Article

Establishment of the Proteolysis Condition of Raw Material Soybeans for Tempeh Production

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＜Abstract＞

Salt-free fermented food of soybeans is full of nutritive value, and tempeh, which is Indonesian traditional fermented food, is a superior health-function food. There are neither mucilage substances, nor the unique fermentation smell and taste such as occurs in Japanese natto. Its characteristics are a soft texture and plain taste such as the cheese. We assumed that the reason why tempeh has not spread into Japanese markets is due to the inferiority in umami taste that Japanese like, compared to natto. Thus we have tried various approaches to improve the umami taste of tempeh. As the part, we tried hydrolysis of the protein of the raw material soybean in protease, aiming to provide an umami taste to tempeh in this study. Specifically, in a stage before fermenting with *Rhizopus*, we examined the optimum condition to hydrolyze the protein of the raw material of soybean with an industrial-use enzyme. Under the conditions of different heat treatments on the raw materials of soybeans (such as non-heating, boiling and steaming), eight kinds of industrial proteases were examined. The appropriate condition based on enzyme treatment times and the enzyme concentrations were examined in this study. The degree of protein digestion was observed with a migration pattern of SDS-PAGE as an index. As a result, papain and bromelain were effective for digesting the soy protein. Regarding the manufacturing method, firstly, a 50-minute steaming process was performed on the raw materials of soybeans after the immersion process. Then, soybeans were immersed in a 0.5% sodium acetate solution in which an enzyme was dissolved in the concentration of 0.2% (w/v), and left for 1 hour at 25℃, and then observing to what degree the soy protein was hydrolyzed. When the soybean was treated with protease by such a technique, it was confirmed that the growth of *Rhizopus* became faster than when using a conventional method, and the tempeh could be produced normally. The industrial production method of tempeh to hydrolyze the protein of the raw materials of soybeans was established in this study, and further study is planned to carry out a sensory examination to confirm association with the umami taste in future.

Keywords: tempeh, soybean, protein hydrolysis, industrial protease, umami

Tempeh is a fermented soybean food with filamentous fungus (*Rhizopus oligosporus*). It is one of the staple foods of Java central and eastern part inhabitants having the history of several hundred years as Indonesian traditional fermented food (Okada, 1988). In Japan, it is said to be the Indonesian natto, but the mucilage substance such as the Japanese natto is not produced.

The Indonesian representative manufacturing

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process of tempeh has been already introduced to Japan by the academic issues (Kato, 2004; Takamine, 1984), but in Japan, the industrial manufacturing method has established to keep a fixed quality with the addition of various laborers (Takamine, 1984).

The outline of a domestic industrial tempeh manufacturing method is as follows (Kato, 2004; Takamine, 1984). The ideal characteristics for the raw material of soybean are the high ability to absorb water and a large grain size. Soybean with the sweetness is good when boiled. The decortication of the soybean is performed by mechanical shocks such as the abrasion. The soybean is boiled in water for 30-50 minutes successively, but boiling time varies by the kind of the soybean. At this process, use of the acidic water is indispensable for various germs pollution restraint. In general, lactic acid, or acetic acid, or citric acid is used for the purpose, but mostly, low-priced acetic acid that gives moderate slight acidic taste to soybean is used. Then the centrifugal moisture separation is performed at 1,000-1,500rpm for 2-5min. 1-3g of *Rhizopus* starter is added to 1kg of soybean. Fermentation is performed within the plastic bag in which an eyelet is emptied. Since *Rhizopus* is aerobic, oxygen of the appropriate amount is required for the growth. Spore is formed before white spawn extends all over the soybean when there are too many quantities of oxygen, thus it is with an important key when size and the quantity of the eyelet to empty into the bag in order to produce high quality tempeh. Appropriate fermentation temperature is about thirty degrees Celsius, and usual fermentation time is about 20hrs. When too much quantity of the starter is added, the fermentation time is shortened, but spore is formed with the extension of the white spawn being insufficient. Because lack in quantity of addition of the starter urges the propagation of various germs, the quantity of the starter is also important. After fermentation, the tempeh is cooled off promptly and should be frozen when it is stored for a long term. A flavor deteriorates rapidly when left at room temperature and sporulation makes tempeh black, then tempeh loses the food value.

The tempeh is placed as a functional food in Japan. As functionality of soybean, there are 〝rich in protein〞, 〝rich in essential fatty acid such as the linoleic acid〞, 〝not to include cholesterol〞, 〝palliation of menopausal disorders and osteoporotic protective efficacy with the isoflavone〞, 〝reduction of blood cholesterol with soy protein〞, and these functionalities of soybean remain to tempeh (Kato, 2004). Furthermore, the new functionality that there was not in the raw soybean is endowed by fermentation. Those new functionalities are 〝peptic improvement〞 〝antibacterial active appearance (Kiers *et al.*, 2002)〞, 〝palliation of the diarrhea symptom (Kiers *et al.*, 2007)〞 〝increase in various free amino acids and soybean peptide including the γ-aminobutyric acid (GABA) (Watanabe *et al*,. 2007)〞 〝absorbent improvement of the soy isoflavone (Murata, 1986)〞, 〝strong antioxidative occurrence (Watanabe *et al.*, 2007)〞.

It was 1962 in the United States and 1983 in Japan that tempeh came up in an Indonesian foreign territory (Takamine, 1984). The expansion of the tempeh market was planned led by a flour milling company at first and several food companies including the natto manufacturers in Japan, but the major food companies withdrew successively without being accepted by Japanese consumers (Kato, 2004). It is produced on a small scale regionally now in various parts of Japan. We assume that it is the main factor that tempeh does not have umami taste why tempeh was not accepted by Japanese consumers. Tempeh has plain flavor and is tasteless where the taste of natto is strong comparably (Kato, 2004). Because it is tasteless, in Indonesia, it is used mainly as one of the materials of the dish with various seasonings. For example, that is to fry the tempeh in oil which is seasoned with salt and spice, and to boil it with soup of the coconut milk, and to season it, and to bake it like kebab (Shurtleff and Aoyagi, 2001). Tempeh is used for a great

variety of dishes and is generally used as dish material mainly, and is cooked with heating mostly in the case (Shurtleff and Aoyagi, 2001). On the contrary, natto is eaten as it is without being cooked.

In the case of Japan, people catch tempeh in comparison with natto by all means. Since natto is Japanese old traditional food, many researchers have been investigating the functionality for a long time. As the result of that, superior functionalities of natto are well known widely among people (Sumi, 2013). Therefore, like natto, it becomes one of the important conditions for tempeh to be edible easily without cooking in order to be received by Japanese consumers. Besides, a preferable characteristic taste unlike the natto should be found. From this viewpoint, at first, we have performed various examinations to endow tempeh with the umami taste assuming eating tempeh as it is without heat-cooking. One of the succeeding in endowing tempeh with a unique nice smell and taste is to roast raw materials soybean (Kai, 1999).

We examined the optimum condition to hydrolyze the protein of the raw materials of soybeans with an industrial use protease to endow soybean with the umami taste by free amino acids caused by hydrolysis. It is the technique that has been done with some soybean fermented food such as natto, soy sauce, miso and so on to produce umami taste with amino acids derived from hydrolysis of soy protein, where the chief ingredient of the umami taste is glutamic acid (Hirano., 1989; Oonishi *et al.*, 2012). There is the most content of glutamic acid in soybean according to the Japanese food composition table (5^{th} supplementary edition). 220mg of glutamic acid is contained per 100g soybean by the table. Because glutamine changes to glutamic acid usually during fermentation, it is thought that it is to considerable quantity as for the glutamic acid as the gross weight. Therefore, it is fairy presumable that the umami taste appears in tempeh like other fermented soybean foods mentioned above with hydrolysis of soy protein. Because protease activity of *Rhizopus* is very weak (Matsumoto and Imai, 1990), appropriate protease should be utilized in the production of tempeh to hydrolyze soy protein. Thus, the industrial use protease is focused and the most suitable method to hydrolyze soy protein was examined in this study.

MATERIALS AND METHODS

Soybean

The soybean brand, High Pro (U.S.A.) was used in this study. This soybean is mostly utilized brand for tofu production in Japan and also is used for tempeh production. The characteristics of the soybean are higher content of protein and the big grain size. The general composition of the soybean used in this study was as follows: protein content was 33.0%, crude fat content was 19.9%, moisture content was 10.3% and ash content was 4.4%.

Industrial proteases

Following eight industrial proteases were used in this study. Orientase 90N (HBI Enzymes Inc., Hyogo, Japan) is metalloproteinase and neutral proteinase derived from *Bacillus subtilis*. Nucleicin (HBI Enzymes Inc., Hyogo, Japan) is neutral proteinase derived from *Bacillus subtilis*. Sumizyme LP50D (Shin-nihon Kagaku-kogyo KK., Aichi, Japan) is serine protease and neutral proteinase derived from *Aspergillus oryzae*. Sumizyme FP-G (Shin-nihon Kagaku-kogyo KK., Aichi, Japan) is metalloproteinase and neutral proteinase derived from *Aspergillus oryzae*. Newlase is acid proteinase (Amano Enzyme Co., Ltd., Aichi, Japan) derived from *Rhizopus sp.* Papain (BIOCON LTD., India) cysteine protease and thiol protease derived from immature papain. Bromelain (BIOCON LTD., India) is cysteine protease derived from pineapple stem. Morusin F (Kikkoman Biochemifa Company, Tokyo, Japan) is acid protease derived from *Aspergillus saitoi.*

Heat treatment of raw soybean and preparation of sample suspension

Raw soybean 16g was soaked in 200ml of distilled water at 25℃ and at room temperature. Non-heated soybean was used in this state. Boiled soybean was boiled as this state for five minutes. Steamed soybean was steamed in a steamer for 50 minutes after water was drained off through a colander. Each of heat treated and non-heated soybean by 16g was immersed in to 200ml of distilled water and was triturated with POLY TRON PT10-35 (Kinematica AG., Switzerland) at power 5 for 5min to get smooth suspension. Sample solution was obtained as filtrate with a filter paper (Whatman, No.1).

Protease treatment of soybean

0.1% and 1.0% enzyme solution was prepared with sterilized water. 0.2ml of this enzyme solution was mixed with 0.4ml of sample solution and 0.4ml of the buffer solution adapted to each enzyme in a microtube of 2ml volume. For control sample, distilled water was replaced with enzyme solution. Therefore final concentration of enzyme solution reached at 0.2% and 2.0%, respectively. The buffer solutions used in this study were Sφrensen buffer (5.2ml of 0.1M glycine, 4.8ml of 0.1M NaCl, pH2.0 at 18℃), Walpole buffer (5.9ml of 0.2N acetic acid, 14.1ml of sodium acetate, pH5.0 at 18℃) and Phosphate buffer (3.89ml of 1/15M KH_2PO_4, 6.11ml of 1/15M Na_2HPO_4, pH7.0 at 18℃). Enzyme reaction was performed by floating tubes on a water bus set to optimum temperature for each enzyme activity.

SDS-PAGE

After enzyme reaction finished, 15$\mu\ell$ of sample solution was mixed with 5$\mu\ell$ of sample buffer solution (0.0625M Tris･Cl6.8/5%β･ME/10%Glycerol/2.3%SDS). 1$\mu\ell$ of dye (0.25%BPB, 0.25%XC, 15%Ficoll-Type400) was added to 4 $\mu\ell$ of this mixture. This solution was heated at 95℃ for 5min and was applied to SDS-PAGE.

SDS-PAGE was performed according to the method of Laemmli (1970). A 12.5% polyacrylamide gel (e･PAGEL E-T12.5L, ATTO Co., Tokyo, Japan) was used. Electrophoresis was conducted at CC125V for 90min in electrophoretic buffer solution (192mM glycine, 25mM Tris-Cl, 0.1% SDS) using the electrophoretic apparatus AE-6000 (ATTO Co., Tokyo, Japan). Gels were stained with 0.025% Coomassie Brilliant Blue R-250 (Merck, Darmstadt, Germany) in methanol/acetic acid/water (5:10:85%, v:v:v), then destained in methanol/acetic acid/water (30:10:60%, v:v:v). DynaMarker Protein MultiColorⅢ (Funakoshi Co., Ltd., Tokyo, Japan) ranging from 17.2kDa to 229.3kDa was used as a protein size marker.

Tempeh starter preparation

Rhizopus oligosporus NBRC8631（＝NRRL 2710） was used in this study. This strain was isolated by K.H. Steinkraus and is the most widely used experimental strain (Nowak and Szebiotko, 1992). This strain was cultured on the potato dextrose agar plate (Merck KGaA, Germany) until spore grew co-fluently. 1ml of sterilized water was added into the plate (9cm in diameter) and the spore suspension was moved into a sterilized tube. This spore suspension was assumed as the starter. The starter was always freshly made for each experiment. The number of spores in the starter was approximately 1×10^9/ml.

Preparation of tempeh in a test tube and a plastic bag

40g of soybean was placed in a beaker and 200ml of 0.5% sodium acetate solution was added. After 18hrs at 25℃, the hull was peeled manually and moisture was dropped off with a colander for five minutes. A heat treatment, that is, non-heating, or boiling, or steaming was performed to this immersed soybean, then protease treatment was conducted to the soybean and it was fermented in vitro after tempeh starter was inoculated.

In the case of non-heating test, a protease was solubilized to be 0.2% (w/v) concentration to 100ml of 0.5% sodium acetate solution, then

the immersed soybean was added and left in an incubator for 1hr at 25℃. After moisture was dropped off with a colander for 5min, the sample was served for following fermentation test in a test tube and for SDS-PAGE analysis. In the case of boiling test, 200ml of 0.5% sodium acetate solution was added to the immersed soybean, and then boiled for 5min. After boiling was finished, moisture was dropped off with a colander for 5min, and the enzyme treatment was conducted as mentioned above. In the case of steaming test, soybean was steamed in a steamer for 50min and the enzyme treatment was conducted as mentioned above.

For the fermentation test in a test tube, 4g sample was put into a test tube of 15cm length which was sterilized and the inner soybean head was pushed with a sterilized glass stick so that height maintained the same level in each test tube. 20$\mu\ell$ of starter was put on a sterilized filter paper of 5mm in diameter and the filter paper was placed on the top of soybean in a test tube. The test tubes corked with a silicon were incubated at 35℃ and the growth of tempeh was observed every day. Five test tubes by the same experiment were incubated and the average growth was observed.

For the preparation of applied sample for SDS-PAGE, 2ml of sterilized water was added to two soybeans in a sterilized tube, and the soybean was triturated with POLY TRON PT10-35 (Kinematica AG., Switzerland) at power 5 for 5min to get smooth suspension. Sample solution was obtained as supernatant after centrifuged at 5,000 rpm for 5min at room temperature in a microtube. Following procedure of SDS-PAGE was as same as mentioned above.

In the experiment of the tempeh production in a fermentation bag, 100$\mu\ell$ of tempeh starter suspension was added to enzyme treated soybean, mixed well in a plastic bag, and then put into a fermentation plastic bag (5×7cm size and 40 pinholes on one side). The fermentation bag was fixed with a flat board evenly and was fermented at 35℃ for 20hrs.

RESULTS AND DISCUSSION

Selection of industrial protease that hydrolyzes soybean protein effectively

Firstly we tried to select the proteases that hydrolyze soy protein effectively from eight kinds of industrial use protease. Here, mashed soybean was served for proteolysis, because it is easy to be affected by the enzyme as a substrate, resulting in more accurate selection of the enzyme. At the same time, the most suitable conditions such as enzyme concentration, pH of enzyme solution, temperature of reaction were examined. Specifically, the experiment was conducted under the following conditions. Three heating methods (non-heating, boiling in water, steaming) of the raw material soybean were studied. The boiling and the steaming methods could be applicable to real process of manufacture. The concentration of enzyme was tested at 0.2% and 2.0% in reaction liquid. An appropriate buffer was used to set the reaction liquid to the optimum pH for each enzyme. About the reaction temperature, optimum temperature for each protease was adopted and the reaction time was tested in 1hr and 5hrs. The reaction conditions of the enzyme were

Table 1. Experimental reaction conditions of the industrial use protease

Name of Protease Product	Optimum pH	Experimental pH	Optimum Temperature (℃)	Experimental Temperature (℃)
Orientase 90N	7.2 (5.8-9.0)	7.0	55 (50-60)	55
Sumizyme LP50D	5.0-8.0	7.0	50 (45-60)	50
Sumizyme FP-G	4.0-8.0	7.0	45-55	50
Newlase	3.0-6.0	5.0	45	45
Nucleicin	7.2 (5.8-9.0)	7.0	55 (50-60)	55
Papain	6.5-8.0	7.0	55-75	65
Bromelain	4.5-5.2	5.0	40-65	50
Morusin F	2.0-3.0	2.0	50-55	50

summarized in Table 1. The resolution degree of soy protein was observed by SDS-PAGE.

Figure 1 shows the result of SDS-PAGE when non-heated soybean was hydrolyzed. In 0.2% enzyme solution, most of the protein was not broken down by 1hr enzyme reaction. When the enzyme reaction time was 5hrs, the hydrolysis of some protein was confirmed with bromelain. In 2.0% enzyme solution, proteolysis considerably progressed in Orientase 90N and papain by 1hr reaction. When the reaction time was 5hrs, proteolysis by papain and Orientase 90N progressed further, and hydrolysis with bromelain considerably progressed. It was predictable easily that proteolysis progressed with the specific enzyme so that enzyme concentration was high so that the enzyme reaction time was long.

In raw soybean, it was unexpected that soy protein is hydrolyzed with an enzyme because of presence of the protease inhibitor in soybean. But the fact that raw soybean protein can be digested enzymatically means production process can be simplified by neglecting the heat treatment of raw soybean. However, production cost would be higher, because 2.0% enzyme concentration is necessary to hydrolyze raw soybean protein. Taking the production cost into account, around 0.2% of enzyme concentration will be the target concentration to hydrolyze soy protein almost completely. In addition, it is unknown whether enzymatic hydrolysis is applicable to whole grain of soybean, since mashed soybean was tested in this experiment.

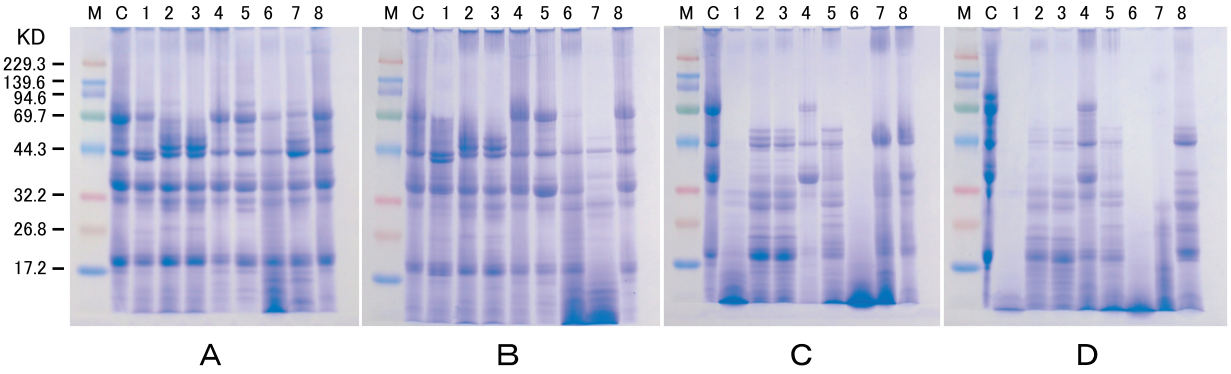

Figure 1. The result of SDS-PAGE when non-heated soybean was hydrolyzed. 0.2% protease was used for A and B. 2.0% protease was used for C and D. Enzyme reaction time was 1hr for A and C, 5hrs for B and D. Lane number indicates the protease used for the hydrolysis of soy protein as follows. 1: Orientase 90N, 2: Sumizyme LP50D, 3: Sumizyme FP-G, 4: Newlase, 5: Nucleicin, 6: Papain, 7: Bromelain, 8: Morusin F. "C" is the control where enzyme treatment was not performed. "M" is a protein size marker.

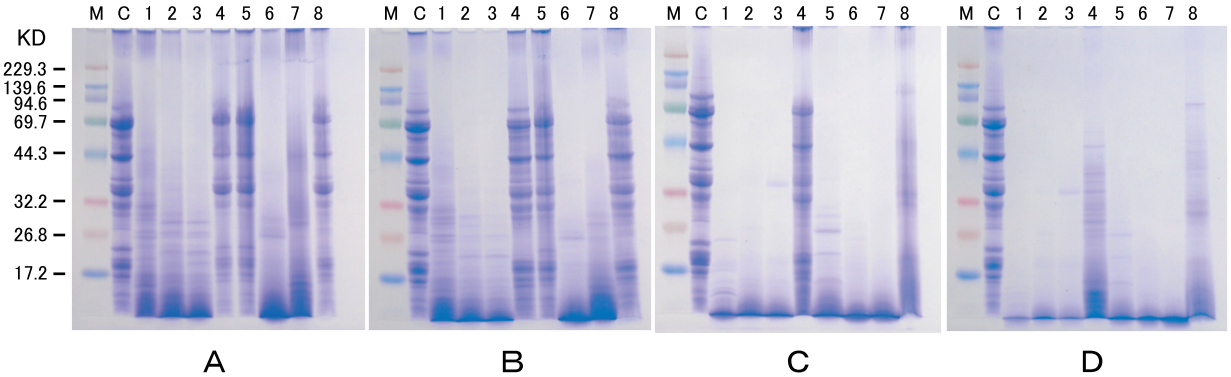

Figure 2. The result of SDS-PAGE when boiled soybean was hydrolyzed. 0.2% protease was used for A and B. 2.0% protease was used for C and D. Enzyme reaction time was 1hr for A and C, 5hrs for B and D. Lane number indicates the protease used for the hydrolysis of soy protein as follows. 1: Orientase 90N, 2: Sumizyme LP50D, 3: Sumizyme FP-G, 4: Newlase, 5: Nucleicin, 6: Papain, 7: Bromelain, 8: Morusin F. "C" is the control where enzyme treatment was not performed. "M" is a protein size marker.

Figure 2 shows the result of SDS-PAGE when boiled soybean was hydrolyzed. In 0.2% enzyme concentration, considerable proteolysis progressed in Sumiteam LP50, Sumiteam FP-G and papain, though it remained without some bands being digested. By 5hrs reaction, proteolysis progressed further more in Sumiteam LP50, Sumiteam FP-G and papain. Proteolysis progressed in bromelain to some extent. Considering with the result of Figure 1, bromelain may require some extent of reaction time for hydrolysis action. In 2.0% enzyme concentration and 1hr reaction, proteolysis progressed in Orientase 90N and Nucleicin, and more proteolysis progressed in Sumiteam LP50, papain and bromelain. Although a band slightly stays near 32KDa, proteolysis progressed most in Sumiteam FP-G. In 5hrs reaction, most protein was broken down to less than 32KDa in Orientase 90N, Sumiteam LP50, papain, and bromelain. In newlase and molsin F, hydrolysis has been low, but some proteolysis was observed at this state of reaction.

By the experiment using the boiled soybean here, Orientase 90N and bromelain showed the strongest power of proteolysis.

Figure 3 shows the result of SDS-PAGE when steamed soybean was hydrolyzed. In 0.2% enzyme concentration and in 1hr treatment, the considerable proteolysis progressed with Orientase 90N, Sumiteam LP50, papain, bromelain. In 5hrs reaction, the proteolysis progressed also about Sumiteam FP-G and Morusin F. In 2.0% enzyme concentration and 1hr reation, proteolysis progressed to a large extent in order of bromelain, papain, Orientase 90N, Sumiteam LP50, Sumiteam FP-G and Nucleicin. Proteolysis in Newlase was weak, and protein hydrolysis was not seen in Morusin F. There was no significant difference in the degree of proteolysis between 1hr and 5hrs enzymatic reaction.

With the comparison of the result of 0.2% enzyme concentration and 1hr reaction, there was no significant difference observed in the degree of proteolysis. Depending on the kind of the enzyme, one hydrolyzed boiled soybean protein well and other hydrolyzed steamed soybean protein well.

When think about practical tempeh production, from the viewpoint of troublesome in production process, shorter heating time is preferable. In addition, less waste fluid in production process is preferable, since expensive and complex equipment is necessary to dispose the waste fluid. In the point, heat treatment of steaming is better than boiling in water which causes a lot of waste fluid. Enzyme consumption should be less, since it is expensive. From the results of these experiments, it is obvious that steaming is the best way of heat treatment on raw soybean, and it is very possible to find out

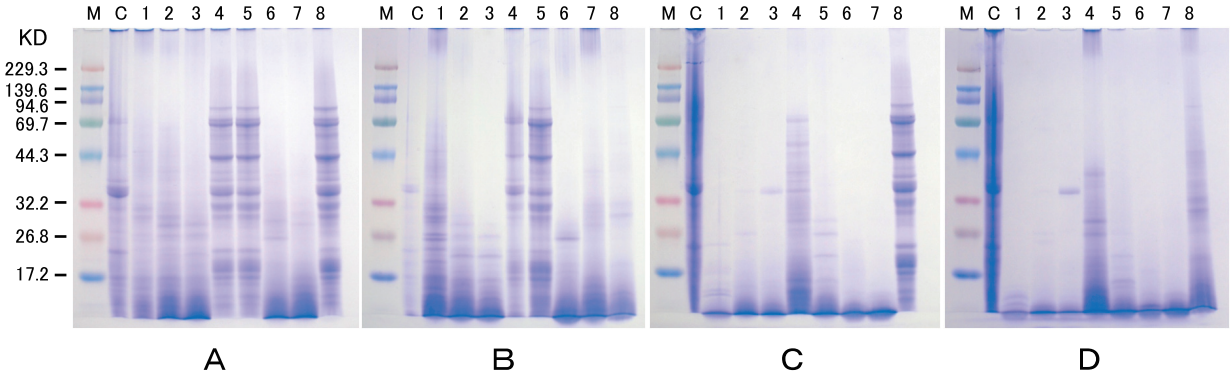

Figure 3. The result of SDS-PAGE when steamed soybean was hydrolyzed. 0.2% protease was used for A and B. 2.0% protease was used for C and D. Enzyme reaction time was 1hr for A and C, 5hrs for B and D. Lane number indicates the protease used for the hydrolysis of soy protein as follows. 1: Orientase 90N, 2: Sumizyme LP50D, 3: Sumizyme FP-G, 4: Newlase, 5: Nucleicin, 6: Papain, 7: Bromelain, 8: Morusin F. "C" is the control where enzyme treatment was not performed. "M" is a protein size marker.

the accurate least level of enzyme usage to hydrolyze soy protein well between 0.2% and 2.0% with Bromelain, or Papain, or Orientase 90N, or Sumizyme LP50D, or Sumizyme FP-G, or Nucleicin.

Effect of protease treatment on whole grain of soybean

According to the result of the previous experiment in this report using mashed soybean as substrate for protease, it was shown that soybean protein is possibly hydrolyzed enzymatically. Therefore, by this experiment, it was examined whether an enzyme hydrolyze granular soybean under a condition same as practical tempeh production. In addition, industrial use enzymes are crude derived from microbes in many cases, and various impurities are included, and they can have possibilities to inhibit the growth of *Rhizopus*. Thus, here, growth inhibition of *Rhizopus* by the industrial use enzymes was examined. In the same manner as the previous experiment in this report, the heating condition of the raw material of soybean assumed boiling and steaming as control as non-heating. Then, protease was acted on the heat-treated soybean, *Rhizopus* starter was inoculated followed by fermentation successively, and the degree of proteolysis was examined by SDS-PAGE. As before, the enzyme treatment was performed after soybean was immersed in the conventional liquid (0.5% sodium acetate solution) followed by heat treatment. Because it was not realistic on the practical tempeh production to use pH buffer solution for enzymatic reaction liquid, the enzymatic reaction was worked in soybean dipping liquid (0.5% sodium acetate solution). This is the significant and different point from the previous experiment. The quantity of enzyme to dissolve in dipping liquid was 0.2%. The same industrial enzymes of eight kinds tested as the previous experiment were examined.

Figure 4 shows the effect of eight industrial proteases on whole grain. As shown on A, protein was not digested by any proteases for non-heated soybean. In the previous experiment, the protein of mashed non-heated soybean was digested slightly, although the degree of hydrolysis was different by the kind of the enzyme. There are two possibilities for this difference. One possibility is that pH is not adjusted to suitable state for each enzyme in the experiment here.

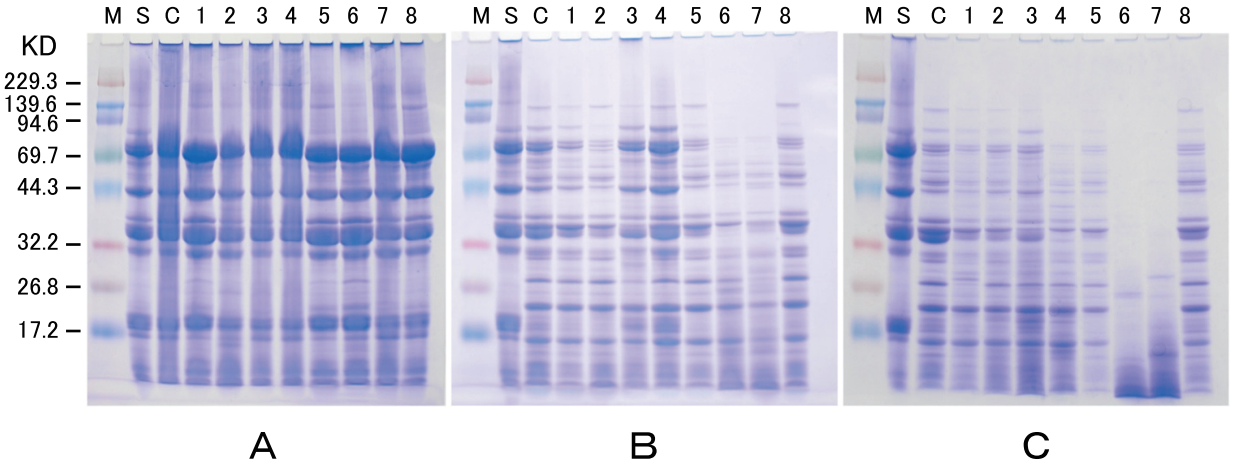

Figure 4. The result of SDS-PAGE when protease was reacted to the whole grain of soybean. Each enzyme concentration in the reaction solution was 0.2% and reaction time was 50min at 25℃. In A, non-heated soybean was hydrolyzed with enzymes. In B, boiled soybean was hydrolyzed with enzymes. In C, steamed soybean was hydrolyzed with enzymes. Lane number indicates the protease used for the hydrolysis of soy protein as follows. 1: Orientase 90N, 2: Sumizyme LP50D, 3: Sumizyme FP-G, 4: Newlase, 5: Nucleicin, 6: Papain, 7: Bromelain, 8: Morusin F. "S" is the standard where a raw soybean was not immersed. "C" is the control where enzyme treatment was not performed. "M" is a protein size marker.

One other possibility is that enzymes could not immerse into the inside of the whole grain. By comparing the result of both boiled soybean (B) and steamed soybean (C), papain and bromelain hydrolyzed soybean protein to fairly high degree in this experimental condition. Thus, this experimental result indicates the strong possibility that soy protein can be hydrolyzed by the treatment of papain or bromelain when steamed soybean is used in the industrial level production of tempeh.

Figure 5 shows the growth condition of *Rhizopus* on enzyme treated soybean. The photograph shown is the representative sample selected from five experimental test tubes at 48hrs after fermentation started. Growth was earlier than control about all other samples for some unknown reason. With the soybean of non-heated, the growth was extremely slow. Growth on boiled soybean seemed to slightly faster than steamed soybean. From the result of the previous experiment, attention should be addressed on papain and bromelain, but any specific problem was not seen in growth condition for those two enzymes.

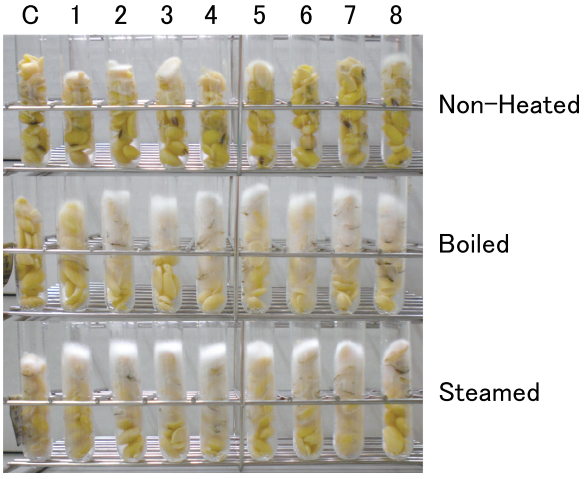

Figure 5. The growth condition of *Rhizopus oligospurus* NBRC8631 on enzyme treated soybean where three different heat treatments were worked. Photographs were taken at 48hrs after *Rhizopus* starter innoculation. Numerous number indicates the protease used for the hydrolysis of soy protein as follows. 1: Orientase 90N, 2: Sumizyme LP50D, 3: Sumizyme FP-G, 4: Newlase, 5: Nucleicin, 6: Papain, 7: Bromelain, 8: Morusin F. "C" is the control where enzyme treatment was not performed.

At the industrial level production of tempeh, soybean is squeezed in a plastic fermentation bag and fermented after having got the form of the bag fixed evenly. Therefore we needed to confirm whether *Rhizopus* grew on enzyme treated soybean in a fermentation bag normally or not. Thus we confirmed this question using papain and bromelain which are selected as the influential candidates for hydrolyzing soy protein in industrial tempeh production. Figure 6 shows the state at 48hrs after fermentation started in a fermentation bag. As shown, *Rhizopus* successively grew on enzyme treated soybeans by both papain and bromelain. There was no significant difference was observed between Papain and Bromelein. The growth of *Rhizopus* was slightly slower for control in the middle stage from the early stage of the fermentation. At 20hrs of the fermentation end, the significant difference was not observed by three experimental tempeh samples. As for the evaluation of the taste by five experimenters, umami taste was strongly felt to each tempeh which was enzyme treated than control. There was no difference in the umami taste between the tempeh treated with Papain and Bromelein. A large-scale sensuality examination is planned and the result should be evaluated statistically to confirm the relation between the enzymatic proteolysis on soybean and the umami taste.

The study whether or not the umami taste increases in a manufacturing process of the tempeh has not yet been studied until now. However, about the protein hydrolysis of the raw materials of soybeans, about half quantity of the soluble protein is shown to be a small molecule during fermentation (Fujio and Hayakawa, 1990). On the other hand, there is the report that there is hardly the increase and decrease about all amino acid contents during fermentation (Matsumoto and Imai, 1990). Therefore, this report showed the umami taste of the tempeh and relations of the proteolysis in PAGE for the first time, and it is considered to be significanct. Interest is held strongly

whether the umami taste of tempeh prepared by the technique that we built this time is proved in a sensuality examination.

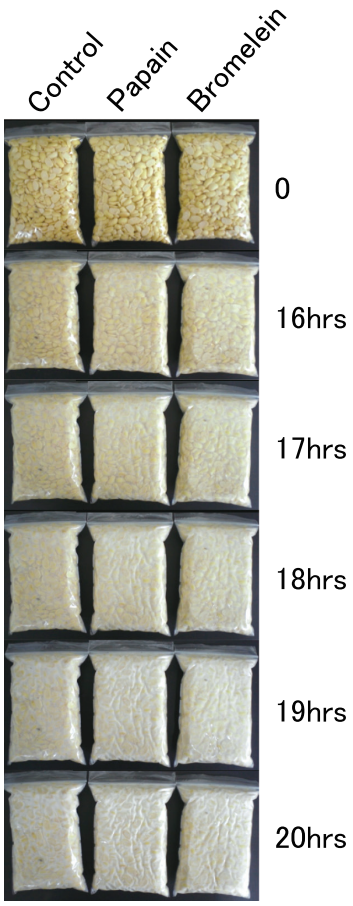

Figure 6. The change in growth condition of *Rhizopus oligospurus* NBRC8631 in a fermentation bag. Steamed soybean was treated with Papain or Bromelein. Control was untreated with any enzyme. The elapsed time after the fermentation start is indicated to the right of the photograph.

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テンペ製造用原料大豆のプロテアーゼ処理条件の確立

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＜要　旨＞

インドネシアの伝統的発酵食品であるテンペは、栄養価に富む大豆の無塩発酵食品であり優れた健康機能食品である。日本の納豆のような粘質物はなく、独特の発酵臭や旨味もない。チーズのような柔らかな食感と淡白な味が特徴である。納豆に比べ、日本人が好む旨味が劣るため、日本市場に普及できないでいると我々は考え、旨味を向上させるためのさまざまなアプローチをこれまで試みてきた。その一環として、本研究では、テンペの原料大豆のタンパク質をプロテアーゼで加水分解して旨味を賦与することを試みた。具体的には、テンペ菌で発酵する前の段階において、産業用酵素で原料大豆のタンパク質を加水分解するための最適条件を検討した。原料大豆を生、水煮、蒸煮した場合において８種類の産業用プロテアーゼで処理を行った。その際、処理時間と酵素濃度の検討も併せて行った。タンパク質の分解度はSDS-PAGEの泳動パターンを指標にして観察した。その結果、パパインとブロメラインが大豆タンパク質の酵素分解に有効であることが分かった。製造方法については、最初に、原料大豆を浸漬後、50分間の蒸煮処理を行う。その後、0.5％酢酸ナトリム水溶液に酵素濃度が0.2％（w/v）となるよう酵素を溶解させた浸漬液に漬けて25℃で１時間酵素処理を行うことで、原料大豆のタンパク質が相当量分解することが認められた。このような手法でプロテアーゼ処理を施した大豆を用いた場合、テンペ菌は常法に比べ生育が早くなることが確認され、正常にテンペが製造できることが分かった。本研究によってテンペの原料大豆のタンパク質を分解する工業的生産方法が確立したので、今後、官能試験を実施して旨味との関連性を確認する。

キーワード：テンペ、大豆、タンパク質の加水分解、産業用プロテアーゼ、旨味

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