant after 3 h, in addition to a slight decrease of Se extraction between 3 and 5 h. For both technical and economical reasons, 3 h was chosen for optimal Se-containing protein extraction from the rice. **Figure 3D** depicts the effect of extraction temperature on Se-containing protein extraction method. In this experiment, 0.08 M NaOH was used as the solvent, the extraction time was fixed to 1 h, and the volume/weight ratio was fixed to 10:1. In general, a higher temperature proved to be advantageous for enhancing rice protein extraction. Extreme high temperatures, however, can lead to starch gelatinization and thus a decrease in the extraction of protein. As for the Se extraction rate, it was apparent that the rate of extraction increased drastically when the temperature was raised from 20 to 30 °C, followed by negligible changes after 30 °C. Similarly, the protein extraction rate displayed the same pattern, with the exception of the peak of 35 °C. In conclusion, the most appropriate temperature for extracting Se-containing proteins is in the range of 25−45 °C.

Optimum Extraction of Se-Containing Protein from Rice. To further optimize the extraction conditions, an orthogonal experiment L_9 (3^4) was designed to correlate the above four extraction parameters on Se-containing protein yield, as determined for the alkaline method (**Table 2**). The extraction rates of protein and Se for each experiment are shown in **Table 2**, where *k* is the

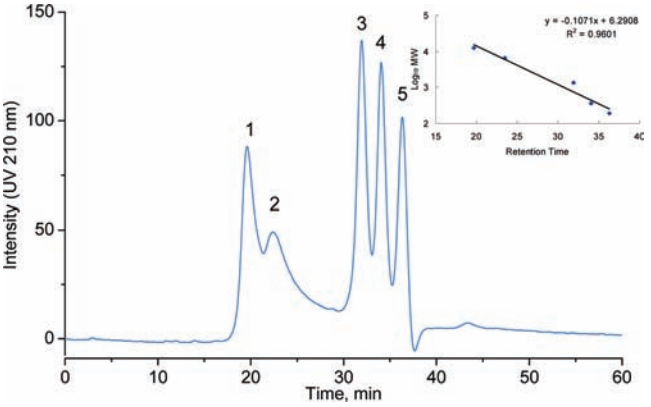

Figure 4. SEC-HPLC-UV chromatogram of mix standards for molecular weight. (Inset) Calibration of standards molecular weight for SEC column (Superdex Peptide 10-300 GL). Numbers correspond to the standards of known molecular mass: 1, 12.5 kDa (19.7 min); 2, 6.5 kDa (23.5 min); 3, 1.35 kDa (31.9 min); 4, 0.36 kDa (34.1 min); 5, 0.189 kDa (36.3 min).

average extraction rate of the parameter at a chosen level and *R* represents the range between the three *k* values. A higher *k* value indicates a preferred level for the chosen parameter, whereas a higher *R* value would indicate a greater influence of the parameter. For example, on the basis of the magnitude order of the *R* value, the effect of four factors on the protein yield is in the following order: *A* (volume/weight ratio) > *C* (time) > *D* (temperature) > *B* (concentration). As for the Se extraction rate, however, the effect of the four factors decreased in the following order: *B* > *D* > *A* > *C*.

The maximum yield value of k_1, k_2, and k_3 of each column, the optimal condition for improving protein yield was determined to be $A_3B_2C_2D_3$, whereas the optimal condition for enhancing Se extraction was determined as $A_2B_3C_2D_3$. Accordingly, temperature and time of extraction could be determined as 35 °C and 3 h. With respect to the *R* value, the volume/weight ratio was the most important factor for protein yield and the less important factor for Se extraction. Thus, the higher protein yield could be obtained at 1:20. As a result, the optimal condition for extraction of Se-containing protein was determined as $A_3B_3C_2D_3$, although this optimal condition did not occur in the orthogonal test. Therefore, another experiment with the optimal conditions of a volume/weight of 20:1, 0.08 M NaOH, extraction time of 3 h, and temperature of 35 °C was carried out for confirmation. Results conclude that the mean protein yield and Se extraction rates were 79.1 ± 2.3 and 61.4 ± 2.9% (n = 3), respectively, which are all higher than those presented in the orthogonal experiments in **Table 2**. This demonstrates that the optimal condition $A_3B_3C_2D_3$ was efficient and reliable for the extraction process.

Se Availability Assessment of Se-Containing Protein in Vitro. In vitro gastrointestinal digestion is a useful method to evaluate the potential elemental availability fraction from food and has been widely accepted for Se bioavalability assessment (*17*, *18*). For this work, the Se fractions of digestion and extraction were separated by SEC-HPLC. Calibration of the SEC column was accomplished using a standard mixture of cytochrome *c* (125 kDa, 0.4 mg mL^{-1}), aprotinin (6.5 kDa, 0.6 mg mL^{-1}), vitamin B_{12} (1.35 kDa, 0.01 mg mL^{-1}), $(Gly)_6$ (0.36 kDa, 0.05 mg mL^{-1}), and $(Gly)_3$ (0.189 kDa, 0.05 mg mL^{-1}), which produced a range with good linear response for the $\log_{10}$ molecular weight versus retention time (min) ($y = -0.1071x + 6.2908$, $R^2 = 0.9601$) (**Figure 4**). Because SeMet was the major selenocompound found in Se-enriched rice as our previous study proved, SeMet standard was used for characterization (**Figure 5A**). As illustrated in **Figure 5B**, the major

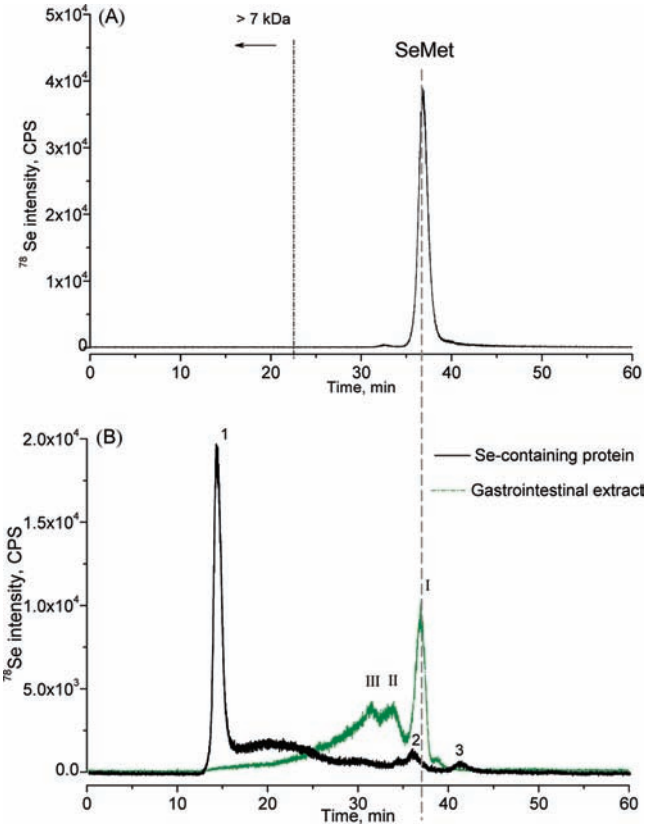

Figure 5. Analytical SEC-HPLC-ICPMS chromatograms of (**A**) SeMet standard (250 μg g^{-1}) and (**B**) Se-containing protein from Se-enriched rice and soluble enzymatic extract after in vitro gastrointestinal digestion: 1 (>7 kDa), 2 (0.182−0.317 kDa), 3 (<0.179 kDa), I (0.179−0.297 kDa), II (0.332−0.646 kDa), III (0.646−1.457 kDa).

Se-containing protein corresponds to the high molecular weight (HMW) fraction (fraction 1, > 7 kDa), eluted near void volume. Moreover, the minor compounds eluting at 36.9 and 41.5 min (fractions 2 and 3) were likely to be SeMet and other low molecular weight (LMW) Se species, respectively. The HMW fraction was broken down to Se-containing LMW fractions (fractions I, II, and III) following an in vitro gastrointestinal digestion (**Figure 5B**). The chromatogram displays a major peak at 37.1 min, which matches the retention time of SeMet standard and represents 52.3% of total Se injected. Two other peaks eluted at 31.4 and 34.0 min and further accounted for 35.8% of the Se injected (fraction II, 0.332 kDa < M_r < 0.646 kDa; fraction III, 0.646 kDa < M_r < 1.457 kDa). These Se fractions are likely to consist of LMW Se peptides, which result from digestion of Se-containing proteins by two enzymes commonly present in human gastrointestinal fluid. All of these potentially bioaccessible fractions accounted for almost 90% of total Se present in Se-containing protein (Se recovery for the SEC column was calculated as 92.3 ± 0.9% of total Se via flow injection without a column). The results proved that the efficiency of human gastrointestinal digestion is sufficient to break down the Se-containing protein extracted from rice, which is then able to convert the protein into LMW fractions with ideal availability, especially SeMet.

DISCUSSION

Se-enriched rice has been widely studied and produced to improve the Se nutrition of people in Se-deficient areas in China (*7*, *19*, *29*). However, before introducing Se-enriched rice to a functional food market, we produced Se levels of rice at 0.06−0.28 μg g^{-1} by controlling the Se supplementation. To further understand the Se distribution, the present study was performed on Se-enriched rice with a higher Se concentration (1.10 μg g^{-1} Se). Rice proteins contain mostly glutelin (about 80%), which is a HMW protein composed of subunits bound by disulfide linkages and soluble only in dilute acid or alkali solutions (*31*). The fractionation procedure revealed that the glutelin contained the largest amount of Se, approximately 31.3% of the total Se in the rice (**Table 1**). Therefore, dilute sodium hydroxide was applied to release the most Se-containing protein, which was then analyzed with an orthogonal experiment. On the basis of our results, the suggested optimal conditions are a volume/weight of 20:1, 0.08 M NaOH, an extraction time of 3 h, and a temperature of 35 °C for the highest extraction efficiency of Se-containing storage protein (**Table 2**). Additionally, enzymes have been considered as an effective agent for protein extraction in several studies (*22*, *32*). However, the proteins recovered by proteolysis are protein hydrolysates that may not maintain their original structural and functional properties (*31*). Moreover, low extraction efficiency and high cost of enzymatic methods limit its application in the food industry. Future studies will be focused on resolving this problem.

Utilizing the optimal extraction conditions, a Se-containing rice protein product containing 9.09 μg g^{-1} Se was produced, which displays a significantly higher extraction than other rice products (P < 0.01) (**Table 3**). The protein contents of the regular rice and Se-enriched rice protein products, however, were markedly increased to 83.5 and 82.9% (P < 0.01), respectively. To further evaluate the nutritional value of this protein product, the amino acid composition of the rice protein from regular rice and Se-enriched rice was compared to the composition of soybean protein amino acids (**Table 4**). As illustrated, glutamic acid is the

Table 3. Selenium and Protein Contents of Regular Rice, Se-Enriched Rice, and Their Protein Products Using Optimized Extraction[a]

rice product	Se content (μg g^{-1})	protein content (%)
regular rice	0.03 ± 0.00 d	7.02 ± 0.01 b
Se-enriched rice	1.10 ± 0.03 b	6.99 ± 0.01 b
regular rice protein	0.44 ± 0.02 c	83.52 ± 2.52 a
Se-enriched rice protein	9.09 ± 0.15 a	82.91 ± 1.73 a

[a] Data are expressed as the mean of three determinations ± standard deviation. Within each column, values followed by different letters are different (P < 0.01) from one another.

Table 4. Amino Acid Profile of Se-Containing Protein from Se-Enriched Rice and Regular Rice (Milligrams per Gram of Protein)

amino acid	regular rice protein	Se-enriched rice protein	soy protein[a]
aspartic acid	6.85	6.70	9.9
glutamic acid	16.00	16.48	17.0
serine	4.05	4.06	4.2
glycine	3.44	3.32	2.4
histidine	2.12	2.63	2.3
threonine	3.25	3.29	3.0
alanine	4.71	4.73	3.4
proline	2.00	2.80	3.8
tyrosine	3.81	3.76	3.2
valine	4.86	4.75	1.1
methionine	1.74	1.62	1.1
isoleucine	3.33	3.09	4.1
leucine	6.56	6.44	6.8
phenylalanine	4.15	3.76	5.2
cystine	0.80	0.80	4.5
lysine	2.49	2.22	5.2
arginine	7.04	6.82	6.6

[a] The amino acid data for soy protein was from the paper by Shen et al., which was compared to the amino acid profile of rice protein in this study.

most abundant amino acid in rice proteins, followed by aspartic acid, leucine, and arginine, which is similar to the order of soy protein. However, of the essential amino acids, the lysine content of rice at 2.49% is lower than that of soy protein. On the other hand, the methionine content of rice, at 1.74%, is substantially higher than the 1.1% of soy protein. Generally, lysine is the limiting amino acid of the protein in rice, and methionine is limiting in legume proteins including soybean proteins (*32*). The combination of Se-containing rice protein and soy protein to produce a high -protein mixture with nutritive value for use in functional foods is an attractive option. Methionine, which contains the element sulfur, in Se-enriched rice protein is lower than that of regular rice protein and can be attributed to replacement of sulfur by Se via the Se uptake, transport, and assimilation pathways (*33*).

The term bioavailability can be used in a wide concept including digestion, absorption, and incorporation into metabolic processes. It can also be used in a narrow sense, meaning that any potentially available part of a nutrient after gastrointestinal digestion should be attributed to its bioavailability (*34*). To avoid confusion, we proposed to use the term availability in the restricted sense of the word. In vitro experiments provide an alternative for human studies, because they are faster, cheaper, and simpler than in vivo experiments, especially when there is increasing interest recently to reduce the use of laboratory animals for testing (*35*). SEC-HPLC is an attractive analytical scheme for the investigation of large biomolecules (*36*) and coupled with ICPMS can be used to explore overall differences in the molecular weight distributions of Se-containing proteins and gastrointestinal extract as accomplished in this study. Our availability results showed that LMW peptides and SeMet were the major Se-containing moieties found in the extract following in vitro gastrointestinal digestion. SeMet is the major nutritional source of Se for animals, and it is known to be highly bioavailable. SeMet is retained for a longer period of time in humans and animals after supplementation; thus, it is an excellent chemical form for Se supplementation compared to other Se species (*18*). A supplementation trial in a Se-deficient population in China showed that Se as SeMet had nearly twice the bioavailability of Se as selenite (*37*). Therefore, the results of our simulation obtained from this study should be validated by in vivo experiments in future studies.

In conclusion, this work demonstrated that Se was predominately present as organic Se and that glutelin protein contained the largest amount of Se. Utilizing orthogonal analysis, it was found that the maximum extraction rates of Se-containing protein (79.1% for protein and 61.4% for Se) could be achieved at optimal extraction conditions. The Se-containing protein proved to be digestible by the simulated gastrointestinal digestion; however, the results of simulation should be validated by in vivo experiment. Therefore, further studies are needed for the determination of bioavailability of Se in Se-containing storage protein in vivo, using an animal model.

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