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Changes in isoflavone contents and composition of sufu (fermented tofu) during manufacturing

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Abstract

Sufu is a fermented tofu product which is popular in China. Manufacturing procedures of sufu significantly affected the isoflavone contents and composition of sufu. The recovery of isoflavones in sufu from soybean was 16.9%. The loss of isoflavones was mainly attributed to the preparation of tofu and salting of pehtze (fresh bean curd overgrown with mould mycelia). The isoflavone composition was altered during sufu processing. The levels of aglycones increased, while the corresponding levels of glucosides decreased. ''Former fermentation'' corresponded to the fastest period of isoflavone conversion. The isoflavones in sufu, in the form of aglycones and in the form of glucosides, accounted for 99.7% and 0.3% of the total, respectively. The changes in the isoflavone composition were significantly related to the activity of β-glucosidase during sufu fermentation, which was affected by the NaCl content.

Keywords: Sufu; Isoflavone; Glucoside; Aglycone; β-glucosidase

1. Introduction

In Asian countries, soybean is consumed in many forms, including soy milk, tofu and fermented products, such as miso, soy sauce, tempeh and sufu. Sufu is a traditional and highly flavoured fermented tofu preparation in China, and it resembles a soft creamy-type cheese (Steinkraus, 1996). There are many types of sufu, but red sufu is one of the most popular types of sufu due to its attractive colour and strong flavour. Red sufu which has been consumed widely in China as an appetizer for more than 1000 years, is becoming increasingly popular in western countries (Han, Rombouts, & Nout, 2001a).

Soybeans and soybean products contain isoflavones, referred to as phytoestrogens due to their estrogenic activities (Bickoff, Livingston, Hendrickson, & Booth, 1962), and recently considerable attention has been focussed on their physiological functions. Based on epidemiological studies, it was suggested that the much lower incidence of breast and prostate cancer in East Asian regions than in western countries could be attributed to the higher amount of soybean food consumption (Adlercreutz et al., 1991; Lee, Gourley, Duffey, Esteve, Lee, & Day, 1991). It was also reported that soybean isoflavones may contribute to lower rates of osteoporosis (Ishida et al., 1998; Ishimi et al., 1999). Furthermore, reports of animal experiments (Baggott, Ha, Vaughn, Juliana, Hardin, & Grubbs, 1990; Sharma, Adlercreutz, Strandberg, Zirkin, Coffey, & Ewing, 1992) and in vitro experiments (Adlercreutz et al., 1992; Wei, Wei, Frenkel, Bowen, & Barnes, 1993) have shown that isoflavones may have a preventive effect on the development of cancer of the breast, intestines, liver, bladder, skin and stomach.

Isoflavones occur in the form of aglycones (daidzein, genistein and glycitein) and corresponding glucosidic conjugates, which include glucosides (daidzin, genistin and glycitin), malonyl-glucosides and acetyl-glucosides (Kudou et al., 1991). Isoflavone aglycones showed a different absorption pattern from that of glucosides in the

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stomach. Daidzein and genistein, unlike their glucoside form, were absorbed in the rat stomach (Piskula, Yamakoshi, & Iwai, 1999). Genistein was reported to have a higher antiproliferative activity in the growth of human breast carcinoma and prostate cancer cells than genistin (Onozawa, Fukuda, Ohtani, Akaza, Sugimura, & Wakabayashi, 1998; Peterson & Barnes, 1991). Therefore, not only the contents but also the composition of isoflavones may influence the physiological functions in food.

The total recovery and the distribution profile of isoflavones in soybean products depend on processing techniques, such as heat treatment, defoaming, enzyme hydrolysis and fermentation (Anderson & Wolf, 1995; Jackson et al., 2002; Wang & Murphy, 1994a, 1994b, 1996). Heat treatment was found to convert some malonyl isoflavones to acetyl forms (Farmakalidis & Murphy, 1985), and defoaming during the heating process allowed removal of isoflavones (Okubo, Kobayzshi, & Takahashi, 1983). It was also reported that the glucoside conjugates of isoflavones were converted to isoflavone aglycones during soybean processing by the effect of β-glucosidase (Toda, Sakamoto, Takayanagi, & Yokotsuka, 2001).

Although there are some reports relating to sufu manufacturing, few reports have paid attention to the changes in the isoflavone contents and composition of sufu during manufacturing or the cause of these changes. In the present study, we determine the isoflavone contents and composition of sufu using HPLC. We also analyze the changes in the β-glucosidase activity during manufacturing, and the relationship between the β-glucosidase activity and the changes in the isoflavone composition were investigated.

2. Materials and methods

2.1. Materials

Daidzein, genistein, daidzin, genistin and *p*-nitrophenyl-β-D-glucoside (*p*-NPG) were purchased from Sigma Chemical Company. Glycitein and glycitin were purchased from ICN Pharmaceuticals Company.

2.2. Sufu preparation

Red sufu was prepared at the Wang-Zhihe sufu manufacturing company in Beijing. Sufu preparation was performed as described by Han et al. (2001a). Steps of and parameters for the preparation of red sufu were as follows:

1. preparation of tofu by salt precipitation from boiled soymilk;
2. preparation of a pure culture fermentation starter, *Actinomucor elegans* As3.227 (fungus solid). Spraying of the starter onto the surface of diced tofu. Fermentation at 28 °C for 48 h, relative humidity around 90%, with air circulation to ensure adequate aeration;
3. salting for 5 days until the salt content of pehtze reached about 16%;
4. ripening for 2 months in a closed bottle with a dressing mixture consisting of kojic red rice, alcohol beverage, sugar, Chiang (wheat-based miso) and spices.

Step (2) is referred to as ''former fermentation'', and steps (3) and (4) as ''latter fermentation''.

Tofu, intermediates product of sufu prior to ripening and sufu were sampled for analyses. Fresh pehtze (bean curd overgrown with mould mycelia) samples were obtained after being fermented for 12, 24, 36 and 48 h and salted pehtze samples were obtained after being salted for 1, 2, 3, 4 and 5 days, respectively. The dressing mixture was decanted and sufu pieces were tested.

2.3. Extraction of isoflavones

All the tofu and sufu samples were freeze-dried and each sample was ground to uniformity in a coffee mill. Isoflavones were extracted from the samples with 10 volume of 80% methanol at 80 °C for 4 h. Extractants were filtered through a 0.45 μm filter unit.

2.4. High performance liquid chromatography (HPLC) of isoflavones

Isoflavones were analyzed quantitatively by HPLC, carried out using a Dikma Diamonsil C_{18} column (250×4.6 mm, 5 μm) with a linear gradient of acetonitrile containing 0.1% (v/v) acetic acid from 15% to 35% in 50 min. The solvent flow rate was 1.0 ml/min and absorption was measured at 254 nm. The column temperature was 40 °C. Quantitative data for daidzein, daidzin, glycitein, glycitin, genistein and genistin were obtained by comparison with known standards.

2.5. Determination of NaCl content

The NaCl content of the samples was determined by a modified method of A.O.A.C. Official Method (937.09) (AOAC, 2000). Ten grammes of the sample were put into a 250 ml beaker. More than a sufficient volume of a 0.1 M $AgNO_3$ solution to precipitate all Cl as AgCl was added, and then 20 ml HNO_3 was added to the mixture. The mixture was boiled until all the solids, except for AgCl were dissolved. Then the mixture was cooled, 50 ml of H_2O and 5 ml of indicator were added, and titration was carried out with a 0.1 N NH_4SCN solution. The volume of 0.1 M NH_4SCN used was subtracted from the volume of 0.1 M $AgNO_3$ added, and the difference was calculated as NaCl. In a 10 g test sample, each 0.1 N volume of $AgNO_3$ was equivalent to 0.058% NaCl.

2.6. Determination of β-glucosidase activity

The modified procedure of Bahl and Agrawal (1968) was used to determine the β-glucosidase activity. Eight grammes of sample were homogenized with 25 ml of 0.2 M acetate buffer, pH 4.5 at 4 °C. The slurry was centrifuged at 25,000*g* for 10 min at 4 °C, and the supernatant was used as a crude enzyme solution. Then 2 ml of a 1 mM *p*-NPG solution and 0.5ml of a crude enzyme solution were mixed and incubated at 45 °C for 5 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 μm of *p*-nitrophenol per min.

2.7. Statistical analysis

Analysis of variance, using the general linear models (GLM), was conducted. Significant differences between the sample means were determined at the $p < 0.05$ levels by ANOVA, followed by Student's *t*-test.

3. Results and discussion

3.1. Changes in the total contents of isoflavones during sufu manufacturing

The yield of isoflavones for soybean, tofu, pehtze, salted pehtze and sufu, normalized for molecular weight differences of the isoflavone isomers, is presented in Table 1, which shows that each step of sufu manufacturing resulted in the loss of total isoflavones. The total recovery of isoflavones in sufu compared with raw soybeans was as low as 16.9%.

Tofu production seemed to be the principal cause of the loss of isoflavones during sufu manufacturing, resulting in 68.7% of the isoflavones being leached from materials. Fermentation generated some differences in the total isoflavone contents in pehtze (303 mg) compared with tofu (321 mg). It was also shown that the loss of isoflavones during salting was the second major cause of the decrease of the total recovery of isoflavones. The isoflavone loss from pehtze to salted pehtze was 8.1%. When the salted pehtzes were mixed with a dressing mixture for ripening, 18.5% of the isoflavones were dissolved in the dressing mixture. The contents of total isoflavones of sufu and the dressing mixture were almost the same as that of salted pehtze, suggesting that ripening did not appreciably decrease the total isoflavone contents.

The addition of other ingredients may possibly have affected the composition of dry matter and the isoflavone concentrations. Isoflavone concentrations were 0.57 mg/g dry matter in tofu and 0.54 mg/g dry matter in pehtze, indicating that the dilution of isoflavones by the addition of a nonsoybean component was not appreciable. However, after salts were added to pehtze, the isoflavone concentration was only 0.26 mg/g dry matter in salted pehtze. The total weight of isoflavones in salted pehtze decreased by 27.3% compared with that of pehtze, whereas the concentration of isoflavones decreased by more than 50%. The addition of the dressing mixture also decreased the isoflavone concentration from 0.26 mg/g dry matter to 0.23 mg/g dry matter.

Wang and Murphy (1996) reported that the recovery of total isoflavones of tofu was 33%. They also stated that the loss of isoflavones during tofu preparation was significantly related to the discarded whey after curd production. Their results of isoflavone recovery (33%) were similar to those in the present study (31.3%). However, the isoflavone recovery of tofu in our study was much lower than that reported by Dwyer, Goldin, Saul, Gualtieri, Barakat, and Adlercreutz (1994), presumably due to the difference in the type of tofu.

Table 1
Yield, moisture, and total contents of daidzin, glycitin and genistin in processing

Sample	Yield (g)[a]	Moisture (%)[a]	Daidzin[b] (mg)	Glycitin[b] (mg)	Genistin[b] (mg)	Total (mg)	Conc (mg/g dry matter)
Raw soybeans	1000 ± 0	11.03 ± 0.17	471	55.6	498	1025	1.15
Tofu	1920 ± 52	70.76 ± 0.81	130	19.3	172	321	0.57
Pehtze	1892 ± 38	70.24 ± 0.46	129	15.9	158	303	0.54
Salted pehtze	1759 ± 37	52.03 ± 1.03	104	5.7	110	220	0.26
Salted water	NC[c]						
Sufu	1827 ± 76	58.82 ± 0.26	73.0/63	4.2	95.9	173	0.23
Dressing mixture	920 ± 61	78.96 ± 2.08	17.4	1.2	20.7	39.3	0.20

Yield, %moisture represents the mean ± standard deviation; $n = 3$. Daidzein, glycitin, genistein and total isoflavone contents in each column with different superscripts are significantly different ($p < 0.05$).

[a] Percentage of moisture was calculated from the difference between wet and freeze-dried samples.

[b] In order to estimate the total isoflavone amounts, the amounts of individual glucoside and aglycone forms of isoflavones were normalized for their molecular weight differences and summed. Values were calculated on a dry basis.

[c] NC, not collected.

Table 2
Effect of sufu processing on the redistribution profile of individual isoflavones

Sample	Isoflavone glucosides				Isoflavone aglycones			
	Daidzin (%)	Glycitin (%)	Genistin (%)	Total (%)	Daidzein (%)	Glycitein (%)	Genistein (%)	total (%)
Raw soybean	53.1	3.8	41.8	98.7	0.4	0.2	0.7	1.3
Tofu	20.1	2.7	59.2	82.0	13.6	3.6	0.8	18.0
Pehtze	2.5	1.2	15.5	19.2	28.7	5.6	46.5	80.8
Salted pehtze	2.5	0.8	12	15.3	30.8	6.1	47.9	84.7
Sufu	ND[a]	0.3	Nd[a]	0.3	33.5	7.8	58.4	99.7

Values in a column with different superscripts were significantly ($p < 0.05$).
[a] ND, not detected.

Dwyer's tofu was coagulated by δ-gluconolactone (GDL), where soybean protein was coagulated in the package and whey was not removed.

3.2. Changes in the composition of isoflavones during sufu manufacturing

The relative concentrations of individual isoflavones for soybean, tofu, pehtze, salted pehtze and sufu are shown in Table 2, which indicates that the composition of the isoflavones changed significantly during sufu processing. The contents of aglycones gradually increased, whereas the contents of glycosides decreased from raw soybean to sufu. Most of the isoflavones (99.7%) in sufu occurred in the form of aglycones. Although the amount of glycitin was lower than those of daidzin and genistin in tofu, pehtze and salt pehtze, glycitin was still detectable in sufu, while daidzin and genistin could not be detected, suggesting that the mechanism of structural change in glycitin was different from that in daidzin and genistin.

The effects of "former fermentation", salting and ripening time on the composition of isoflavones are shown in Figs. 1–3, respectively, which indicate that the composition of each isoflavone and the changing speed

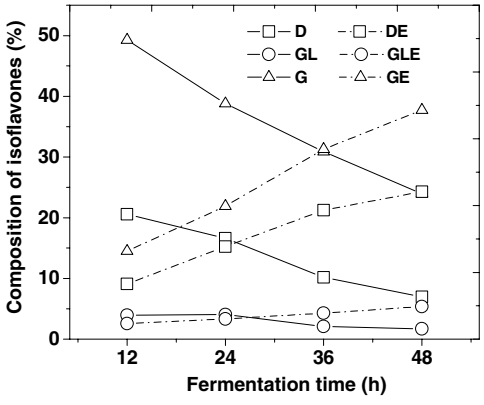

Fig. 1. Effect of fermentation time on the contents and composition of isoflavones D, daidzin; GL, glycitin; G, genistin; DE, daidzein; GLE, glycitein; GE, genistein.

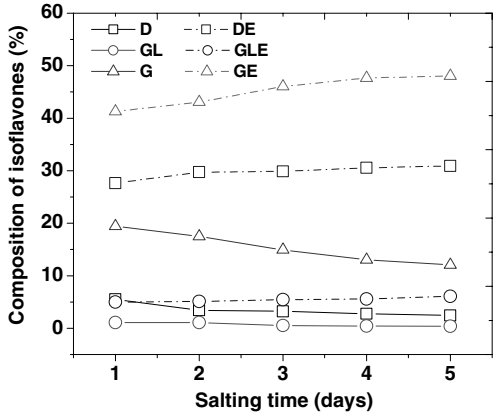

Fig. 2. Effect of salting time on the contents and composition of isoflavones D, GL, G, DE, GLE, GE, same as in Fig. 1.

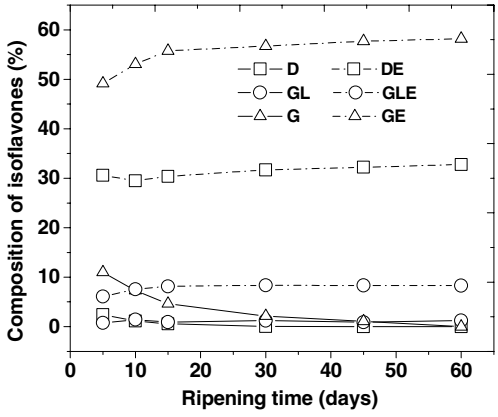

Fig. 3. Effect of ripening time on the contents and composition of isoflavones D, GL, G, DE, GLE, GE, same as in Fig. 1.

of the composition were different in each step of sufu manufacturing.

Fig. 1 shows that the fermentation time significantly affected the isoflavone composition. During the "former fermentation", the ratio of isoflavone aglycones such as daidzein, glycitein and genistein, increased clearly with a decrease of the amounts of corresponding glucosides. Figs. 2 and 3 show that, during the "latter fermentation", which included salting and ripening, despite

changes in the ratio of glucosides and aglycones being observed, the changing speed was much slower than that in the "former fermentation". In our study, changes in the composition of isoflavones of sufu were also detected during the 15-month period of storage. No significant changes in the composition of isoflavones were observed after sufu ripening (data not shown).

In several reports, it was mentioned that isoflavones, in the form of aglycones, displayed a higher bioavailability than isoflavones in the form of glucosides (Onozawa et al., 1998; Peterson & Barnes, 1991; Piskula et al., 1999). Our study suggested that sufu was rich in isoflavone aglycones and that sufu manufacturing might be beneficial to the enhancement of the physiological function.

3.3. Changes in the activity of β-glucosidase during sufu manufacturing

The changes in the activity of β-glucosidase and the content of NaCl during sufu manufacturing are shown in Fig. 4. The β-glucosidase activity was relatively low in tofu, but it significantly increased during the "former fermentation".

The β-glucosidase enzyme present in soybean was able to convert isoflavone glucosides into aglycones, and the increase in the amount of isoflavone aglycones during soaking in water, accompanied with a decrease in the amount of isoflavone glucosides, was generated by β-glucosidase in soybean (Matsuura, Obata, & Fukushima, 1989; Toda et al., 2001). Ha, Morr, and Seo (1992) confirmed that this conversion associated with endogenous β-glucosidase in soybeans, was only observed prior to heat treatment. A low β-glucosidase activity in tofu was detected in our study. Although the hydrolysis from isoflavone glucosides to aglycones could be caused by endogenous β-glucosidases in soybean, most of the endogenous enzymes had been presumably inactivated by heat treatment during tofu prepatation.

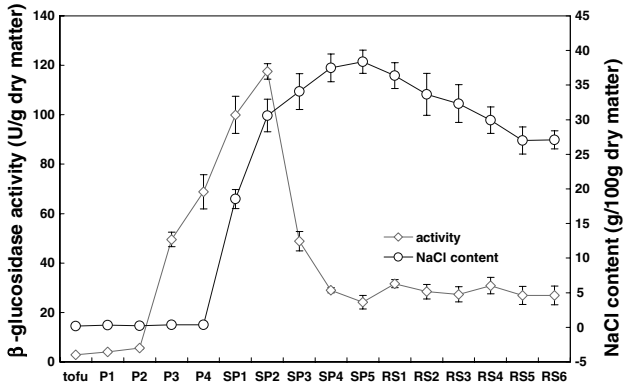

Fig. 4. Changes in the β-glucosidase activity and salt content for sufu processing P1 to P3, fermented pehtzes for 12, 24, 36, and 48 h; SP1 to SP5, pehtzes subjected to salting for 1, 2, 3, 4, and 5 day(s); RS1 to RS6, ripening of sufu for 5, 10, 15, 30, and 45 days, respectively.

The release of aglycones from glucosides appeared to be due to the hydrolysis by β-glucosidase during sufu fermentation. Because "former fermentation" corresponded to the fastest period of isoflavone conversion and also to the period when the activity of β-glucosidase increased, it was assumed that the enzyme hydrolyzing isoflavones from glucosides to aglycones was derived from the microorganisms involved in sufu fermentation. As mould starters, a pure culture of *Actinomucor elegans* AS3.227 was used in sufu manufacturing. It is possible that the fungi produced β-glucosidase which hydrolyzed isoflavone glucosides to aglycones, although the properties of this fungi were not analyzed in the present study.

The content of NaCl in each sample was determined, because the activity of β-glucosidase is known to be inhibited by NaCl. In our study, the β-glucosidase activity was considerably affected by the content of NaCl (Fig. 4). The highest β-glucosidase activity (118 U/g dry matter) was recorded on the second day of the salting procedure. It is possible that the level of the β-glucosidase activity could remain high if the content of NaCl was lower than 30 g/100 g dry matter during salting. Han, Beumer, Rombouts, and Nout (2001b) reported that the 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 the ripening and storage steps because of the high levels of salt and/or ethanol in the dressing mixtures. Our study showed that β-glucosidase generated by *Actinomucor elegans* AS3.227 could tolerate NaCl to some extent, but that, at a higher concentration of NaCl in pehtze, the β-glucosidase activity tended to decrease. The β-glucosidase activity was only 29 U/g dry matter when the percentage of NaCl increased to 38 g/100 g dry matter. Although the content of NaCl decreased to some extent during sufu ripening due to dissolution in the dressing mixture, the concentration of NaCl still remained high, above 27 g/100 g dry matter. The β-glucosidase activity was not significantly different before and after ripening. In our study, the activity of β-glucosidase could still be detected after storage during a 15-month period.

A high NaCl content in sufu may result in inhibiting the β-glucosidase activity and preventing isoflavone glucosides from being converted into aglycones. Furthermore, high NaCl content of food could increase the dietary sodium intake (Baggott et al., 1990). Therefore, sufu with a low NaCl content may be preferable from the viewpoint of public nutritional health. In order to decrease the amount of NaCl used for sufu preparation, further investigation is needed to clarify the flavour, taste, texture, consumer acceptability and shelf life of sufu with a lower NaCl content.

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