There are difficulties in detecting and separating rice prolamin polypeptides by 2D-PAGE analysis because prolamin polypeptides are insoluble, and the amino acid sequences show high homology among them. In this study, we improved the prolamin extraction method and the 2D-PAGE procedure, and succeeded in separating prolamin polypeptide species by 2D-PAGE and in identifying major prolamin polypeptide sequences.

Key words: rice seed; prolamin; 2D-PAGE; amino acid sequencing

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is a powerful tool for the separation, quantification, and identification of proteins. Considerable research effort has been applied to the use of rice proteomic analysis to predict the functions of proteins, and much insight into the yield and quality of rice grain has been acquired.1,2

Rice seed storage proteins consist mainly of glutelins and prolamins.3) 1D-PAGE analysis of prolamin polypeptides from isolated type I protein body (PB-I) indicated that PB-I contains 10 kDa, 13 kDa, and 16 kDa prolamins.4) Two groups of 13 kDa prolamins have been defined: 13b prolamins were extracted in the absence of any reducing reagent, while the remaining 13a prolamins were extracted under reducing conditions similar to those used for the 10 kDa and 16 kDa prolamins. Several studies involving proteomic analysis of rice seed proteins have been reported, and albumins, globulins, and glutelins in rice seeds were well separated and identified by 2D-PAGE.5–7) However, little has been reported regarding the analysis of prolamins by 2D-PAGE.

Recently, proteomic analysis of rice seeds by 2D-PAGE showed 400 protein spots, but only one spot was identified as a prolamin polypeptide.8) The reason is that the prolamins were less solubilized by a conventional lysis solution consisting of urea and non-ionic detergent for use in 2D-PAGE due to their hydrophobic property. There have been several advances recently in the methodologies of proteomic analysis, including improved protein extraction techniques.9,10) These studies indicate that a combination of urea, thiourea, and CHAPS was efficient for the extraction of highly insoluble proteins. These methods are expected to be useful in the extraction of prolamins from rice seeds. In addition, prolamin polypeptides are encoded by multi-gene families and have high homology. An NCBI database search indicated that there are three copies of 10 kDa prolamin, one copy of 16 kDa prolamin, four copies of 13a prolamin, and 13 copies of 13b prolamin. Due to this close sequence identity, it is difficult to separate prolamin polypeptides by isoelectric focusing (IEF)/SDS–PAGE. Hence we analyzed prolamin polypeptides by high-resolution nonequilibrium pH gradient gel electrophoresis (NEPHGE)/SDS–PAGE.11) In this study, we succeeded in detecting and separating several prolamin polypeptides by 2D-PAGE and in sequencing major prolamin polypeptides using an amino acid sequencer. Fifty mg of brown rice (Oryza sativa L. cv Nipponbare) was homogenized in 1 mL of urea/thiourea buffer consisting of 5 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT, and 0.8% v/v biolyte (pH 3–10). The homogenates were centrifuged at 288,000 × g for 4 h to obtain the protein extracts as supernatant solutions. 2D-PAGE was carried out by the method of Abe et al.5) with some modifications. The gel components for first-dimension NEPHGE consisted of 8 M urea, 3.5% (T) acrylamide, 2% v/v nonidet P-40 (NP-40), and 2% v/v biolyte (pH 5–8). The lower reservoir was filled with 20 mM sodium hydroxide, and was connected with a cathode, and the upper reservoir was filled with 10 mM phosphoric acid and connected with an anode. A total of 450 μg of protein extracts was loaded onto a disc gel (Nihon Eido, Tokyo), and then 20 μL of overlay buffer (1/5 diluted urea/thiourea buffer) was placed in a glass tube. Electrophoresis in NEPHGE was carried out at 400 V over 4 h. After electrophoresis in the first dimension, the gel was removed from the glass tube and placed in an equilibration solution consisting of 62.5 mM Tris–HCl (pH 6.8), 2.5% w/v SDS, and 10% DTT for 20 min and gently shaken twice. The gel was placed on a 3% acrylamide stacking gel on an 18% acrylamide separating gel (24.0 × 21.5 cm, 1 mm in thickness) and sealed with 1% w/v agarose dissolved in equilibration solution without DTT. Electrophoresis was
conducted at a constant current of 50 mA per gel. After SDS–PAGE, the gel was stained by the colloidal Coomassie Brilliant Blue (CBB) staining method. The staining solution consisted of 20% ethanol, 1 M ammonium nitrate, 20 mM phosphoric acid, and 0.08% CBB G-250. The gel was stained for 12–18 h and destained with distilled water until a clear background appeared.

The 2D-PAGE profile of the separated polypeptides is shown in Fig. 1A. A number of spots were spread widely on the gel, and major spots were detected in a molecular mass range of 30–40 kDa, which corresponds to glutelins. Furthermore, 11 spots were detected in the low molecular mass area (10–18 kDa), corresponding to prolamins (Fig. 1B). To determine whether these spots were prolamins, they were manually picked from CBB stained gels, and the amino acid sequences were determined. Because the NH$_2$-terminals of many polypeptides in the rice seeds were blocked, which interferes with amino acid sequence analysis, the internal sequences of the suspected prolamin polypeptide spots (10–18 kDa) were analyzed by Edman sequencing.

To obtain the internal amino acid sequences of the various protein spots, in gel digestion with V8 protease or lysyl endoproteinase was performed by published procedures, with some modifications. The picked gel spots were incubated with destained buffer containing 50% acetonitrile and 25 mM ammonium bicarbonate, and then were reduced with 10 mM DTT and alkylated by 55 mM iodoacetate. The dehydrated gel spots were washed with 25 mM ammonium bicarbonate. The gel spots were dehydrated with acetonitrile and dried in a vacuum centrifuge. They were further rehydrated for 1 h at 4°C in a digestion solution containing V8 protease or lysyl endoproteinase, the excess digestion solution was aspirated, and the samples were digested overnight at 37°C. After digestion, the samples were separated in Tris-tricine SDS–PAGE. The digested polypeptides were electroblotted onto PVDF membranes (0.5 mA/cm$^2$) over 2 h, and the amino acid sequences were determined by the automated Edman sequencing system (PPSQ-21, Shimazu, Kyoto). Eleven spots were sequenced, and the amino acid sequences were obtained from the protein database of Oryza sativa using the BLAST program at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Eight (spots #3, #4, #5, #7, #8, #9, #10, and #11) of the 11 spots were identified as prolamins from the low molecular mass area based on the protein database (Table 1). As shown in Table 1, spot #3 corresponded to a 16-kDa prolamin encoded by gene locus Os06g0507200, and #11 corresponded to a 10-kDa prolamin encoded by gene locus Os03g0766100. Spots #4 and #5 were identified as 13a prolamins encoded by gene loci Os07g0206500 and Os07g0206400 respec-
tively. Spot #8 was identified as a 13b prolamin encoded by Os07g0219300. The internal amino acid sequences of spots #7, #9, and #10 were the same, and were also identified as 13b prolamins (gene loci, Os05g0329400, Os05g0329100, and Os05g0330600). The 13b prolamins are encoded by a multigene family and are highly homologous within a given family.15) For the 13b prolamins, additional proteomic characterizations such as mass analysis might clarify which of these spots correspond to the protein encoded by each gene locus. The spot intensities of 13a and 13b prolamin polypeptides were apparently higher than that of the 10 and 16kDa prolamin polypeptides in Nipponbare. This is consistent with the findings obtained by 1D-PAGE analysis of the rice prolamins. Thus, in this study, a number of prolamin species were extracted and separated by 2D-PAGE, and many prolamin spots were identified by amino acid sequencing.

As for protein spots other than prolamin, spots #1 and #2 corresponded to RAG2 and RA17 respectively, which are known to be rice allergenic proteins.16) Spot #6 was identified as an α-amylase/trypsin inhibitor family protein encoded by gene locus Os07g0216700 or Os07g0216700 and is homologous with RAG2.5) Spot #3 contained another fragment in addition to a 16-kDa prolamin polypeptide, one that was identified as 18kDa oleosin, a component of the membrane protein from the oil body in the rice aleurone layer and embryo.17)

Next, by the 2D-PAGE method used in this study, we tried to analyze the details of the composition changes in the seed storage protein in a rice mutant. LGC1 is a typical, well characterized mutant that shows low glutelin and high prolamin content. Kusaba et al.18) have reported that the low glutelin content of LGC1 is due to reduction in GluB by RNA silencing, but it is unclear whether the high prolamin content of LGC1 is caused by an increase in all classes of prolamin or by one in a specific prolamin. As Fig. 2 shows, several spots of LGC1 (Fig. 2B, D) in a molecular mass range of 30–40 kDa, which corresponds to glutelins, were re-produced in comparison to Nipponbare (Fig. 2A, C). It is noteworthy that the 13b prolamin (#7, 8, 9, and 10) content of LGC1 increased approximately 2-fold as compared to Nipponbare (Fig. 2E), as determined by densitometric analysis of the spots using Multi Gauge software (Fujifilm, Tokyo). The contents of the other classes of prolamins (#3, 4, 5, and 11) were the same as between Nipponbare and LGC1. It has been reported that a reduction in a particular seed storage synthesis process resulted in a compensatory increase in other storage proteins.19,20) Thus the cereals maintained a process resulted in a compensatory increase in other that a reduction in a particular seed storage synthesis classes of prolamins (#3, 4, 5, and 11) were the same as between Nipponbare and LGC1. It has been reported that a reduction in a particular seed storage synthesis process resulted in a compensatory increase in other storage proteins.19,20) Thus the cereals maintained a process resulted in a compensatory increase in other that a reduction in a particular seed storage synthesis

in this study should provide a useful tool for understanding the composition changes of storage proteins as between various rice cultivars.

Acknowledgments

We thank Dr. K. Hirooka, Mr. N. Tomari, and Mr. Y. Yamamoto of the Kyoto Municipal Industrial Research Institute, for kindly providing the technique for 2D-PAGE analysis. This work was supported by a grant from the Japan Science and Technology Agency (JST), Innovation Plaza Niigata, the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Grant-in-Aid for Scientific Research (C) to T.M., no. #21580417).

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