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Anti-mutagenic and anti-oxidative activities found in Chinese traditional soybean fermented products *furu*

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Abstract

Furus, Chinese traditional soybean products, which are salted, fermented and seasoned bean curd preserved in dipping sauce, were studied for their anti-mutagenic and anti-oxidative activities. There are many varieties of *furu* by region, depending on their ingredients and bacterial strain used. *Furus*, not only have peculiar palatable taste and aroma, but also present strong anti-oxidative activity which ordinary Japanese soybean curd, *tofu*, never shows. Against benzo(α)pyrene, all samples, regardless of solid or dipping sauce, showed anti-mutagenic activity. In general, red *furus* had higher activity than white. All red *furus* inhibited more than 50% of the mutagenicity and this high suppressive ratio suggested that it was raised mainly by secreted substances discharged from the bacterial body rather than by functional compounds originating from soybeans. In the case of white *furu*, which showed wide differences in activity, the functional role of each spice and condiment added needs to be further studied, in addition to the soybean and white *furu* ferment-rice malt.

Keywords: *Furu*; Fermented soybean product; Anti-mutagenicity; Anti-oxidative activity

1. Introduction

Soy beans have been well-recognized as an excellent source of high-quality protein and lipid. In most Asian countries, a meat diet was not common until the 19th century, except for a narrow region and an era. In eastern Asia, soybeans have been grown as one of the most important protein resources. Soybeans are a good source of dietary fibres, which accelerate excretion of harmful chemical compounds and regulate the function of the intestines. At the same time, soybeans have attracted the attention of scientists as a supply source for different kinds of valuable minerals (Fonseca & Ward, 2004) such as calcium, phosphorus, and iron.

The importance of biologically active substances, such as saponin and isoflavones (Ohsawa, 1998; Watanabe, 2001), in soybeans and their products has been recognized from the standpoint of pathological dietetics. Saponin has been known as a suppressor of the formation of hydroperoxides. Due to an analogous chemical structure to female hormone, the latter was demonstrated as a preventative against losing calcium reserved in bone, as well as an accelerator of bone formation. The functional components (Wang, Saito, Tatsumi, & Li, 2003) of soybeans and the mechanism of their function have been well studied by many researchers.

Chinese people have consumed *furu* for a long time (Liu, Li, & Liu, 1995) as a side dish or seasoning. There are many varieties by region, depending on their ingredients and bacterial strain used. A recent review (Hana, Romboutsa, & Robert Nout, 2001) discusses compositional characteristics and nutritional values of many

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Table 1
Manufacturing process of *furus*

1	Raw materials: soybean (protein content ca. 40%, 170–200 g/1000 grains), wheat meal, rice malt, salt, spices and condiment different from product to product
2	Water soak (eight times as much water as soybean) at 15 °C for 24 h
3	Preparation of bean curd (thickness 1.9–2.1 cm, moisture 69–70%)
4	Steam for 20 min and cool for 30 min
5	Salting (Sprinkle salt on the bean curd evenly, upside down 24 h later, and sprinkle additional salt. Keep for another 24 h to make final salt concentration to 6.5–7.0%)
6	Washing with water (40 °C in winter, 20 °C in summer)
7	Inoculation of *Aspergillus* fungi (stuff bean curd and spray *bacterial* suspension)
8	Previous fermentation (Ferment at 28–30 °C for about a week, upside down when the surface becomes yellow after 2–3 days)
9	Late stage fermentation dehydrate at 50–60 °C for 12 h to make moisture and salt concentration at 45–48% and 8–9%, respectively. Pile up in an earthen pot, sprinkle a mixed spices and condiment solution layer by layer, and seal the mouth of the pot
10	Additional fermentation (Leave the pot in a fermentation room, keep at 28–30 °C for 50–60 days, upside down, then wait 30 more days to finish all process)

Chinese fermented soybean foods. A typical process flow of *furu* production, shown in Table 1, is carried out in the same manner, regardless of the region in which it is manufactured. However, addition of ingredients, spices, condiments, and fermentation auxiliaries results in differences of taste, aroma, colour and biological activities. (Surh, 2002) The colour of *furu* depends on the species of bacterial strain engaged in the fermentation process; the most popular ones are white, pale-white and red in colour. In general, red *furus* are preferred in northeastern and northern regions, and pale-white in central and southern regions of China.

In this study, we purchased 20 *furus*, mostly manufactured in China and some in Hong Kong and Taiwan. Anti-mutagenicity was measured by the forward mutation assay method against three authentic mutagens and DPPH radical-scavenging activity, determined by DPPH–HPLC method.

2. Methods and materials

2.1. Furus studied

Twenty *furus* were purchased in several different stores in Tokyo, which sell mainly imported foods from Asian countries. The names of the samples, ingredients, and the places of manufacture are listed in Table 2.

2.2. Authentic mutagenic compounds

Four indirect mutagens, purchased from Sigma Co. (St. Louis, USA), were chosen as authentic mutagenic compounds for the anti-mutagenic test. They were 3-amino-1-methyl-5*H* -pyrido [4,3-*b*]indole acetate (Trp-P-2), benzo(α)pyrene (BaP), 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), and 2-amino-3,8-dimethyl imidazo[4,5-f] quinoxaline (MeIQx), that are reported

Table 2
Locality, raw materials, and the colour of *furus* studied

Sample No.	Place of manufacture	Locality in China	Raw materials	Colour of the products
1	Heilongjiang	Northeast	Soybean, green bean, salt, wheatmeal, ginger, white grain wine	Red
2	Beijing	North	Soybean, salt, wheatmeal, white grain wine, sugar	Red
3	Beijing	North	Soybean, salt, wheatmeal	Red
4	Beijing	North	Soybean, salt, yellow rice wine, red hot pepper, sesame seed oil	White
5	Beijing	North	Soybean, salt, yellow rice wine, sesame seed oil	White
6	Beijing	North	Soybean, salt, white pepper	White
7	Sichuan	Southwest	Soybean, salt, red hot pepper, wine, sugar, sesame seed oil	White
8	Sichuan	Southwest	Soybean, salt, red hot pepper, mixed spices, sesame seed oil	White
9	Sichuan	Southwest	Soybean, salt, wine, sugar, garlic, sesame seed oil	White
10	Guiyang	Southwest	Soybean, salt, red hot pepper, vegetable oil, sesame seed oil, sugar	White
11	Guiyang	Southwest	Soybean, salt, red hot pepper, yellow rice wine, condiments	White
12	Guangdong	South	Soybean, salt, yellow rice wine, red hot pepper, sesame seed oil	White
13	Guangdong	South	Soybean, salt, red hot pepper, wine, sesame seed oil	White
14	Hong Kong	South	Soybean, salt, red rice malt	Red
15	Hong Kong	South	Soybean, salt, red hot pepper, sesame seed oil	White
16	Hong Kong	South	Soybean, water, salt, sesame seed oil	White
17	Taiwan	South	Soybean, salt, red rice malt, ethanol	Red
18	Taiwan	South	Soybean, salt, ethanol, chili powder, sesame seed oil	White
19	Taiwan	South	Soybean, salt, yellow rice wine, sesame seed oil	White
20	Taiwan	South	Soybean, salt, rice, yellow rice wine, sugar	White

to be generated from overheating of protein rich food or found in smoked products.

2.3. Preparation of test samples for anti-oxidative and anti-mutagenic tests

Bottled *furu* samples were separated into solids and dipping sauces, and then homogenized using a mortar and pestle. Active fraction was extracted with 80% ethanol three times and the extract combined and centrifuged at 4000 rpm at 4 °C for 20 min to remove insoluble particles. The supernatant was applied to the anti-oxidative test without any further treatment. For an anti-mutagenic test, ethanol was removed by evaporation under reduced pressure. Then the test sample was dried under a nitrogen stream, and dissolved in dimethylsulfoxide (DMSO) to make a final concentration 20 mg (of each sample equivalent)/ml (of 5% DMSO).

2.4. Anti-oxidative test

Among various anti-oxidative tests available, we employed the 1,1-diphenyl-2-picrylhydrazyl (DPPH)–HPLC method, using a comparatively stable radical (DPPH·) as a scavenging target. This method measures the ratio of radical elimination and is a measure of the process of carcinogenesis. In other words, this chromatographic method can estimate the radical control ability, which directly acts on cells. Moreover, this method will not be interrupted by the colour of the samples tested, because the interfering substances are separated chromatographically from the radical fraction. The principle of the method is explained as follows: when the DPPH radical is scavenged by reaction with the test sample, the peak area on the chromatogram is then decreased in proportion to the ability of anti-oxidative activity of the sample; i.e. the peak becomes smaller.

The DPPH–HPLC method was carried out according to the method of Yamaguchi, Takamura, Matoba, and Terao (1998). An aliquot of sample solution (200 μl) was mixed with 100 mM Tris–HCl buffer (pH 7.4, 800 μl) and then added to 1 ml of 500 μM DPPH in ethanol (final concentration of 250 μM). The mixture was shaken vigorously and allowed to stand for 20 min at room temperature in the dark. A blank test was run with the Tris–HCl buffer instead of the sample solution. The absorbance at 517 nm, by DPPH, was measured during reversed-phase HPLC analysis. The HPLC equipment consisted of a Shimadzu LC-9A pump, a Rheodyne injector fitted with a 20 μl sample loop and a Shimadzu SPD-6AV UV–Vis detector set at 517 nm. Analyses were performed with a TSK gel Octyl-80Ts column (4.6 × 150 nm; Tosoh, Tokyo, Japan) at ambient temperature using a mobile phase of methanol/water (70:30,v/v) at a flow rate of 1 ml/min. The DPPH radical-scavenging activity was calculated by the following equation:

$$\text{Radical scavenging activity } (\%) = [A_b - A_s]/A_b \times 100,$$

where A_b is the peak area of the blank and A_s is the peak area of the sample.

2.5. Anti-mutagenic test

The Ames method judges the mutagenicity by the change of histidine requirement of the test strain, but is not adequate for high free histidine-containing samples. It also consumes a lot of the expensive reagent S9, a rat liver crude extract, and test sample itself too. We employed the FM assay method proposed by Skopek, Liber, Kaden, and Thlly (1978), modified by Takagi, Goto, Murata, Lewtas, and Matsushita (1988), and reduced in size by Ren, Endo, and Hayashi (2001), for these reasons.

According to the results of the preliminary examination, the highest concentrations of authentic mutagenic compounds were as follows: BaP, 50.6 ng, Trp-P-2, 12.8 ng, and IQ, 0.88 ng per plate, which showed sufficient mutagenicity with no growth inhibition. The test samples (*furu* or its dipping sauce) and one of the three mutagens were mixed in each well of the micro-titre plate (Corning, 96 wells, NY, USA). They were incubated on a rotary shaker (Tuple-mixer, Iwaki Glass Co. Ltd., Funabashi, Japan) for 2 h at 37 °C and treated in the same manner as reported by Ren et al. (2001). The mutation frequency and the mutagenicity suppression ratio were obtained by the formula shown below.

$$\text{Mutation frequency ratio} = (C_m - C_n)/C_v,$$

where C_m is the number of colonies of mutants, C_n is the number of spontaneous colonies for negative control, and C_v is the number of colonies of viable cells.

$$\text{Mutagenicity suppression ratio}(\%) = (1 - M_{b+s}/M_b) \times 100,$$

where M_{b+s} is the mutation frequency of an authentic mutagen upon treatment of test samples, and M_b is mutation frequency of an authentic mutagen alone.

3. Results and discussion

Anti-oxidative activities of *furu* were determined by the HPLC method against the metastable radical, DPPH·. The anti-oxidative activities of the solids and dipping sauces, shown in Table 3, indicate that three out of 20 sample solids eliminated more than 50% of the DPPH radical and 12 samples removed 30–50%; the remaining five reduced less than 30%. In contrast to the solids, the dipping sauce showed lower activity,

Table 3
Anti-oxidative activity of *furus* (solid and dipping sauce) by DPPH radical-scavenging ratio(%)

Sample No.	Solid	Dip sauce
1	54	53
2	65	67
3	50	52
4	21	14
5	32	31
6	27	21
7	32	26
8	37	29
9	31	27
10	36	17
11	35	20
12	31	22
13	31	25
14	44	44
15	24	25
16	27	22
17	31	24
18	36	29
19	26	23
20	52	52

i.e. only samples 4 and 2 indicated more than 50% and 30–50% elimination, respectively, and the other 14 inhibited less than 30% of the original DPPH·.

The colour of the *furu* correlated with the degree of anti-oxidative activity and all five red *furus*, including both solid and dipping sauce, exhibited a high elimination ratio of more than 40% of the DPPH· in the test system. On the other hand, comparatively high anti-oxidative activity (more than 35% elimination) was detected in 5 solid samples, out of 15 white *furus*, compounded with more than two spices and condiment. In the case of red *furus*, almost similar radical-scavenging abilities were found in both solid and dipping sauces, except for the 1 sample manufactured in Taiwan. Most of the white *furus* had higher activity in the solids than in the dipping sauces, with the exception of one Hong Kong brand. By comparison of the ingredients of the products, red *furus* tested were made with simple ingredients. Evidently the differences in anti-oxidative activities among *furus* manufactured in various localities derived mainly from the raw materials, such as soybeans, bacterial strain, and fermentation parameters (temperature and humidity), because any spices and condiments used for manufacturing red *furus* had no apparent anti-oxidative activities (Hana et al., 2001).

From the experimental data shown above, fermented soybean products, *furus*, manufactured with rice malt, sesame seed oil, and red hot pepper as spices and condiment, not only had peculiar taste and aroma, but also presented strong anti-oxidative activity which ordinary Japanese soybean curd, *tofu*, hardly shows (Yin, Li, Li, Tatsumi, & Saito, 2004).

Anti-mutagenic activities of *furus* against BaP, Trp-P-2, IQ, and MeIQ$_x$ are summarized in Table 4. Regardless of their colour, higher anti-mutagenic activity was found in the solid than in the dipping sauce, against the four authentic mutagenic compounds. From the results given in this table, the causative agents responsible for the activity are probably not water-soluble com-

Table 4
Anti-mutagenic activity of *furus* by the suppressive ratio against four authentic mutagens[a]

Sample No.	BaP		Trp-P-2		IQ		MeIQ$_x$	
	Solid	Dip sauce	Solid	Dip sauce	Solid	Dip sauce	Solid	Dip sauce
1[b]	57	21	77	11	95	26	100	13
2	73	15	42	0	88	37	70	18
3	77	19	35	0	75	32	63	11
4	25	7	28	0	70	58	88	19
5	44	25	60	11	94	28	100	77
6	19	5	46	11	72	37	56	22
7	18	5	92	0	90	50	100	67
8	58	35	93	0	90	33	100	10
9	35	13	93	44	86	38	100	68
10	25	29	68	0	74	25	72	19
11	44	7	77	5	80	28	72	58
12	71	36	80	0	88	13	100	62
13	44	10	56	0	92	35	100	55
14	59	37	30	0	92	12	100	24
15	27	8	26	0	89	56	100	52
16	59	25	71	24	90	57	82	73
17	52	13	87	0	78	17	98	34
18	47	26	66	0	97	13	96	19
19	61	30	59	67	97	18	100	18
20	36	15	70	54	72	29	46	32

[a] Concentration of each authentic mutagen and furus poured in each plate(ng/plate). BaP; 50.6 ng, Trp-P-2; 12.8 ng, IQ; 0.80ng, MeIQ$_x$; 2.85 ng.
[b] Every mutagen was mixed with 375 μg of each sample furu in one plate.

pounds, but soy proteins which are in the solid portion of the *furu*.

From the individual suppressive rate data against four authentic mutagens, the following tendencies were observed. Toward BaP (Edenharder, Worf-Wandelburg, Decker, & Platt, 1999), all samples, regardless of solid or dipping sauce, showed anti-mutagenic activity in forward mutation assay with little differences. In general, red *furus* had higher activity than white, i.e. average inhibitory ratio of red *furu* (solid) was 64 ± 10% and that of white ranged from 18% to 71% and averaged 41 ± 16%. These averaged data show significant difference at the 95% level by the *t*-test. In the case of dipping sauce, even the red *furus* revealed less than one-third (21 ± 9%) and alike the white ones, exhibited less than one half (18 ± 11%) of the activities of the of solid.

According to the compositional information displayed on the labels, all red *furus* were manufactured without addition of any spices or condiments, except sample No. 1 to which some of traditional Chinese herbal leaves were added. A recent review (Surh, 2002) suggested that the spices and condiment added to white *furus* may have an important role in affecting some of their bioactive functions. All these red *furus* inhibited more than 50% of the authentic mutagenicity caused by BaP, and this high suppressive ratio suggested that it was raised mainly by secreted substances discharged from the bacterial body or its wall component (Kriková, Urakováb, Andulac, Sasinkovác, & Krajovia, 2001) rather than functional compounds originating from soybeans. In white *furus*, the inhibitory ratio broadly ranged from product to product, from lowest at 18% to highest at 71%. This wide difference in the activity suggests that the functional role of each spice and condiment needs to be further studied, with respect to soybean and white *furu* fermentrice malt.

The anti-mutagenic study against Trp-P-2 (Thompson, Carrano, Salazar, Felton, & Hatch, 1983) revealed characteristic patterns of activity. Though the suppressive rate ranged widely (26–93%), the anti-mutagenic activity of dipping sauce showed only one forth to one fifth of that of the solid part. The average inhibition ratio of the solid varied widely (54 ± 26% in red *furus* and 66 ± 21% in white), with no significant difference detected at the 95% confidence limit. Differing from the results against BaP shown above, white *furus* were determined to have stronger activities than red ones. Dipping sauces of all *furus* showed no definite suppressive associations between their constituents and the activity. Since marked anti-mutagenic activity was detected in *furus* manufactured with several different spices and condiments, such as sample Nos. 7–13, 16, and 18–20, this activity was enhanced considerably by the presence of hot pepper, garlic, yellow rice wine, and sesame seed oil, most of which are thought to include bio-functional constituents, rather than compounds originally in soybean itself. To pursue additional bio-functional activities of *furus*, the mutual chemical reactions between effective compounds in soybeans and other raw materials need to be further studied.

The anti-mutagenicity of *furus* against IQ (Thompson et al., 1983) ranged from 13% to 18% showing a large variation among samples. For the solid fraction, mutagenic suppression ratio of red *furus* was from 75% to 95% (ave. 86%), and for white 70–97% (ave. 85%). In contrast to the results for the solids, the dipping sauce fraction of red and white *furus* showed, respectively, 12–37% (ave. 25%) and 13–58% (ave. 38%). Since white *furus* were blended with many different spices and condiments, these side ingredients, such as red hot pepper, garlic, yellow rice wine, and sesame seed oil, added at the starting stage of the fermentation process, are thought to be involved in the development of the anti-mutagenic activity of white *furus*.

In the last anti-mutagenic test against $MeIQ_x$, the mutagen suppressive ratio was calculated as 10–100% in 40 test samples. Ten *furus* completely inhibited (100%) mutagenic activity of $MeIQ_x$, suggesting that the suppressive effect of *furus* was the most effective against $MeIQ_x$ among the four authentic mutagens tested. The solid fraction of red and white *furus* inhibited 63–100% and 46–100%, respectively, and dipping sauce fraction of red and white ones suppressed 11–34% and 10–77% as well. Like the result against IQ, *furus* treated with MeIQ revealed almost similar suppressive tendencies as showing higher inhibitory ratios in the solid.

One of the major raw materials of all *furus* was soybeans (Liu et al., 1995) except for sample No. 1, in which green bean was also used, along with a soybean. According to the results of anti-mutagenic tests against the four authentic mutagens, higher suppressive ratios were obtained in sample No. 1, either in the solid fraction (57–100%) or dipping sauce (11–25%), than in the remaining 19 *furus* from soybean.

The most bioactive components of *furus*, which contain many different active ingredients, were not identified, because the parameters which contribute to each function are very complicated. As might be expected, the anti-oxidative and anti-mutagenic activities of *furus* we studied differed with locality, ingredients added, and manufacturer. However, in conclusion, these traditional fermented soybean products are supposed to improve their organoleptic characteristics and functionality by rearrangement of the recipe or manufacturing process. Further study is planned on the process of *furu* production to create newly designed functional food products.

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