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Anti-α-glucosidase activity of Chinese traditionally fermented soybean (douchi)

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Abstract

Anti-α-glucosidase activity of aqueous douchi extracts was investigated in this study. Thirty-one douchi samples collected from different parts of China exerted various degree of inhibitory activity against rat intestinal α-glucosidase. Among them, three samples, sourcing from Hunan, Sichuan and Jiangxi province, respectively, showed a significant higher anti-α-glucosidase activities than other samples ($p < 0.05$). Moreover, three fungal strains, namely *Aspergillus oryzae*, *Actinomucor elegans* and *Rhizopus arrhizus* were then used to prepare douchi in our laboratory. The α-glucosidase inhibitory activities of all soybeans increased slightly and no apparent differences were found in anti-α-glucosidase activity among the soybeans at the end of pre-fermentation. For maturation, different salt levels (5.0%, 7.5%, 10.0% and 12.5%) were then added to the douchi qu resulted from pre-fermentation. The anti-α-glucosidase activity of douchi qu fermented with *A. oryzae* were higher than those of *A. elegans* and *R. arrhizus* and the highest anti-α-glucosidase activities was observed in douchi qu fermented with *A. orzyzae* at 5.0% and 7.5% salt levels. The results indicated that *A. oryzae* could utilize cooked black soybean to generate certain α-glucosidase inhibitor more effectively than *A. elegans* and *R. arrhizus*.

Keywords: Anti-α-glucosidase; Douchi; Fermented soybean; α-Glucosidase inhibitor; Anti-hyperglycemic

1. Introduction

Diabetes mellitus has become a common disease not only in developed countries but also in developing countries due to the changes in people's lifestyle and dietary habits (Horton, 1995). α-Glucosidase inhibitor is usually used to prevent or medically treat type II diabetes (Non-insulin dependent mellitus, NIDDM) (Alain, 1998; Floris et al., 2005; Holman, 1998; Patricia, Steven, Jennifer, & Bryan, 2005). These inhibitors combine with intestine α-glucosidase and block the uptake of postprandial blood glucose (Holman, 1998). Although powerful synthetic α-glucosidase inhibitors (i.e. voglibose) are available, they usually can cause hepatic disorders and other negative gastrointestinal symptoms (Murai et al., 2002). Hence, natural α-glucosidase inhibitors from food sources have become an attractive therapeutic approach for treating post-prandial hyperglycemia (Gallaher & Schneeman, 1986; Heacock, Hertzler, Williams, & Wolf, 2005; Kyung & Moo, 2000; Murai et al., 2002; Ye, Shen, & Xie, 2002).

Soybean and its products have been appreciated by consumers as health foods due to their valuable nutritional and medicinal attributes. In particular, the intake of soybean foods has been associated with the prevention and treatment of chronic diseases, such as cardiovascular disease and cancers. Douchi (or touchi) is a popular fermented soybean product among Chinese community worldwide. Many historical medical books have described douchi being able to prevent and cure diseases. Zhang Zhongjing of the Han Dynasty recorded that soup cooked with cape jasmine and douchi was useful to relieve tiredness, weakness, insomnia, and poor appetite (Li, Yin, Zhang, Zhang,

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& Zhao, 2002). Recent studies show that fermented soybeans products possess anti-diabetic properties (Fujita, Yamagami, & Ohshima, 2001; Fujita, Yamagami, & Ohshima, 2003; McCue, Kwon, & Shetty, 2005). The douchi extract, for example, elicited significant anti-hyperglycemic effect at a minimum effective dose of 0.3 g in human (Fujita et al., 2003). Moreover, the douchi extract demonstrates excellent anti-hyperglycemic effect without causing any side effects such as diarrhea, retching and flatulence, which are commonly encountered with the use of currently available α-glucosidase inhibitory therapeutic drugs (Fujita et al., 2003).

The douchi processing is mainly made up of fungal solid-state fermentation (pre-fermentation), followed by salting and maturation (post-fermentation). Based on the microorganisms used, douchi products can be classified into *Aspergillus*-type (i.e. Liuyang douchi and Yangjiang douchi etc.), *Mucor*-type (i.e. Yongchuan douchi), *Rhizopus*-type (i.e Indian tempe) and *Bacterial* type (i.e. Qianxi douchi, Babao douchi & Japanese natto) (Liang, Cheng, & Ma, 2004; Niu & Ma, 2005). Salt plays multiple roles in the fermentation of various soybean products. In sufu, for instance, besides imparting salty taste to the final product, salt is important in controlling microbial growth and enzymatic activities as well (Han, Wang, Rombouts, & Nout, 2003). On the other hand, salts supplemented to douchi during post-fermentation lowers the antioxidative activity of douchi (Zou, Wang, Cheng, Li, & Tatsumi, 2006). Fifty percent salt added in douchi could deduce to the loss of 61% isoflavone in raw soybean during the fermentation (Wang et al., 2007). Although microorganisms and salts are important in endowing unique sensory properties and enhancing the nutritional values of the fermented soy products, their roles in generating α-glucosidase inhibitors have not been investigated. In this study, we analyzed the α-glucosidase inhibitory activity of 31 douchi samples collected from various parts of China. The anti-α-glucosidase activity of douchi samples prepared using different fungal strains (namely, *A. oryzae*, *A. elegans* and *R. arrhizus*) at different salt levels (5.0–12.5%, w/w) were also studied. The first objective of this study is to investigate the anti-α-glucosdiase activity of Chinese douchi. The second is to investigate the factors which are related to the anti-α-glucosdiase activity of douchi and sufu.

2. Materials and methods

2.1. Materials

Thirty-one douchi samples were purchased directly from douchi manufacturers from different parts of China (Table 1). Fungal strains, namely *Aspergillus oryzae* 3.951, *Actinomucor elegans* 3.118 and *Rhizopus arrhizus* 3.078 were kindly provided by the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). Intestinal acetone powders of rat and 4-nitrophenyl α-D-glucopyranoside (4-NPG) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade.

2.2. Fungal strains and culture conditions

Fungal strains of *A. oryzae* 3.951, *A. elegans* 3.118 and *R. arrhizus* 3.078 were grown individually on malt extract agar (pH 4.0) at 28 °C for 2–3 days. Then, 25 ml sterile distilled water was added to each of the 3-day-old fungus grown on malt-extract agar plates and the plates were scraped aseptically with inoculating loop. The resulted spore suspensions were then allowed to propagate on a sterilized substrate consisting of wheat bran (50.0 g), wheat flour (10.0 g) and sterilized distilled water (50 ml) in an incubator at 28 °C for three days. After the complete growth of the organisms, spores of the three fungal strains were harvested by adding sterilized water to the fermenting substrate, shaking the flask and filtration to make homogenous spore suspensions. The number of spores in each suspension was enumerated using a thrombocytometer.

2.3. Douchi preparation

The preparation of douchi is illustrated in Fig. 1. Black soybeans were washed, soaked in water (1:3, w/w) at 25 ± 2 °C for 8–10 h, and steamed at 121 °C for 30 min in a retort (YMQ.L31.400, Beijing Jiangtai Medical Instrument Co., Beijing, China). After cooling to 30 °C, the cooked soybeans were inoculated either with the spores of *A. oryzae*, *A. elegans* or *R. arrhizus* (10^6 spores per gram of cooked soybean) and fermented in an incubator (LTI-601SD, Tokyo Rikakikai Co., Tokyo, Japan) for 60 h at 28 °C and 90% relative humidity. Semi-finished products were called douchi qu (koji). For post-fermentation treatment on douchi, different levels of salt (5–12.5%, w/w) were added to the douchi qu and the douchi qu was allow to age for one month.

2.4. Preparation of aqueous extract of douchi

Douchi samples were minced and lyophilized with a freeze-dryer (EYELA Co., Tokyo, Japan). Exactly 4.000 g of each sample was mixed with 40 ml distilled water, homogenized (20 000 rpm, 2 min) and centrifuged (3800 rpm, 10 min). The resulted supernatant of each sample was collected and freeze-dried again. Finally, the powder was diluted with 10 ml distilled water, filtered and stored at 4 °C until use.

2.5. Measurement of α-glucosidase inhibitory activity

The inhibitory activity of douchi extracts against rat α-glucosidase was determined by measuring the formation of 4-nitrophenol by α-glucosidase after the reaction with 4-nitrophenyl α-D-glucopyranoside (4-PNP) as described by Yamaki and Mori (2006). The inhibitory activity of

Table 1
Brand names, origins, type of soybean used and anti-α-glucosidase activities of commercial douchi samples

Sample no.	Brands	Origins	Type of soybean	Anti-α-glucosidase activities[a]
1	Y.J.Q	Guangdong(south)	Black	8.474
2	S.S	Guangdong (south)	Black	7.526
3	X.Q	Guangdong (south)	Black	5.131
4	H.S.Q	Guangdong (south)	Black	5.705
5	Q.G	Hunan (middle)	Black	13.063
6	T.P.Q	Hunan (middle)	Black	7.467
7	L.Y.P.X	Hunan (middle)	Black	6.04
8	Q.W	Hunan (middle)	Black	4.739
9	R.M	Sichuan (middle-west)	Black	1.812
10	A.J	Sichuan (middle-west)	Black	8.930
11	Y.C	Sichuan (middle-west)	Black	4.300
12	Y.C.J.X	Sichuan (middle-west)	Black	5.300
13	C.Q.W.Z	Sichuan (middle-west)	Yellow	2.856
14	F.W	Sichuan (middle-west)	Black	5.746
15	W.N.H	Sichuan (middle-west)	Black	13.863
16	J.X.Z.Z	Jiangxi (middle)	Yellow	0.790
17	D.X.Y	Jiangxi (middle)	Black	3.200
18	J.X	Jiangxi (middle)	Black	8.060
19	M.W	Jiangxi (middle)	Yellow	12.230
20	Q.X	Guizhou (middle)	Yellow	2.376
21	G.Y	Guizhou (middle)	Yellow	8.212
22	L.Y	Shandong (east)	Yellow	6.651
23	X.Y	Beijing (north)	Yellow	2.203
24	Y.M	Guangxi (south)	Black	5.240
25	N.N	Guangxi (south)	Black	3.060
26	Z.J	Hebei (north)	Yellow	6.238
27	J.L.D.J.1	Jilin (east-north)	Yellow	4.780
28	J.L.D.J.2	Jilin (east-north)	Yellow	1.182
29	T.J	Tianjin (east)	Yellow	4.135
30	K.M	Yunnan (west-south)	Yellow	3.476
31	X.J.D.J	Xinjiang (west)	Yellow	2.400

[a] Anti-α-glucosidase activities of douchi samples were computed as the slope values from the curves of absorbance versus the concentration of aqueous douchi extract. The higher the slope value, the stronger the anti-α-glucosidase activity of the aqueous douche extract.

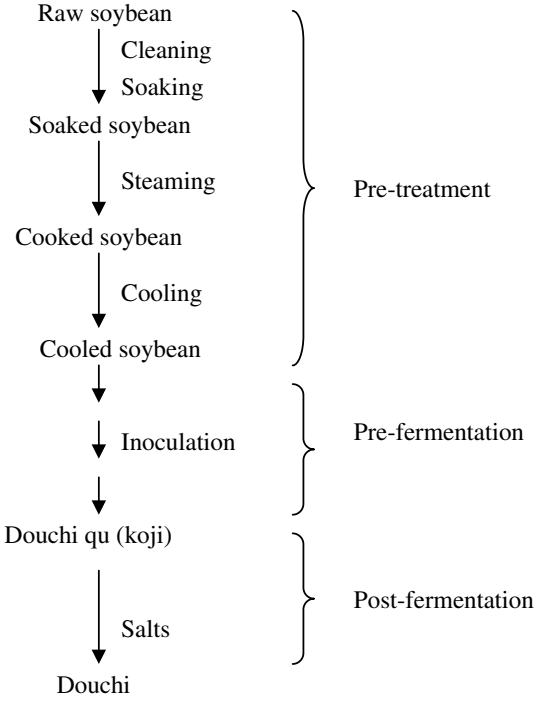

Fig. 1. Flow-chart of producing douchi from raw soybean.

douchi extract was measured according to the protocol of a micro-well kit (0.4 ml × 96 wells Flat bottom, Sumitomo Bakelite Co., Ltd., Tokyo, Japan). A serial two-fold dilution of the aqueous douchi extracts in microtiter flat-bottom 96-well plates were mixed with 50 μl α-glucosidase (25 mg/ml), 50 μl 4-NPG (0.9133 mg/ml) and 120 μl 0.5 M phosphate buffer (pH 6.7). The mixtures were incubated at 37 °C for 60 min. Sodium carbonate (50 μl, 0.67 M) was then added to stop the reaction. Absorbance of the reactants was measured at 405 nm using a microplate reader (Model 550, BIO-RAD Lab., Tokyo, Japan). The α-glucosidase inhibitory activity of douchi extract was then computed as the slope value from the curve of absorbance versus concentration of the douchi extract. The higher the slope value, the stronger the anti-α-glucosidase activity of the aqueous douchi extract.

2.6. Statistical analysis

Data collected were analyzed using SPSS software version 12.0. Analyses of variances were performed using general linear model (GLM) procedure determine differences within treatment for independent variable assessed. Comparision of means was performed using Student–New-

man–Keuls (S–N–K) test when F-value ($p < 0.05$) was significant.

3. Results

3.1. Anti-α-glucosidase activities of commercial douchi samples

In this study, we collected 31 douchi samples from various parts of China. The brand names and origins the douchi samples as well as types of soybean used for douchi preparation are listed in Table 1.

Anti-α-glucosidase activities of the douchi samples are shown in Table 1. All the aqueous douchi extracts showed anti-α-glucosidase activities *in vitro*. Among these samples, sample No. 5, No. 15 and No. 19, collected from Hunan, Sichuan and Jiangxi, respectively, showed a significant higher anti-α-glucosidase activities than other samples ($p < 0.05$). Moreover, the anti-α-glucosidase activity of aqueous douchi extracts varied not only between different regions but also within the same region.

3.2. Anti-α-glucosidase activities of soybeans during pre-fermentation

To determine the effect of microorganism on the anti-α-glucosidase activity of soybeans during pre-fermentation, the anti-α-glucosidase activities of soybeans inoculated with *A. oryzae*, *A. elegans* and *R. arrhizus* were monitored (Table 2). All soybeans inoculated with the fungi showed a gradual increase in anti-α-glucosidase activity during pre-fermentation. However, the total increments in anti-α-glucosidase activity of all the soybeans were very little and no apparent difference in anti-α-glucosidase activity was observed among the soybeans at the end of pre-fermentation.

3.3. Anti-α-glucosidase activities of douchi during post-fermentation

Table 3 shows the effect of salt on the anti-glucosidase activities of douchi samples inoculated with *A. oryzae*, *A. elegans and R. arrhizus* during the post-fermentation period. Anti-α-glucosidase activities of all douchi qu samples rose gradually during the post-fermentation. The anti-α-glucosidase activity of douchi qu fermented with *A. oryzae* was higher than those of *A. elegans* and *R. arrhizus*. The highest anti-α-glucosidase activity was observed in douchi qu fermented with *A. oryzae* at 5.0% salt level. However, increment in anti-glucosidase activity of douchi samples became smaller when salt level was increased in douchi qu fermented using *A. oryzae*. On the other hand, compared to douchi fermented by *A. oryzae*, only slight increases were observed in the anti-α-glucosidase activity of douchi samples fermented using *A. elegans* and *R. arrhizus* for all salt levels tested.

4. Discussion

Douchi is a very popular fermented soy food product throughout China, especially at the Southern and Central regions. This study shows that there is a great variation

Table 2
Anti-α-glucosidase activity of douchi during pre-fermentation

Microorganism	Time (h)					
	0	12	24	36	48	60
Aspergillus oryzae 3.951	0.116 ± 0.01	0.29 ± 0.07	0.273 ± 0.05	0.389 ± 0.06	0.296 ± 0.05	0.412 ± 0.02
Actinomucor elegans 3.118	0.166 ± 0.04	0.19 ± 0.08	0.207 ± 0.04	0.311 ± 0.06	0.320 ± 0.10	0.393 ± 0.06
Rhizopus arrhizus 3.078	0.301 ± 0.03	0.222 ± 0.02	0.312 ± 0.06	0.305 ± 0.04	0.235 ± 0.06	0.378 ± 0.03

Table 3
Effect of microorganisms, levels of salt and fermentation time on the anti-α-glucosidase activity of douchi during post-fermentation

Microorganism	Level of salt (%)	Fermentation time (week)			
		1	2	3	4
Aspergillus oryzae 3.951	5.0	2.55 ± 0.44	2.98 ± 0.18	5.64 ± 0.15	6.85 ± 0.41
	7.5	1.78± 0.12	2.85 ± 0.35	3.68 ± 0.12	5.45 ± 0.49
	10.0	0.160 ± 0.05	1.80 ± 0.09	3.88 ± 0.30	3.97 ± 0.63
	12.5	0.345 ± 0.14	1.58 ± 0.20	3.43 ± 0.09	3.88 ± 0.46
Actinomucor elegans 3.118	5.0	0.756 ± 0.04	0.899 ± 0.05	1.03 ± 0.21	0.944 ± 0.05
	7.5	0.343 ± 0.02	1.35 ± 0.05	1.01 ± 0.08	1.40 ± 0.13
	10.0	0.344 ± 0.08	0.999 ± 0.03	1.22 ± 0.06	1.30 ± 0.15
	12.5	0.455 ± 0.07	0.765 ± 0.04	1.00 ± 0.20	0.987 ± 0.08
Rhizopus arrhizus 3.078	5.0	0.673 ± 0.06	0.734 ± 0.02	1.04 ± 0.09	1.321 ± 0.07
	7.5	0.956 ± 0.07	1.38 ± 0.02	1.01 ± 0.04	1.60 ± 0.13
	10.0	0.578 ± 0.03	1.00 ± 0.06	1.17 ± 0.23	1.31 ± 0.09
	12.5	0.455 ± 0.05	0.765 ± 0.07	1.20 ± 0.14	1.34 ± 0.11

in anti-α-glucosidase activity of collected douchi samples. These differences are most probably arisen from the variation in processing techniques and microorganisms used in douchi manufacturing. During fermentation, soybean isoflavones from the glucosides in soybean are converted into the corresponding aglycons under hydrolysis by fungal β-glucosidase. Lee and Lee (2001) isolated and identified genistein, an aglycon form of isoflavone found in soybean, as a candidate for α-glucosidase inhibitor from fermentation broths of *Streptomyces* sp. Genistein was shown to be a reversible, slow-binding, non-competitive inhibitor of yeast α-glucosidase (Lee & Lee, 2001). In a more recent study, Chen, Sugiyama, Abe, and Kuroto-Niwa (2005) isolated four phenol compounds, one isoflavanone, eight isoflavones and one 4-pyrone from douchi (Chen et al., 2005). Among these fourteen compounds, 3′-hydroxydaidzein showed as high DPPH radical-scavenging activity as that of α-tocopherol while 6-hydroxydaidzein had mushroom tyrosinase inhibitory activity with an IC_{50} value of 10 μM (Chen et al., 2005). Nonetheless, the relationships between these compounds with the α-glucosidase inhibitory activity of douchi have not been investigated. The isoflavone contents of fermented soy products could be affected by variety of soybean, processing technique, and dilution with nonsoy ingredients. Heat processing, enzymatic hydrolysis and fermentation significantly alter the isomer distribution of the soy isoflavonoids (Wang & Murphy, 1994).

Our study shows that douchi extract from soybean fermented with *A. oryzae* possess higher α-glucosidase inhibitory activity than those of *A. elegans* and *R. arrhizus* at all salt levels tested. Similarly, McCue et al. (2005) found that the aqueous extract of soybean fermented with *Lentinus edodes* possessed lower anti-α-glucosidase than those of *R. oligosporus*. This could be due to the different ability of the fungi to generate active compounds from soybean during fermentation. Wang and Murphy (1994) analyzed the concentrations of aglycons in tempeh, bean paste, miso, and fermented bean curd. The total contents of aglycons (namely, daidzein, genistein and glycetein) for tempeh, bean paste, sufu and honzukuri miso were 625, 593, 390 and 294 mg/g, respectively. Wang and Murphy (1994) attributed the differences in aglycon level to the variation in hydrolysis extent caused by each inoculants used for fermenting soybean.

Salt exerted different effects on the anti-α-glucosidase activity of douchi prepared with different fungi. This variation in anti-α-glucosidase activity most probably was caused by the ability of each fungus to adapt to habitats with low water activity. The approximate a_w limit for *Aspergillus*, *Rhizopus* and *Mucor* is 0.70, 0.90, and 0.90, respectively (Prescott, Harley, & Klein, 1993). Furthermore, Song, Nah, Han, and Han (2001) found that salts, such as KCl, NaCl and $MgCI_2$ promote conidial head formation in *A. oryzae*. Hence, the ability of *A. oryzae* to survive at the presence of salt explains partly the higher anti-α-glucosidase observed in douchi using this fungal strain. Han, Cao, Rombouts, and Robert (2004) studied the microbial changes during sufu fermentation. Their results shows that fungi were absent in all sufu samples after 30 days of ripening. They concluded that the fungi, particularly the mould starters, do not survive after the pehtze preparation, owing to the combination of salt and ethanol in the dressing mixtures applied for the maturation of sufu.

5. Conclusion

This study shows that douchi, in general, possess α-glucosidase inhibitory activity. Among them, three samples, sourcing from Hunan, Sichuan and Jiangxi province, respectively, showed a significant higher anti-α-glucosidase activities than other samples ($p < 0.05$). Moreover, douchi fermented with three fungal strains, namely *Aspergillus oryzae*, *Actinomucor elegans* and *Rhizopus arrhizus* also exerted anti-α-glucosidase activities. Douchi produced with *A. oryzae* showed a stronger inhibition activity on α-glucosidase than those samples manufactured with *A. elegans* and *R. arrhizus*. The anti-α-glucosidase inhibitory activities of self-produced douchi with *A. oryzae* increased slightly and no apparent differences were found at the end of pre-fermentation. During the maturation period, the highest anti-α-glucosidase activities were observed in douchi qu fermented with *A. oryzae* at 5.0% and 7.5% salt levels. It was indicated that α-glucosidase inhibitory activity of self-produced douchi depends partly on the fungal strains and salt used for fermentation. *A. oryzae* could be potential candidate as inoculant for producing douchi with strong anti-α-glucosidase activity.

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