

Reduction of proteins in rice flour by proteases in kiwifruit and pineapple

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On average, rice contains 6% of proteins. Low-protein rice is used by people that need to limit their intake of dietary protein because of kidney malfunction. This study evaluated the fruit extract of kiwifruit and pineapple, which contain proteolytic enzymes, to reduce the amount of proteins in rice.

By treatment with the fruit crude extracts, glutelin (which is the most abundant protein in rice), as well as α -globulin and 14-16 kDa albumin (both of which are allergy-causing rice proteins), were successfully degraded and reduced. Protein quantification analysis indicated that proteases from the two fruits are highly effective in reducing proteins in rice flour. The sensory evaluation of the processed food products prepared from the fruit extracts-treated low-protein rice flour showed no significant difference from untreated rice-based products. These results demonstrated that proteolytic enzymes in kiwifruit and pineapple extracts are effective for reducing the protein content of rice.

Key words: rice protein, allergenic protein, protease, pineapple, kiwifruit.

INTRODUCTION

Rice is one of the most important cereal crops in the world, particularly in Asian countries. In Japan, rice has been eaten as a staple diet since ancient times. Recently, as new techniques for making fine particles have advanced, rice flour is also being used in a variety of processed foods including confectionery. Polished rice contains approximately 6% protein; specifically, acid-/alkali-soluble glutelin accounts for about 60% of total endosperm protein in rice, while alcohol-soluble prolamin accounts for 20%, and globulin and albumin account for 5-10% each^{1,2)}. Glutelin is a seed storage protein that is composed of two subunits; namely, the 22-23 kDa basic subunit and the 37-39 kDa acidic subunit³⁾. Albumins in rice seed have α -amylase inhibitory functions and have molecular masses ranging from 14 to 16 kDa⁴⁾. Previous studies have shown that these albumins are potentially allergenic for patients with rice allergies^{5,6)}. A major component of globulin, called α -globulin, and another globulin, with a molecular mass of 33 kDa that has the enzyme activity of glyoxalase I, are also allergenic proteins found in rice^{7,8)}. Recently, another rice protein, granule-bound starch synthase with a molecular mass of 56 kDa, was identified as an allergy patients' IgE-binding protein, and the protein was shown to be responsible for rice-induced anaphylaxis^{9,10)}. In some cases, patients allergic to rice must avoid eating rice grains and its processed products;

hence, instead of rice, they must choose products processed from wheat or non-allergenic minor cereals, such as millet or amaranthus.

In Japan, rice is one of the most important sources of starch in food. However, rice intake is occasionally restricted in people that must limit their intake of dietary proteins, such as patients with kidney failure. Usually, the daily consumption of rice is high, as rice tends to be present in every meal. Because polished rice contains approximately 6% protein, the total amount of protein intake through rice consumption is not considered negligible.

In addition to eating rice grains, rice flour, such as "Joshin-ko," which is made from non-waxy rice, and "Shiratama-ko," which is made from waxy rice, has been used in traditional Japanese confectionery, such as "Dango" (skewered rice dumplings in a sweet soy glaze) since ancient times. In Japan, the food self-sufficiency rate has decreased over the last 40 years; the food self-sufficiency rate was 39% in 2012. To improve this rate, the Japanese Government has run various campaigns, one of which is to promote rice consumption in Japan, because all rice for domestic food use is produced in Japan. Recently as new technique for making fine particles have advanced, rice flour has also been used in a variety of processed foods except confectionery. The flour milling technique of rice has been improved, with the establishment of certain practices, such as milling by an air stream, leading to fine and constant particle sizes. These improvements have facilitated the use of rice flour as a substitute for wheat flour in the production of cakes and bread.

Low-protein rice products have been developed for

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people who must limit their intake of dietary protein at daily meals, such as patients with chronic kidney disease. One example is products made from polished rice in which the surface is scraped off. Scraping removes a large amount of proteins, because they are mainly localized on the seed surface. Another product that has been developed is the low-protein product called “starch rice,” which is an imitation of rice that is made from starch particles. Certain rice cultivars have also been established by mutagenesis and crossbreeding¹¹⁾ methods to select varieties in which glutelin content (the major protein in rice) is relatively low.

Rice, wheat, and corn are the most important cereal crops worldwide. Wheat and corn also contain proteins, with contents of less than 10%. Wheat contains functional proteins, such as gluten-related glutenin and gliadin. It is not advisable to remove glutenin and gliadin from wheat, because gluten formation is necessary to make dough for processed foods, such as bread and noodles. In contrast, since rice does not contain gluten-related proteins, so it is expected that there are no characteristic changes in its functions even if the amount of proteins in rice seed decreases.

In this study, we aimed to reduce the protein content of rice flour by using extracts of kiwifruit (*Actinidia deliciosa*) and pineapple (*Ananas comosus*). These fruits contain proteolytic enzymes; specifically, actinidin in kiwifruit¹²⁾ and bromelain in pineapple¹³⁾. Actinidin is a cysteine protease that is present in the fruit tissue of kiwifruit, and it is the predominant soluble protein, representing up to 40% of soluble proteins. Bromelain is also a cysteine protease that is present in the fruit and stem of pineapple. Quantitative analyses confirmed a decline in the amount of protein in rice flour treated with the fruit extracts. Furthermore, we conducted a sensory evaluation of processed rice products that were made from rice flour with lower protein content. Rice flour obtained by this technique might provide some benefits to people that must limit their intake of dietary protein.

MATERIALS AND METHODS

Rice flour. Commercially available rice flour was obtained from Namisato Co. (Tochigi, Japan) and used for our analyses.

Preparation of the fruit extract. Commercially available kiwifruit (Hayward cultivar) and pineapple (Smooth Cayenne cultivar) were used for extraction. The peel was first removed from the two fruits, and then the fruits were cut into 1-cm cube pieces each. These pieces of the fruits (100 g) were suspended in 100 ml of water, and mixed using a homogenizer (ULTRA-TURRAX T25 basic, IKA-WERKE) until the blocks disappeared. The homogenized suspension was then filtered through double layers of gauze. The filtrate was centrifuged at $2,000 \times g$ for 10 min, and the supernatant was collected. The supernatant was again filtered through double layers of gauze. The filtrate was collected and used for the subsequent analyses.

Treatment of rice flour with the fruit extract. To determine the appropriate reaction time, rice flour (0.1 g) was suspended in 1 ml of the kiwifruit or pineapple extract for 0, 10, 20, 30, 40, 50, 60, 90, and 120 min at 35°C . For each analysis, leupeptine was added to the solution at a final concentration of 0.1 mM, and mixed. The

suspension was then centrifuged at $13,000 \times g$ for 10 min, and the supernatant was collected. For the preparation of fruit extract-treated rice flour for the processed products, rice flour (100 g) was suspended in 400 ml of kiwifruit or pineapple extract for 1 hour at 35°C . The suspension was centrifuged at $1,600 \times g$ for 10 min, and the supernatant was collected. Distilled H_2O was added to the rice flour precipitate and suspended. The suspension was then centrifuged at $1,600 \times g$ for 10 min, and the supernatant was removed. These procedures were repeated a total of three times. The resulting rice flour precipitate was spread on filter paper, and dried at 50°C in a convection oven. The treated rice flour was used for the subsequent analyses.

For the heat treatment, the fruit extract of pineapple and kiwifruit was incubated at 100°C for 10 min, and cooled. The treated extract was used for a comparison with the non-heated extract to determine the influence of heat treatment. The condition of treatment was the same above; rice flour was suspended with fruit extract for 1 h at 35°C .

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Rice flour treated with fruit extract was suspended in SDS-PAGE sample buffer (20 mM Tris-HCl, pH 6.8, 1% SDS, 6% glycerol, 0.012% bromophenol blue) and sonicated three times for 3 min with 1-min intervals. Centrifugation was carried out at $16,000 \times g$ for 10 min, and the resulting supernatant was transferred to new tubes. For the supernatant of the extract, SDS-PAGE sample buffer and β -mercaptoethanol were added to each sample, followed by heating at 90°C for 3 min. SDS-PAGE was performed according to the method by Laemmli et al.¹²⁾, using 4.5% stacking gel and 15% or 18% separating gel, with a constant current of 10 amperes (A) and 20 A, respectively. The gel was later stained with Coomassie brilliant blue (CBB)-staining solution (2-propanol:acetic acid:distilled $\text{H}_2\text{O} = 5:1:5$, 0.25% CBB-R250) for 20 min, followed by destaining in a buffer solution (7% methanol and 5% acetic acid), and gently shaken overnight using an electric shaker.

Protein quantification. The amount of protein in rice flour treated with fruit-extract was quantified by the bicinchoninic acid (BCA) method¹³⁾. Protein was extracted from rice flour with an extraction buffer (20 mM Tris-HCl, pH6.8, 1% SDS), and quantified using the BCA Protein Assay Kit (PIERCE) following the manufacturer's protocol. BSA (bovine serum albumin) solution was used as a standard protein. The absorbance values were measured at 562 nm using a spectrophotometer (Shimadzu UV-1600). The amount of each protein in each sample was calculated from the standard curve.

Processing rice flour products. Cookies were made from rice flour using the following recipe. Non-salt butter (15 g) was left at room temperature and creamed, with a tablespoon of sugar being mixed in. Twenty-five grams of treated- or untreated- rice flour and a half-teaspoon of baking powder were added and mixed. Approximately 10 ml water was added and kneaded to form a dough, which was formed in the shape of a column and stored at 4°C for 20 min. The columns were cut into 5-mm-thick slices, and then baked for 10 min at 180°C in the oven.

Sensory evaluation. A seven-scale evaluation sheet was prepared for eight panelists who joined the sensory evaluation (-3; “extremely bad” to +3; “extremely

good”) of the processed products. The results were evaluated using a two-factor analysis of variance (ANOVA). A 5% test of significance was applied.

Analyses of rice flour physical properties. The water content of rice flour was analyzed by the normal pressure drying method. Weighing bottles and caps were heated for 30 min at 135°C and were weighed. Each rice flour sample (1g) was placed in the weighing bottles, and incubated for 3 h at 135°C. After cooling, the samples were weighed and repeatedly incubated for 1 h at 135°C until a constant weight was obtained.

For the analyses of physical properties, dumplings were made at the same level of water content of rice flour. For 3 g of each rice flour sample, 0.5 ml of water was added and mixed quickly. A total quantity of 2 ml water was added, and the rice flour was hardened. They were then put into a mold (15 mm diameter and 15 mm height). The dumplings were boiled for 6 min, and then immediately transferred to cold water. The samples were placed back into the same mold, and the excess part was removed with the edge of a razor. The condition of the texturometer (TPU-2SB, Yamaden Co.) was set to 5 mm/sec speed and 10 mm distance, and two times of measurement. For each sample, elasticity was measured three times.

RESULTS AND DISCUSSION

Treatment of rice flour with the fruit extract. The protein content of polished rice is approximately 6%, according to the Standard Tables of Food Composition in Japan. Some essential amino acids, including lysine, are insufficient in rice proteins; therefore, rice proteins are not as important as a nutrient in Japan. Among rice proteins, albumin (molecular weight of 14-16K)^{5, 6}, α -globulin (molecular weight of 26K)⁷, and glyoxalase I (molecular weight of 33K)⁸ have been identified as allergenic proteins. In addition, the protein content of rice is related to the taste; specifically, rice varieties with low protein content tend to have better taste compared to those with high protein content. In this study, we aimed to reduce the protein content of rice flour. We used the fruit extract of kiwifruit (*A. deliciosa*) and pineapple (*A. comosus*) in the reduction process. Kiwifruit contains proteolytic enzymes such as actinidin¹², while pineapple fruit contains proteolytic enzymes such as bromelain¹³.

The rice flour was treated with the crude fruit extract for each time-period. The supernatant was then centrifuged and subjected to electrophoresis. It was found that over 30 min and 50 min was required for the kiwifruit (Fig. 1A) and pineapple fruit extract (Fig. 1B), respectively, for the effective reduction of α -globulin and albumin. The gel electrophoresis of the rice flour treated with fruit extract showed that proteins, particularly glutelin, were clearly reduced compared to ordinary rice flour, indicating that these proteins had been degraded by the contents of the fruit extract (Fig. 2).

To confirm that the rice proteins had been reduced by enzymatic action, we subjected the the fruit extract to heat treatment before using it to treat the rice flour. The heat treatment of both of fruit extracts resulted in no reduction in α -globulin and albumins, even though the unheated extract produced a remarkable reduction in these proteins (Fig. 3). This result indicates that the decrease in proteins content was caused by degradation through the enzymatic activity of proteases in these fruits.

Quantitative analyses of the rice flour treated with fruit extract. To clarify the reductive effect of fruit-protease on the protein content of rice flour, protein quantification by the BCA method was carried out. Rice flour treated with fruit-extract was used for the analysis. Quantification analyses confirmed that the amount of proteins in rice flour treated with fruit extract decreased to 23-37% of the ordinary rice (Fig. 4). Hence, the protease in fruit extract might cause a reduction in the proteins in rice flour. Rice flour proteins appeared to be degraded by protease in the fruit extract, with digested products that had low molecular weights being retained; thus, influenc-

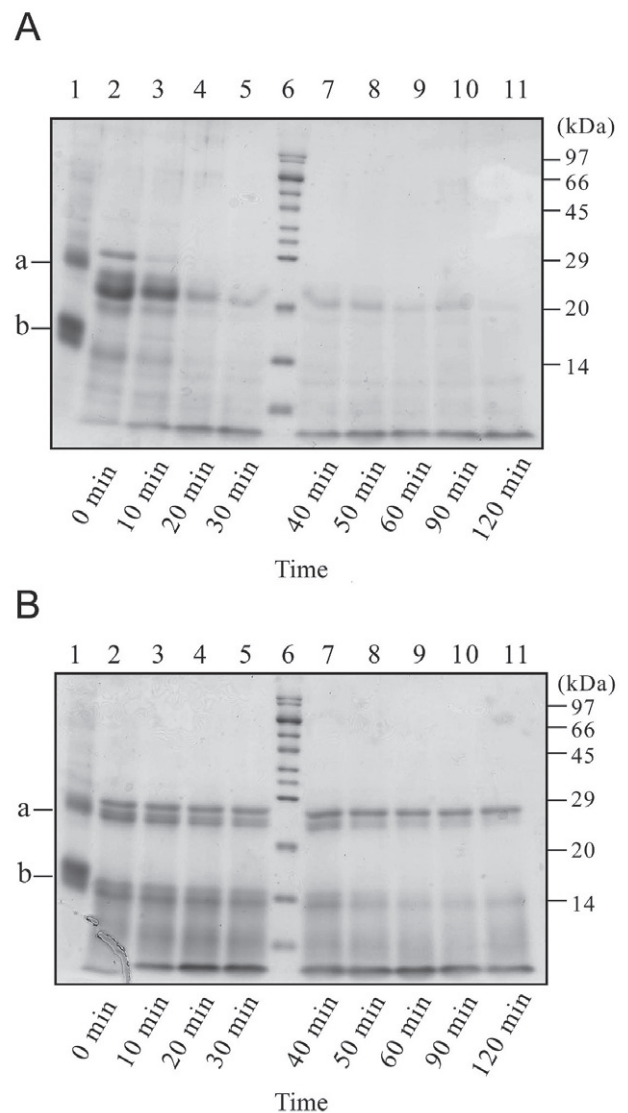


Fig. 1. Reduction of proteins in rice flour by treating with fruit extract. Fruit extract of kiwifruit (A) or pineapple (B) was added to rice flour and incubated at each time as follows. Proteins in the fruit extract containing soluble- and digested- proteins were detected by CBB staining in SDS-PAGE. α -Globulin (26K) and albumin (14-16K) were denoted by “a” and “b”, respectively. Lane 1: PBS-soluble proteins of ordinary rice flour (control); lane 2: 0 min; lane 3: 10 min; lane 4: 20 min; lane 5: 30 min; lane 6: Molecular weight marker; lane 7: 40 min; lane 8: 50 min; lane 9: 60 min; lane 10: 90 min; lane 11: 120 min.

ing the quantitative results. Gel electrophoresis and the quantification analyses indicated that treating the rice flour with the fruit extract reduced the protein content to

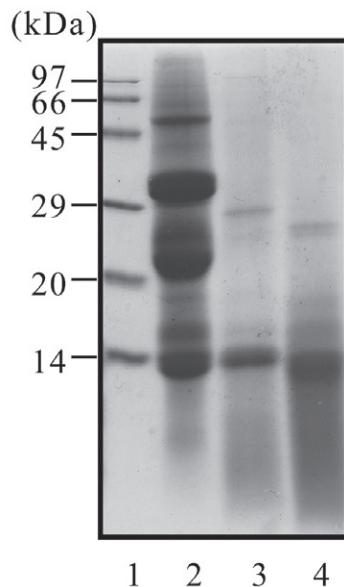


Fig. 2. Electrophoresis of proteins in fruit extract-treated rice flour. Proteins were extracted from rice flour treated with kiwifruit/pineapple extract for 1 h and were subjected to SDS-PAGE. Lane 1: Molecular weight marker; lane 2: SDS-soluble proteins of ordinary rice flour (control); lane 3: SDS-soluble proteins of kiwifruit extract-treated rice flour; lane 4: SDS-soluble proteins of pineapple extract-treated rice flour.

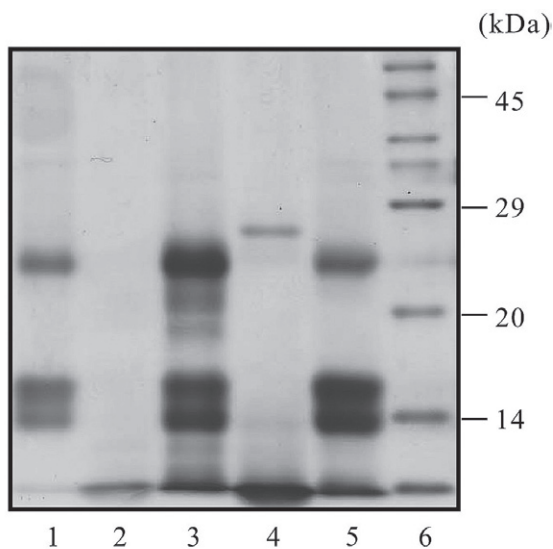


Fig. 3. Effect of heat treatment for efficiency of protein reduction in rice flour. Fruit extract of kiwifruit and pineapple was heated in boiling water and was mixed with rice flour for 1 h. After centrifugation, supernatant was subjected to SDS-PAGE. Lane 1: PBS-soluble proteins of ordinary rice flour (control); lane 2: unheated kiwifruit extract-treated rice flour; lane 3: heated kiwifruit extract-treated rice flour; lane 4: unheated pineapple extract-treated rice flour; lane 5: heated pineapple extract-treated rice flour; lane 6: molecular weight marker.

relatively low levels. The proteolytic enzymes related to the degradation of rice proteins might be actinidin in kiwifruit and bromelain in pineapple. Alternatively, proteins might be degraded by other proteases present in these fruits. When commercially available purified bromelain was used for rice flour, the degree of rice protein degradation was lower compared to the fruit extraction method. This method of treating rice flour with fruit extract might prove useful in the preparation of low-protein rice-based products. Hence, it is necessary to examine the similarity between low-protein rice flour and ordinary rice flour, and to determine whether the former is suitable for processing.

Evaluation of processed products prepared from low-protein rice flour. When the rice flour treated with fruit extract was dried, the outward appearance was almost identical to that of ordinary rice flour to the naked eye, except for the slight coloring of the stem by the fruit pigments.

To determine whether the treated rice flour may be used for processing, cookies were produced using rice flour as the main ingredient, and were then evaluated. A sensory evaluation based on the outward appearance, aroma, taste, and texture of the cookies, in addition to an overall evaluation, was completed by eight panelists to compare the quality of the processed products. A seven-scale score sheet (-3, extremely bad to +3, extremely good) was used for the evaluation. The results of the evaluation for outward appearance and texture revealed no significant differences compared to untreated samples. However, the average evaluation points for aroma and taste decreased after treatment with the fruit extract; particularly, points for pineapple were lower than those of kiwifruit. These changes may have been caused by the remaining contents of the fruit extract in treated rice flour, in addition to a decrease in, or the removal of, certain compounds in rice, which may be related to aroma and flavor. The results of the overall evaluation were determined statistically by

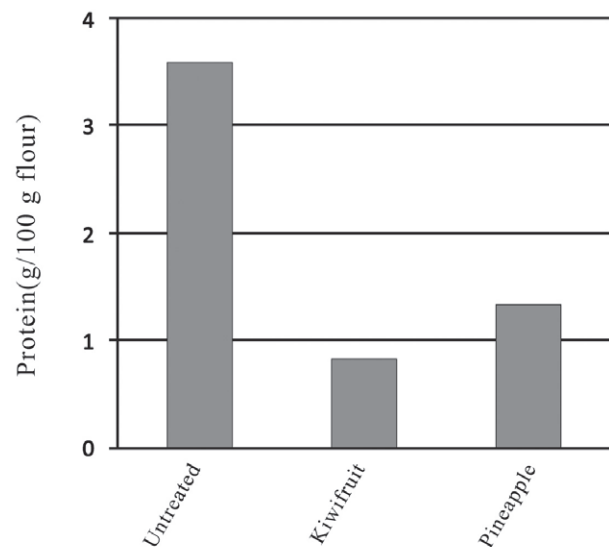


Fig. 4. Protein quantification of rice treated by fruit extract. Proteins were extracted from rice flour for 1 h followed by BCA protein assay. Standard curve was obtained using standard solutions of bovine serum albumin. Protein content per 100 g of rice flour was calculated.

Table 1. Summary of sensory evaluation of cookies made from fruit extract-treated rice flour

	Outward appearance	Aroma	Taste	Texture	Overall evaluation
Untreated	0.875	1	0.625	0.125	0.625
Kiwifruit	1	-0.75	-0.25	0.125	-0.125
Pineapple	0.875	-1.125	-0.625	-0.125	-0.5

A seven-scale evaluation sheet (-3; "extremely bad" to +3; "extremely good") was prepared for eight panelists of the processed products. The average values of eight panelists for each item were shown.

two-factor ANOVA. The average overall evaluation points for cookies made from ordinary rice, kiwifruit extract-treated rice flour, and pineapple extract-treated rice flour were 0.625, -0.125, -0.5, respectively (Table 1). ANOVA indicated no significant differences among samples based on the scores of the panelists for overall estimation. These results demonstrate that rice flour treated with the fruit extracts could be used as a food material in the manufacture of low-protein processed foods. Cookies made from treated rice flour had lower average values for aroma and taste compared to ordinary rice flour. It is possible that taste was influenced by the presence of protease-digested peptides. These issues could be solved by removing excess ingredients related to odor and taste by using improved washing methods of the treated rice flour, or by purifying the fruit extract.

Evaluation of low protein rice flour physical properties. The physical properties of rice flour treated with fruit extract were examined. When water content was analyzed by the normal pressure drying method, lower water content values were obtained for rice flour treated with fruit extract compared to ordinary flour. This result might have been influenced by the wash and firm dry technique used after treatment with the fruit extract. Physical property tests using the texturometer were carried out. Rice dumplings were made after the water content of the flour was standardized. Values of the maximum loading, stress, gumminess stress, and cohesiveness were measured. The maximum loading, stress, gumminess stress of rice flour treated with fruit extract were less than half of those of ordinary rice flour (Fig. 5A-C). These results indicate that dumplings made from treated rice flour were softer and easier to bite compared to those made from ordinary rice flour. However, the cohesiveness of the dumplings made from treated rice flour was slightly higher than that of dumplings made from ordinary rice flour (Fig. 5D). These results indicate that this rice protein reduction method enhances the cohesiveness and softness of dumplings.

Significance of protein reduction and future prospects. Protein in food is one of the most important nutrients, particularly in animal food products, which are rich in essential amino acids, and serve as important protein sources. However, the intake of food protein is sometimes restricted in patients with kidney failure. Rice and wheat also contain small amounts of protein (less than 10%). Since rice is a staple food in Japan and other Asian countries, it is important to consider the protein intake from rice, particularly for patients with kidney disease. Hence, rice products have been developed for those patients. For example, the outer portion of the endosperm of rice grains is removed in some rice products. This method is similar to that of developing rice for brewing sake (Japanese rice

wine); specifically, rice varieties used to brew sake contain lower protein content, with the outer portion of the grain being removed because proteins in rice influence the quality of sake. Because most rice proteins are localized in the outer portion of the endosperm in the grain, the protein content of surface-removed rice grains may be reduced.

In previous studies, we succeeded in reducing the protein content of rice flour based on the solubility of rice seed proteins by using diluted organic acid solutions, such as ascorbic acid, citric acid, malic acid, and gluconic acid¹⁶). The four types of organic acid solutions caused 12-20% reduction in rice proteins compared to ordinary rice flour. In this study, as an alternative approach, we reduced the protein content of rice flour by using fruit extract, which contains proteases. We found that glutelin, α -globulin, and albumin were degraded to low molecular weight molecules by proteases in the fruit extract. Most of the digested products were solubilized by the fruit extract solution, and rice flour after the treatment had reduced the amount of proteins present.

In recent years, rice flour has been increasingly used in Japan as one of the main materials in various types of food products, in addition to traditional Japanese sweets. Rice flour treated by the method presented in this study could be applied to processed foods intended for people whose intake of protein is restricted. Rice might provide a good alternative for people that require/prefer a gluten-free diet, such as those with wheat allergies because it does not contain gluten-related proteins for the viscoelasticity of foods. Furthermore, wheat allergy patients are sometimes also allergic to other cereals such as rice and barley. Rice allergenic proteins, α -globulin and 14-16 kDa albumin, were reduced in rice flour treated with fruit extracts in this study; hence, this treated rice flour might have versatility in the preparation of many food products, in addition to providing to patients allergic to various foods.

Furthermore, it would be interesting to develop products processed from rice that are formulated to have viscoelasticity or gluten-like properties. The processing properties of low-protein food materials may be supplemented by auxiliary materials, such as starch or other agricultural products. Moreover, the method presented in this study may be applied to wheat flour, which contains gluten-related proteins, for people with kidney failure. In this case, auxiliary materials supplementing the absence of gluten would be necessary to supplement viscoelasticity.

In conclusion, rice flour that has a lower protein content following treatment with fruit extract is expected to prove useful for people with restricted protein intake. Further studies are necessary to improve the quality of treated rice flour, and to clarify whether these rice products are beneficial for people with kidney failure or rice allergies.

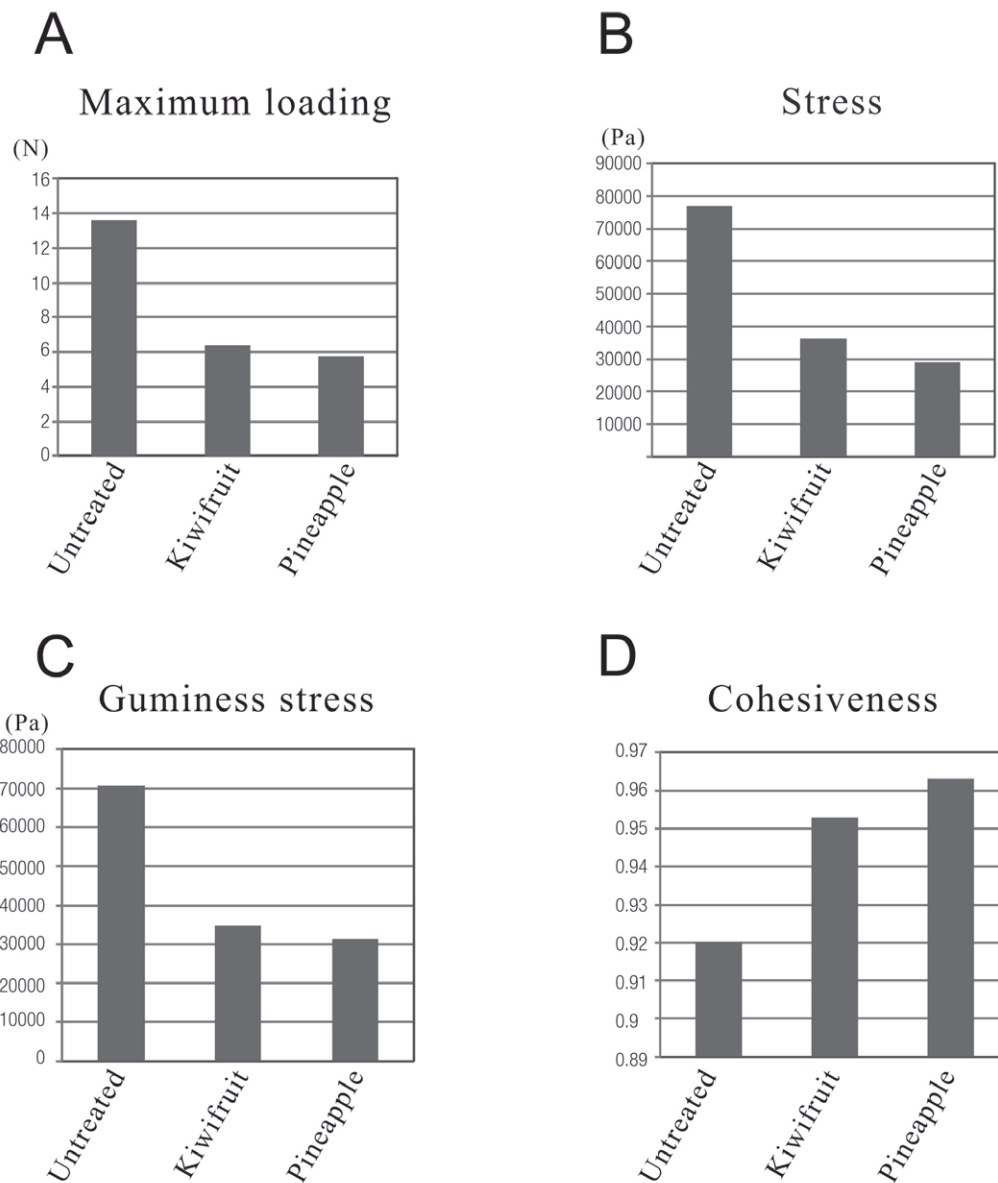


Fig. 5. Physical properties of dumplings made from fruit extract-treated rice flour. Rice flour treated with fruit extract was processed into dumplings and was subjected to analyses of physical properties.

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