



## Evaluation of Extraction Solutions for Biochemical Analyses of the Proteins in Rice Grains

Gang-hua LANG, Yukari KAGIYA, Mayumi OHNISHI-KAMAYAMA, and Kazumi KITTA<sup>†</sup>

National Food Research Institute, National Agriculture and Food Research Organization,  
2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

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**The influence of different extraction solutions on the proteins extracted from rice grains was investigated. The largest amounts of salt-soluble proteins were extracted with solutions supplemented with Tris-HCl at pH 8.0. Rice allergens were analyzed by multiplex immunodetection. Except for  $\alpha$ -globulin extracted with the solutions at pH 8.0, which showed a low-molecular-weight band besides the main band, no significant solution-dependent difference among the allergens was found. Total proteins were extracted with four kinds of solution. The extraction of the basic subunit of glutelin was found to be SDS-dependent, and more protein was obtained with extraction solutions supplemented with SDS. The contents of  $\alpha$ -globulin and  $\alpha$ -amylase/trypsin inhibitors were higher in the extracts without SDS than with SDS. We conclude from the present data that, in order to obtain comparable data from rice grain salt-soluble and total protein analyses, differences in the protein extraction efficiency of solutions used should be taken into consideration.**

**Key words:** salt-soluble protein; total protein; rice grain; multiplex immunodetection; allergen

Rice is consumed as a staple food by more than half of the world's population,<sup>1)</sup> and the proteins in rice grain have an important impact on both the nutritional and textural quality of rice. Protein is the most commonly analyzed nutrient in rice, and its content has been found to differ significantly by variety. Analyses of the soluble proteins are widely used to characterize rice varieties. Rice protein contents range from 4.5% to 15.9% for *Oryza sativa* varieties and from 10.2% to 15.9% for *Oryza glaberrima* varieties.<sup>2)</sup> The soluble fractions of rice proteins consist of water-soluble albumin, salt-soluble globulin, alcohol-soluble prolamin, and acid- and alkali-soluble glutelin. In order to characterize rice varieties, several rice-soluble proteins have been analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE),<sup>3–11)</sup> 2-dimensional (2D) gel electrophoresis,<sup>12)</sup> gel filtration chromatography,<sup>13)</sup> and high-performance liquid chromatography.<sup>14)</sup> A 2D-PAGE-based database of rice proteins has also been established.<sup>15)</sup>

The development of genetically modified (GM) rice has been advancing because of such potential benefits

as increased crop yield, high protein or vitamin content, insect and/or disease resistance, and herbicide tolerance.<sup>16)</sup> Analyses of the rice protein profile and allergenic protein content to guard against unintended effects of genetic modification on rice storage proteins have recently attracted attention from both nutritional and toxicological viewpoints. Teshima *et al.* have examined the natural variation of rice salt-soluble proteins among different rice cultivars by 2D fluorescence difference gel electrophoresis (2D-DIGE).<sup>17)</sup> Rice allergens in the salt-soluble proteins of a transgenic rice and its non-transgenic counterpart have also been compared by 1D and 2D immunoblotting analyses.<sup>18)</sup>

Analyses of rice total proteins have also been widely used in rice composition studies;<sup>16,19–23)</sup> however, possibly because most reported rice allergens have been included in salt-soluble proteins, rice salt-soluble proteins have always been used for allergen analyses.<sup>24–28)</sup> Even so, among the available reports, the composition of solutions for extracting both salt-soluble and total proteins varies from laboratory to laboratory (Table 1). To evaluate the possible influence of the extraction solution on the amount and composition of extracted rice proteins, we extracted the salt-soluble, salt-insoluble and total proteins from grains of Koshihikari (*Oryza sativa* cv. Koshihikari), one of the most popular varieties of rice cultivated in Japan, by using representative extraction solutions. The extracted proteins were quantitatively and qualitatively analyzed. Several rice proteins with molecular masses of 9, 14–16, 26, 33 and 60 kDa have so far been reported to be recognized by the serum IgE of patients showing hypersensitive reactions to rice ingestion. These rice allergens have been identified as non-specific lipid transfer protein-1 (nsLTP1),<sup>29)</sup>  $\alpha$ -amylase/trypsin inhibitors,<sup>25)</sup>  $\alpha$ -globulin,<sup>30)</sup> glyoxalase I (GlyI),<sup>24)</sup> and granule-bound starch synthetase (GBSSI).<sup>26)</sup> Most of these allergens have been used to assess the safety of GM rice.<sup>18,31)</sup> In order to evaluate the influence of various extraction solutions on the extraction of rice allergens, we prepared an antiserum against each of these allergens, and developed a multiplex immunodetection method to analyze the rice allergens in the extracts.

<sup>†</sup> To whom correspondence should be addressed. Tel/Fax: +81-29-838-7369; E-mail: kaz@affrc.go.jp

**Abbreviations:** SDS-PAGE, SDS-polyacrylamide gel electrophoresis; 2D-DIGE, 2-dimensional fluorescence difference gel electrophoresis; nsLTP1, non-specific lipid transfer protein-1; Gly I, glyoxalase I; GBSSI, granule-bound starch synthetase I; DTT, DL-dithiothreitol; PVDF, polyvinylidene difluoride; HSD, honest significant difference; pI, isoelectric point; MALDI-TOF MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry

**Table 1.** Solutions Applied to Extract Proteins from the Rice Grains

| Protein              | Extraction solution                                                                                  | References                                                                                                                         |
|----------------------|------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------|
| Salt-soluble protein | 1 M NaCl                                                                                             | Satoh <i>et al.</i> , 2011, <sup>18)</sup> Teshima <i>et al.</i> , 2010, <sup>17)</sup> Usui <i>et al.</i> , 2001 <sup>24)</sup>   |
|                      | 0.7 M NaCl                                                                                           | Perdon and Juliano, 1978 <sup>5)</sup>                                                                                             |
|                      | 0.5 M NaCl                                                                                           | Limas <i>et al.</i> , 1990, <sup>6)</sup> Matsuda <i>et al.</i> , 1991 <sup>25)</sup>                                              |
|                      | 5% NaCl                                                                                              | Iwasaki <i>et al.</i> , 1982 <sup>13)</sup>                                                                                        |
|                      | 2% NaCl                                                                                              | Stenson and Sathe, 1995 <sup>7)</sup>                                                                                              |
|                      | 0.5 M NaCl, 50 mM PBS (pH 6.8)                                                                       | Asano <i>et al.</i> , 2000, <sup>8)</sup> Ikezawa <i>et al.</i> , 1999, <sup>26)</sup> Kumamaru <i>et al.</i> , 1988 <sup>9)</sup> |
|                      | 0.5 M NaCl, 20 mM Tris-HCl (pH 8.0)                                                                  | Momma, 2005 <sup>10)</sup>                                                                                                         |
|                      | 1 M NaCl, 50 mM Tris-HCl (pH 8.0)                                                                    | Chen <i>et al.</i> , 1987 <sup>12)</sup>                                                                                           |
|                      | 10 mM PBS (pH 7.0)                                                                                   | Yamada <i>et al.</i> , 2005 <sup>27)</sup>                                                                                         |
|                      | 50 mM Tris-HCl (pH 7.5), 2% (w/v) SDS, 0.6% (v/v) 2-mercaptoethanol and 4 M urea                     | Chen <i>et al.</i> , 1987 <sup>11)</sup>                                                                                           |
| Total protein        | 62.5 mM Tris-HCl (pH 6.8), 10% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 4 M urea | Shyur <i>et al.</i> , 1988 <sup>19)</sup>                                                                                          |
|                      | 50 mM Tris-HCl (pH 6.8), 20% (w/v) glycerol, 4% SDS, 5% (v/v) 2-mercaptoethanol and 8 M urea         | Takagi <i>et al.</i> , 2005, <sup>16)</sup> Kawagoe <i>et al.</i> , 2005 <sup>20)</sup>                                            |
|                      | 50 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 4% SDS, 5% (v/v) 2-mercaptoethanol and 8 M urea         | Teda <i>et al.</i> , 2003 <sup>4)</sup>                                                                                            |
|                      | 1% (v/v) Triton X-100, 2.5% (w/v) DTT and 8.5 M urea                                                 | Lin <i>et al.</i> , 2005 <sup>21)</sup>                                                                                            |
|                      | 4% (w/v) CHAPS, 40 mM Tris, 65 mM DTT and 8 M urea                                                   | Zhang <i>et al.</i> , 2012 <sup>22)</sup>                                                                                          |
|                      | 4% (w/v) CHAPS, 10 mM phenylmethylsulfonylfluoride, 7 mM DTT and 8 M urea                            | Xu <i>et al.</i> , 2012 <sup>23)</sup>                                                                                             |
|                      |                                                                                                      |                                                                                                                                    |
|                      |                                                                                                      |                                                                                                                                    |

## Materials and Methods

**Materials.** The cDNA clones of nsLTP1 (006-212-H12 and AK061288), protein RAG2 (a member of the rice 14–16-kDa  $\alpha$ -amylase/trypsin inhibitor family proteins) (002-126-F01 and AK10738),  $\alpha$ -globulin (J090087L24 and AK242943), Gly I (J033136J17 and AK103694) and GBSSI (J023052C23 and AK070431) were obtained from the National Institute of Agrobiological Sciences (Tsukuba, Japan). The protease inhibitor cocktail tablet, Complete Mini, was obtained from Roche Diagnostics (Indianapolis, IN, USA). Can Get Signal Solutions 1 and 2 were from Toyobo (Osaka, Japan), and DL-dithiothreitol (DTT) was from Sigma Chemicals (St. Louis, MO, USA). The ECL Plex fluorescent rainbow markers, ECL Plus Western blotting detection reagents, 2-D Clean-Up kit and 2-D Quant kit were from GE Healthcare UK (Buckinghamshire, England). The MagicMark XP Western protein standard and Novex Sharp pre-stained protein standard were obtained from Invitrogen (Carlsbad, CA, USA). HRP-labeled goat anti-rabbit IgG (H + L), Quick Start Bradford protein assay kit and Bio-safe Coomassie stain were from Bio-Rad Laboratories (Hercules, CA, USA). Unless otherwise stated, all other materials and reagents used for SDS-PAGE and immunoblotting were obtained from Bio-Rad Laboratories. Brown rice grains of Koshihikari (*Oryza sativa*, japonica) produced in Ibaraki (Japan) were purchased at a local market.

**Extraction of the rice salt-soluble and salt-insoluble proteins.** Brown rice grains were pulverized three times for 30 s each time with a 1-min resting interval by an IFM-800DG lab mixer (Iwatani, Japan). The following six kinds of salt solution were prepared to extract the salt-soluble proteins: solution I, 1 M NaCl; solution II, 0.5 M NaCl; solution III, 30 mM Tris-HCl (pH 6.8) and 1 M NaCl; solution IV, 30 mM Tris-HCl (pH 6.8) and 0.5 M NaCl; solution V, 30 mM Tris-HCl (pH 8.0) and 1 M NaCl; and solution VI, 30 mM Tris-HCl (pH 8.0) and 0.5 M NaCl. Complete Mini protease inhibitor cocktail tablets were supplemented to each solution immediately before use.

The salt-soluble proteins were extracted three times from each 100 mg of rice flour. In the first extraction, for each of the six kinds of salt solution, 1 mL of the salt solution was added to 100 mg of rice flour in a 2-mL microcentrifuge tube and mixed by vortexing. The tube was rotated on a rotary mixer at room temperature (25 °C) for 2 h. After centrifuging at 15,000 *g* for 10 min at 4 °C, 860  $\mu$ L of the resulting supernatant was transferred to a new tube. In the second extraction, 860  $\mu$ L of each salt solution was added to the corresponding precipitate. The precipitate was suspended by vortexing which was

continued for another 20 s. The tube was then centrifuged, and 860  $\mu$ L of the supernatant was transferred to a new tube. The third extraction followed the same procedure as the second. To extract the salt-insoluble proteins, 1 mL of a solution containing 55-mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol and 11 mg/mL of DTT were added to the remaining precipitate. The tube was incubated at 90 °C for 3 min after the precipitate had been suspended by vortexing, and then centrifuged at 15,000 *g* for 10 min at 4 °C. In the last step, 700  $\mu$ L of the supernatant was transferred to a new tube.

**Extraction of the rice total proteins.** The following four kinds of extraction solution that had been used in previous studies were selected to extract the total proteins from Koshihikari rice flour: solution A,<sup>11)</sup> 50 mM Tris-HCl (pH 7.5), 2% (w/v) SDS, 0.6% (v/v) 2-mercaptoethanol and 4 M urea; solution B,<sup>4)</sup> 50 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol and 8 M urea; solution C,<sup>21)</sup> 1% (v/v) Triton X-100, 2.5% (w/v) DTT, and 8.5 M urea; and solution D, one of the lysis buffers recommended by GE Healthcare, 4% (w/v) CHAPS, 60 mM DTT and 8 M urea. With each kind of solution, 1 mL was added to 25 mg of rice flour in a 2-mL microcentrifuge tube and mixed by vortexing. The tube was rotated on a rotary mixer at room temperature (25 °C) for 2 h. The resulting supernatant was transferred to a new tube after centrifuging at 15,000 *g* for 10 min at 4 °C. Whenever necessary, the proteins in the extracts by solutions A and B were purified with the 2-D Clean-Up kit according to manufacturer's protocol, and then re-dissolved in solution D.

**SDS-PAGE and multiplex immunodetection analysis.** The extracted rice proteins were resolved by SDS-PAGE, using a 15% (w/v) separating gel with a 4% (w/v) stacking gel. The gels were stained with Bio-safe Coomassie stain after the electrophoresis. Otherwise, for immunoblotting, the resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting with a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories). To detect the extraction efficiency of the rice allergens, we developed a multiplex immunodetection method with a mixture of rabbit antisera against rice nsLTP1 (1:4,000 dilution),  $\alpha$ -amylase/trypsin inhibitors (1:8,000 dilution),  $\alpha$ -globulin (1:20,000 dilution), Gly I (1:4,000 dilution) and GBSSI (1:4,000 dilution) to simultaneously detect the respective rice allergens. Full-length proteins of nsLTP1, RAG2,  $\alpha$ -globulin, GlyI and a peptide (379–582 residues) of GBSSI were respectively expressed in *Escherichia coli*, and the recombinant proteins were respectively used as antigens. The rabbit antisera were prepared by immunizing rabbits with the respective recombinant proteins as previously described.<sup>28,32)</sup>

Immunoblotting and signal detection were also carried out as previously described.<sup>28)</sup> The chemiluminescent signal was detected by the PharoFX molecular imager system (Bio-Rad Laboratories), the signal intensity being quantified by using Quantity One 1-D Analysis software (Bio-Rad Laboratories).

**In-gel digestion and protein identification by MALDI-TOF MS.** After the rice proteins had been resolved by SDS-PAGE and stained with Bio-safe Coomassie stain, the protein in the main band corresponding to  $\alpha$ -globulin and the fraction in the band just below it were excised and treated by tryptic digestion. The obtained peptide mixtures were subjected to MALDI-TOF MS analyses as previously described.<sup>33)</sup>

**Determination of the protein concentrations.** A Quick Start Bradford Protein Assay kit was used with bovine serum albumin as a standard to determine the concentration of salt-soluble proteins, and the 2-D Quant kit was used to determine the concentrations of salt-insoluble proteins and total proteins.

**Statistical analysis.** The data were subjected to a one-way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test by one-way-between-groups ANOVA statistics software ([http://www.wessa.net/rwasp\\_OneFactorANOVA.wasp](http://www.wessa.net/rwasp_OneFactorANOVA.wasp)). Differences between groups were considered significant at  $p < 0.05$ .

## Results and Discussion

### *Amounts of salt-soluble and salt-insoluble proteins extracted from rice grains*

Among the salt solutions reported by other groups for extracting the proteins from rice grains, 1 M and 0.5 M NaCl have been widely used, and either a Tris-HCl or phosphate buffer was supplemented in many cases. The pH value was adjusted to 7.8 or 7.0 in the phosphate buffer and 8.0 in the Tris buffer. To evaluate the effects of NaCl concentration and pH on the extraction efficiency in the present study, we designed six kinds of salt solution supplemented with NaCl (1 M or 0.5 M) and with or without Tris-HCl to extract the proteins from rice flour. After a three-times extraction of the salt-soluble proteins, we extracted the salt-insoluble proteins in the remaining sediment with a solution that had been used to extract starch-binding proteins.<sup>34)</sup> Since the same volume of supernatant was collected from each tube after each extraction, the protein concentrations were proportional to the recovered proteins content and were used to compare the extraction efficiency of the salt solutions.

Table 2 shows that the largest amounts of salt-soluble proteins were extracted by the first extraction; the

protein concentrations by the second extraction were about one-seventh of those by the first extraction, while those by the third extraction were undetectable. The results of Tukey's HSD test suggested that, in the first extraction, the concentrations of proteins extracted with the salt solutions supplemented with Tris-HCl at pH 8.0 (solutions V and VI) were significantly higher than those extracted with the other salt solutions ( $p < 0.05$ ). No significant difference was apparent in protein concentrations between extracting with 1 M NaCl and 0.5 M NaCl supplemented or not with Tris-HCl at the same pH value ( $p > 0.05$ ).

The pH value of the salt solutions without Tris-HCl (solutions I and II) was 4.3 before extraction and increased to 5.9 after being mixed with rice flour, which showed the influence of rice flour on the pH value of the extraction solution, and suggested that a buffer system was necessary to maintain a constant pH value for the extraction solutions. Since most salt-soluble proteins in rice grains had an isoelectric point (pI) ranging from approx 5.0 to 8.0 (data not shown), more salt-soluble proteins might have been electrically charged and dissolved in the salt solutions at pH 8.0 than at pH 6.8 or pH 5.9. No significant difference was apparent in protein concentrations ( $p > 0.05$ ) by the second extraction of salt-soluble proteins. The salt-insoluble proteins were extracted from the remaining sediment after extracting the salt-soluble proteins. Among the extracts of salt-insoluble proteins, only the extract from the sediment of the 0.5 M NaCl (solution II) showed a significantly higher protein concentration than those of the other extracts ( $p < 0.05$ ).

### *SDS-PAGE and multiplex immunodetection of rice allergens from salt-soluble and salt-insoluble proteins*

We analyzed the influence of extraction solution on the composition of the extracted salt-soluble and salt-insoluble proteins by subjecting these proteins to SDS-PAGE and conducting multiplex immunodetection of the rice allergens. Among the salt-soluble proteins resolved on the SDS-PAGE gels (Fig. 1A), an obvious difference was found in the main bands corresponding to  $\alpha$ -globulin and the bands just below them. Judged by the signal intensity, the content of  $\alpha$ -globulin in the main band was lower, while the content in a band just below the main band was higher in the salt-soluble proteins extracted with solutions at pH 8.0 supplemented with either 1 M (solution V) or 0.5 M NaCl (solution VI) when

**Table 2.** Concentration of Salt-Soluble and Salt-Insoluble Proteins Extracted from Grains of Koshihikari Brown Rice

| Solution | Composition <sup>1</sup>            | Protein concentration ( $\mu\text{g/mL}$ ) <sup>2</sup> |                          |                                 |
|----------|-------------------------------------|---------------------------------------------------------|--------------------------|---------------------------------|
|          |                                     | Salt-soluble 1st extract                                | Salt-soluble 2nd extract | Salt-insoluble <sup>3</sup>     |
| I        | 1 M NaCl                            | 1187.7 $\pm$ 66.8 <sup>a</sup>                          | 158.6 $\pm$ 13.9         | 4193.1 $\pm$ 281.2 <sup>a</sup> |
| II       | 0.5 M NaCl                          | 1206.4 $\pm$ 52.9 <sup>a</sup>                          | 164.4 $\pm$ 17.1         | 4947.5 $\pm$ 233.4 <sup>b</sup> |
| III      | 1 M NaCl, 30 mM Tris-HCl (pH 6.8)   | 1247.2 $\pm$ 67.9 <sup>a</sup>                          | 190.8 $\pm$ 36.9         | 4088.7 $\pm$ 494.6 <sup>a</sup> |
| IV       | 0.5 M NaCl, 30 mM Tris-HCl (pH 6.8) | 1258.4 $\pm$ 117.8 <sup>a</sup>                         | 182.7 $\pm$ 10.1         | 4259.9 $\pm$ 341.3 <sup>a</sup> |
| V        | 1 M NaCl, 30 mM Tris-HCl (pH 8.0)   | 1383.7 $\pm$ 54.2 <sup>b</sup>                          | 197.0 $\pm$ 44.7         | 4372.2 $\pm$ 16.4 <sup>a</sup>  |
| VI       | 0.5 M NaCl, 30 mM Tris-HCl (pH 8.0) | 1443.4 $\pm$ 130 <sup>b</sup>                           | 190.3 $\pm$ 12.6         | 4288.3 $\pm$ 192.0 <sup>a</sup> |

<sup>1</sup>Complete Mini tablets were dissolved in each salt solution immediately before use.

<sup>2</sup>Each value is the mean  $\pm$  standard deviation (SD) of data from three independent experiments. Values in the same column followed by different letters are significantly different ( $p < 0.05$ ).

<sup>3</sup>The salt-insoluble proteins were extracted from the remaining sediment of rice flour after being extracted with the respective salt solution three times.

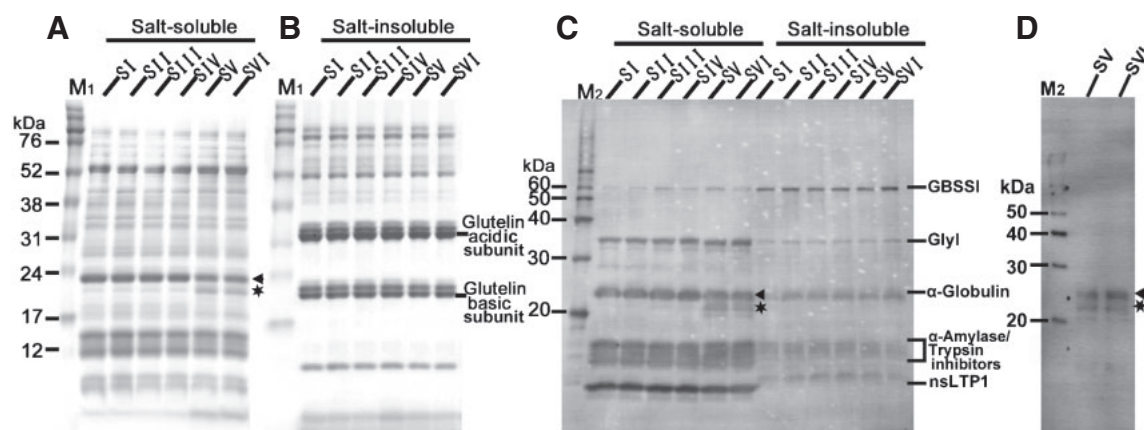


**Fig. 1.** Representative SDS-PAGE Images of the Salt-Soluble and Salt-Insoluble Proteins Extracted from Grains of Koshihikari Brown Rice and Multiplex Immunodetection of the Rice Allergens.

A and B, stained with Bio-safe Coomassie stain; C, multiplex immunodetection; D, immunodetection of  $\alpha$ -globulin in the salt-soluble proteins extracted with solutions V and VI. Four micrograms of salt-soluble proteins extracted with six kinds of salt solution (indicated by SI to SVI) and salt-insoluble proteins extracted from the respective remaining sediment was loaded in each lane. The rice allergens (nsLTP1,  $\alpha$ -globulin, Gly I,  $\alpha$ -amylase/trypsin inhibitors and GBSSI) were multiplex-immunodetected with a mixture of polyclonal antisera against the respective allergens. Both  $\alpha$ -globulin in the main band and the fraction just below the main band in extracts of SV and SVI immunoreacted with the antiserum against  $\alpha$ -globulin (1:20,000 dilution). Triangles show the position of the main band for  $\alpha$ -globulin; asterisks show the position of the fraction just below the main band of  $\alpha$ -globulin; M<sub>1</sub>, ECL Plex fluorescent rainbow markers; M<sub>2</sub>, MagicMark XP Western protein standard.

compared with the other extracts. With regard to the salt-insoluble proteins, the acidic and basic subunits of glutelin constituted the main fractions among the proteins resolved by SDS-PAGE (Fig. 1B). No obvious difference was apparent in the SDS-PAGE patterns of the salt-insoluble proteins.

Multiplex immunodetection showed that nsLTP1,  $\alpha$ -amylase/trypsin inhibitors,  $\alpha$ -globulin and Gly I were mainly detectable in the salt-soluble proteins, while GBSSI was mainly detectable in the salt-insoluble proteins (Fig. 1C). A weak signal of GBSSI was also observed in the salt-soluble proteins, and weak signals from trace amounts of nsLTP1,  $\alpha$ -amylase/trypsin inhibitors,  $\alpha$ -globulin and Gly I were also detected among the salt-insoluble proteins. Both  $\alpha$ -globulin in the main band and the fraction in the bands just below the main band were found to immunoreact with the antiserum against  $\alpha$ -globulin (Fig. 1D). Despite the lower signal intensity in the main band of  $\alpha$ -globulin and the higher signal intensity of the fraction in the band just below the main band of  $\alpha$ -globulin in the salt-soluble proteins extracted with solutions V and VI, no obvious difference was apparent in either the SDS-PAGE pattern or signal intensity of the detected rice allergens.

The proteins in the main band corresponding to  $\alpha$ -globulin and in the band just below it were respectively subjected to tryptic digestion and a MALDI-TOF MS analysis. The peptide mass and MS/MS fragment mass lists were used for a search of the NCBI nr.20110610 database to identify the candidate proteins. The results show that the proteins in the two bands were both  $\alpha$ -globulin. When  $\alpha$ -globulin in the main band was digested by trypsin, seven peptides were observed in the range of  $m/z$  700–4000. Three peptides were identified in the mass spectrum of the fraction in the band just below the main band for  $\alpha$ -globulin: E39-R49, M76-R87 and Q162-R172. In addition to these three peptides, the main band of  $\alpha$ -globulin gave another peptide (V173-Y186). This result indicates that  $\alpha$ -globulin had been partially cleaved at the carboxyl

**Table 3.** Total Proteins Extracted from Grains of Koshihikari Brown Rice with Four Kinds of Solution

| Solution   | Protein concentration ( $\mu\text{g/mL}$ ) | Obtained extract ( $\mu\text{L}$ ) |
|------------|--------------------------------------------|------------------------------------|
| Solution A | 1852.3 $\pm$ 259.5 <sup>a</sup>            | 860                                |
| Solution B | 1632.0 $\pm$ 172.4 <sup>a,b</sup>          | 860                                |
| Solution C | 1407.0 $\pm$ 188.4 <sup>b</sup>            | 700                                |
| Solution D | 1174.5 $\pm$ 223.5 <sup>c</sup>            | 450                                |

Each value is the mean  $\pm$  SD of data from three independent experiments. Values with different letters differ significantly ( $p < 0.05$ ).

terminus to form a fraction with a lower molecular mass, at least in solutions V and VI. This conclusion is supported by the immunodetection result in which both types of protein immunoreacted with the antiserum against  $\alpha$ -globulin (Fig. 1D).

#### Extraction and analysis of the rice total proteins

We used four kinds of solution to extract total proteins from the rice flour. Solutions A to C have been used in previous studies,<sup>4,11,21)</sup> and solution D is one of the lysis buffers recommended by GE Healthcare Life Sciences to extract proteins for a 2D-PAGE analysis. We obtained 860  $\mu\text{L}$  of supernatant by extracting with solutions A and B. However, the gelatinization of starch in solutions C and D resulted in only 700  $\mu\text{L}$  and 450  $\mu\text{L}$  of supernatant being respectively collected by extracting with solutions C and D.

The concentrations of total proteins extracted with these four solutions are shown in Table 3. Tukey's HSD test suggested that the concentration of proteins extracted with solution A was significantly higher than those extracted with solutions C and D ( $p < 0.05$ ), while a significantly lower protein concentration was obtained with solution D ( $p < 0.05$ ). Although no significant difference was apparent in concentration between extracting with solutions A and B and between extracting with solutions B and C, a greater extract volume was

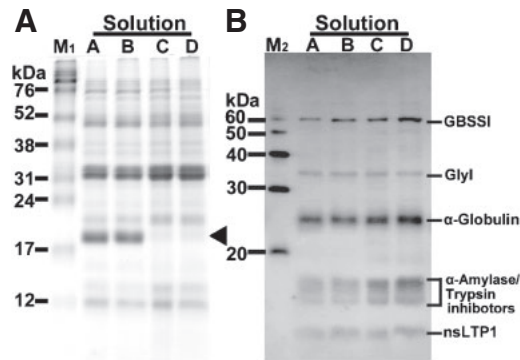


**Fig. 2.** Representative SDS-PAGE Images of Total Proteins Extracted from Grains of Koshihikari Brown Rice and Multiplex Immunodetection of the Rice Allergens.

A, stained with Bio-safe Coomassie stain; B, multiplex immunodetection. Four micrograms of total soluble proteins extracted from Koshihikari with four kinds of solution (indicated by A to D) were loaded into each lane. The rice allergens were multiplex-immunodetected with a mixture of polyclonal antisera against the respective allergens. The triangle indicates the position of the basic subunit of glutelin.

obtained with both solutions A and B than solution C. Consequently, more total protein was obtained with solutions A and B than with solutions C. These proteins were subjected to SDS-PAGE and multiplex immunodetection. Figure 2A shows that the proteins extracted with solutions A and B had similar SDS-PAGE patterns, while the proteins extracted with solutions C and D had similar patterns. The signal intensity of the band corresponding to the basic subunit of glutelin was obviously higher in the extracts by solutions A and B than in those by solutions C and D.

In order to further evaluate the influence of the solvents on the solubility of the basic subunit of glutelin, the solutions were exchanged, after purifying the total proteins extracted with solutions A and B with the 2-D Clean-Up kit, by re-dissolving them in solution D. The basic subunits of glutelin were found to be still dissolvable after the solution exchange (Fig. 3A). In respect of the composition difference among the solutions, Tris-HCl contained in both solutions A and B had no effect in dissolving the basic subunit of glutelin when it was used in the extraction of salt-soluble proteins. 2-Mercaptoethanol contained in solutions A and B, and DTT contained in solutions C and D had a similar effect, *i.e.*, breaking the disulfide bonds, whereas SDS, a widely used anionic surfactant, was present only in solutions A and B. We speculated that SDS in solutions A and B was essential for extracting the basic subunit of glutelin; this was confirmed by extracting the basic subunit with solutions of solution C or D supplemented with SDS (Fig. 3B). Glutelin is the major protein of rice endosperm, comprising approx. 80% of the total rice protein. The combination of SDS and 2-mercaptoethanol has been reported to increase the extraction efficiency of total glutelin from rice.<sup>35)</sup> The present study is the first to prove that dissolution of the basic but not the acidic subunit of glutelin is dependent on SDS.

All of the rice allergens were multiplex-immunodetected from the rice total proteins (Fig. 2B). The intensity of the signals for the  $\alpha$ -globulin and  $\alpha$ -amylase/trypsin

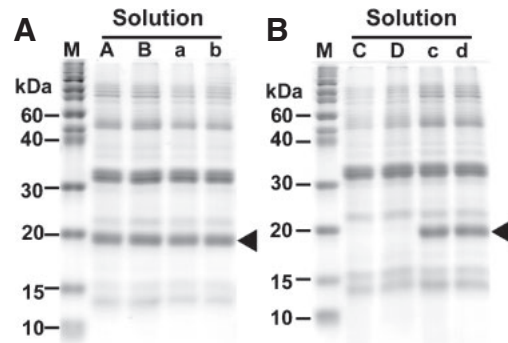


**Fig. 3.** Effect of SDS on the Solubility of the Basic Subunit of Rice Glutelin.

Images of SDS-PAGE gels stained with Bio-safe Coomassie stain. A, the proteins extracted with solutions A and B (indicated by A and B) were purified with the 2-D Clean-Up kit and respectively re-dissolved in solution D (indicated by a and b). B, the basic subunit of glutelin was extracted with solutions C and D supplemented with SDS (indicated by c and d). Protein (4  $\mu$ g) was loaded in each lane. Triangles show the position of the basic subunit of glutelin; M, Novex Sharp pre-stained protein standard.

inhibitors in solutions C and D was obviously higher than that in solutions A and B. Excepting GlyI, signal-intensity variations were also apparent in nsLTP1 and GBSSI among the extraction solutions. This result suggests that differences in the composition of the extraction solutions for total proteins would result in differences in the extraction efficiency for the basic subunit of glutelin as well as in the content of rice allergens.

## Conclusion

The largest amounts of salt-soluble proteins were extracted from rice grains by the first extraction. Larger amounts of salt-soluble protein were extracted by using the salt solutions supplemented with Tris-HCl at pH 8.0 (solutions V and VI) than by the other salt solutions, and no significant difference was found in the extraction efficiency between 1 M NaCl and 0.5 M NaCl in the solutions supplemented or not with Tris-HCl at the same pH value. The rice allergens, including nsLTP1,  $\alpha$ -amylase/trypsin inhibitors,  $\alpha$ -globulin, and Gly I, were mainly contained in the salt-soluble proteins, whereas GBSSI was mainly detected in the salt-insoluble proteins. Moreover, in the extracts with solution V and solution VI,  $\alpha$ -globulin showed an SDS-PAGE pattern for the main band and for a fraction with lower molecular mass which was different from that in each of the other salt solutions.

Extracting of total proteins from the rice grains resulted in larger amount of protein being extracted with solutions A and B in which SDS had been supplemented, and significantly the least amount of protein was extracted with solution D. The basic subunit of glutelin was only extracted with solutions A and B containing SDS; the solubility of the basic but not the acidic subunit of rice glutelin was proved to be dependent on the presence of SDS. Compositional differences in the solutions for extracting of total protein resulted in differing release efficiency for the basic subunit of

glutelin as well as variation in the content of rice allergens.

In conclusion, selecting the most appropriate extraction solutions should be considered in order to obtain comparable and reliable data for rice protein analyses.

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