Effects of Fermentation Temperature on the Content and Composition of Isoflavones and \( \beta \)-Glucosidase Activity in Sufu

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Sufu is a popular fermented tofu product in China. The low quality of sufu produced in the hot summer is a big problem in sufu manufacture, so we prepared sufu at two different temperatures, 26 \(^\circ\)C as normal and 32 \(^\circ\)C as high temperature, and the effects of temperature on isoflavones and \( \beta \)-glucosidase activity were investigated. Fermentation temperature did not cause significant differences in the recovery of isoflavones, but resulted in a different redistribution of isoflavone isomers in sufu. Sufu fermented at 26 \(^\circ\)C was richer in isoflavone aglycones than at 32 \(^\circ\)C; the enrichment of isoflavone aglycones might have the advantage of enhancing the physiological function. No 6\(^\prime\)-O-malonyl-glucosides were detected in sufu fermented at 26 \(^\circ\)C, whereas some 6\(^\prime\)-O-malonyl-glucosides were found at 32 \(^\circ\)C. A fermentation temperature of 26 \(^\circ\)C benefited the \( \beta \)-glucosidase production by fungi, which contributed to valid conversion from glucosides to aglycones. It was also found that \( \beta \)-glucosidase converted \( \beta \)-glucosides more effectively than 6\(^\prime\)-O-malonyl-glucosides and 6\(^\prime\)-O-acetyl-glucosides into aglycones.

Key words: sufu; fermentation; isoflavone; conversion; \( \beta \)-glucosidase

In Asian countries, soybean is consumed in many forms, including soymilk, tofu products, and fermented products such as miso, soy sauce, tempeh, and sufu. Sufu is a traditional fermented tofu product in China that resembles a soft creamy-type cheese. There is a similar product called tofuyo in Okinawa, Japan. China has many different colors of sufu, such as red sufu, white sufu, and gray sufu, but red sufu is the most popular type due to its attractive color and fine flavor, and has been consumed widely in China as an appetizer for centuries.1

Sufu is produced by fungal solid-state fermentation of tofu, followed by salting and maturation. During sufu fermentation, most of the components are hydrolyzed, which is of benefit to improve flavor and taste.2 Subunits of soybean protein have not been detected in sufu, and most proteins are degraded into peptides and amino acids.3 It has been reported that sufu contains 22 esters, 18 alcohols, 7 ketones, 3 aldehydes, 2 phenols and other volatile compounds.4 The esters contribute a characteristic flavor to red sufu.5

Sufu fermentation converts soybean isoflavones from the glucosides in tofu into the corresponding aglycones under hydrolysis by \( \beta \)-glucosidase.6 Isoflavones are referred to as phytoestrogens due to their estrogenic activities in soybean and soybean foods.7 Considerable attention has been paid to their health protective effects including reducing the risk of cardiovascular disease, lowering rates of prostate, breast, and colon cancer, and improving bone health, related to their estrogenic activities.8,9 The lower incidence of breast and prostate cancer in Asian countries than in western ones has been attributed to the differences in soybean food consumption in epidemiological studies.10,11

Genistein, daidzein, and glycitein are isoflavone aglycones with three glucoside forms, \( \beta \)-glucoside, 6\(^\prime\)-O-malonyl-glucoside, and 6\(^\prime\)-O-acetyl-glucoside. The content and composition of these isoflavones varies in soybean food depending on processing techniques, such as heat treatment, defoaming, soaking in water, enzyme hydrolysis, and fermentation.12–14 Heat treatment has been found to convert some malonyl isoflavones into acetyl forms,15 and defoaming reduces isoflavone contents.16 The glucoside conjugates of isoflavones are converted into aglycones during soybean processing by the effect of \( \beta \)-glucosidase.6,17 \( \beta \)-Glucosidase is considered to be a key enzyme for the conversion of isoflavone forms in fermented soybean foods. Our previous study confirmed that fermentation was the major contributor to the conversion of isoflavones in sufu under \( \beta \)-glucosidase hydrolysis,6 but the relations

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Abbreviations: Ace-glu, 6\(^\prime\)-O-acetylglucoside; Agl, aglycone; Dein, daidzein; Din, daidzin; Gein, genistein; Gin, genistin; Glein, glycitein; Glin, glycitin; Glu, \( \beta \)-glucoside; Mal-glu, 6\(^\prime\)-O-malonylglucoside; p-NPG, p-Nitrophenyl-\( \beta \)-D-glucoside
among β-glucosidase, isoflavone forms, and fermentation conditions such as temperature are not clear. It is difficult to produce sufu of high quality during the hot summer period in a factory without air-conditioning, where the temperature rises higher than 30 °C. The low quality of sufu produced in the summer is a big problem in sufu manufacture in China. Fermentation temperature strongly affects the growth of microorganisms and the enzyme activities produced by microorganisms. These enzymes are thought to have a close relation to sufu quality. Han evaluated the growth of *Actinomucor elegans* between 20 °C to 35 °C and reported that the optical growth temperature for *A. elegans* was 25 °C to 30°C, which is also the normal temperature for sufu fermentation. The formation rate of *A. elegans* declined sharply at temperatures exceeding 30 °C. Not enough enzyme amount or activity, such as protease, lipase, α-amylase, glutaminase, and β-galactosidase, were produced during pehtze fermentation at high temperatures. As an important physiological component, the isoflavones in sufu are converted from glucosides into the corresponding aglycones under hydrolysis by β-glucosidase. The differences in the content and composition of isoflavones perhaps do not dramatically decrease the quality of the final sufu, but affect the nutritional property and flavor. In this study, the effects of fermentation temperature, 26 °C as normal temperature and 32 °C as high temperature in the summer season, on β-glucosidase activity and its relation to the content and mass distribution of isoflavones in sufu were investigated.

**Materials and Methods**

*MATERIALS.* Daidzein, glycine, genistein, daidzin, glycitin, genistin, 6’-O-malonyl-daidzin, 6’-O-malonyl-glycitin, 6’-O-malonyl-genistin, 6’-O-acetyl-daidzin, 6’-O-acetyl-glycitin, and 6’-O-acetyl-genistin were purchased from Japan LC Services Corporation. *p*-Nitrophenyl-β-D-glucoside (*p*-NPG) was purchased from Sigma Chemical Company. *Actinomucor elegans* (Academia Sinica (AS) 3.227) was kindly donated by the Beijing Wang-Zhi-He sufu manufacturer.

*Red sufu preparation.* Processing of red sufu is illustrated in Fig. 1. The steps of and parameters were as follows:

1. Tofu was prepared by CaSO\textsubscript{4} precipitation from boiled soymilk and cut into rectangular pieces, approximately 3.2 × 3.2 × 1.6 cm.

2. Fresh tofu pieces were inoculated with dispersion of *A. elegans* by spraying onto their surface, and incubated for 48 h under 90% relative humidity and air circulation to ensure adequate aeration. Semifinished products were called pehtze.

3. Pehtzes were piled up in a container and salt was spread between the layers. Pehtzes absorbed salt until the salt content reached about 15%.

4. Twelve pieces of salted pehtzes were matured in glass bottles with a dressing mixture consisting of koji red rice, an alcohol beverage, sugar, Chiang (wheat-based miso), and spices. Red sufu was obtained after ripening for 2 months.
Sufu fermentation was divided into earlier fermentation (step 2) and later fermentation (steps 3 and 4). Sufu fermentation from steps (2) to (4) was controlled at two temperatures, 26°C and 32°C.

Tofu, pehtze, salted pehtze, and red sufu were sampled for analysis. Fresh pehtzes were sampled after being fermented for 12, 24, 36 and 48 h and salted pehtzes were sampled after being salted for 5 d. Sufu was drawn randomly from each batch.

**Isoflavones extraction.** Samples of tofu, pehtze, salted pehtze, and sufu were freeze-dried and finely ground. Approximately 0.5 g of sample was weighed into 125 ml flat-bottom flasks, and 12 ml of extraction solution (acetonitrile:0.1 N HCl in 5:1 ratio) was added to each flask. The solution was mixed using a rotary shaker (120 rpm) overnight at room temperature. Extracts were suction filtered into 250 ml round bottom flasks through Whatman no. 42 filter paper and washed twice with filtered Milli Q water, and (B) 0.1% (v/v) acetic acid in acetonitrile. The solvent gradient was as follows: Solvent B was increased from 15 to 25% over 35 min, then increased to 26.5% within the next 12 min, and then increased to 26.5% within the next 12 min, and finally to 50% within 30 s, and held for the next 14.50 min. The flow rate was 1 ml/min up to 48 min and was increased to 1.3 ml/min within 30 s and held until 63 min. Quantitative data for each isoflavone were obtained by comparison to known standards.

**Crude enzyme extract and determination of β-glucosidase activity.** A modified procedure of Bahl and Agrawal\(^1\) was used to determine β-glucosidase activity. Eight grams of sample was homogenized with 25 ml of 0.2 M acetate buffer (pH 4.5) at 4°C. The slurry was centrifuged at 10,000 x g for 15 min at 4°C, and the supernatant was used as a crude enzyme solution. Then 2 ml of 1 mM p-NPG solution and 0.5 ml of a crude enzyme solution were mixed and incubated at 45°C for 30 min. The reaction was stopped by the addition of 2.5 ml of 1 M sodium carbonate. The resultant color was immediately measured at 400 nm. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 µmole of p-nitrophenol per min.

**Statistical analysis.** Analyses of variance using the general linear models were conducted. Significant differences between the sample means were established at the p < 0.05 level by ANOVA, followed by student’s t-test.

### Results and Discussion

**Effects of fermentation temperature on content and composition of isoflavones in sufu**

The yields, moisture content, and isoflavone contents for tofu, pehtze, salted pehtze, and sufu are shown in Table 1. The total isoflavone amounts were normalized for the differences of mole of isoflavone isomers.

There were no significant differences in the yields of sufu between that fermented at 26°C and that fermented at 32°C. Water leached from pehtze under high salt concentrations, as was observed from an obvious decrease in the moisture in salted pehtze. Salting was the main factor affecting the yield of sufu.

The total recovery of isoflavones was 1.61 mol in sufu fermented at 26°C and 1.65 mol in sufu fermented at 32°C, whereas tofu contained 2.55 mol of isoflavones, which indicates that fermentation temperature did not generate significant differences in the recovery of total isoflavone in sufu. In our previous study, it was shown that some amounts of the isoflavones were dissolved into

### Table 1. Yields, Moisture, and Isoflavone Contents\(^a\) in Sufu Fermentation

<table>
<thead>
<tr>
<th>Name of sample</th>
<th>Yield (g)</th>
<th>Moisture (%)</th>
<th>β-glucoside (mol)</th>
<th>6″-O-malonylglucoside (mol)</th>
<th>6″-O-acetylglucoside (mol)</th>
<th>Aglycone (mol)</th>
<th>Total (mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tofu</td>
<td>2000</td>
<td>72.8 ± 2.1</td>
<td>1.19</td>
<td>0.35</td>
<td>0.39</td>
<td>0.62</td>
<td>2.55</td>
</tr>
<tr>
<td>Pehtze (26°C)</td>
<td>1992 ± 38</td>
<td>70.2 ± 0.5</td>
<td>0.28</td>
<td>0.21</td>
<td>0.28</td>
<td>1.70</td>
<td>2.47</td>
</tr>
<tr>
<td>Salted pehtze (26°C)</td>
<td>1859 ± 37</td>
<td>52.0 ± 1.0</td>
<td>0.11</td>
<td>Nd</td>
<td>0.19</td>
<td>1.67</td>
<td>1.97</td>
</tr>
<tr>
<td>Sufu (26°C)</td>
<td>1927 ± 76</td>
<td>58.8 ± 0.3</td>
<td>0.04</td>
<td>Nd</td>
<td>0.09</td>
<td>1.48</td>
<td>1.61</td>
</tr>
<tr>
<td>Pehtze (32°C)</td>
<td>1978 ± 46</td>
<td>72.0 ± 3.0</td>
<td>0.90</td>
<td>0.27</td>
<td>0.36</td>
<td>0.89</td>
<td>2.42</td>
</tr>
<tr>
<td>Salted pehtze (32°C)</td>
<td>1849 ± 65</td>
<td>51.7 ± 0.2</td>
<td>0.62</td>
<td>0.18</td>
<td>0.30</td>
<td>0.84</td>
<td>1.94</td>
</tr>
<tr>
<td>Sufu (32°C)</td>
<td>1923 ± 39</td>
<td>58.9 ± 2.2</td>
<td>0.58</td>
<td>0.15</td>
<td>0.29</td>
<td>0.63</td>
<td>1.65</td>
</tr>
</tbody>
</table>

\(^a\) In order to estimate total isoflavone amounts, individual isoflavone glucosides were normalized according to their aglycone form and summed up. Values were calculated on a dry basis. The isoflavone contents in each column are significantly different (p < 0.05). Values represent the mean ± standard deviation; n = 3.

\(^b\) Percent moisture was calculated from the difference between wet and freeze-dried samples.
the dressing mixture during sufu maturation, but the total isoflavone contents of sufu and the dressing mixture were almost the same as that of salted pehtze, indicating that maturation did not result in appreciable losses of isoflavones. Our present study indicates that salting resulted mainly in a decrease in the total recovery of isoflavones. When sufu were fermented at 26 °C or 32 °C, 0.5 mol and 0.48 mol isoflavones respectively were leached from salting.

The mass distribution profiles of individual isoflavone isomers are shown in Table 2. Fermentation temperature caused significant differences in the redistribution of isoflavone isomers. Although the amounts of isoflavone aglycones increased with decreases in the corresponding glucoside both at 26 °C and at 32 °C, the extent of increase was found to be significantly different. More than 90% of isoflavones took the form of isoflavone glucosides in sufu fermented at 26 °C, whereas aglycones existed at only 39.2% in sufu fermented at 32 °C. The total amounts of isoflavones in β-glucoside, 6'-O-glucosidase, and 6'-O-acetyl-glucoside conjugates were 0.58, 0.15, and 0.29 mol in sufu fermented at 32 °C respectively. But only 0.04 mol of glucoside, and 0.09 mol of 6'-O-acetyl-glucosides were detected and no 6'-O-malonyl-glucosides were detected in sufu fermented at 26 °C. It has been reported that loss of isoflavones during salting is a major cause of the decrease in the total recovery in isoflavones. Since 6'-O-malonyl-glucosides possess polar and hydrophilic properties, the significant decrease of 6'-O-malonyl-glucosides from phetze to salted phetze might be caused by loss into the salt water. These results indicate that the conversion of isoflavones from glucosides to aglycones was influenced by fermentation temperature. Sufu fermentation at 26 °C is preferable for the conversion of isoflavones from glucosides to aglycones.

It has been reported that the endogenous β-glucosidase present in soybean is able to convert isoflavone glucosides into aglycones, in correspondence with the increase in the amounts of isoflavone aglycones accompanied by the decrease in isoflavone glucosides during soybean soaking in water. This conversion associated with the endogenous β-glucosidase in soybeans was observed only prior to heat treatment of soybean. It has been confirmed that most of the endogenous enzymes are inactivated by heat treatment during tofu preparation. Therefore, exogenous β-glucosidase must be the major enzyme for the conversion of isoflavones from glucosides into aglycones during sufu fermentation. We assumed that the predominance of isoflavone aglycones in sufu was converted from isoflavone glucosides by β-glucosidase that derived from the microorganisms involved in sufu fermentation.

Changes in β-glucosidase activity and isoflavone composition in pehtze during earlier fermentation

Earlier fermentation was carried out at 26 °C or 32 °C to produce pehtze. The changes in β-glucosidase activity during earlier fermentation are shown in Fig. 2. The β-glucosidase activities in pehtze were considerably affected by fermentation temperature. It was observed that β-glucosidase activities at 26 °C were 2 times higher than at 32 °C after pehtze was fermented for 48 h. The β-glucosidase activity of 97.2 U/g dry matter was recorded at 26 °C, whereas β-glucosidase activity was only 43.2 U/g dry matter at 32 °C. Therefore, earlier fermentation at 26 °C was beneficial for β-glucosidase

<table>
<thead>
<tr>
<th>β-glucosidase</th>
<th>6'-O-malonylglycoside</th>
<th>6'-O-acetylglucoside</th>
<th>aglycone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tofu</td>
<td>0.30</td>
<td>0.12</td>
<td>0.76</td>
</tr>
<tr>
<td>Salted pehtze (26 °C)</td>
<td>0.09</td>
<td>0.01</td>
<td>0.18</td>
</tr>
<tr>
<td>Sufu (26 °C)</td>
<td>0.03</td>
<td>0.08</td>
<td>0.04</td>
</tr>
<tr>
<td>Pehtze (32 °C)</td>
<td>0.18</td>
<td>0.11</td>
<td>0.07</td>
</tr>
<tr>
<td>Sufu (32 °C)</td>
<td>0.12</td>
<td>0.14</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 2. Effect of Fermentation Temperature on Mass Distribution Profile of Isoflavone Isomers (mol)

Values in a column with different superscripts were significantly different (p < 0.05).

Abbreviations: Den, daidzein; Din, daidzin; Gein, genistein; Gin, genistin; Glein, glycitein; Glin, glycitin

Fig. 2. Changes in β-Glucosidase Activity During Earlier Fermentation at 26 °C and 32 °C.
production by fungi. It is possible that the fungi were less active at 32°C than at 26°C and produced smaller amounts of β-glucosidase. Han et al.\(^\text{18}\) reported that the fungi involved in sufu fermentation grew well at 25–30°C and produced considerable enzymes, such as protease, lipase, α-amylase, glutaminase, and α-galactosidase. Our results are in agreement with that report. Our previous study showed that earlier fermentation corresponded to the fastest period of isoflavone conversion. During earlier fermentation, the ratio of isoflavone aglycones clearly increased with decreases in the amounts of corresponding glucosides. Although changes in the ratio of glucosides to aglycones during later fermentation were observed, speed of change was much slower than in earlier fermentation due to the β-glucosidase activity inhibited by added salt.\(^\text{6}\) Han et al.\(^\text{23}\) reported that fungi, particularly the mould starters in sufu, were not metabolically active after the salted pehtze phase, and that the generated enzyme was not active during maturation steps because of the high levels of salt and/or ethanol in the dressing mixtures. Therefore, the increase in β-glucosidase activity during earlier fermentation might be the main contributor to the conversion of isoflavones from glucosides to aglycones.

The relative concentrations of individual isoflavone isomers, retained in the samples at 26°C and 32°C, are shown in Fig. 3. It was shown that the distribution pattern of isoflavones and the speed of isoflavone conversion from three types of isoflavone glucosides to aglycones were significantly different in pehtze under the two different temperatures. The conversion of isoflavones at 26°C was more dramatic than at 32°C, which probably resulted from higher β-glucosidase activity production at 26°C.

Figure 3 also shows that higher amounts of β-glucosides were converted to the corresponding aglycone than 6′-O-malonyl-glucosides and 6′-O-acetyl-glucosides both at 26°C and at 32°C, suggesting that the mechanisms of structural change in malonyl-glucoside and acetyl-glucoside might be different from those of β-glucosides. β-Glucosidase had more remarkable activity on the hydrolysis of β-glucosides to aglycones than on 6′-O-malonyl-glucosides or 6′-O-acetyl-glucosides. Isoflavone aglycones in sufu perhaps derived mainly from β-glucosidases hydrolyzed by β-glucosidase, so we deduced that it was difficult for β-glucosidase to react to 6′-O-malonyl-glucosides and 6′-O-acetyl-glucosides due to a space barrier in the larger molecular structure of those isoflavones. It has been reported that 6′-O-malonyl-glucosides and 6′-O-acetyl-glucosides are easily induced to deesterificate by heating and toasting.\(^\text{12}\) suggesting that 6′-O-malonyl-glucosides and 6′-O-acetyl-glucosides perhaps convert their structures more easily by thermal treatment than by β-glucosidase hydrolysis.

It has been reported that isoflavones in the form of aglycone display higher bioavailability than isoflavones in the form of glucoside.\(^\text{24–26}\) Our study suggests that sufu fermented at 26°C is richer in isoflavone aglycones than that fermented at 32°C. Therefore, sufu manufacturing under suitable temperatures might be beneficial to the enhancement of the physiological function. In addition, soybean isoflavones are also responsible for the astringent and main bitter flavor in soybean food.\(^\text{27–29}\) The change in isoflavone content and composition can affect the taste, flavor and nutritional values in sufu. In this study, it was found that when the fermentation temperature increases to about 32°C, sufu might be of lower quality. Strict control of temperature in the hot season during earlier fermentation is important to reduce the variability in quality of the final product in sufu manufacture.

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