

The higher temperature SDS-PAGE and assessment for nutritional quality of rice seed storage protein glutelin in wild species

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Abstract

Asian cultivated rice (*Oryza sativa* L.) seed contains around 8-9% protein and contributes about 28-54% of the consumed proteins in the Asian diet. The rice seed protein is, however, deficient in the essential amino acid, lysine. The major storage protein accounting for 70-80% of the total seed protein is glutelin, which is comprised of two subfamilies GluA and GluB. The major subunits of GluB have 20% more lysine in average than GluA subunits. In order to evaluate nutritional quality of rice seed in wild species, we assessed the diversity of glutelin polypeptides by unique SDS-PAGE method and subunit-specific antibodies. SDS-PAGE performed at higher temperature (45°C) than generally employed temperature (4-25°C) resulted in improved separation of microheterogeneous glutelin α polypeptides, which is a prerequisite for the diversity evaluation. Seven anti-peptide antibodies raised against subunit-specific epitope sequences showed high specificity when examined using rice glutelin mutants. The western blot analysis in combination with the higher temperature SDS-PAGE demonstrated considerable variation in amino acid sequence and accumulation level of glutelin subunit in wild species. The degree of the variation was, however, changed according to the site of epitope sequences and the type of subunits. Some wild species accumulated higher amount of nutritious GluB subunits than cultivated rice. And the wild species *O. longiglumis* and *O. brachyantha* had glutelin with low reactivity against most antibodies examined in this study, reflecting the significant divergence. Such wild species may hopefully serve as important genetic resources for nutritional improvement of cultivated rice.

Media summary

The higher temperature SDS-PAGE method was devised to evaluate the nutritional quality of rice seed glutelin in wild species in combination with subunit-specific antibodies.

Key Words

Wild species of rice, Seed storage protein, Glutelin, Higher temperature SDS-PAGE, Nutritional quality

Introduction

The major storage protein of rice seed, glutelin, is encoded by a small multigene family. The typical glutelin of Asian cultivated rice ssp. *japonica* is composed of six major subunits (GluA1, GluA2, GluA3, GluB1, GluB2, and GluB4) (Takaiwa et al 1991) and minor ones. The difference in the characteristics of the glutelin subunits is significant, so that the compositional change of glutelin by means of genetic and agronomic approaches has successfully resulted in the increased content of the most limiting amino acid, lysine (Katsube-Tanaka et al 2007). The continuing research about the glutelin of cultivated rice by Katsube-Tanaka et al (2001, 2004ab, 2005), however, suggest the necessity of exploration concerning new superior genetic resources for further nutritional improvement. The wild species of rice is important reservoir of genes and not only has played a vital role in crop improvement by contributing valuable genes for resistance to biotic and abiotic stresses but also is presumed to contain promising genetic resources for quality improvement. In this study, we examined thirteen self-fertile wild and two cultivated rice species to evaluate the diversity of glutelin polypeptides for the nutritional improvement using unique SDS-PAGE condition and subunit-specific antibodies. On the way to optimize an electrophoresis condition, we found it effective to heat a gel for better glutelin band separation for the first time. The principle of the method, higher temperature SDS-PAGE or temperature controlled SDS-PAGE, was discussed and assumed to be useful for the fractionation of other polypeptides with microheterogeneity. In addition, glutelin subunit-specific antibodies were raised and its reactivity was examined. Our SDS-PAGE system coupled with subunit-specific antibodies enabled us to reveal the details of diversity of glutelin and would be a useful tool for superior gene isolation and characterization.

Methods

Plant materials

The species names and chromosome type of the plant materials used in this study are respectively shown as follows (Khush 1997, Vaughan 1994); *O. sativa*, AA; *O. nivara*, AA; *O. rufipogon*, AA; *O. glaberrima*, AA; *O. barthii*, AA; *O. meridionalis*, AA; *O. punctata* (2X), BB; *O. punctata* (4X), BBCC; *O. minuta*, BBCC; *O. latifolia*, CCDD; *O. alta*, CCDD; *O. grandiglumis*, CCDD; *O. australiensis*, EE; *O. brachyantha*, FF; *O. longiglumis*, HHJJ. Germplasms of African cultivated rice *O. glaberrima* and all wild species except *O. nivara* were obtained from the National Institute of Genetics (Plant Genetics Laboratory and Experimental Farm), Mishima, Japan. *O. nivara* was from the National Institute of Agrobiological Sciences Genebank, Tsukuba, Japan. Glutelin mutants developed by Iida et al (1993, 1997), Type1, Type2, Type3, a-123less, and LGC-1, were from the National Agricultural Research Center for Western Region, Fukuyama, Japan.

Higher temperature SDS-PAGE and Western blotting

Electrophoresis was performed at constant voltage of 200V with low concentration of running buffer (16.7 mM Tris, 127.9 mM glycine, 0.07% (w/v) SDS) to reduce the Joule heat generation. The temperature of the running buffer was equilibrated and kept to 45°C unless otherwise described specifically (higher temperature SDS-PAGE), and 25°C and 4°C for comparison purpose. Electrophoresis was continued until smaller molecular size of proteins (prolamin) or glutelin α polypeptides were nearly reached to the bottom of gel. Sample preparation for SDS-PAGE and Western blotting were essentially according to Katsube et al (1999).

Subunit-specific anti-peptide antibody

Glutelin subunit-specific antibodies (polyclonal) were prepared against specific peptide sequences of glutelin α polypeptides against five sites (I, II, III, IV-a, and IV-b) from four variable regions within 11S globulin family (I, II, III, and IV) (Utsumi 1992, Utsumi et al 1997, Katsube et al 1998). Seven peptide sequences were chosen for epitope; A1(No.2), GQAQLTES in the site II of GluA1; A3(No.3), LYRYEARDN in the site III of GluA3; B1(No.4b), SERQQTSSRW in the site IV-b of GluB1; B4(No.1), GPNVNPWHN in the site I of GluB4; B4(No.3), EQQMYGRSIE in the site III of GluB4; B4(No.4a), KLLRPAFA in the site IV-a of GluB4; B4(No.4b), SEEQPSTRC in the site IV-b of GluB4. Extra cysteine residue was attached to the N-terminus of all peptide sequences except B4(No.4b) for peptide synthesis.

Results

SDS-PAGE is usually carried out at room temperature with low electrical current or even in a cold room with cooling apparatus in order to reduce heat generation which has been unpleasantly thought to cause protein band diffusion. We found for the first time that heating a polyacrylamide gel during electrophoresis is good for achieving improved protein band separation (Fig. 1). Possible reasons for improved band patterns at higher temperature are likely to be the conformational change in glutelin higher order structure even in denatured condition by SDS and/or the change in the dissociation degree of charged amino acid residues.

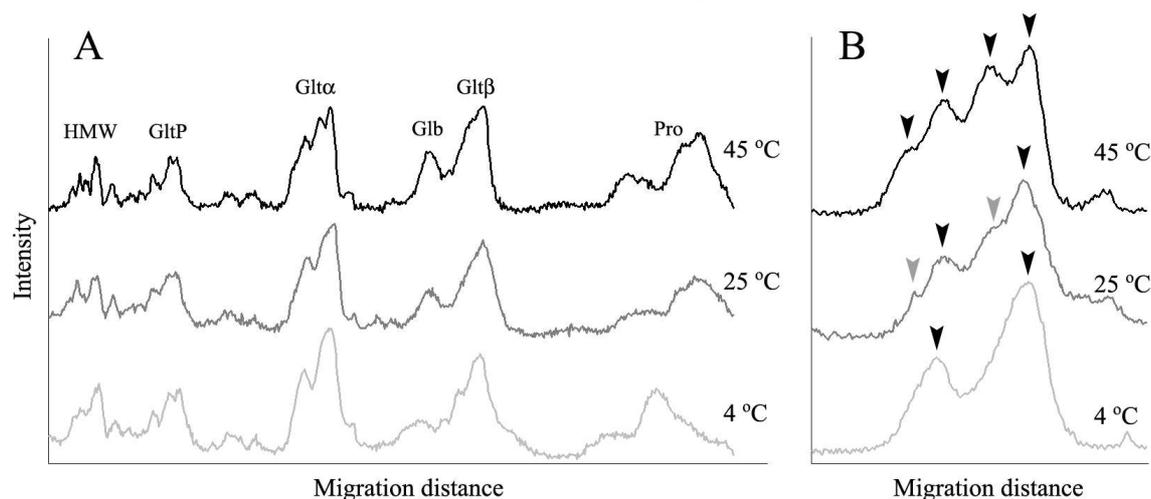


Figure 1. Densitogram of band patterns for rice glutelin α polypeptides resolved by SDS-PAGE at various temperatures. Total rice seed proteins extracted from *O. sativa* cv. Koshihikari were resolved by SDS-PAGE pre-equilibrated and kept at 4°C, 25°C, and 45°C. Band patterns were compared under the condition in which (A) all seed proteins were evenly dispersed within a gel and (B) glutelin α polypeptides were nearly reached to the bottom of gel. HMW, high molecular weight proteins; GltP, glutelin precursors; Glt α , glutelin α polypeptides; Glb, globulin; Glt β , glutelin β polypeptides; Pro, prolamin. Downward black and grey arrowheads indicate major and minor bands, respectively. Electrophoretic migration was in the direction from left to right.

The specificity of the anti-peptide antibodies examined in this experiment was confirmed to be generally high except that of anti-B4(No.1) using standard *japonica* type variety, Koshihikari and five glutelin mutants, Type1 lacking $\alpha 1$ (*GluB4*), Type2 lacking $\alpha 2$ (*GluA2*), Type3 lacking $\alpha 3$ (*GluA1*), a-123less lacking $\alpha 1$ - $\alpha 3$, and LGC-1 in which the accumulation levels of $\alpha 1$ (*GluB4*), $\alpha 5$ (*GluB2*), and $\alpha 6$ (*GluB1*) were drastically decreased (Iida et al 1993, 1997) (data not shown). The reactivity of the anti-peptide antibodies against wild and cultivated species glutelin was different among the types of antibodies and among wild and cultivated rice species, suggesting the diversity of glutelin in terms of amino acid sequences and accumulation levels (Fig. 2). Comparison of the reactivity among the four antibodies against *GluB4* suggested that the site IV-a is most surface-exposed and tolerable to evolutionary structural change. When compared at the site III, *GluA3* was more divergent than *GluB4* in terms of the size and number of the corresponding polypeptide among non-AA genome species. Likewise, comparison at the site IV-b indicated *GluB4* is more divergent than *GluB1* in terms of the accumulation level. In contrast, either *GluA1* or *GluA2* recognized by anti-A1(No.2) was constantly detected in all species, resulting in less variation in terms of the size and accumulation level.

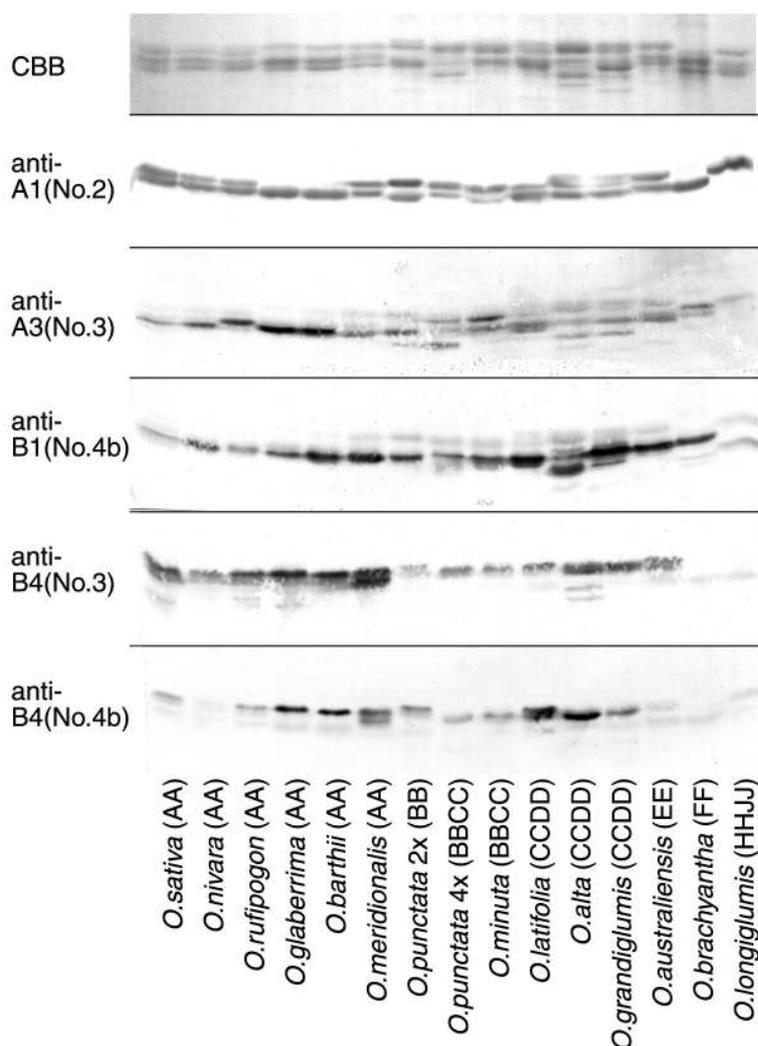


Figure 2. Evaluation for the diversity of glutelin polypeptides in wild species of rice. Glutelin α polypeptides extracted from the thirteen wild and two cultivated rice species were separated by higher temperature SDS-PAGE method and detected with CBB staining or immunoreaction with glutelin subunit-specific anti-peptide antibodies. Anti-peptide antibodies against A1(No.2), A3(No.3), B1(No.4b), B4(No.3), and B4(No.4b) were compared. The results of anti-B4(No.1) and anti-B4(No.4a) are not shown. The name and chromosome type of the rice species are shown under the figure.

Although precise evaluation for the accumulation level of each subunit using our current system needs further improvement, interspecific introgression of *GluB* genes the products of which can accumulate at higher level than that of cultivated rice will be one of promising approaches for the nutritional improvement of cultivated rice seeds, such as *GluB1* from *O. barthii*, *O. meridionalis*, *O. latifolia*, *O. alta*, *O. grandiglumis*, *O. australiensis*, and *O. brachyantha* and *GluB4* from *O. glaberrima*, *O. barthii*, *O. meridionalis*, *O. latifolia*, and

O. alta. On the other hand, *O. longiglumis*, having HHJJ chromosome type, had very weak reactivity against all antibodies examined in this study except anti-A1(No.2). Similarly, *O. brachyantha*, having FF chromosome type, had low reactivity against all anti-B4 antibodies designed over the four variable regions spanning whole glutelin α polypeptide. The data coupled with the fact both species have multiple bands detected by CBB suggest that both species have quite unique glutelin polypeptides and might be promising candidates for the quality improvement effort on seed protein of cultivated rice as another approach.

Conclusion

The higher temperature SDS-PAGE method in combination with subunit-specific antibodies successfully identified wild species accumulating high amount of nutritious GluB subunits and unknown subunits. Such wild species may hopefully serve as important genetic resources for nutritional improvement of cultivated rice.

References

- Iida S, Amano E and Nishio T (1993). A rice (*Oryza sativa* L.) mutant having a low content of glutelin and a high content of prolamine. *Theor. Appl. Genet.*, 87, 374-378.
- Iida S, Kusaba M, Nishio T (1997). Mutants lacking glutelin subunits in rice: mapping and combination of mutated glutelin genes. *Theor. Appl. Genet.*, 94, 177-183.
- Katsube T, Maruyama N, Takaiwa F and Utsumi S (1998). Food protein engineering of soybean proteins and development of soy-rice. In *Engineering Crops for Industrial Uses* (P. R. Shewry et al. eds.) Portland Press, London, 65-76.
- Katsube T, Kurisaka N, Ogawa M, Maruyama N, Ohtsuka R, Utsumi S and Takaiwa F (1999). Accumulation of soybean glycinin and its assembly with the glutelins in rice. *Plant Physiol.* 120, 1063-1074.
- Katsube-Tanaka T, Kimura Y, Hiroyama N, Iida S, Yamaguchi T, Nakano J, Takaiwa F and Utsumi S (2001). Analysis of rice seed storage protein glutelin using *Escherichia coli* expression systems. *Proc. Asian Agriculture Congress 2001* (Virmani SS et al. eds.), Asian Crop Science Association, Manila. 343-347.
- Katsube-Tanaka T, Duldulao JBA, Kimura Y, Iida S, Yamaguchi T, Nakano J, Utsumi S (2004a). The two subfamilies of rice glutelin differ in both primary and higher-order structures. *Biochim. Biophys. Acta.*, 1699, 95-102.
- Katsube-Tanaka T, Endo T, Iida S, Yamaguchi T and Nakano J (2004b). Quality assessment of rice seed protein using the capillary electrophoresis method. *Proc. 4th International Crop Science Congress* (Fischer T et al. eds.), Regional Institute, Brisbane. www.cropscience.org.au.
- Katsube-Tanaka T, Iida S, Yamaguchi T and Nakano J (2005). Qualitative and Quantitative Analyses of Seed Storage Protein Glutelin for the Food Quality Improvement of Rice. *Proc. Int. Symp. "The recent status and perspectives of agricultural environment and biotechnology 2005"* (Ohmura H et al. eds.). Kyushu University, Fukuoka. 66-72.
- Katsube-Tanaka T, Nakashima H, Iida S, Okita TW, Washida H, Yamaguchi T, Nakano J (2007). Nutritional improvement of rice seed protein glutelin by genetic and agronomic approaches. In: *Abstracts of the 2nd International Conference on Rice for the Future*. 5-9 November 2007, Bangkok, Thailand, p.189
- Khush GS (1997). Origin, dispersal, cultivation and variation of rice. *Plant Mol. Biol.*, 35, 25-34.
- Takaiwa F, Oono K, Wing D and Kato A (1991). Sequence of three members and expression of a new major subfamily of glutelin genes from rice. *Plant Mol. Biol.* 17, 875-885.
- Utsumi S (1992). Plant food protein engineering. In *Advances in Food and Nutrition Research*; Kinsella JE Eds.; Academic Press, pp 89-208.
- Utsumi S, Katsube T, Ishige T and Takaiwa F (1997). Molecular design of soybean glycinins with enhanced food qualities and development of crops producing such glycinins. In "Food proteins and lipids 1st edition" (Damodaran S and Paraf A eds.). Plenum Press, New York. 1-15.
- Vaughan DA (1994). Wild relatives of rice. In *Genetic Resources Handbook*; IRRI, Los Banos, Philippines, pp 1-137.