Original article

Chemical components of *Aspergillus*-type Douchi, a Chinese traditional fermented soybean product, change during the fermentation process

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Summary Douchi, a traditional fermented soybean product that originated in China, has been consumed since ancient times as a food seasoning. The influence of fermentation on the chemical components of naturally fermented douchi and *Aspergillus egypticus* pure-cultured douchi was investigated. Changes in per cent and/or concentration of amino-type nitrogen, total acid, reducing sugar, organic acid, amino acids (AA) and isoflavone, along with the neutral protease and β-glucosidase activities during the fermentation, were analysed. The results indicated that fermentation had a significant effect on the concentration of chemical components. The concentration of all free amino acids (FAA) increased gradually during fermentation, to a maximum of 109.54 mg g^{-1} in 15-day fermented products. The main organic acids in douchi are 7.788 and 17.778 mg g^{-1}, respectively. During fermentation, the contents of daidzin and genistin decreased from 160.7 and 207.9 to 7.54 and 24.12 μg g^{-1} respectively. Daidzein and genistein increased from 18.2 and 16.9 to 63.4 and 84.6 μg g^{-1}, respectively.

Keywords Component change, Douchi, fermentation.

Introduction

Douchi, a traditional fermented product that originated in China before the Han dynasty (206 BC),was the first soy food to be described in written records (Liu, 1999). It has been used as a food seasoning since ancient times, soy paste and soy sauce were developed from it. Different types of douchi result from fermentation by three strains: *Mucor*, *Bacteria* and *Aspergillus*. Among them, *Aspergillus*-type douchi is the earliest type and is the most widely produced. Douchi products made by workshops and families are shown in Fig. 1.

Douchi is made from soybeans by pretreatment and a two-step fermentation process (primary fermentation and secondary fermentation). Black soybeans are often used since they give the douchi a bright black colour. Proteins are denatured and both proteins and starch are dissolved during the pretreatment process. During primary fermentation, where douchi qu is produced, enzymes such as proteinase, peptidase and amylase hydrolyse part of the protein and starch to simpler components, which become nutrients for the yeast and lactic bacteria in the secondary brine fermentation.

Secondary fermentation with yeast and bacteria improves the aroma of the douchi. While the microorganisms can tolerate a salt concentration of 18–20%, the brine effectively prevents the growth of undesirable microorganisms. The primary change during this fermentation step is the conversion of sugars and proteins by salt-tolerant microorganisms into lactic acid, ethanol, amino acid and various aroma components (Zhang & Liu, 2000).

Dan douchi, a naturally fermented product used as a Chinese medicine, is formed primarily by *Bacteria sp.* and *Aspergillus sp.*; it is produced without the secondary fermentation step. Although primarily used as a food additive, *Aspergillus* pure-cultured douchi qu extract has been found to elicit a dose-dependent antiglycemic effect in humans (Fujita *et al.*, 2001a,b), suggesting that *Aspergillus* pure-cultured douchi might also have a health benefit.

While douchi has been produced in small workshops in China for thousands of years, few studies have published about the changes in chemical composition

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Figure 1 Bacterial-type (left), *Aspergillus*-type (middle, TianMaShan douchi) douchi products made by Chinese workshop and northern China family-produced douchi product (right).

that occur during fermentation. This paper describes various biochemical changes that occur during natural fermentation in a pure *Aspergillus egypticus* culture, including fluctuations in protein, free amino acids (FAA), organic acid and isoflavone profiles.

Materials and methods

Materials

TianMaShan (TMS) douchi and qu (TaiPinQiao Douchi Factory, LiuYang, China), YiPinXiang (YPX) douchi and qu (YiPinXiang Food Factory, LiuYang, China) were provided by the manufacturers. Genistein, genistin, daidzein, daidzin and acetonitrile (chromatographic grade) were obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). All other chemicals used were analytical grade.

Aspergillus inoculum

A *Aspergillus egypticus* strain was screened from YPX douchi qu and used to make pure-cultured douchi qu. The spore suspension was prepared as previously described (Sparringa & Owens, 1999). The fungus was grown on Potato Dextrose Agar (PDA) medium and suspensions were stored at 5 °C until use.

Preparation of douchi qu and douchi

Aspergillus-type douchi was prepared as described by Kang (2001). Steps and parameters for the preparation were as follows:

1 Black soybeans were soaked in three times tap water for 4 h at 30 °C. Once drained, they were steamed at 121 °C for 50 min.
2 Primary Fermentation. Soybeans were inoculated with 1% (v/w) *A. egypticus* inoculum (10^7 spores mL^{-1}) as a pure culture fermentation starter. The soybeans were cultured at 30 °C and 90% humidity for 48 h in an incubator (LTI-601SD; Tokyo Rikakikai Co. Ltd, Tokyo, Japan). The product is douchi qu.
3 Secondary Fermentation. Douchi qu was washed with tap water and then combined with 16% salt, 10% water, and a powder mixture of ginger, shallot, and garlic. The mixture was sealed in bottles and ripened at 37 °C in an incubator for 15 days. The product is douchi.

Chemical analysis of douchi and douchi qu

The moisture, lipid and protein contents of samples were determined by the official methods of AOAC (2000). Amino-type nitrogen and total acid contents were determined by the formol titration method described by Judoamidjojo (1986). The reducing sugar content was measured using the dinitrosalicylic acid method (Miller, 1959).

Enzyme assay

Enzyme extraction
Five grams of finely ground sample were mixed with 50 mL of distilled water. After shaking at 50 rpm for 1 h, the sample was centrifuged for 10 min at $5000 \times \boldsymbol{g}$. Distilled water (20 mL) was added to the residue and the mixture was centrifuged again. The combined supernate was used as the enzyme assay sample.

Neutral protease assay
Neutral protease activity was determined by a modification of the procedure of Yang *et al.* (2000). An enzyme solution (2 mL) was mixed with 2 mL of 2% casein solution in 0.2 M phosphate buffer at pH 7.5; both solutions had been incubated at 40 °C for 10 min. After 10 min at 40 °C, the reaction was terminated by adding 4 mL of 0.4 M trichloroacetic acid. The reaction mixture was filtered and the soluble peptide in the filtrate was measured by a spectrophotometer (model

1240; Hitachi Co. Ltd, Kyoto, Japan) at 275 nm. One unit of enzyme activity was defined as the activity of 1 mL enzyme solution that produced the equivalent of 1 μg tyrosine in 1 min under the same conditions.

β-*Glucosidase assay*

β-Glucosidase activity was measured using *p*-nitrophenyl-α-D-glucopyranoside (*p*-NPG) as the substrate, with a minor modification of the procedure of Matsuura & Obata (1993). The enzyme sample (0.5 mL) was added to 2 mL of 1 mM p-NPG in 0.1 M phosphate-citrate buffer at pH 4.5, which had been incubated at 45 °C for 5 min. After a 10 min incubation period at 45 °C, the reaction was stopped by the addition of 2.5 mL of 1 M sodium carbonate. Absorbance was immediately determined at 400 nm with a spectrophotometer. Hydrolysed *p*-nitrophenol was by comparison with a calibration curve prepared with 10–50 μM of *p*-nitrophenol. One unit of β-glucosidase activity was defined as the amount of enzyme which released 1 μmol of *p*-nitrophenol per minute at the same conditions.

Sample preparation and analysis of amino acid and free amino acid

Free amino acids were determined according to the method described by Sarkar *et al*. (1997). A 10 g sample was homogenised with 10 mL of 8% sulphosalicylic acid. The slurry was then diluted to 50 mL with the same solution and centrifuged at 10 000 × ***g*** for 20 min followed by filtration of the supernatant through a 0.45 μm filter (Millipore, Milford, MA, USA).

Amino acids were extracted using a minor modification of the procedure described by Abiodun *et al*. (1999). Eight millilitres of 6 N HCl were added to a 100 mg sample in a sealed tube, which was held for 24 h in an oil bath at 110 °C. A Hitachi 835–50 amino acid autoanalyser (Hitachi Co. Ltd) was used for separating the amino acids.

Sample preparation and HPLC analysis of organic acid

Two grams of douchi or douchi qu were mixed with 20 mL of a HCl (pH 1.0) solution and extracted at 40 °C for 10 h. The supernatant was diluted 50 times after centrifugation at 10 000 × ***g*** for 10 min, and 5 mL of the diluted solution was dried in a water bath at 75 °C. The dried sample was dissolved with HPLC mobile phase to a volume of 5 mL, and the final solution was used as the analysis sample after filtering with a 0.45 μm filter. A mixture of formic acid, acetic acid, propionic acid, butyric acid, lactic acid, citric acid, malic acid, amber acid and L-pyroglutamic acid was used as the standard sample, in which each acid was 0.10 g L^{-1}. The methods were the same as those used by Ding *et al*. (1995).

Sample preparation and HPLC analysis of isoflavone

Isoflavones were extracted using the methods described by Robin *et al*. (2001). Quantification of isoflavones was completed using a Shimadzu HPLC system equipped with two LC-10AT pumps, a Lichrosper 100RP-18 (4.6 mm × 25 cm × 5 μm) column and a UV detector (SPD-10AVVP) (Yin *et al*., 2004).

Statistical analysis

Analysis of variance, using general linear models (GLM), was conducted. Significant differences ($P < 0.05$) between the sample means were determined by ANOVA, followed by Duncan's multiple range test.

Results and discussion

Total acid, protein, fat and reducing sugar

During the fermentation process, the per cent of crude fat decreased slightly during the first 24 h from 23.78% to 23.28% then dropped rapidly to 21.04% at 48 h. At

Table 1 Per cent of crude protein and crude fat; concentration of reducing sugar and total acid during the fermenting process for douchi qu

Fermentation interval (h)	Crude protein (%)	Crude fat (%)	Reducing sugar (mg g^{-1})	Total acid (mg g^{-1})
0[a]	35.96 ± 0.34	23.78 ± 0.65	10.98 ± 0.65	0.645 ± 0.030
12	ND	ND	16.61 ± 0.43	0.702 ± 0.031
24	36.54 ± 0.49	23.28 ± 0.67	24.62 ± 0.11	0.783 ± 0.022
36	ND	ND	21.9 ± 0.35	0.945 ± 0.03
48	39.14 ± 0.60	21.04 ± 0.51	17.37 ± 0.11	1.109 ± 0.032
60	40.44 ± 0.75	21.36 ± 0.72	11.08 ± 0.07	1.164 ± 0.031
72	40.05 ± 0.08	20.86 ± 0.57	5.35 ± 0.03	1.295 ± 0.036
96	39.58 ± 1.27	19.87 ± 0.42	ND	ND

Values represent the mean ± SD ($n = 3$).

ND, not detected. The fermentation was processed from 2002-4-6 to 2002-4-10.

[a]0 h represents boiled soybeans. Values represent the mean ± SD ($n = 3$).

96 h, the per cent of crude fat had fallen to 19.87% (Table 1).

Conversely, the per cent of crude protein increased during the fermentation process (Table 1). Protein rose from 35.96% to 40.44% after 60 h. This increase may be associated with the loss of solid mass (such as fats). After 60 h the protein content decreased gradually, illustrating a trend similar to that of tempeh (Francisco & Owens, 1996; Sparringa & Owens, 1999).

Following primary fermentation, the concentration of total acid increased continuously from 0.645 to 1.295 mg g^{-1} during the first 72 h (Table 1). Reducing sugar concentration also increased for the first 24 h, but then began to decrease (Table 1). The final sugar concentration (5.35 mg g^{-1}) was approximately half the initial level.

Amino-type nitrogen

Per cent of amino-type nitrogen in douchi qu increased steadily for 72 h (Fig. 2). The per cent of amino-type nitrogen in the douchi products was $>1.2\%$, with the pure-cultured douchi being the highest at 1.76%. The levels of amino-type nitrogen in the douchi products were much higher than in the corresponding qu, which suggests protein hydrolysis occurred mainly during secondary fermentation.

Organic acid

The concentrations of nine organic acids (formic, propionic, butyric, citric, malic, amber, acetic, lactic and L-pyroglutamic acid) were quantified in naturally fermented douchi, douchi qu and black bean, but only the last three acids were detectable (Table 2). The concentration of L-pyroglutamic acid was much lower than that of acetic acid and lactic acid, which suggests that the latter two were the main organic acids in douchi. Levels were similar to that of soy sauce (Luh, 1995). The sums of the three organic acids in TMS qu and YPX qu were 4.34 and 1.98 mg g^{-1}, respectively, while in douchi, the levels were 27.16 and 21.03 mg g^{-1}, respectively, indicating that organic acids were mainly produced during secondary fermentation.

Table 2 Concentration of organic acid in different douchi products (mg g^{-1})

Sample	Acetic acid	L-pyroglutamic acid	Lactic acid	Total
TMS qu	1.740	0.722	1.882	4.344
TMS douchi	7.788	1.596	17.778	27.162
YPX qu	1.174	0.220	0.588	1.982
YPX douchi	3.848	1.021	16.164	21.032
Black soybean	0.302	0.844	0.360	1.506

Values are the mean of three trials.

Neutral protease and β-glucosidase activities

During primary fermentation, both neutral protease and β-glucosidase activity rose for the first 60 h (Fig. 3). Neutral protease and β-glucosidase activities at 60 h were 221.8 and 0.259 U g^{-1}, respectively. These two enzymes are important in contributing to the flavour and healthy functions of douchi. If the biomass produced is excessive, it can be difficult to wash it away, thus imparting a bitter taste. In order to avoid this, 48 h was chosen as the primary fermentation time point.

Amino acid and free amino acid

The concentrations of all FAA increased gradually following the fermentation process (Table 3). The ratio of total FAA in YPX qu and YPX douchi was 0.219: 1 and that of *Aspergillus egypticus* 72 h-pure-cultured qu to 15 d secondary-fermented douchi (15 d douchi for short) was 0.302:1. These data suggest that, with a few

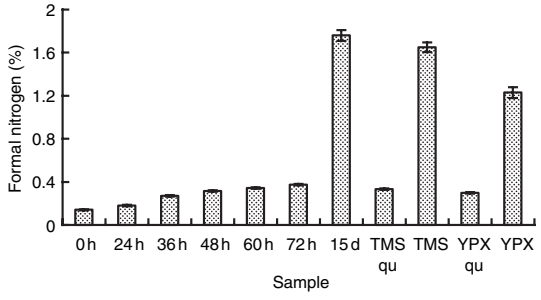

Figure 2 Per cent of amino-type nitrogen in different douchi products 0–72 h: Douchi qu after the given time period; 15 day: Douchi that is pure cultured for 48 h followed by 15 days of secondary fermentation; TMS qu: TianMaShan douchi qu; TMS: TianMaShan douchi; YPX qu: YiPinXiang douchi qu; YPX: YiPinXiang douchi. Values represent the mean ± SD ($n = 3$).

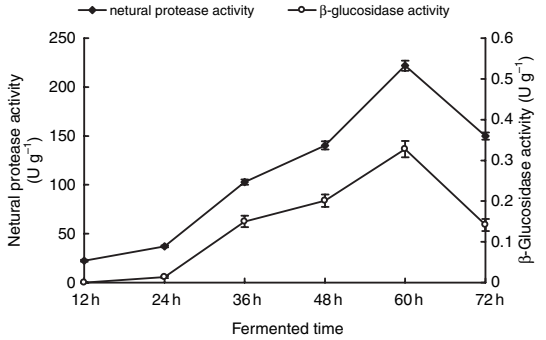

Figure 3 Natural protease and β-glucosidase activities during qu making 12–72 h: Douchi qu after the given time period. Values represent the mean ± SD ($n = 3$).

Table 3 Concentration of free amino acids in different products (mg g^{-1})

	***Aspergillus egypticus* pure-cultured products**								
	Primary fermented time								
Amino acids	**0 h**	**24 h**	**48 h**	**72 h**	**Secondary fermented for 15 day**	**Ratio[b] (%)**	**YPX qu**	**YPX Douchi**	**Ratio[a] (%)**
Asp	0.42	0.57	0.55	1.07	6.42	16.7	1.61	8.65	18.6
Thr	0.39	0.52	1.62	3.14	4.9	64.1	1.52	2.58	58.9
Ser	ND	0.24	0.95	1.71	5.95	28.7	1.15	3.66	31.4
Glu	0.58	1.16	2.19	3.75	25.92	14.5	1.3	17.89	7.3
Pro	ND	ND	0.35	0.95	7.08	13.4	0.68	9.8	6.9
Gly	0.06	0.07	0.26	0.45	2.68	16.8	0.35	2.85	12.3
Ala	0.18	0.31	1.05	1.74	7.2	24.2	1.22	9.8	12.4
Val	0.11	0.25	0.99	2	7	28.6	1.54	7.26	21.2
Met	0.09	0.02	0.49	0.77	2.09	36.8	0.55	1.01	54.5
Ile	0.03	0.1	0.64	1.27	6.32	20.1	0.84	6.48	13.0
Leu	0.06	0.23	2.06	3.82	11.2	34.1	2.29	10.12	22.6
Tyr	0.36	0.49	1.73	2.13	6.33	33.6	1.56	1.09	DD
Phe	0.39	0.85	3.07	4.9	8.68	56.5	3	4.34	69.1
Lys	0.12	0.22	0.9	1.97	5.81	33.9	1.23	4.86	25.3
His	0.06	0.23	0.27	0.48	1.88	25.5	0.27	0.99	27.3
Arg	1.58	1.58	1.83	2.92	0.08	DD	1.21	0.19	DD
Total	4.43	6.84	18.95	33.07	109.54	30.2	20.32	92.63	21.9

ND, not detected; DD, decreased.
[b]Ratio of 72 h concentration to 15 day concentration, expressed as per cent; [a]ratio of YPX qu concentration to YPX douchi concentration, expressed as per cent.

exceptions, most of the FAAs were produced during secondary fermentation. Contrary to most of the FAAs, the concentrations of threonine and phenylalanine in qu were more than half of what was seen in douchi, and the concentration of arginine in qu was more than what it was in douchi. Except for asparagine and proline, the concentrations of FAAs following a 15-day fermentation period were greater than, or very similar to, that of YPX douchi, which had secondary fermented for 2 months.

The main FAA and amino acid (Table 4) in both YPX douchi and *Aspergillus egypticus* pure-cultured douchi was glutamic acid, which improves the flavour of soybean fermented products. The total amino acid content of douchi qu was lower than that in boiled soybeans, and that in douchi was lower than that in qu,

Table 4 Concentration of amino acid in different products (mg g^{-1})

Amino acids	**Black soybean**	**TMS qu**	**TMS douchi**	**YPX qu**	**YPX douchi**	**48 h douchi qu**	**15 day douchi**
Asp	55.12	54.47	50.54	58.06	50.71	46.14	42.23
Thr	18.74	19.79	16.51	20.66	16.33	18.68	16.71
Ser	24.65	25.18	19.90	27.16	21.48	23.15	19.87
Glu	92.99	88.71	83.57	93.97	87.10	88.87	84.14
Pro	25.50	24.30	29.94	23.27	28.64	20.8	17.78
Gly	18.98	20.77	20.34	20.78	18.29	17.51	16.63
Ala	19.10	21.35	25.90	21.28	23.52	19.71	17.55
Val	21.52	24.00	25.03	28.00	28.24	19.35	19.88
Met	2.62	9.75	3.79	4.13	2.67	1.03	ND
Ile	16.88	19.02	19.58	18.92	19.81	17.45	16.37
Leu	33.87	37.06	35.72	36.76	36.47	32.82	28.12
Tyr	14.22	14.59	8.91	15.29	9.56	15.93	12.79
Phe	23.38	25.13	19.77	25.21	21.92	27.06	18.25
Lys	27.37	27.17	23.11	27.72	22.35	23.3	18.41
His	11.45	11.30	9.74	11.63	9.05	10.91	9.07
Arg	38.37	33.21	18.18	34.85	20.76	31.85	11.09
Total	444.76	455.79	410.52	467.69	416.90	414. 6	348.91

ND, not detected.

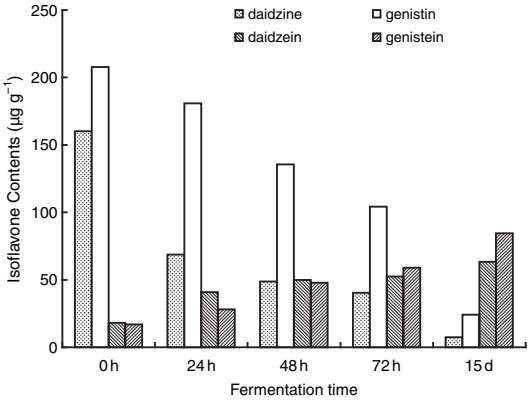

Figure 4 Change in the concentration of isoflavone during douchi qu fermentation 15 day value represents batch that was cultured for 48 h, followed by 15 days of secondary fermentation.

which strongly suggests a loss of amino acids during the fermentation process.

Among all the amino acids, arginine concentration decreased the most, which corresponded to the results of FAA levels. Arginine may be a nitrogen source for secondary fermentation microorganisms, which would explain its rapid decrease. These results are comparable with those reported for tempeh after a long fermentation process (Stillings & Hackler, 1965).

Isoflavone

Isoflavones, one of the functional chemical groups found in soybeans, either increased or decreased during fermentation, depending on the specific chemical (Fig. 4). The concentrations of genistin and daidzine in black soybeans (time 0, no fermentation) were 207.9 and 160.7 μg g^{-1}, respectively, but the corresponding algycon isoflavone concentrations (genistein and daidzein) were only 16.9 and 18.2 μg g^{-1}, respectively. The concentration of the glycoside isoflavones decreased gradually during fermentation while that of the algycon isoflavones increased gradually. In the 15 day secondarily fermented product, the concentrations of genistin and daidzine were only 24.12 and 7.54 μg g^{-1}. The corresponding algycon isoflavones increased to 84.6 and 63.4 μg g^{-1}, respectively, which indicated that almost 90% glycoside isoflavones had been converted to algycon isoflavones or lost during the process.

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