

Degradation of Soluble Proteins Including Some Allergens in Brown Rice Grains by Endogenous Proteolytic Activity during Germination and Heat-Processing

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The effect of germination and subsequent heat-processing on the degradation of soluble proteins, including some allergenic proteins, in brown rice grains was investigated. The content of soluble proteins, including 14–16-kDa and 26-kDa allergens, in the germinated and processed brown rice grains (GPR) was much lower than that of non-germinated brown rice. These proteins in brown rice grains were also much lower after subsequent heat-processing during the manufacturing process. The protease activity of germinated brown rice (GR) was detected and increased 1.5 times after germination. The optimum pH values for degradation of the 26-kDa and 14–16-kDa allergens in the GR grains were 4 and between 5 and 7, respectively. These results suggest that the decrease in the soluble proteins and allergens was induced in part by proteolytic degradation. The presence of a detergent enhanced the proteolytic degradation of the soluble proteins, especially of the 26-kDa allergen, in the brown rice grains. The degradation of the 26-kDa allergen was weakly inhibited by NEM, suggesting cysteine protease(s) may have been involved in its degradation. These results suggest that the two abundant allergens were degraded in a different manner and probably by different proteases in the grains during germination.

Key words: rice; germination; allergen; α -amylase/trypsin inhibitor; food allergy

Cereal grains have been reported to sometimes be causative of food allergy.¹⁾ Several clinical studies have suggested that rice grains are responsible for severe atopic dermatitis in some adult patients.^{2–4)} Some allergenic proteins, including the 14–16-kDa, 26-kDa and 33-kDa types, have been identified and characterized biochemically and immunochemically.^{5–10)} Furthermore, cDNAs encoding these allergenic proteins have

been cloned and their nucleotide sequences determined.^{10–14)} The deduced amino acid sequences show that these 14–16-kDa allergens are structurally homologous to each other. These sequences are also similar to those of the α -amylase/trypsin inhibitors of other cereal species, especially in the number and distribution of cysteine residues.¹⁵⁾ In fact, the isolated 14–16-kDa allergens have been reported to show inhibitory activity toward human salivary α -amylase.⁸⁾ The wheat and barley α -amylase/trypsin inhibitors have been identified as major allergens associated with baker's asthma.^{16,17)}

Some production trials for hypoallergenic rice have been conducted by using enzymatic processing.¹⁸⁾ Hypoallergenic rice produced by the enzymatic method in combination with a detergent has already been clinically evaluated and supplied to patients with rice allergy.^{19,20)}

Pre-germinated brown rice has recently been produced at an industrial level and provided to consumers in Japan. This pre-germinated brown rice can be produced by soaking brown rice in water and then germinating it for only a short time. The amount of some functional elements, including γ -aminobutyric acid, has been greatly increased during germination.²¹⁾ During grain germination and the early-stage of seedling growth, the storage proteins are degraded by proteases and mobilized to supply amino acids that support growth of the embryo. It has been reported that REP-1,²²⁾ a cysteine endopeptidase, digested *in vitro* both the acidic and basic subunits of glutelin, the major seed storage protein of rice, and that the activity of REP-1 increased noticeably 9–18 days after imbibition. However, no report has been published with regard to the degradation of soluble proteins, including allergens, during grain germination.

We describe in the present paper the effect of early-stage germination and subsequent heat-processing on

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rice seed proteins, especially the major allergenic proteins. Germination and subsequent heat-processing of brown rice markedly reduced the content of soluble proteins, including some allergenic proteins in the grains, and this reduction of allergens seems to have been due to proteolytic degradation by endogenous proteases activated during germination. The application of such endogenous protease activity for the production of hypoallergenic rice is also discussed.

Materials and Methods

Preparation of germinated and processed brown rice (GPR). Brown rice grains (*Oryza sativa* L var *japonica* cv. Hoshinoyume, BR) were germinated by imbibition of water at 30 °C for 22 hours. These are termed germinated brown rice (GR). The GR grains were subsequently steamed at 100 °C for 5 min, these being termed germinated and heated brown rice (GHR). Finally, the GHR grains were dried at 100 °C for 30 min, these final products being termed germinated and processed brown rice (GPR). Polished rice grains (PR) were prepared from BR with a rice-polished machine (CBS300AS, Satake Co., Hiroshima, Japan) to remove the bran and embryo.

Extraction of soluble rice proteins. Soluble-protein extraction started with 2 g of rice grains being crushed to less than 48 mesh and then being mixed with 10 ml of a buffer solution. The soluble proteins in the rice grains were extracted with UT205S ultrasonic apparatus (SHARP) at 100 W for 30 min, and separated from the insoluble material by centrifugation at $10,000 \times g$ for 10 min. The supernatant was used for the subsequent analyses.

Determination of the nitrogen and protein contents. The total-nitrogen content of the rice grains was determined by the Kjeldahl method.²³⁾ The concentration of the soluble proteins extracted from rice grains was determined by the method of Lowry *et al.*,²⁴⁾ using BSA as a standard.

Protease assay. The protease activity in the soluble-protein fraction of the rice grains was measured by using casein as a substrate. The assay mixture containing 1 ml of the soluble protein extract and 1 ml of 1% casein was incubated at 35 °C for 120 min, and the enzyme reaction was stopped by adding 3 ml of 10% TCA. After being kept at room temperature for 60 min, the mixture was centrifuged at $2,800 \times g$ for 10 min. The absorbance at 280 nm of the resulting supernatant (the TCA-soluble component) was determined. As a control analysis, a mixture containing all components of the reaction was treated with 10% TCA before being incubated.

Incubation of the rice grains. Two grams of rice grains were incubated with 10 ml of a 10 mM citrate

buffer (pH 3, 4, 5 and 6), 10 mM phosphate buffer (pH 7), or 10 mM Tris-HCl buffer (pH 8 and 9) at 40 °C for 24 hours. In some experiment, the grains were incubated with 10 ml of the citrate buffer (pH 6) containing 0.1% SDS and several protease inhibitors: 10 mM *N*-ethylmaleimide (NEM), 0.2 mM *L*-trans-epoxysuccinyl-leucylamido-(4-guanidinobutane) (E-64), 0.2 mM pepstatin, 10 mM diisopropyl fluorophosphates (DFP) and 10 mM ethylenediaminetetraacetic acid (EDTA) as respective inhibitors of cysteine, cysteine (papain type), aspartic, serine and metal proteases. The soluble proteins in the rice grains were extracted by the same methods as those just described.

SDS-PAGE, immunoblotting and densitometric analyses. SDS-PAGE (15% acrylamide gel) and immunoblotting were conducted by the methods of Laemmli²⁵⁾ and Towbin *et al.*,²⁶⁾ respectively. Gel sheets were stained with CBB, while blotted PVDF membranes were stained with the monoclonal antibody, 25B9, specific for rice 14–16-kDa allergens, or the mouse antiserum against the 26-kDa allergen, as described previously.²⁷⁾ The staining intensity of each protein band was densitometrically analyzed with a densitograph (ATTO, Tokyo).

Results

Protein analyses of the GPR grains

The total nitrogen and soluble protein contents of the PR, BR and GPR grains were first analyzed. Figure 1 shows the contents of total nitrogen determined by the Kjeldahl method and of the soluble proteins determined by the method of Lowry *et al.* in the three kinds of rice grain. No difference in the total nitrogen content was apparent among the three. On the other hand, the content of soluble proteins differed substantially. The soluble protein content of GPR was much lower (about 20%) than that of the original grains (BR), while the soluble protein content of GPR was even lower than that of PR.

The soluble proteins in the three types of rice grain were then analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 2A, the CBB-stained bands, including the 26-kDa and 14–16-kDa bands of GPR were much weaker than those of BR. However, no difference in the CBB-stained bands of total proteins, except for the low-molecular-weight proteins, was observed among the three types of rice grain (Fig. 2D). Immunoblotting analyses of these extracts by using monoclonal antibody 25B9 specific for the 14–16-kDa allergens (Figs. 2B and E) and the mouse antiserum against the 26-kDa allergen (Figs. 2C and F) also showed that the contents of 14–16-kDa and 26-kDa allergens in GPR were low.

Changes in the soluble protein components in brown rice during the manufacturing process were analyzed by SDS-PAGE (Fig. 3A), and the 14–16-kDa allergens were analyzed by immunoblotting (Fig. 3B). The soluble proteins analyzed by SDS-PAGE were not greatly changed by the germination treatment, but were mark-

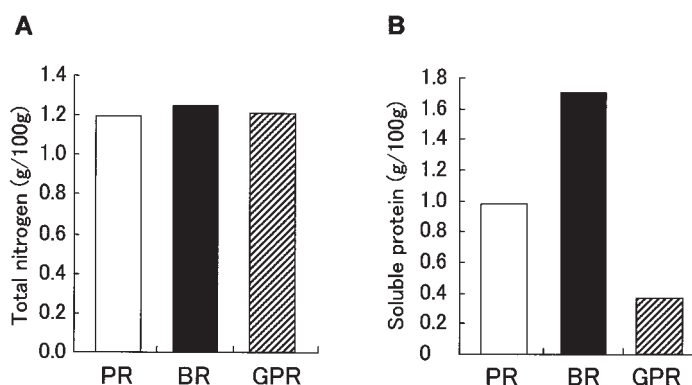


Fig. 1. Comparison of Total Nitrogen and Soluble Protein Contents among the Three Types of Rice Grains.

The total nitrogen contents (panel A) of brown rice (BR; closed bar), and germinated and processed brown rice (GPR; striped bar) were determined by the Kjeldahl method, and the soluble protein contents (panel B) of the three were determined by the method of Lowry *et al.* Each value for polished rice (PR; open bar) is also shown for comparison.

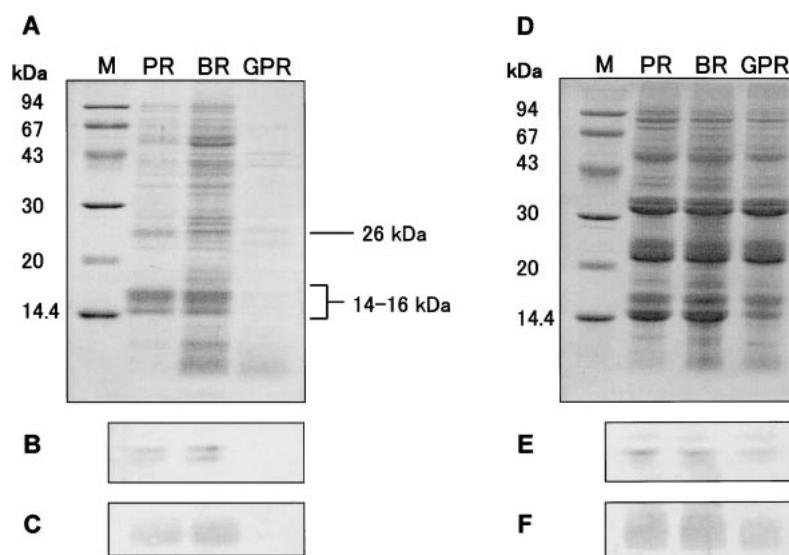


Fig. 2. SDS-PAGE and Immunoblotting Analyses of the Total and Soluble Proteins among the Three Types of Rice Grains.

The soluble (panels A, B and C) and total (panels D, E and F) proteins were respectively extracted with PBS and the SDS-containing buffer from polished rice (PR), brown rice (BR), and germinated and processed brown rice (GPR) and subjected to the analyses. The gel sheets were stained with CBB (panels A and D), and the blotted membranes were immunostained with monoclonal antibody 25B9 specific for the 14–16-kDa allergen (panels B and E) and with mouse antiserum against the 26-kDa allergen (panels C and F). A molecular mass standard (M) was also applied to the same gel.

edly decreased by the subsequent heat processing (steaming). The relative staining intensity of the immunoblotted bands as estimated by the densitometric analysis is shown in Fig. 3C. The 14–16-kDa allergens were decreased to about 70% of the original content by the germination treatment, and became undetectable after heat processing.

Protease activity of BR, GR and GHR

Such a marked decrease in the content of rice proteins led us to expect some unknown endogenous factors, so we analyzed the protease activity of these rice grains. The protease activity of the BR grains as well as PR grains was detected by using casein as a substrate

(Fig. 4). The protease activity of GR was markedly higher (1.5-fold) than that of non-germinated brown rice (BR), and remained even after heat processing at 50 °C. The protease activity of GHR (treatment at 90 °C or 98 °C) was low, but still weakly detectable.

Effect of pH and detergent on the decrease in content of soluble proteins including the 14–16-kDa and 26-kDa allergens in the GR grains

The GR grains were incubated with each buffer (pH 3–9) at 40 °C for 24 hours. The content of the major soluble proteins, except for the 14–16-kDa proteins, was markedly reduced by incubating in the acidic pH range (3 to 5), while the 14–16-kDa proteins remained

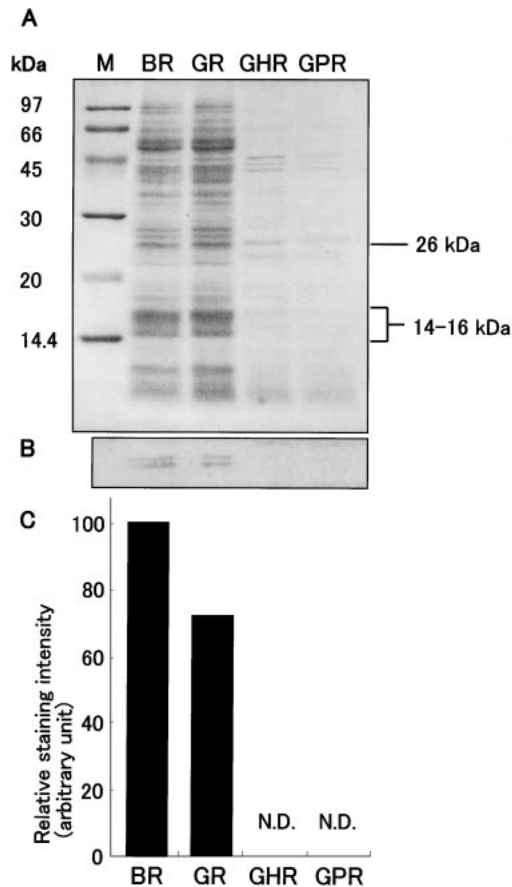


Fig. 3. SDS-PAGE of the Soluble Proteins and Immunoblotting Analyses of the 14–16-kDa Allergens in Brown Rice Grains (BR, GR, GHR and GPR). The soluble proteins were extracted with PBS from each brown rice sample with a different treatment, and subjected to the SDS-PAGE (panel A) and immunoblotting analyses for 14–16-kDa allergens (panel B). The intensity of each 14–16-kDa band from immunoblotting was determined by a densitometric analysis (panel C). The samples in each lane are from brown rice (BR), germinated brown rice (GR), germinated and heated brown rice (GHR), and germinated and processed brown rice (GPR).

unchanged after incubating at any pH value tested. The intensity of the CBB-stained band of 26-kDa was reduced by incubating at pH 4. On the other hand, the content of the 14–16-kDa allergens as estimated by immunoblotting was decreased by incubating at pH 5–7 (Fig. 5). The content in non-germinated brown rice (BR) grains was also decreased by incubating at pH 6, but the decrease was much smaller than that of GR.

Some proteases are known to be activated in the presence of a detergent.²⁸⁾ Therefore, the BR and GR grains were incubated at 40 °C for 24 hours with a citrate buffer (pH 6) in the presence of 0.1% SDS. The content of some proteins was markedly decreased by incubating in the presence of 0.1% SDS (Fig. 6A). The respective contents of the 14–16-kDa allergens and 26-kDa allergen as estimated by immunoblotting were decreased to about 45% and 30% by the incubation (Figs. 6B, C, D and E). The decrease of the 26-kDa allergen was greater than that of the 14–16-kDa allergens.

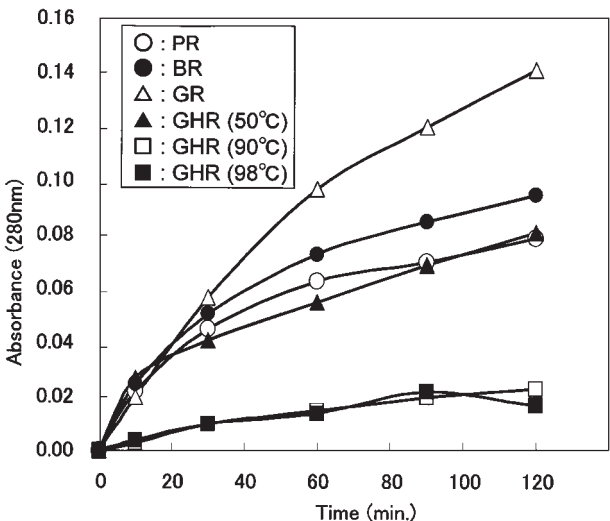


Fig. 4. Protease Activity in Brown Rice Grains during the Manufacturing Process. Protease activity was measured by using casein as a substrate. The symbols represent polished rice (PR; ○), brown rice (BR; ●), germinated brown rice (GR; △), and germinated brown rice heated at 50 °C (▲), 90 °C (□), or 98 °C (■).

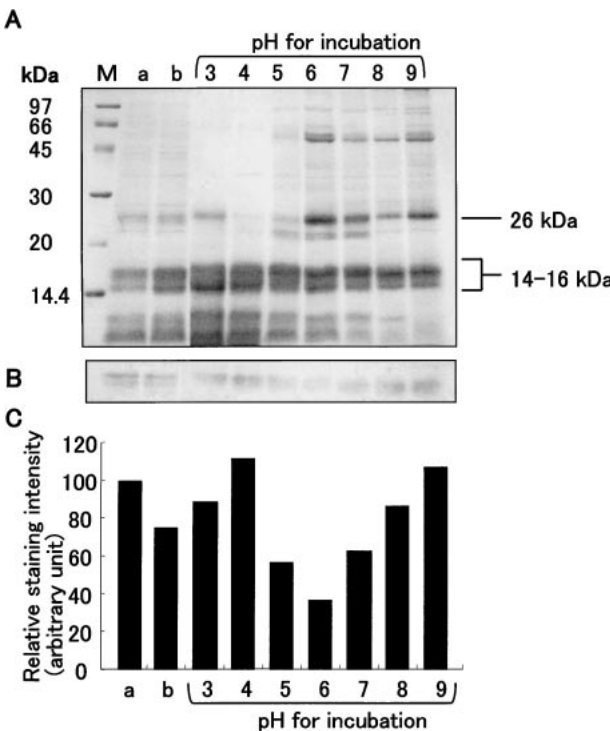


Fig. 5. Effect of pH on the Degradation of Soluble Proteins Including the 14–16-kDa Allergens in Germinated Brown Rice Grains. The germinated brown rice grains were incubated at 40 °C for 24 hours with a citrate buffer (pH 3, 4, 5, or 6), phosphate buffer (pH 7), or Tris–HCl buffer (pH 8 or 9), before the soluble proteins in rice grains were extracted and subjected to SDS-PAGE (panel A) and immunoblotting analyses for the 14–16-kDa allergens (panel B). The intensity of each 14–16-kDa band from immunoblotting was determined by a densitometric analysis (panel C). The extracts of non-germinated brown rice without incubation (a) and incubated with the citrate buffer at pH 6 (b) were also analyzed for comparison.

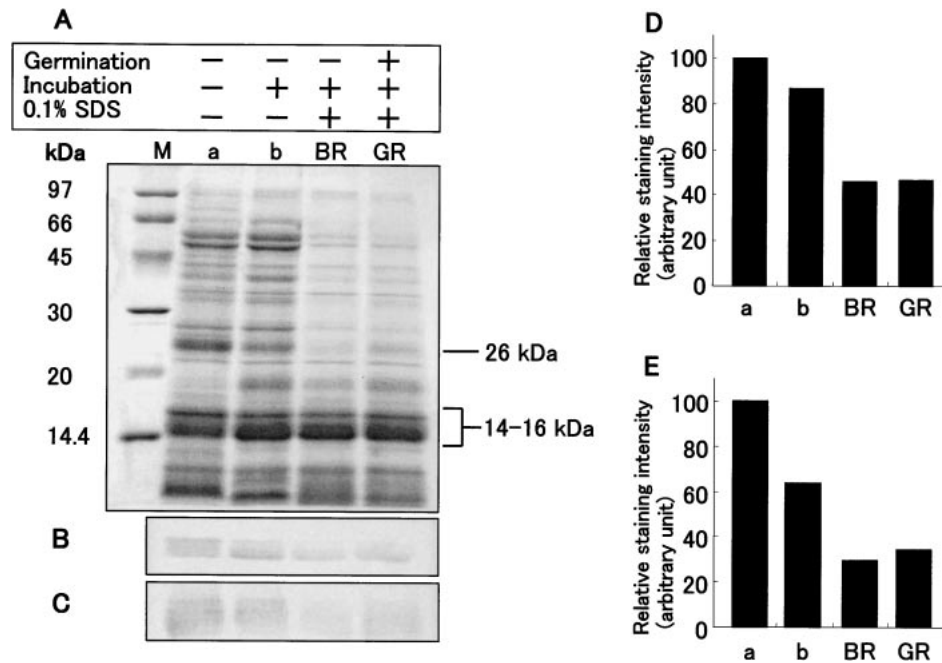


Fig. 6. Effect of a Detergent on the Degradation of Soluble Proteins Including the 26-kDa Allergen in Brown Rice Grains.

The brown rice (BR) and germinated brown rice (GR) grains were incubated with a 10 mM citrate buffer (pH 6) containing 0.1% SDS at 40 °C for 24 hours, before the soluble proteins in the grains were extracted and subjected to SDS-PAGE analysis (panel A). The blotted membranes were immunostained with monoclonal antibody 25B9 specific for the 14–16-kDa allergen (panel B) and with the mouse antiserum against the 26-kDa allergen (panel C). The intensity of each 14–16-kDa band (panel D) and 26-kDa band (panel E) from immunoblotting was determined by a densitometric analysis. The brown rice extracts without incubation (a) and incubated without SDS (b) were also analyzed for comparison.

The GR grains were then incubated with citrate buffer (pH 6) containing 0.1% SDS at 40 °C for 24 hours in the presence of several protease inhibitors. The soluble proteins were analyzed by SDS-PAGE and immunoblotting (Fig. 7). Four kinds of inhibitor, including the specific inhibitor of the aspartic protease, pepstatin, did not markedly affect the protein electrophotogram. Only NEM, which is an inhibitor of cysteine protease, was found to weakly affect the protein degradation. The immunoblotting band of the 26-kDa allergen in the grains treated with NEM was slightly but significantly stronger than those of the grains incubated with the other inhibitors. These results suggest that cysteine protease(s) might have been involved in part in the degradation of some rice proteins, especially 26-kDa allergen, during germination.

Discussion

No difference in the total nitrogen concentration in the three kinds of rice grain, PR, BR and GPR, was observed. On the other hand, the contents of soluble proteins in the GPR grains were lower than those in BR and PR (Fig. 1). The bands of 26-kDa and 14–16-kDa in the GPR grain extract could not be clearly detected by SDS-PAGE or immunoblotting (Figs. 2B and C). These results show that the major allergens were almost completely absent in the GPR grains, but that the nitrogen content remained unchanged in GPR. It is considered that all of the decrease in contents of these

proteins was not due to insolubilization by interaction between starch and the proteins during heat processing, because 14–16-kDa and 26-kDa in the total protein extract from GPR were immunoblotted more weakly than those allergens in BR (Figs. 2E and F).

These allergens were found to be degraded during germination and subsequent heating in the manufacturing process (Fig. 3). Such a dramatic decrease in the contents of the allergens could be explained in part by the contribution of one or more proteases which are activated during germination. In fact, the protease activity of the GR grains was increased (Fig. 4), while the 14–16-kDa allergens were decreased by incubating the GR grains (Fig. 5). The 14–16-kDa allergens are known to be α -amylase/trypsin inhibitors in rice grains.^{11,12} Therefore, it would be reasonable to consider that these proteins with inhibitory activity toward digestive enzymes might function as a defense system against predators such as insects. On the other hand, starch and storage proteins must be digested during germination for sprout growth. These events might explain that the 14–16-kDa allergens with amylase inhibitory activity were inactivated or degraded during germination and that starch in the endosperm would then be smoothly digested during sprout growth.

The presence of a detergent (0.1% SDS) enhanced the proteolytic degradation of the 26-kDa allergen in the GR grains (Fig. 6). The results of inhibition assay (Fig. 7) using several protease inhibitors suggest that the protease involved in the degradation of some proteins,

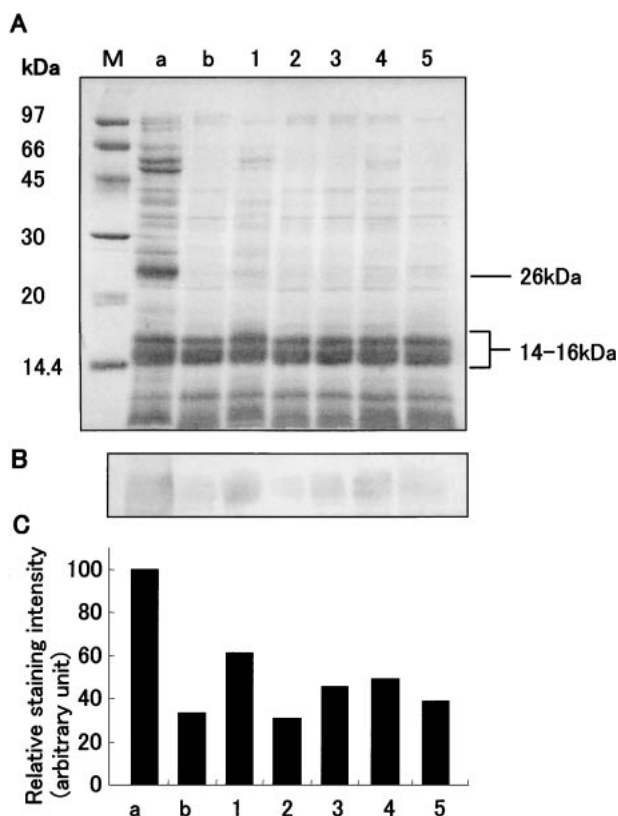


Fig. 7. Effect of Several Protease Inhibitors on Degradation of the Soluble Proteins in Germinated Brown Rice.

The germinated brown rice grains were incubated at 40 °C for 24 hours with a citrate buffer (pH 6) containing 0.1% SDS in the presence of several protease inhibitors: NEM (1), E-64 (2), pepstatin (3), DFP (4), or EDTA (5). The soluble proteins in the rice grains were then extracted and subjected to SDS-PAGE (panel A) and immunoblotting analyses for the 26-kDa allergen (panel B). The intensity of the 26-kDa band from immunoblotting was determined by a densitometric analysis (panel C). The extracts of germinated brown rice without incubation (a) and incubated without a protease inhibitor (b) were also analyzed for comparison.

but not the 14–16-kDa allergens, during germination was a cysteine protease. It has already been reported that the cysteine proteases in rice seeds, REP-1 and REP-2, were activated during germination, 18 days after imbibition, and that these proteases digested the major storage protein, glutelin, within an optimum pH range of 5–6.²²⁾ These cysteine proteases remain in an inactive form in harvested and dried seeds. It is therefore suggested that the REPs or other cysteine proteases were activated and affected the degradation of seed proteins in the early stage of germination. In fact, the soluble proteins were found to be digested prior to the major storage proteins, glutelin and prolamin, during germination (Yamada *et al.* unpublished data). The degradation of the two abundant allergens, 26-kDa and 14–16-kDa, was found to occur in an independent manner, suggesting that the allergens might be degraded by different proteases activated in the grains during the early stage of germination.

Hypoallergenic rice has already been produced commercially by treating polished grains with microbial enzymes in combination with a detergent.¹⁸⁾ In the case of GPR, the allergens were reduced by some endogenous factors including the proteolytic activity activated by germination. Furthermore, the germination treatment and subsequent heat and dry processing are simple and economically advantageous, and can be easily accomplished on an industrial scale. Thus, an artificial germination or pre-germination treatment in combination with heating could be utilized as a novel processing tool for reducing the allergenicity of plant seed-based food such as cereals and beans.

GPR is also a safe and functional food, because no microbial enzymes or detergent are used and functional elements, including γ -aminobutyric acid,²¹⁾ increase during germination. The present results provide a useful method for producing hypoallergenic rice by the combination of germination and heat processing.

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