Changes of Protein in Natto (a fermented soybean food) Affected by Fermenting Time

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The purpose of this study was to investigate effect of fermentation time on the proteins of steamed soybean inoculated with Bacillus subtilis (natto).

The result was found that the contents of trichloroacetic acid soluble nitrogen (TCA-N) and degree of hydrolysis (DH) of protein increased with the fermentation time increased, but the protein solubility was declined with increasing the fermentation time in advance, then increased. The most bands of soy proteins were found to disappear from the Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoretogram after 24 h of fermentation. Some of nonessential amino acids were found to remarkably decline, but some of them were remained unchanged after 24-48 h of fermentation. However the most essential amino acids were found to declined after 36h of fermentation, then all of them increased after 48 h of fermentation except histidine.

Keywords: Natto, soy protein, Bacillus subtilis, SDS-PAGE

Introduction

Natto is a traditional fermented soybean food product with venerable history in Japan. By steaming soybeans and inoculating with Bacillus subtilis, natto spores to ferment it, and then it would become a characteristic odor, flavor and slimy food.

The processing of natto includes soaking, steaming, cooling, inoculating, fermenting and aging (Ohta, 1986; Maruo and Yoshikawa, 1989). During fermentation the steamed soybeans will produce a large amount of sticky substances which include glutamic acid, fructan, amino acids (such as glutamic acid, phenylalanine, tyrosine) and volatile fatty acids (such as butanediol, ethanoic acid, pentanoic acid) (Shih and Van, 2001). Natto contains isoflavones, dietary fiber, vitamins, linoleic acid and some minerals which are originated from soybeans, in addition, it also contains some functional compounds such as enzymes, bioactive peptides, natto kinase (fibrinolytic agent), gama-polyglutamic acid (γ-PGA) etc. which are produced by B. subtilis, natto (El-Safey and Abdul-Raouf, 2004; Ma et al., 2006).

Perhaps because of it possesses characteristic musty odor and slimy appearance, natto, even though it is well known in Japan, is not so popular so widely consumed as compared with other fermented soybean foods. However, there seems to be an upswing in consumption in Taiwan, recently. In Taiwan, natto is seasoned with soy sauce and mustard like Japanese. Natto has a short shelf life, partly because it has a moisture content of 59.5% (Ohta, 1986) and partly because it is usually prepared in small scale plants with poor quality control. Cold storage has been used to extend its shelf life. Because it contains some functional compounds such as isoflavones, γ-PGA, nattokinase and oligopeptides, so, besides it is consumed as wet and sticky form, some biotechnical or functional foods companies try to dry it into powder form or make capsulized form sold as functional foods. Proteases which are produced by B. subtilis, natto can hydrolyze soy protein into polypeptides. The higher proteins are hydrolyzed the more peptide bonds are broken and the more free amino acids and lower molecular weight of oligopeptides are produced (Spellman et al., 2003). Shih (2006) considered that increase in steaming and fermenting time would accelerate the solubility of protein. When the fermenting time and inoculum of B. subtilis, natto number are not enough, the bacteria are unable to utilize the protein of soybeans to raise the solubility of protein. If the fermentation time and the number of inoculum increased it will be able to raise the solubility of the protein (Chou, 2004). Liu (2005) also found that the steamed soybeans were inoculated with B. subtilis, natto would increase in protein solubility and amino nitrogen

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content. Wu (2006) reported that *B. subtilis*, natto inoculated in soy protein added with different concentrations of sucrose and sodium glutamate and incubated for different times and produced different contents and types of amino acids. The conditions such as fermentation time and temperature have been established. Ohta et al. (1986) investigated the effects of fermentation time on the organoleptic quality of natto, they found that natto made with 8 h fermentation had the highest overall scores, whereas natto made with the traditional 16 h fermentation received a poor rating. In general, when the ammonium production exceeds a level of 1%, the product becomes obnoxious. This research is to study the changes in TCA soluble nitrogen, protein solubility, amino acid composition and electrophoretic patterns of protein, when the steamed soybeans inoculated with *B. subtilis*, natto and fermented at 43°C for 0, 24, 36 and 48 h.

Materials and Methods

**Natto preparation**  *B. subtilis* obtained from Nattomoto (Yuko Takahashi Laboratory, Japan) was used as inoculum. The organisms were grown in brain heart infusion broth (Difco 0037-17) at 37°C for 18 h. The culture was diluted in sterile distilled water containing 0.85% NaCl and 0.1% peptone to a concentration of approximately 10⁹ colony forming units (cfu)/mL.

**Soybean fermentation**  Soybeans were cleaned and soaked in fresh water overnight at 4°C. The soaking water was then discarded, and the beans were cooked in fresh tap water for 20 min (ratio of bean:water = 1:3), cooled to 40°C, inoculated with 5 mL of the diluted culture, and fermented at 43°C for 48 h. The samples were taken for analysis after 0, 24, 36 and 48 h of fermentation.

**TCA soluble nitrogen content**  TCA soluble nitrogen was determined according to the method described by Nagai (1983) and modified. A 5 g dried samples were placed into the volumetric bottle and added the deionized water to 100mL and blended with magnetic stirrer. Next 10 mL of blended solution was added with 20 mL of 10% trichloroacetic acid (TCA) solution and stored at 4°C to extract for 16 h, then filtrated with Whatman #1. A 5mL of the filtrate sample were added 15 mL of the concentrated sulfuric acid and one granule of Kjeldahl tecator (FOSS Analytical, Sweden) and placed into digestive flask for carried on Kjeldahl method analysis.

TCA soluble nitrogen content was figured out according to the following equation:

\[
\text{TCA soluble nitrogen content (\%)} = \frac{(A-B) \times N \times 14.01 \times f}{\text{g of sample}} \times 10
\]

where A is the 0.05 M HCl volume (mL) used for sample titration, B the 0.05 M HC volume (mL) used for blank titration, N:Normality of titration solution (0.1N), f :Nitrogen factor in soybean and its products = 5.71

**Protein solubility**  Protein solubility was determined according to the method described by Machado et al. (2008), based on the soluble nitrogen determination in 0.2% sodium hydroxide solution and the results were expressed as percent of soluble nitrogen in relation to total nitrogen in sample. Natto (2 g) was stirred with 100 mL of 0.2% sodium hydroxide solution at 150 rpm for 20 min at 25°C and centrifuged at 3840 × g for 15 min.

Supernatant (15 mL) was taken for protein determination using the Kjedhal method. The soluble nitrogen percentage (SN%) was determined according to the formulas:

\[
\text{Soluble nitrogen\% (SN\%)} = \left(\frac{(V_s - V_b) \times 0.05 \times f \times 1.4}{SW}\right) \times 100
\]

Protein solubility (%): \(\frac{\text{SN\%}}{\text{TN\%}}\) × 100

where VS is the 0.05 M HCl volume (mL) used for sample titration, VB the 0.05 M HCl volume (mL) used for blank titration, f the HCl standardization factor, SW the sample weight and TN is the sample total nitrogen.

**Determination of the Degree of Hydrolysis**  Sample preparation: 1 g of natto was mixed with 9 mL distilled water and stood for one day at 4°C. The sample was then centrifuged at 9,000 × g for 30 min. The supernatant was used for hydrolysis degree determination. The hydrolysis degree was determined by quantification of o-phthaldialdehyde (OPA) spectrophotometric assay, as described by Church et al. (1983). The OPA solution was a combination of 25 mL of 100 mM in 1 mL methanol and 100 μL of β-mercaptoethanol to create a total volume of 50 mL. An aliquot of 50 μL of the hydrolysates was added to 1 mL OPA solution. The solution was mixed by inversion, incubated for 2 min at room temperature, and then measured the absorbance of sample by a spectrophotometer (Hitachi, U-2800).

The content of peptides was calculated by regression equation obtained from standard dipeptides (Leu-Gly) as follows: \(y = 0.33x + 0.0366, R^2 = 0.9979\). Degree of hydrolysis is defined as the percentage of the number of peptide bonds in a protein which have been cleaved during hydrolysis (Spellman et al., 2003). DH values were calculated as follows: \(\text{DH\%} = \frac{n \times 100}{N}\), Where n is the average number of peptide bonds hydrolysed, N the total number of peptide bonds per protein molecule.

**Amino acid composition**  Amino acid composition was determined as the modified procedures from the methods described by Konosu et al. (1974). A 10 g natto sample was added with 20 mL 12 N HCl and hydrolyzed at 100°C vacuum oven for 24 hr. The hydrolysate 5 mL was added 15 mL 7% TCA solution and mixed and centrifuged by 4000 × g.
g for ten min at 10°C. The supernatant was added 7% TCA solution to make 50 mL volumetrically. A 20 mL of the solution was filtrated through funnel filter and added 20 mL ether to remove TCA solution. This procedure was repeated three times. Finally, the collected solution was freeze dried or evaporated by vacuum distillation to remove ether residue. Then the solution was added extra-pure water to make 20 mL and stored at -20°C after filtrated by 0.45 μm filter. To take the sample solution or standard solution 25 μL and then added 25 μL distilled water, 50 μL 100 mM NaHCO₃ buffer (pH 8.3) and 200 μL DABSYL-CL solution and mixed well. Then the mixture was heated at 70°C for 15 min., and mixed with 700 μL 50 mM NaHPO₄ and filtrated through 0.45 μm filter. Finally, a 20 μL solution was taken for high performance liquid chromatography analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as the procedures described by Mujoo et al. (2003). The total proteins were extracted using the procedures modified from the method described by De Mejia et al. (2004). A 20 g milligrams of samples were extracted with 0.5 mL 0.03 M Tris [Tris (hydroxy-methyl) aminomethane] buffer (pH 8.0) containing 0.01 M β-mercaptoethanol (β-ME) for 1 h with vortexing every 10 min. Samples were then centrifuged at room temperature for 20 min at 11,000 × g. The protein content of the supernatant was determined according to the Bradford method (Bradford, 1976). The protein concetration of the supernatant was diluted to 4 mg/mL with distilled water, and 20 μL of the distilled extract were mixed with 20 μL of SDS-sample buffer (0.15 MTris-HCl, pH 6.8, 4% w/v SDS, 5% v/v β-ME) and heated at 96°C for 5 min. A 20 μL of the solution cooled to room temperature (20°C), containing 40 μg of protein, were loaded onto a gel containing 12% polyacrylamide. SDS-PAGE was performed in a vertical electrophoresis unit (Mini-Protein 3 Electrophoresis Cell, Bio-Rad) at 100 V constant voltage for 1 h, followed by 125 V constant voltage until the tracking dye migrated to the bottom edge of the gel (6 h). Gels were stained with Coomassie Brilliant Blue R-250 (0.05%,v/v/v) and destained in the same solution without the dye.

Results and Discussion

TCA soluble nitrogen and protein solubility Table 1 shows effect of fermentation time on TCA soluble nitrogen (TCA-N), protein solubility of soy protein in natto. The result showed TCA-N increased with fermentation time increased at first and then decreased. Generally, natto fermentation time takes 24 h, thus we inferred 36 h of fermentation may be the time of stationary phase of growth of starter. After 36h of fermentation non protein nitrogen compounds may be utilized by the starter and resulting in TCA-N decrease. Thirty six hours of fermentation time although TCA-N content being the highest, the product may have ammonia odor. Thus, this may be the reason why natto fermented for 20 to 24 h. The protein solubility from fermented soybeans ranged from 32.71% to 13.69% after 36 h of fermentation, about 1.5 to 3 times lower than the protein solubility of non-fermented soybean. This could be due to the fact that microorganisms and enzymes involved in the fermentation process can easily hydrolyze soluble proteins as the inference of Song et al. (2008). After 36 h of fermentation the protein solubility increased again, which suggests protein hydrolysis occurred mainly secondary fermentation.

Degree of hydrolysis (DH) of soy protein in natto Fig. 1 showed the degree of hydrolysis of soy protein of natto. The results indicated the peptide content and DH of soy protein increased with the fermentation time. DH did not increase too much during the first 24 h of fermentation. This result was in accordance with the report of Gibbs et al. (2004). They pointed out in fermented soy foods, the proteins were only partly hydrolyzed because of the inability of most proteases to cleave glycoproteins, phosphoproteins, other post

![Image of Table 1](https://example.com/table1.png)

Table 1. Effect of fermentation time on TCA-N and Protein solubility of soy protein in natto.

<table>
<thead>
<tr>
<th>fermentation time(h)</th>
<th>TCA-N(%D.B.)</th>
<th>Protein solubility(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.98</td>
<td>36.96</td>
</tr>
<tr>
<td>24</td>
<td>2.98</td>
<td>32.71</td>
</tr>
<tr>
<td>36</td>
<td>3.96</td>
<td>13.69</td>
</tr>
<tr>
<td>48</td>
<td>4.93</td>
<td>39.00</td>
</tr>
</tbody>
</table>

1. Data represent the average of triplicate determination.  
2. D.B.: Dry basis
translationally modified species, or domains that contained a higher number of disulfide bridges. Terlabie et al. (2006) reported that the first 36 h of fermentation, protease activity of the spontaneously fermented soybean (contain more than one species of Bacillus) was lower, however, sharp increase in the protease activity occurred at the advanced stages of the fermentation.

Contents of amino acids of natto at different fermentation time In non-fermented cooked soybean, the highest content of essential amino acid was leucine (7.36 g/100 g dry matter), and the next was lysine, phenylalanine, threonine, tyrosine, valine, histidine, and isoleucine in the descending order. The lowest content of essential amino acid was methionine plus cysteine. The result in sequence of content of amino acid was the same trend as the content of amino acids of the fermented soybean meal which was reported by Song et al. (2008). And the highest content of nonessential amino acid was glutamic acid (15.57 g/100 g dry matter), and next was aspartic acid, proline, arginine, Ser, Ala and glycine in the descending order. The lowest content of nonessential amino acid was methionine plus cysteine.

Table 2. Effect of fermentation time on amino acid composition of soy protein in natto.

<table>
<thead>
<tr>
<th></th>
<th>Unfermented Soybean</th>
<th>Fermented Soybean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Non essential amino acids b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu</td>
<td>15.57</td>
<td>(-83.5)</td>
</tr>
<tr>
<td>Asp</td>
<td>9.0</td>
<td>(-87.8)</td>
</tr>
<tr>
<td>Arg</td>
<td>7.17</td>
<td>(-54.8)</td>
</tr>
<tr>
<td>Ala</td>
<td>5.65</td>
<td>(-26.4)</td>
</tr>
<tr>
<td>Gly</td>
<td>5.44</td>
<td>(-44.0)</td>
</tr>
<tr>
<td>Ser</td>
<td>5.88</td>
<td>(-65.8)</td>
</tr>
<tr>
<td>Pro</td>
<td>7.62</td>
<td>(+3.3)</td>
</tr>
<tr>
<td>Essential amino acids b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>7.36</td>
<td>(+2.7)</td>
</tr>
<tr>
<td>Lys</td>
<td>5.24</td>
<td>(-45.6)</td>
</tr>
<tr>
<td>Ile</td>
<td>3.34</td>
<td>(+5.1)</td>
</tr>
<tr>
<td>Val</td>
<td>3.57</td>
<td>(+12.9)</td>
</tr>
<tr>
<td>Thr</td>
<td>3.79</td>
<td>(-48.5)</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.74</td>
<td>(+15.8)</td>
</tr>
<tr>
<td>Phe</td>
<td>5.02</td>
<td>(+11.2)</td>
</tr>
<tr>
<td>His</td>
<td>3.40</td>
<td>(-59.1)</td>
</tr>
<tr>
<td>Cys</td>
<td>0.9</td>
<td>(+1.1)</td>
</tr>
<tr>
<td>Met</td>
<td>2.46</td>
<td>(+6.9)</td>
</tr>
</tbody>
</table>

a: The figures in parentheses represented increase or decrease ratio (%) of the amino acids after fermentation as compare with those of the sample before fermentation.
b: grams of amino acids per 100 g of dry matter.
Changes of Protein in Natto Affected by Fermenting Time

acid in this study was tyrosine which was different from that of the fermented soybean meal was proline which was reported by Song et al. (2008).

After 36 h of fermentation all of the content of amino acids decreased over 46% except for the cystein decreased 22%, and among which the glutamic acid and serine decreased 71%. When the soybean was fermented for 48 h, most of the essential amino acids increased significantly, except for the histidine, and among which the valine increased 93.6% and glycine increased 50%. By the increase of amino acids in this study we inferred that it could increase the nutrition values and functions of the steamed soybean after 48h of fermentation.

Changes in SDS-PAGE patterns of soya protein  Fig. 2 shows the electrophoretic patterns of proteins in the fermented soybeans (natto) at different times. Lane 1 presents marker of protein, lane 2 presents soy beans (original sample), lane 3 presents soaked soy beans, lane 4 presents soaked and steamed soybeans, Lanes 5-8 presents the steamed soybeans fermented for 12, 24, 36 and 48 h. From the fig 2 it can be seen the soy protein components with molecular weight above 20 Kd disappeared from the electrophoretograms for the samples fermented for 24-48 h. However, the electrophoretic patterns for the uncooked, soaked and cooked samples and the sample fermented for 12 h did not change. The result was the same as the report of Kiers et al. (2000). In our previous work we also met the trouble with the majority of the soy protein from the sample did not appear on the electrophoretogram. We found the reason for the very poor separation of soy protein subunits was not due to autoclave of soybeans (Kiers et al., 2000). It was due to the process of the extraction of protein. At presence, we soaked the sample in the extraction buffer solution overnight at 4°C before extraction by ultrasonic treatment. The electrophoretic patterns showed the most of soy protein components in natto disappeared after 24 h fermentation. This result may provide for industry reference. Several studies have reported the degradation of soybean allergens (glycinin and β-conglycinin) during fermentation by microbial proteolytic enzymes such as in soy sauce, miso, soybean ingredients and feed grade soybean meals improving their nutritional and functional properties (Hong et al., 2004; Ito et al., 2005; Kobayashi, 2005; Ogawa et al., 2000; Yamanishi et al., 1995). In this study, those allergens of soy protein subunits all degraded after 24 h fermentation. However, what changes of soy protein subunits at the fermenting time between 12 and 24 h may need further more study in the future.

References

Fig.2. Change of SDS-PAGE electrophoretogram of soya protein in natto at different fermentation.
Lane 1:Marker, lane 2: Soybean, lane 3: Soaked soybean without inoculation, lane 4-8 are the steamed soybean fermented with B. subtilis for 0, 12, 24, 36, 48 hr.


