

Angiotensin I-Converting Enzyme Inhibitory Activities of Extracts from Commercial Chinese Style Fermented Soypaste

Feng-Juan LI^{1,†}, Li-Jun YIN^{2,†}, Yong-Qiang CHENG², Masayoshi SAITO^{3,4}, Kohji YAMAKI^{3,5} and Li-Te LI^{2,*}

¹ College of Food Engineering and Biotechnology, Tianjin University of Science and Technology (Taida, Tianjin 300457, China)

² College of Food Science & Nutritional Engineering, China Agricultural University (Haidian, Beijing 100083, China)

³ Post-harvest Science and Technology Division, Japan International Research Center for Agricultural Sciences (Tsukuba, Ibaraki 305–8686, Japan)

[†] These authors contributed equally to this work.

Abstract

Angiotensin I-converting enzyme (ACE) inhibitory activities of thirty commercial Chinese style soypaste were determined. All the aqueous sample extracts exhibited ACE inhibitory activities. Nine samples produced in Guangdong Province showed significantly higher ACE inhibitory activities than other samples ($p < 0.0001$) and the strongest activity was detected with an extraordinarily low IC_{50} value of 0.012 mg/mL. Furthermore, these nine samples contained significantly lower peptide contents in line with the slighter proteolysis degrees and showed significantly lighter colors as evidenced by the higher L^* (lightness) values than other samples ($p < 0.0001$). It was indicated that special processing techniques depending on production regions might, to a large extent, affect the ACE inhibitory activities of Chinese soypaste. The findings in this work would be useful in exploring ACE inhibitors derived from Chinese style soypaste and designing functional products with potential anti-hypertensive effects.

Discipline: Food

Additional key words: angiotensin I-converting enzyme (ACE) inhibitory activity, Chinese soypaste, peptide, production region

Introduction

Angiotensin I-converting enzyme (ACE) plays an important physiological role in the regulation of blood pressure by converting angiotensin I into the powerful vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin¹³. For the sake of health and safety, ACE inhibitors from food resources have received a great deal of attention in connection with the prevention and remedy of hypertension.

It is well known that fermentation is one of the

popular food processing steps to generate biologically active substances derived from the raw materials and/or the products of fermentation. Soybean is rich in economical and high quality vegetable protein. It has been reported that many fermented soybean foods exerted ACE inhibitory activities^{8,11,12,15-17}. In China, soypaste is one of the popular traditional fermented soybean foods, which has been consumed as a seasoning for thousands of years. Generally, it is a semisolid mushy product obtained by three steps: (1) pretreatment, (2) preparing *Koji* by inoculating *Koji* mold such as *Aspergillus oryzae* on the raw material mixture of steam-cooked whole/defatted

Present address:

⁴ Agriculture, Forestry and Fisheries Research Council, Ministry of Agriculture, Forestry and Fisheries of Japan (Chiyoda, Tokyo 100–8950, Japan)

⁵ National Food Research Institute, National Agriculture and Food Research Organization (Tsukuba, Ibaraki 305–8642, Japan)

*Corresponding author: e-mail jircas2000@yahoo.cn

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soybeans and wheat flour, and (3) salting and ripening for several months. It is thought that soypaste was developed from douchi, which is another famous Chinese traditional soybean food manufactured by using soybeans as the raw material with the intact soybean grains remaining in the final product. Actually, there are some products similar to Chinese soypaste in Korea and Japan, that is, doenjang and miso, which have developed with special local texture and flavor. Okamoto et al.¹² reported the ACE inhibitory activities of miso produced by using different raw materials. Shin et al.¹⁵ obtained an ACE inhibitory peptide from Korean soybean paste. There are many kinds of soypaste products with various processing techniques in China. However, little information is available on their physiological properties.

In this study, we investigated the ACE inhibitory activities of Chinese commercial soypaste collected from nine different regions of the country and attempted to elucidate the relationships between the ACE inhibitory activities and the chemical parameters of soypaste. It was hoped this will be useful in improving the potential antihypertensive effects of Chinese style soypaste as a subject for further study.

Materials and methods

1. Materials

Thirty commercial soypaste samples were collected from various parts of China (Table 1). Hippuryl-His-Leu (HHL), angiotensin I-converting enzyme (ACE, from rabbit lung), *o*-phthalaldehyde (OPA), β -mercaptoethanol, and glutathione were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). All other reagents were of analytical grade.

2. Preparation of soypaste extracts

Samples were lyophilized with a freeze dryer (EYELA FDU-540, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and ground. One gram of soypaste powder was suspended in 10 mL of distilled water, followed by homogenization (T25 basic, IKA Labortechnik, Staufen, Germany) at 15,000 rpm for 2 min and sonication for 5 min. The mixture was extracted in an orbital shaker for 1 h at room temperature. After boiling for 15 min, the suspension was centrifuged at 12,000 \times g for 15 min. The resulting supernatant was collected after filtration by a 0.45 μ m membrane and then used for assaying. The concentration was labeled as 100 mg/mL.

3. ACE inhibitory activity assay

ACE inhibitory activity assay was measured by using the modified method described by Horie⁵. A 20 μ L of

aqueous extract was mixed with 40 μ L of ACE solution (12.5 munits/mL) and 40 μ L of substrate solution (4.66 mM HHL in 400 mM phosphate buffer containing 600 mM NaCl, pH 8.5) and then incubated at 37°C for 1 h. The reaction was terminated by adding 150 μ L of 1.2 M NaOH solution. The mixture was incubated for 20 min at room temperature after the addition of 40 μ L of 2% OPA dissolved in methanol. The derivation reaction was terminated by adding 40 μ L of 6 M HCl solution. The fluorescence intensity was measured using a spectrofluorometer (RF-5300PC, Shimadzu Co., Kyoto, Japan) under the following conditions: Ex, 340 nm; Em, 455 nm; slit width, 5 nm. ACE inhibitory activity was calculated as follows:

$$\text{ACE inhibitory activity (\%)} = [1 - (a - c) \div (b - d)] \times 100,$$

where *a* is the fluorescence intensity of the ACE solution with the addition of ACE inhibitors to the buffer; *b* is the fluorescence intensity of the ACE solution in the buffer; *c* is the fluorescence intensity of the ACE inhibitors in the buffer; and *d* is the fluorescence intensity of the buffer. IC₅₀ value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the Laemmli method⁹ using 15% acrylamide separating gel and 4.5% acrylamide stacking gel containing 0.1% SDS. Each sample (10%, w/v) was centrifuged at 12,000 \times g for 10 min, and the supernate was then heated at 100°C for 5 min in treatment buffer (pH 6.8) containing 1% SDS. An aliquot of 12 μ L of each sample was applied. Electrophoresis was carried out at a constant current of 20 mA for 3 h using a vertical electrophoresis apparatus (AE-6500, ATTO Co., Tokyo, Japan). Then the gel sheet was stained for proteins with Coomassie Brilliant Blue R250.

5. Chemical analysis

The peptide contents of freeze-dried powder were measured according to the OPA method³ with slight modifications. Briefly, 50 μ L of sample extract were added to 1 mL of OPA reagent and incubated for 4 min at ambient temperature. Then, the absorbance at 340 nm was measured on a spectrophotometer (UV 1240, Shimadzu Co., Kyoto, Japan). Glutathione was used as the standard. The total soluble carbohydrate contents were analyzed by using the phenol-sulfuric method⁴ with glucose as the reference compound.

6. Color determination

Soypaste color was determined by using a

Table 1. ACE inhibitory activities, chemical parameters and colors of commercial Chinese style soypaste^a

Sample No.	Brand Origin ^b		IC ₅₀ (mg/mL) ^c	Peptides (g/100 g dry matter) ^c	Total soluble carbohydrate (g/100 g dry matter) ^c	Color values ^d		
						L*	a*	b*
1	HTK	BJ	1.009 ± 0.043	17.31 ± 0.13	13.17 ± 0.07	65.80	6.70	25.25
2	JD	BJ	0.920 ± 0.030	12.52 ± 0.04	12.49 ± 0.14	63.16	6.23	20.06
3	LBJ	BJ	0.514 ± 0.017	16.27 ± 0.04	9.19 ± 0.30	63.25	6.43	20.50
4	LCC	BJ	0.599 ± 0.003	17.72 ± 0.10	12.30 ± 0.24	53.14	9.44	23.32
5	WZH	BJ	1.093 ± 0.025	15.47 ± 0.30	10.63 ± 0.28	66.71	6.59	25.58
6	LBJG	BJ	0.477 ± 0.013	17.01 ± 0.02	12.25 ± 0.11	54.85	7.80	21.30
7	CBL	SD	1.961 ± 0.305	21.72 ± 0.21	12.29 ± 0.23	43.96	6.17	10.38
8	WDF	SD	3.241 ± 0.539	15.52 ± 0.16	10.67 ± 0.21	48.45	8.04	18.82
9	SQ	LN	0.494 ± 0.010	27.77 ± 0.22	8.09 ± 0.03	61.07	5.70	17.97
10	TR	LN	0.786 ± 0.018	31.09 ± 0.46	7.12 ± 0.30	53.09	5.71	18.08
11	GH	TJ	1.014 ± 0.035	15.59 ± 0.17	15.18 ± 0.06	46.82	8.06	15.47
12	BQ	HLJ	0.737 ± 0.011	21.50 ± 0.39	9.92 ± 0.25	48.90	8.58	19.20
13	XQJ	HLJ	1.330 ± 0.016	17.93 ± 0.09	10.36 ± 0.09	46.13	8.40	16.06
14	BQL	HLJ	1.073 ± 0.026	14.19 ± 0.06	17.19 ± 0.03	49.41	8.44	19.07
15	TYD	HLJ	0.995 ± 0.018	20.99 ± 0.04	12.56 ± 0.14	50.03	7.65	17.78
16	BK	JL	0.825 ± 0.033	23.08 ± 0.33	9.18 ± 0.28	61.70	7.79	29.05
17	SWZ	JL	0.761 ± 0.017	23.68 ± 0.12	12.61 ± 0.15	50.80	6.12	18.21
18	SR	JL	0.712 ± 0.005	23.77 ± 0.20	3.71 ± 0.25	76.20	2.56	15.15
19	HP	GD	1.626 ± 0.037 ^g	19.39 ± 0.05	13.94 ± 0.10	79.41	2.21	20.47
20	ZSX ^e	GD	0.034 ± 0.003 ^{f,g}	15.55 ± 0.20 ^f	10.23 ± 0.37	80.31 ^f	0.72 ^f	25.54
21	WJ	NMG	1.298 ± 0.105	25.44 ± 0.08	9.53 ± 0.33	56.28	5.28	19.07
22	LFJ	HB	0.983 ± 0.029	16.73 ± 0.12	15.56 ± 0.10	48.08	8.28	17.95
23	ZK	GD	0.196 ± 0.023 ^{f,g}	12.51 ± 0.23 ^f	8.92 ± 0.14	82.61 ^f	1.27 ^f	19.09
24	LRX	GD	0.250 ± 0.035 ^{f,g}	7.99 ± 0.03 ^f	11.66 ± 0.28	89.99 ^f	0.17 ^f	16.14
25	JX	GD	0.296 ± 0.021 ^{f,g}	8.19 ± 0.01 ^f	10.60 ± 0.29	89.12 ^f	0.26 ^f	15.80
26	CT	GD	0.137 ± 0.004 ^{f,g}	8.71 ± 0.09 ^f	12.37 ± 0.33	89.94 ^f	0.04 ^f	15.04
27	YH	GD	0.109 ± 0.003 ^{f,g}	8.50 ± 0.03 ^f	12.70 ± 0.35	89.31 ^f	0.01 ^f	15.91
28	ZSX ^e	GD	0.053 ± 0.003 ^{f,g}	15.42 ± 0.18 ^f	12.12 ± 0.26	85.70 ^f	1.31 ^f	21.31
29	ZSX ^e	GD	0.012 ± 0.001 ^{f,g}	15.83 ± 0.07 ^f	4.73 ± 0.23	87.81 ^f	0.70 ^f	16.75
30	ZSX ^e	GD	0.171 ± 0.004 ^{f,g}	13.05 ± 0.16 ^f	11.75 ± 0.26	86.45 ^f	0.51 ^f	18.31

^a: The raw materials of the samples were soybeans and wheat flour except samples No. 9, 10 and 11 with defatted soybeans and wheat flour.

^b: BJ, Beijing (capital of China); SD, Shandong (north of China); LN, Liaoning (north-east); TJ, Tianjin (north); HLJ, Heilongjiang (north-east); JL, Jilin (north-east); GD, Guangdong (south); NMG, Neimenggu (north); HB, Hebei (north).

^c: Values were means ± standard deviations from triplicate analysis.

^d: Values were means of four determinations.

^e: Four kinds of products from the same manufacturer.

^f: Values in the same column with the superscript are significantly ($p < 0.0001$) different from other values.

^g: Values in the same column with the superscript are significantly ($p < 0.01$) lower than the others.

chroma meter (CR-300, Minolta Co., Ltd., Osaka, Japan) and recorded in the $L^*a^*b^*$ color system which consists of a luminance component (L^*) and two chromatic components, that is, the a^* value represented green (-a) to red (+a) and the b^* value represented blue (-b) to yellow (+b) colors. Dry sample powder was used for measurement. The chroma meter was calibrated using a standard white plate ($L^* = 96.82$, $a^* = 0.65$, $b^* = 2.32$).

7. Statistical analysis

Data were analysed by analysis of variance (ANOVA) using the general linear model (Version 8.0, SAS Institute Inc., USA). Duncan's multiple range test was used to determine the differences among samples. After a rejection test, correlation analysis was carried out by Spearman's rank correlation test. Significant levels were defined as probabilities of 0.05 or less.

Results and discussion

1. ACE inhibitory activities of soypaste extracts

As shown in Table 1, all the aqueous extracts of commercial Chinese style soypaste exhibited ACE inhibitory activities with the lowest and the highest IC_{50} values of 0.012 and 3.241 mg/mL, respectively. Samples No. 20 and 23-30 exhibited significantly stronger ACE inhibitory activities than other samples ($p < 0.0001$) with IC_{50} values ranging from 0.012 to 0.296 mg/mL, and the strongest activity was recorded in a ZSX sample produced in Guangdong Province of China. Compared with the literature data for the ACE inhibitory activities of various fermented soybean foods, such as the IC_{50} values of 0.51 mg/mL for tempeh, 0.66 and 1.77 mg/mL for tofuyo, 3.44 and 0.17-17.80 mg/mL for soy sauce, 2.38, 5.35 and 1.27 mg/mL for miso paste, and 0.16, 0.19, 0.40, and 0.27-0.44 mg/mL for natto, respectively^{6,8,11,12}, some Chinese style soypaste exerted much more powerful activities.

Figure 1 depicts the production region distribution of samples with different levels of ACE inhibitory activities referenced by reported data. Combined with results from Table 1, it is noticeable that samples produced in Guangdong Province possessed significantly higher ACE inhibitory activities than those collected from other regions ($p < 0.01$), although sample No. 19 showed a relatively low activity with an IC_{50} value of 1.626 mg/mL. Meanwhile, the two samples produced in Shandong Province, namely, samples No. 7 and 8 with IC_{50} values of 1.961 and 3.241 mg/mL, showed weak ACE inhibitory activities. Furthermore, although we might not have collected enough products, it seemed that samples manufactured in the south, north and north-east of China tended to possess relatively high, low and moderate ACE inhibitory activi-

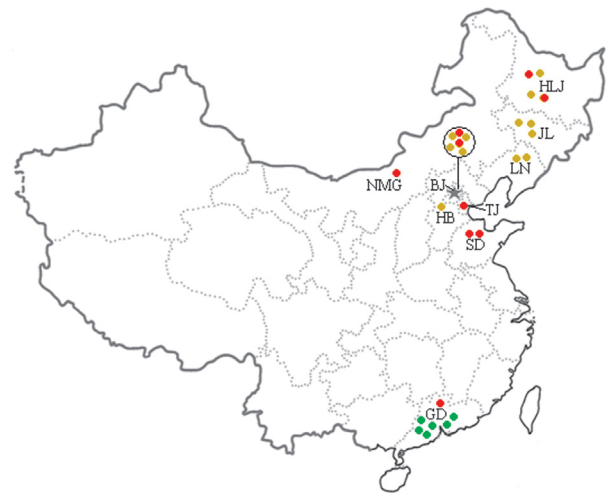


Fig. 1. Production region distribution of samples with different levels of ACE inhibitory activities

Green dots represent samples with IC_{50} values lower than 0.300 mg/mL, red dots represent samples with IC_{50} values higher than 1.000 mg/mL and yellow dots represent samples with intervenient IC_{50} values. Region symbols BJ and TJ represent Beijing and Tianjin City respectively, and SD, LN, HLJ, JL, GD, NMG, and HB represent Shandong, Liaoning, Heilongjiang, Jilin, Guangdong, Neimenggu, and Hebei Province, respectively.

ties, respectively. This phenomenon might be attributed to the especial soypaste processing techniques depending on production regions, for example, kinds of raw materials, starter cultures used, duration of ripening, and so on, which would affect the release of ACE inhibitors during fermentation. Further work is now in progress to develop improved processing techniques for producing soypaste with strong ACE inhibitory activity.

2. SDS-PAGE patterns of soypaste

The ACE inhibitors in soy-fermented foods had been mainly recognized as peptides resulting from proteolysis by microorganisms. SDS-PAGE patterns (Fig. 2) demonstrate the molecular weight distribution of proteins and peptides in the soypaste samples. According to Fig. 2, evident bands of 11S protein subunits were observed in the ten samples produced in Guangdong Province, among which almost all samples showed potent ACE inhibitory activities except sample No. 19. However, little evidence of proteins with large molecular weight was detected in other samples, and on the other hand, these samples generally exhibited average ACE inhibitory activities. It was reported that higher levels of the degree of soy protein hydrolysis did not guarantee a high ACE inhibitory activity due to the different properties of the enzymes used². Okamoto et al.¹¹ reported that the inhibitor with a low IC_{50} value in natto was a protein with high molecular weight.

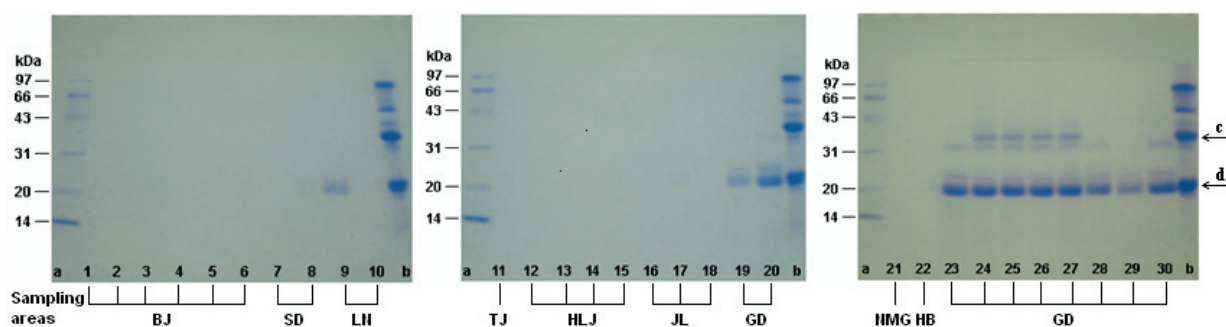


Fig. 2. SDS-PAGE patterns of soypaste samples

Lane *a* represents the molecular weight marker (low range, M.W. 14,400-97,400). Lane *b* represents the soybean protein isolated (SPI). Lanes 1 to 30 represent soypaste samples No. 1 to 30, respectively. Arrows *c* and *d* represent acidic and basic subunits of the 11S soy protein, respectively. Sampling areas BJ and TJ represent Beijing and Tianjin City respectively, and SD, LN, HLJ, JL, GD, NMG, and HB represent Shandong, Liaoning, Heilongjiang, Jilin, Guangdong, Neimenggu, and Hebei Province, respectively.

Moreover, starter cultures played important roles in generating bioactive substances during fermentation¹. The potential possibility might be because the microorganisms, which were used in soypaste manufactured in Guangdong Province, were more helpful in releasing ACE inhibitors than those in other regions. In regard to the four kinds of ZSX samples, i.e., samples No. 20, 28, 29, and 30, in which the manufacturer probably used the same starter, it was found that the ACE inhibitory activities increased with the increasing degrees of proteolysis (Fig. 2), indicating that, for a certain starter, proper protein hydrolysis would be beneficial to produce ACE inhibitory peptides.

3. Chemical parameters and colors of soypaste

According to Table 1, there were no obvious correlations between the ACE inhibitory activities and the chemical parameters of the tested samples. However, paying attention to the nine samples that possessed potent ACE inhibitory activities with low IC_{50} values less than 0.300 mg/mL, it was found that these samples had significantly lower peptide contents ($p < 0.0001$) than other samples, which coincided with the slighter proteolysis degrees in these samples. This result demonstrated that high peptide contents wouldn't lead to high ACE inhibitory activities of soypaste, whereas many ACE inhibitors derived from fermented soybean foods were identified as peptides. After all, it had been reported that the ACE inhibitory activities of peptides fairly depended on the structures of the peptides¹⁰. In addition, it was found that the ratios of peptides to total soluble carbohydrate contents, reflecting the ratios of soybeans to wheat flour in the fermentation mixture, to a certain extent, were increased, that is, 1:0.90, 1:0.79, 1:0.66, and 1:0.30, in the four ZSX samples with the increase in ACE inhibitory activities. Our result suggested that the difference in raw materials might influence the capability of generating ACE inhibitors for the

microorganism used in soypaste processing. Okamoto et al.¹² pointed out that the ratio of raw materials affected the activities of products when they investigated the ACE inhibitory activities of Japanese soy sauce and *miso*. These results were consistent.

Generally, soypaste products showed dark colors resulting from Maillard reactions that occurred during fermentation. Rufián-Henares and Morales¹⁴ reported that melanoidins derived from aqueous Maillard reaction model systems showed some ACE inhibitory activities. Okamoto et al.¹² reported that the soy sauces with thin color showed lower ACE inhibitory activities than those with dark color. In this work, it was found that L^* and a^* values were significantly correlated with the ACE inhibitory activities of the tested samples (Fig. 3). Meanwhile, the darkness and reddish colors of samples No. 20, and 23-30 with potent ACE inhibitory activities were significantly reduced as evidenced by the higher L^* values ($p < 0.0001$) and the lower a^* values ($p < 0.0001$) than other samples (Table 1), which was not consistent with the results described by Okamoto et al.¹². In these samples, protein hydrolysis was evidently insufficient (Fig. 2) and thus produced relatively less amino compounds, which might to some extent reduce the occurrence of Maillard reactions and resulted in the light colors of the products. On the other hand, it was also suggested that colored compounds might not be quite responsible for the ACE inhibitory activities of these kinds of Chinese soypaste products. It was reported that nicotianamine was the ACE inhibitory substance in soy sauce⁷. Whereas we haven't identified the materials that contributed to ACE inhibitory activities of the samples, it was presumed that the total activity might occur from various bioactive substances in different kinds of Chinese soypaste.

Although there is not enough information to make sure how the products with strong ACE inhibitory

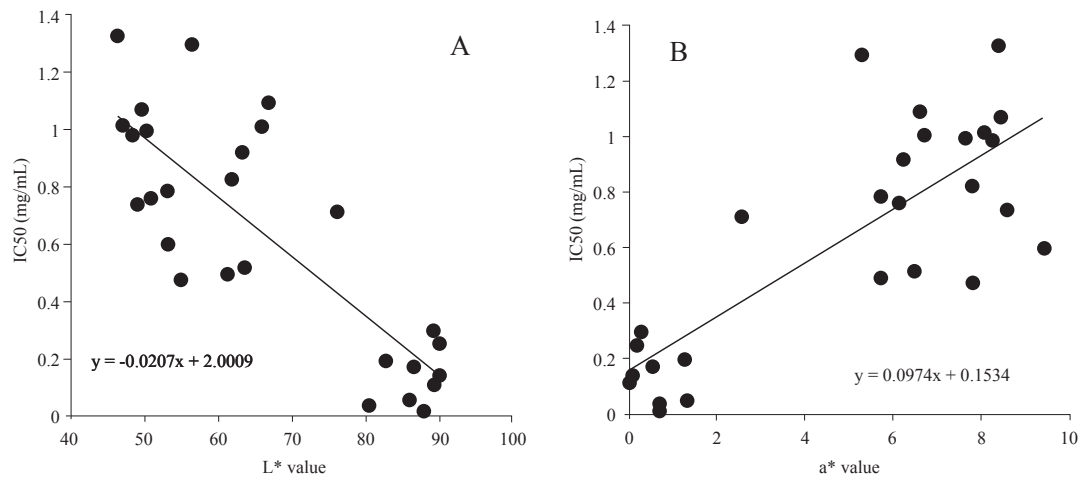


Fig. 3. Linear correlations between ACE inhibitory activities and L* (A) or a* (B) values of soypaste samples
Both showed significant correlations at the $p < 0.01$ level with correlation factors of -0.734 or 0.696 , respectively.

activities were manufactured, particularly those produced in Guangdong Province, further investigations are being conducted to clarify the processing factors that might result in high ACE inhibitory activities during soypaste fermentation and to explore ACE inhibitors derived from Chinese style soypaste in order to design functional products with potential antihypertensive effects.

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