INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–1103/2018/20–5–1005–1011
DOI: 10.17957/IJAB/15.0592
http://www.fspublishers.org

Full Length Article

Breeding of Selenium Rich Red Glutinous Rice, Protein Extraction and Analysis of the Distribution of Selenium in Grain

Yuanke Liang[1†], Muhammad Umer Farooq[1†], Rui Zeng[1†], Zhicheng Tang[1], Yujie Zhang[1], Tengda Zheng[1], Hla Hla Ei[1], Xiaoying Ye[1], Xiaomei Jia[1] and Jianqing Zhu[1*†]
[1]*Rice research institute, Sichuan Agricultural University, Chengdu, Sichuan 611130, China*
[*]For correspondence: zhujianqing1963@163.com
[†]These authors contributed equally to this work

Abstract

Selenium acts as a chemo preventive agent for specific cancers, which can be useful as protective agent against stress. The objective of this study was to breed healthy selenium rich red glutinous rice serving as diet and search for convenient methods to extract protein from grain and measure the protein and selenium concentration. Furthermore, determine the interaction of selenium and protein in different parts of red glutinous rice seeds. After progress of five years breeding, the red glutinous rice achieved the standard (≥40 ng/g) selenium contents of 121.75 ± 3.01 ng/g. Total selenium concentrations in the rice fractions varied in the following order: husk < polished rice < whole grain < bran. Through the analysis of the protein content in different red glutinous rice parts, compared with the selenium concentration, we have made a conclusion that more than 80% selenium in the grain is organic selenium (in selenoprotein form) and more than half of the total selenium is in the endosperm of the red glutinous rice. Overall, the selenium rich red glutinous rice might be used as a natural healthy food, which could be applied as preventive measures against cancer and supplements in medical treatments near future.

Keywords: Selenium protein interaction; Selenium accumulation; Anticancer; Life-protecting agent; *Heterosis*

Introduction

Cancer is generally considered as one of the biggest threat to human health. In previous study, the most commonly diagnosed forms of cancers were lung (1.82 million), breast (1.67 million), and colorectal (1.36 million); while the most lethal were lung cancer (1.6 million deaths), liver cancer (745,000 deaths), and stomach cancer (723,000 deaths) all over the world in 2012 (Ferlay *et al*., 2015). However, the majority of epidemiological studies provided evidence for selenium as a chemo preventive agent for specific cancers: lung (Knekt *et al*., 1998), breast (Cann *et al*., 2000), colorectal (Ghadirian *et al*., 2000), liver (Yu *et al*., 1997), stomach (Scieszka *et al*., 1997) and other multiple cancers (Patrick, 2004; Wallace *et al*., 2009). Cancer is a major public health problem across the world. Besides, previous studies have reported that more than 30% of human cancers could be prevented by an alternative strategy of appropriate dietary modification (Liu, 2004). So, supplement of selenium in food products could be important to the human health.

Selenium (Se), an essential micronutrient for many organisms, is important for preventing disease, improving health, and preventing aging and has been considered a life-protecting agent (Zhao *et al*., 2017), with a recommended dietary allowance (RDA) for healthy adults of 55 μg/d (Institute of Medicine (US) Panel on Dietary Antioxidants and Related Compounds, 2000). But people in many areas of the world are selenium deficient, with the consequence that they are unable to express their selenoproteins fully (Xia *et al*., 2005). For humans, the boundary between inorganic Se deficiency and toxicity is narrow (Augilar *et al*., 2009). So it has a great potential risk to regard inorganic selenium as selenium supplement directlys. Organic Se, selenomethylcysteine (SeMeSeCys) and selenomethionine (SeMet) species are better assimilated by the natural body than inorganic Se and also serves as effective anticarcinogens, leading by SeMeSeCys (Carey *et al*., 2012). Nevertheless, for most people worldwide, as well as for livestock, plants are the main source of dietary organic selenium; thus, plant selenium metabolism is of great importance for the selenium nutrition of humans and animals (Zhu *et al*., 2009).

At present, food health is a burning issue in the world. Avoiding selenium deficiency and toxicity, it is important to monitor and optimize crop selenium quality and concentrations, through it can vary greatly between different crops and regions (Zhu *et al*., 2009). Rice (*Oryza sativa* L) is one of the most principal food crops in the world, serving as the staple food source for more than half of the world's

To cite this paper: Liang, Y., M.U. Farooq, R. Zeng, Z. Tang, Y. Zhang, T. Zheng, H.H. Ei, X. Ye, X. Jia and J. Zhu, 2018. Breeding of selenium rich red glutinous rice, protein extraction and analysis of the distribution of selenium in grain. *Int. J. Agric. Biol*., 20: 1005–1011

population (Gealy *et al.*, 2003), which contributes 40% to the total calories intake of people. Increased healthy rice production can play a vital role to address this issue successfully.

Since long ago, Chinese used Monascus purpureus to ferment red glutinous rice for cuisine and medicinal food owning to its especial function such as promoting blood circulation (Chairote *et al*., 2009), reducing cholesterol (Heber *et al*., 1999) and antioxidant activities (Que *et al*., 2006). Pigmented rice (*Oryza sativa* L.) has been consumed for a long time in Asia, especially China, Japan, Korea and many countries in Southeast Asia (Kanitha and Wanida, 2010). Besides, pigmented rice genotypes have high nutritive value compared to white rice genotypes (Faiz *et al*., 2015). Several varieties of pigmented rice, particularly red rice, have been cultivated in many countries. Many studies have demonstrated antioxidant activity and radical scavenging ability of the pigmented rice or its extract in both *in vitro* and *in vivo* models (Nam *et al*., 2006; Bae *et al*., 2014). Glutinous rice (*Oryza sativa* L.) is popular rice type due to its superior qualities of fineness, aroma, taste and protein content (Loypimai *et al*., 2017). It also contains very high quantities of bioactive components which are beneficial for health such as tocopherols, tocotrienols, c-oryzanol and phenolic compounds especially in the outer pericarp and aleurone layers covering the grains (Loypimai *et al*., 2009). These scientific reports assist us to do research on selenium accumulation and protein contents deviation in red selenium rich glutinous rice than other traditional rice. Hence, breeding of selenium rich red glutinous rice has very high application value.

The beneficial health-related effects of selenium rich red glutinous rice are of great importance to consumers, breeders and the rice industry. The objective of this study was to determine the interaction of selenium and protein in different parts of red glutinous rice seeds. Using *Heterosis* (Huang *et al*., 2016), we cross-fertilized different rice species for five years, then we obtained the natural selenium rich red glutinous rice Z5097A (sterile lines) and Z5097B (maintainer line). The selenium contents in them are high enough to meet the daily selenium intake. Moreover, analysis of successive abrasive brown rice milling fraction has shown that nutrients aren't uniformly distributed in brown rice. In addition, we proved that the selenium and protein are in direct relationship in red glutinous rice, the more protein concentration the more will be selenium contents in different parts of rice. More than 80% selenium in the grain is organic selenium (in selenoprotein form) and more than half of the total selenium is in the endosperm of the red glutinous rice.

Materials and Methods

Plant Material

The rice germplasm (2045B red rice, maintainer line, as female parent) used in this study while local glutinous rice and D62 A (Sterile line) were of Chinese origin. Then conventional hybridization was conducted between local glutinous rice and 2045B. The progenies since F3 hybrids were selfed and then test crossed with D62A. Continuously backcrossed till the material entered into most advanced F7 generation. The red rice sterile and maintainer line materials (numbered 5097A and B) observed thoroughly with identical characters in both parents were preferably selected for backcross, selection and preservation in the Winter of 2013. After years of generations of breeding, being named as Z5097A and Z5097B (Fig. 1).

Preparation of Red Glutinous Rice Powder

The fresh red glutinous rice samples (cleaned with distilled water) were put into the drying oven (DHG-9240B, Shen Xian Co., Ltd, Shanghai, P. R. China) 80℃ for 3 h. Then the brown rice machine (JLG-II, Da Ji Co., Ltd, Hangzhou, P. R. China) was used to separate hulls and brown rice (with episperm, embryo, aleurone layer and endosperm). Then, tweezers and dissecting needles were used to fetch the episperm (red surface), embryo of brown rice and residues with white and a little yellow color. Moreover, we used the rice milling machine (JNM-III, CHINA GRAIN RESERVES CORPERATION) for 45 sec to separate the aleurone layer and endosperm, referred as Lamberts *et al.* (2007), Singh *et al.* (2000). All parts of material were then put into pulverizer (F160, Zhong Xing Co., Ltd, Beijing, P. R. China) to make powder, filtered with 100-grade sifter to obtain the tested flour for different studies.

Protein Extraction

The total protein extracted from red glutinous rice according to the method of Daiana *et al.* (2016) with some modifications. Weighted red glutinous rice powder (filtered with 100-grade sifter) 100 g, added into NaOH (0.05 mol/L, 1500 mL), DTT (DL-Dithiothreitol) 2.5 g and sodium hyposulfite 0.5 g. Then put them inside the ultrasonic cleaners (WD-9415B, LiuYi Co., Ltd, Beijing, P.R. China) with frequency of 100 Hz at 40℃ for 4 h. Afterwards, the suspension was centrifuged (5804R, Eppendorf Co., Ltd, Germany) at 3000×g for 20 min. According to Ju *et al*. (2001), the isoelectric points of albumin (pH 4.1), globulin (pH 4.3 and pH 7.9), and glutelin (pH 4.8), proteins were precipitated from the protein extract with HCl at pH 5.0. Herein after centrifugation at 3000×g for 20 min, precipitate referred to as the isoelectric precipitation protein concentrate (IPPC). Washed with deionized water and packed into dialysis bag for dislodging salt and micro molecular impurities. Then put the protein into ultra-low temperature refrigerator (MDF-U3386S, Panasonic Co., Ltd, Japan) at -80℃ for 6 h. In the end, after vacuum refrigeration protein flour can be obtained.

Determination of Protein Content

The content of protein determined by Kjeldahl method (Ayalew *et al*., 2017) and National food safety standard determination of protein in food (GB 5009.5—2010), with a little modification. First, weigh 1.00 g powder samples, put in glass digestive tube (250 mL), add into $CuSO_4$ (0.2 g), K_2SO_4 (6 g) and H_2SO_4 (10 mL). After gently shaking, put a small funnel on the top of every tube. The mixture was then heated with graphite digestion apparatus (SH220, Hanon Instruments Co., Ltd, Jinan, P. R. China) until the liquid turned into blue-green and clear. Afterwards, we used the *Kjeldahl* nitrogen determination apparatus (K9860, Hanon Instruments Co., Ltd, Jinan, P.R. China) to measure the protein content. Made the blank control group at the same time. The formula for calculating total protein is as follows:

$$protein\ content\ (\%) = \frac{(V_1 - V_2) \times c \times 0.0140}{m \times V_3/100} \times F \times 100$$

Where, V_1 is the volume (mL) of samples' liquid H_2SO_4 consumption standard titration solution; V_2 is the volume (mL) of control group (without sample); V_3 is the volume (mL) of absorbed digestive solution; c is the concentration (mol/L) of H_2SO_4 standard titration solution; 0.0140 is the mass of nitrogen of 1.0 mL H_2SO_4; m is the mass of samples (g); F means the coefficient of nitrogen conversion for protein (rice is 5.95).

Determination of Se Content

The content of Se determined by the National food safety standard determination of selenium in foods (GB 5009.93—2010), Wu *et al*. (2007) with a little modification. Primarily weigh 0.1g flour samples into glass digestive tube, added into HNO_3 (9 mL) and $HClO_4$ (1 mL), mixed up then covered. After ultrasonic cleaners (WD-9415B, LiuYi Co., Ltd, Beijing, P.R. China) at 20℃ for 4 h with frequency 100 Hz, then heated using electric hot plate (EH20A Plus, Labtech, USA), HNO_3 added occasionally. When the solution's volume left less than 2 mL and turned into colorless and became clear with blank smoke, made it cools down. Then added HCl (5.0 mL, 6 mol /L), and heated again until the solution turned into colorless and became clear with blank smoke. Cooled it down, poured into volumetric flask, made the blank control group at the same time. After that it was necessary to make the standard curve by adding 0.00 mL, 0.10 mL, 0.20 mL, 0.30 mL, 0.40 mL and 0.50 mL selenium standard solution in centrifugal tube (15 mL), added deionized water to the volume of total 10 mL, HCL (2 mL, guarantee reagent) and potassium ferricyanide (1 mL, 100 g/L). Afterwards, we used the atomic fluorescence spectrophotometer (RGF-6800, Bo Hui Co., Ltd, Beijing, P. R. China) to determine the Se content and set the parameter as follows: Negative high voltage: 340 V; Lamp current: 100 mA; The atomization temperature: 800℃; High furnace: 8 mm; The carrier gas flow rate: 500 mL/min; Shielding gas flow rate: 1000 mL/min; Measurement methods: standard curve; Reading: peak area; Delay time: 1 s; Reading time: 15 s; Charging time: 8 s; Sample size: 2 mL. Se content (mg/kg) was calculated with the following formula:

$$Se\ content = \frac{(C - C_0) \times V \times 1000}{m \times 1000 \times 1000}$$

Where, C is the sample measured concentration of digestive solution (ng/mL); C0 is concentration of blank control group (ng/mL); m is mass of samples; V is the total volume of digestive solution.

Statistical Analysis

The experiment was replicated thrice under the same conditions. Data were expressed as the mean ± standard error (SEM). One-way ANOVA was carried out with multiple comparisons using Duncan's test to compare the means of different treatments at $p \leq 0.01$. All statistical analyzes were performed using the SPSS 21.0 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Protein Content

Results of protein content determined by *Kjeldahl* method showed that different parts of red glutinous rice have different protein concentrations. Brown red glutinous rice has more total protein than polished red glutinous rice (Fig. 2A). Among all the parts of red glutinous rice, there are significant different protein contents (Fig. 2B). The content of protein is in the following descending order: embryo > aleurone layer > glume > episperm. The protein extracts with protein content 81.65 ± 1.75% is very high compared with others (Fig. 2C). But the result of the protein extract residue illustrated that there is still a little protein remains in the residue after protein extraction.

Se Content

As can be seen from the Fig. 3A, a noteworthy difference ($p \leq 0.01$) was detected in the tested material; the selenium content of brown red glutinous rice is higher than polished red glutinous rice. Brown red glutinous rice have episperm, endosperm, embryo and aleurone layer, while polished red glutinous rice has endosperm and little embryo. So, we divided different parts of rice to measure the selenium content Fig. 3B. Compared with protein content, we observed relatively the similar trend in selenium content of different parts of rice. The embryo has the highest selenium content than other parts, while the episperm with the minimum protein content has the minimum selenium content. Fig. 3C showed that the selenium content in the red glutinous rice protein extract flour is much higher in comparison to the residue.

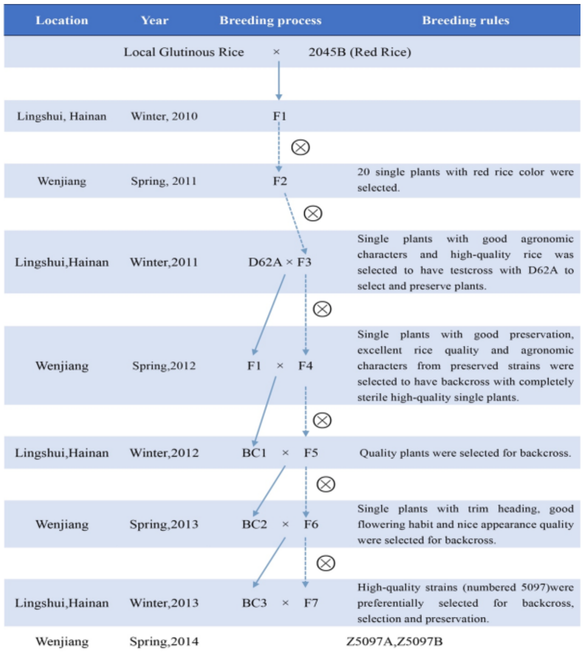

Fig. 1: Breeding process of sterile lines Z5097A and maintainer line Z5097B

Wenjiang, a district in Chengdu, China. Lingshui, Hainan, China. ×means crossing;⊗ means selfing; A means sterile lines; B means maintainer line; F means crossing generation; BC means backcross generation

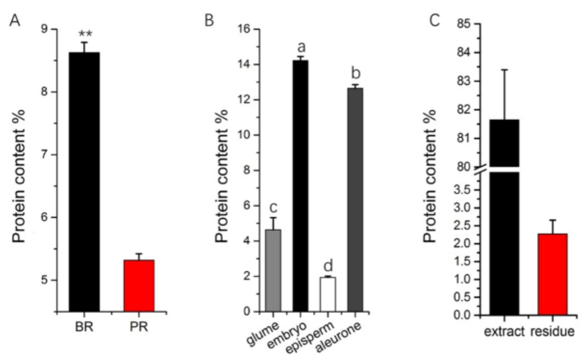

Fig. 2: The protein content from different parts in red glutinous rice

(A) The protein content of brown red glutinous rice and polished red glutinous rice. BR means brown red glutinous rice; PR is polished red glutinous rice. Error bars show the SEM (n = 3). Different letters on the top of column indicate significant differences at $p \leq 0.01$

(B) The protein content in different parts of red rice grain. Error bars show the SEM (n = 3). Lowercase letters on the top of column indicate the statistical significance between different parts of rice according to the Duncan's test ($p \leq 0.01$)

(C) The protein content of protein extracts and residue. Error bars show the SEM (n = 3)

Se Distribution

In red glutinous rice grain, there are five parts making up the whole seed: glume, episperm, endosperm, aleurone layer and embryo (Fig. 4). In a grain of red glutinous rice, the weight of glume, accounts for 17.84%, while endosperm occupies 60.66% weight. Thus, in one-gram red glutinous rice seed, all of the five parts contain Se while endosperm significantly has the most of it (Fig. 5A). Besides, the episperm is the only part that has the red color (Fig. 4), which accounts for 7.35% in a grain of red glutinous rice while it holds Se just 2.76%.

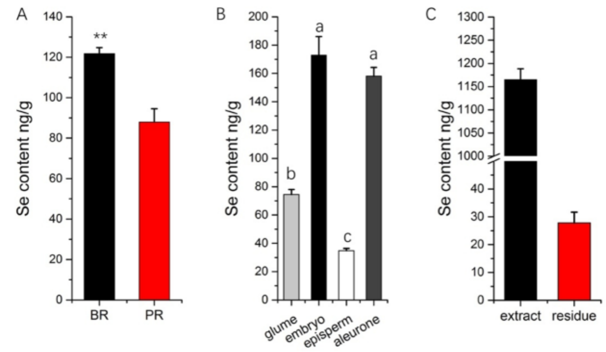

Fig. 3: The Se content from different parts in rice the red glutinous rice

(A) The Se content of brown red glutinous rice and polished red glutinous rice. BR means glutinous rice; PR is polished red glutinous rice. Error bars show the SEM (n = 6). Different letters on the top of column indicate significant differences at $p \leq 0.01$

(B) The Se content in different parts of red rice grain. Error bars show the SEM (n = 6). Lowercase letters on the top of column indicate the statistical significance between different parts of rice according to the Duncan's test ($p \leq 0.01$)

(C) The Se content of protein extracts and residue. Error bars show the SEM (n = 6)

Discussion

Breeding of Red Glutinous Rice

In China, most of the selenium rich rice is produced from inorganic selenium fertilizer by spraying on the leaves such as Na_2SeO_3 (Chen *et al*., 2002; Yong *et al*., 2009). But it is difficult to ensure the selenium content in rice is within safety boundary. As previously reported, excessive inorganic selenium compounds are toxic to human health (Spallholz, 1994). Uptake of Selenium by plant roots depends greatly on rhizospheric conditions of crops such as competing anions, pH, type of clay minerals and hydrous oxides of iron while the form and concentration of Se in the soil also affects the absorption rate of it (Dhillon and Dhillon, 2003). Thus, to ensure healthy food, it is necessary to monitor and optimize crop selenium concentrations (Zhu *et al*., 2009) and electing the rice that has strong ability to uptake selenium from soil is a good way to avoid the selenium deficiency or toxicity problem. After five years breeding, the sterile lines Z5097A and maintainer line Z5097B holds selenium contents as 121.74 ± 3.01 ng/g (National standard of selenium rich rice is ≥40 ng/g). In addition, the total Se concentrations is more than most other rice in China, Japan, Egypt, France and other countries compared with Williams *et al.* (2009) reports.

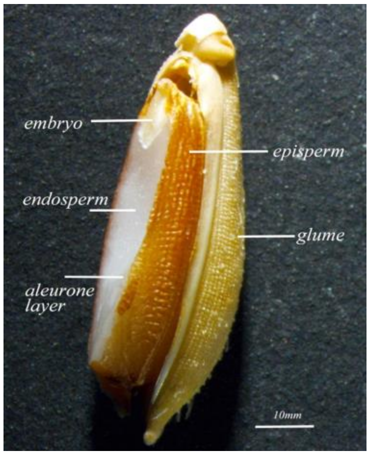

Fig. 4: The endoscopic observation of red glutinous rice
The scale bar represents 10 mm

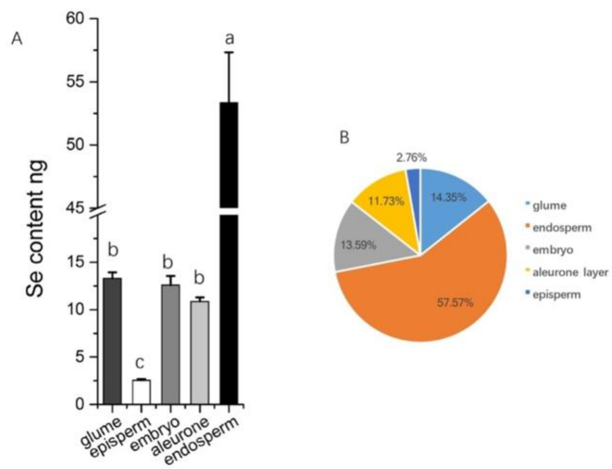

Fig. 5: Se distributes in different part of red glutinous rice grain
(A) The content of Se in different parts of one-gram red glutinous rice seed. Error bars show the SEM (n = 6). Lowercase letters on the top of column indicate the statistical significance between different parts of rice according to the Duncan's test ($p \leq 0.01$)
(B) Proportion of Se in a grain of red glutinous rice seed

So the range of selenium in red glutinous rice is not too toxic or deficient. Without inorganic Se fertilizer, the natural Se rich red glutinous rice has a high application value.

Protein Concentrations

Chaiyakul *et al*. (2009) believed that rice has relatively low protein content ranged between 6–8%. While Xie *et al.* (2014) found a range of 6.7–13.8% for the protein content in 335 samples of milled indica rice grown in China, which is a little different from ours. While the range of protein contents in polished rice is determined by the different degree of milling (Chen and Siebenmorgen, 1997; Lamberts *et al*., 2007; Wang *et al*., 2007). According to our results, we proved that the high protein concentration in glutinous rice different parts is located mostly in the outermost layers. Some previous studies reported that the brown rice accumulates more nutrient components such as protein than polished rice which supports this study (Chen *et al*., 1998; Ohtsubo *et al*., 2005).

In one-gram red glutinous rice seed, the distribution from outermost to innermost layers was in the following order: glume, episperm, aleurone layer, embryo and endosperm (Fig. 4). It is easily observed that only episperm (the surface of brown rice) contains pigments in natural pigmented rice (Zhu *et al*., 2017). Lamberts *et al*. (2007) believed that approximately 80% of the kernel proteins were located in the starchy endosperm (the degree of milling> 12%). In this research, there is a trend that the protein content gradually starts declining from embryo to episperm (Fig. 2A and B), which is in accordance with Itani *et al*. (2002) and Resurrection *et al*. (1979). The protein contents found maximum in the embryo is due to the reason that the embryo is the rich source of nutrients and is the next generation plant (Jia *et al*., 2017). So it is obvious that the maximum protein contents in embryo are needed for plants proper growth and development. Rice protein, with a high nutrition value and good digestion, has a strong ability in antioxidant and antiatherosclerotic (Burris *et al*., 2010). Thus, researching for a fast and simply method to extract red glutinous rice protein can improve the application value in the food industry. Compared with Daiana *et al.* (2016), the extract protein content is approximate equality, but the residue with 2.27 ± 0.38% protein is a little more than 1.30 ± 0.03% as reported (Fig. 2C). So, how to enhance the protein concentration in extract and decline protein in residue deeply needs to be studied in the future.

Se Concentrations and Distribution

As Lamberts *et al*. (2007) reported, minerals were more abundant in the outer bran layers, losses of minerals reached up to 84.7%, during the milling process for brown rice to polished rice. Promuthai *et al*. (2007) believed that milling process resulted in 25–84% iron loss from different brown rice cultivars. Like other nutrients, selenium is lost inevitably from brown rice to polished rice during milling process (Fig. 3A). Embryo and aleurone layer have more Se than polished rice (endosperm). Fig. 3A, B, which coincides with Sun *et al*. (2010) stated that the Se concentration in bran is 1.9 times higher than corresponding polished rice. The Se content of the protein extract has the highest concentration as 1164.86 ± 23.67 ng/g (Fig. 3C) and it demonstrated that Se prevails in the rice grain is organic Se (selenoprotein), which are in settlement with the findings of Sun *et al*. (2010). Above all the results showed that there is positive correlation between selenium and protein contents, the more protein concentration the more selenium content, while the protein content of embryo is nearly 7.3 times to episperm but the Se concentration of embryo is five times to episperm. This revealed that availability of Se in the grain is not only in selenoprotein form; plant also accumulated other forms of Se such as Se-polysaccharide, selenium nucleic acid (Stadtman, 1983; Wang *et al*., 2013).

Total Se concentrations in the rice fractions varied in the following order: husk < polished rice < whole grain < bran. The Se distribution for the one gram of red glutinous rice seed (Fig. 5A and B) showed that organic Se deposited into the embryo, aleurone layer and ultimately in the endosperm. More than half of the total selenium is found in the endosperm of the red glutinous rice as disclosed by Carey *et al*. (2012). Williams *et al*. (2009) demonstrated that mature rice grain's endosperm predominantly has organic form of Se while limited to the bran layer was inorganic Se. Kadasi *et al*. (2010) indicated that the dispersal of S and Se was coincident, evenly distributed throughout the endosperm's aleurone layer, while specifically deposited around the starchy endosperm cells (starch granules) a rich proteinaceous area. Only 5% of the Se was present as inorganic Se in the endosperm. On the contrary, we found more than 80% selenium in the grain as organic Se (selenoprotein).

Conclusion

Based on the results observed in the present study and the aforementioned discussion, it is concluded that the selenium and protein are in direct relationship in red glutinous rice, the more the protein the more will be selenium contents in different parts of rice. Moreover, more than 80% selenium in the grain is organic selenium (in selenoprotein form) and more than half of the total selenium is in the endosperm of the red glutinous rice. Besides, there is still need to identify, what molecular mechanisms or pathways involved in the uptake of selenium, protein and aids their accumulation within specific parts. Meanwhile, the bio-fortified red glutinous rice obtained through genetic improvement by breeding is a natural source of selenium and can be used for further application uses, as natural alternate source to fulfill body selenium demands.

Acknowledgments

This work was supported by the International Cooperation and Exchanges Research Program of the Department of S&T of Sichuan Province (2015HH0028), Technology R&D Program of Sichuan Province (2016NZ0106).

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(Received 04 October 2017; Accepted 02 January 2018)