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Angiotensin I-converting enzyme inhibitory peptides in douchi, a Chinese traditional fermented soybean product

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Abstract

Douchi, a soybean product originating in China, produces angiotensin I-converting enzyme (ACE) inhibitors with the potential to lower blood pressure. The ACE inhibitory activities of douchi qu pure-cultured by *Aspergillus Egyptiacus* for 48 h, and 72 h were compared with douchi secondary-fermented for 15 d. The results showed that ACE inhibitory activities were improved following the fermentation. ACE inhibitory activities of 48 h-primary-fermented douchi qu did not change dramatically after preincubation with ACE, but increased greatly after preincubation with gastrointestinal proteases. The results suggest they were pro-drug-type or a mixture of pro-drug-type and inhibitor-type inhibitors. The ACE inhibitors in 48 h-fermented douchi qu were fractionated into four major peaks by gel filtration chromatography on Sephadex G-25. Peak 2, which had the highest activity, had only one peptide, composed of phenylalanine, isoleucine and glycine with a ratio of 1:2:5.

Keywords: Douchi; ACE inhibitory activity; Peptide

1. Introduction

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxypeptidase I, kinase II, EC. 3.4.15.1) plays a key physiological role in the control of blood pressure, via the rennin–angiotensin system (Lee, Kima, Park, Choi, & Lee, 2004). Oshima, Shimabukuro, and Nagasawa (1979) first reported ACE-inhibitory peptides produced from food protein by digestive proteases. Since then, many other ACE-inhibitory peptides have been discovered from enzymatic hydrolysates of different food proteins. These food protein sources include soybean protein (Shin et al., 2001; Wu & Ding, 2001), casein (Kim & Chung, 1999; Tauzin, Miclo, & Gaillard, 2002), chicken muscle and chicken egg (Fujita, Yokoyama, & Yoshikawa, 2000; Yoshii et al., 2001), whey protein (Mullally, Meisel, & FitzGerald, 1997; Pihlanto-Leppa''la'', 2001), and fish protein (Curis, Dennes, Waddell, Macgillivray, & Ewart, 2002; Wako, Ishikawa, & Muramoto, 1996). Other soybean fermented products have been found that contain antihypertensive peptides, such as sufu (Iwamik & Buki, 1986), soy sauce (Kinoshita, Yamakashi, & Kikuchi, 1993), Korean soy paste, tempeh (Gibbs, Zougman, Masse, & Mulligan, 2004) and natto (Okamoto, Hanagata, Kawamura, & Yanagida, 1995a, 1995b). These findings suggest that such peptides might be utilized to control blood pressure, especially among people with a high risk of essential hypertension (Yoshii et al., 2001).

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ACE-inhibitory peptides must survive gastrointestinal digestion and be absorbed, in order to reach the cardiovascular system in an active form. Therefore, simulation of gastrointestinal digestion can be used to detect the ACE-inhibitory peptides (Ruiz, Ramos, & Recio, 2004).

Peptides showing in vitro ACE-inhibitory activity can be classified into three groups: (1) true inhibitor type, with 50% inhibition (IC_{50}) values of peptides that are not affected by preincubation with ACE or gastrointestinal proteases, (2) substrate type, with peptides that are converted to true inhibitors by ACE or gastrointestinal proteases, resulting in peptides with weaker activity, and (3) pro-drug type, with peptides that are converted to true inhibitors by ACE or gastrointestinal proteases (Fujita et al., 2000; Li, Le, Shi, & Shrestha, 2004).

Douchi is a traditional fermented soybean product originating in China. It has been used as seasoning for foods and for pharmaceutical purposes since before the Han dynasty (206 B.C.); even now it is still added to some Chinese traditional medicines. More recently, soy paste and soy sauce have been developed from douchi. Some researches have showed that douchi has an effect of α-glucosidase inhibitory activity (Fujita, Yamagami, & Ohshima, 2001a, 2001b), but little information has been published about its ACE inhibitory activity.

In this study, ACE inhibitory activities of douchi products fermented for 48 h, 72 h, and 15 d were compared. Peptides with ACE inhibitory activities were extracted from *Aspergillus egypticus*-fermented douchi qu, purified, and analyzed.

2. Materials and methods

2.1. Materials and chemicals

ACE (extracted from rabbit lung acetone powder), hippuric acid–histidine–leucine, OPA (O-phthaldialdehyde), pepsin (EC.3.4.23.1) and trypsinase (EC.3.4.21.4) were bought from Sigma Chemical Co., St. Louis, MO. All other chemicals used were of analytical grade.

2.2. Preparation of douchi qu and douchi

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

1. Black soybeans were soaked at 30 °C with 3 times their volume of water for 4 h. After draining, they were steamed at 121 °C for 50 min.
2. Primary Fermentation. Soybeans were inoculated with a pure culture fermentation starter (1%, v/w) inoculum of 10^7 A. *egypticus* spores/ml. The soybeans were cultured at 30 °C and 90% humidity for 48 h in an incubator (LTI-601SD, Tokyo Rikakikai Co. Ltd., Tokyo). 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 d. The product is douchi.

2.3. Preparation of natto

Natto was produced in Petri dishes, in triplicate, according to the method of Gibbs et al. (2004). Fifty grammes of soybeans were cleaned and soaked overnight until they doubled in weight. The soaked beans were steamed at 121 °C for 30 min, then cooled to 40 °C before inoculation with *Bacillus subtilis* (1 ml/100 g) and incubated at 40 °C for 24 h. Samples were left at 5 °C for 24 h to complete maturation prior to analysis.

2.4. Extraction of ACE inhibitor

Lyophilized douchi and douchi qu samples (5 g) were suspended in 50 ml of distilled water. After agitating at normal temperature for 1 h, the suspension was centrifuged (3000*x* g for 10 min) and the supernatant was filtered. The filtrate was used to assay ACE activity. The concentration was labeled as 100 mg/ml.

2.5. ACE inhibitory activity assay

ACE solution (0.1 ml, 0.025 U/ml) was preincubated with each sample (50 μl) for 15 min at 37 °C. The enzymatic reaction was initiated by adding 100 μl of 4.7 mM of the substrate Bz-Gly-His-Leu in 400 mM phosphate buffer (pH 8.5 with 600 mM NaCl) to the mixture. After incubation for 30 min at 37 °C, the reaction was stopped by adding 1.5 ml of 0.3 N NaOH.

OPA (0.1 ml, 2%) was stirred into the ACE mixture, which was then allowed to sit for 10 min, before adding 0.2 ml 3 M HCl to stop the reaction. The solution was diluted 250 times and the fluorescence intensity was determined in 30 to 90 min with an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The ACE activity was calculated as follows:

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

$$\text{ACE inhibitory activity}\ (\%) = 1 - \text{ACE}\ (\%),$$

where a is the fluorescence intensity in the presence of ACE inhibitor and ACE inhibitor component; b is the fluorescence intensity without inhibitor; c is the fluorescence intensity without inhibitor and ACE; The IC_{50} value is the concentration that inhibits 50% of ACE activity.

2.6. Classification of ACE-inhibitory peptide by preincubation method

The stability of each individual peptide to ACE was determined, following the method described by Fujita et al. (2000). The sample solution was diluted to 20 mg/ml; then a 500 μl diluted sample was added to 500 μl of ACE solution (0.025 U/ml). The mixture was incubated at 37 °C for 4 h and the reaction was stopped by boiling for 10 min. After cooling, the ACE inhibitory activities were assayed. The procedure for sample digestion by gastrointestinal proteases and ACE inhibitory activity assaying (Fig. 1) was as described by Matsui et al. (2002).

2.7. Purification of ACE-inhibitory peptide

ACE-inhibitory peptide was purified by a Sephadex-G25 column (∅10 mm × 750 mm). The mobile phase was: $NaH_2PO_4 \cdot 2\,H_2O$ (3.545 g), $NaH_2PO_4 \cdot 12\,H_2O$ (6.190 g), NaCl (11.688 g), NaN_3 (0.4 g), and H_2O (2 l) with a flow rate of 0.2 ml/min. Elution curves were obtained by measuring absorbance of each fraction at 280 nm and 220 nm using a spectrophotometer (UV-1240, Shimadzu, Kyoto). Separation of the peptides from the elution of fractions showing ACE inhibitory activities was performed by HPLC, using a Vydac 218TP54 column kept at 40 °C.

After elution with 0.1% TFA at a flow rate of 1 ml/min for 3 min, 20 μl of samples, which had filtered through 0.45 μm filters, were injected into the HPLC. A linear gradient of 0– 60% acetonitrile in 0.1% TFA was applied over 60 min at the same flow rate. The elution was monitored at 220 nm with a UV detector (Suetsuna, 1998; Yust et al., 2003).

2.8. Amino acid analysis

Amino acid analysis was performed using a modified procedure described by Abiodun, Anthony, and Omolara (1999). Eight millilitres of 6 N HCl were added to a 100 mg sample in a sealed tube. The tube was placed in an oil bath at 110 °C for 24 h. A Hitachi 835-50 amino acid autoanalyzer (Hitachi Co. Ltd., Japan) was used for separating the amino acids.

2.9. Statistical analysis

Analysis of variance was done using general linear model (GLM) procedures, followed by Duncan's multiple range tests. Significant differences between the sample means were determined at the 5% significance ($p < 0.05$) level.

3. Results and discussion

3.1. The ACE inhibitory activities of products with different fermented time

The ACE inhibitory activities of natto and douchi qu, fermented by *A. egypticus* for 24 h, 48 h and 72 h, and douchi secondary-fermented for 15 d were measured. The ACE inhibitory activities of douchi qu at 24 h, 48 h, 72 h and douchi fermented for 15 d with a concentration of 10 mg/ml were 7.8%, 66.2%, 72.4% and

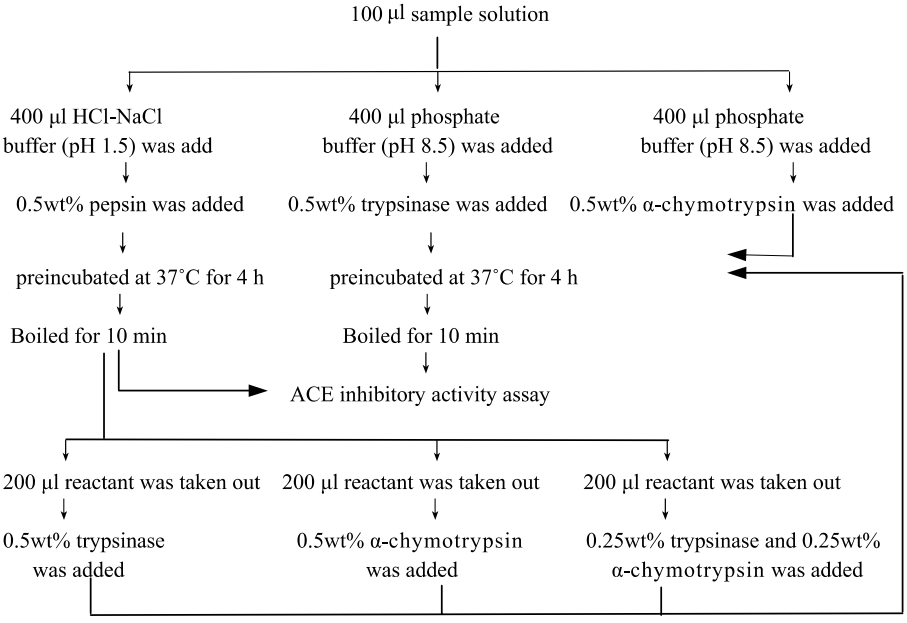

Fig. 1. The procedure of digestion by gastrointestinal protease and ACE activity assaying.

86.3%, respectively. The results (Fig. 2) showed that the inhibitory activity was improved following fermentation and that 24 h-fermented douchi qu was only 7.8% ACE inhibitory, suggesting that the ACE inhibitors were produced during the fermentation.

3.2. Classification of ACE-inhibitory peptide

Extracts of 48 h-fermented and 72 h-fermented douchi qu were preincubated with ACE before measurement of ACE-inhibitory activity (Table 1). After preincubation, the ACE-inhibitory activity of 48 h-fermented douchi qu increased from 56.8% to 60.4%, while 72 h-fermented douchi qu decreased from 76.3% to 72.7%. Both differences were not greater than 5%.

To investigate the resistance of the inhibitors to digestion by gastrointestinal proteases, 48 h-fermented douchi qu extracts were digested with various proteases. All the IC_{50} values of the samples hydrolyzed by gastrointestinal proteases were lower than IC_{50} values of 48 h-fermented douchi qu (Table 2). The highest and the lowest IC_{50} values were 0.61 mg/ml and 0.019 mg/ml, respectively. The former was of sample hydrolyzed with trypsin (less than 12.5% of the unhydrolyzed sample). The latter was of sample hydrolyzed with pepsin, followed by chymotrypsin (only 3.7% of 48 h-fermented douchi qu).

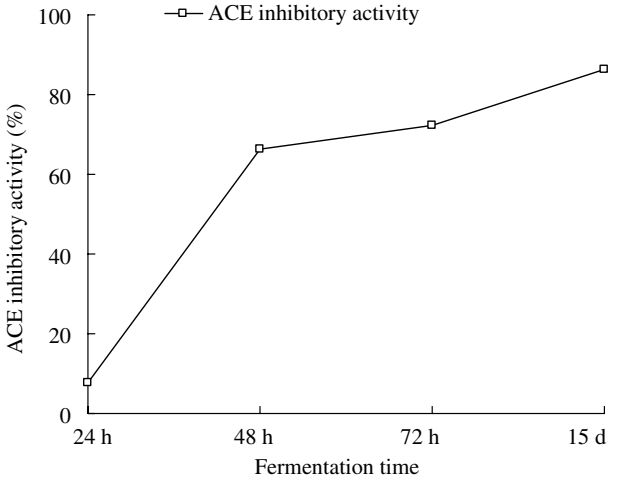

Fig. 2. The ACE inhibitory activities as a function of fermentation time.

Table 1
Changes in ACE inhibitory activity after pre-incubation with ACE

ACE inhibitory activity (%)	Sample	
	48 h qu	72 h qu
Non-preincubation	56.8 ± 4.52	76.3 ± 6.32
Preincubation	60.4 ± 3.78	72.7 ± 5.27

The changes in ACE inhibitory activities of douchi qu when treated with ACE and gastrointestinal proteases suggest that the inhibitors in 48 h-fermented douchi qu were of the pro-drug type or a mixture of the pro-drug type and the true inhibitor type.

3.3. Purification of ACE-inhibitory peptide

Extracts of 48 h-fermented douchi qu were purified with a Sephadex-G25 column. The OD_{220} and OD_{280} values of each fraction were measured and the ACE inhibitory activities of some fractions were assayed. The results (Fig. 3) indicated that fractions with ACE-inhibitory activity correlated with chromatographic peaks observed at OD_{220}. Chromatographic peaks with OD_{280} had a poor correlation with ACE-inhibitory fractions.

Four peaks were observed at OD_{220}: fraction 15–18 (Peak 1), fraction 26–31 (Peak 2), fraction 34–40 (Peak 3), and fraction 56–60 (Peak 4). Peak 2 and Peak 3 show high ACE inhibitory activities, with fraction 29 and fraction 34 at 80.5% and 77.9%, respectively. In contrast, ACE inhibitory activities of fraction 16 and fraction 57 were 42.3% and 53.9%, respectively. The results demonstrated that the inhibitors had a wide range of molecular weight.

The sample hydrolyzed with pepsin with subsequent tryptic and α-chymotryptic digestion was also purified with a Sephadex-G25 column. As illustrated in Fig. 4, only one broad peak, from fractions 19–65, was observed. The volume of each fraction was 8 ml. Fractions 28–54 showed clear ACE-inhibitory activity. Fractions 28, 36, 45 and 54 had ACE-inhibitory values of 45.6%, 68.3%, 51.2% and 30.2%, respectively. The maximum inhibitory activity was 72.1%, observed in fraction 39.

The ACE-inhibitory activity of the 48 h-fermented douchi qu increased from fraction 14 (× 6 ml) to fraction 44, with a minor peak from fractions 54–60. The OD_{220} elution curves of inhibitors digested by gastrointestinal proteases had only one peak corresponding to fractions

Table 2
IC_{50} value of douchi qu digested with gastrointestinal proteases (mg/ml)

Sample	IC_{50}	Sample	IC_{50}
48 h-fermented qu	5.09	Hydrolyzed with pepsin followed by trypsinase	0.057
Hydrolyzed by pepsin	0.16	Hydrolyzed with pepsin followed by chymotrypsin	0.019
Hydrolyzed by chymotrypsin	0.15	Hydrolyzed with pepsin followed by trypsinase and chymotrypsin	0.051
Hydrolyzed by trypsinase	0.61	Natto	4.54

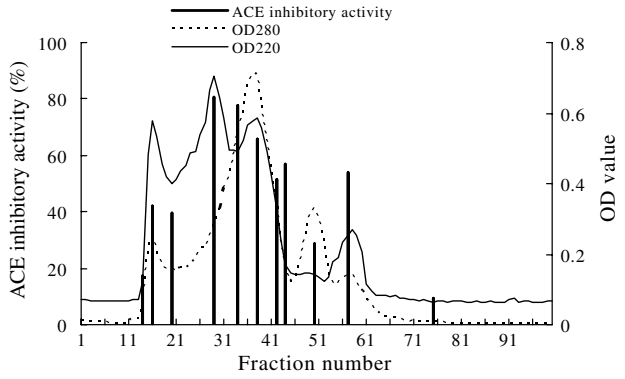

Fig. 3. Elution profile of ACE inhibitor extracted from douchi qu.

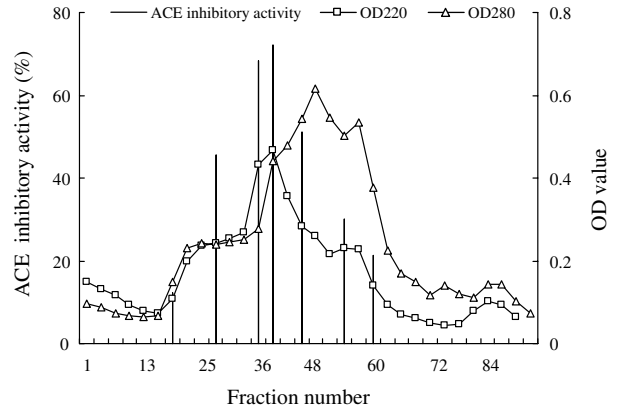

Fig. 4. Elution profile of ACE inhibitor digested with gastrointestinal proteases.

28–54 (8 ml per fraction). Elution volumes required for elution of digested ACE-inhibitors were larger than elution volumes required before digestion, suggesting that the molecular weight of peptides with ACE-inhibitory activity had decreased after hydrolysis.

The peptides in each fraction that showed ACE inhibitory activities were separated by high performance liquid chromatography (HPLC). Figs. 5–8 show the elution patterns of Peaks 1–4. Fraction 26–31 and fraction 56–60 have only one HPLC absorbance peak. This indicates that the inhibitory peptide in these fractions was pure. Conversely, the composition of Peaks 1 and 3 was complex. The HPLC chromatograms of fractions 38 and 39 were very similar to Peak 3 (not shown in figures).

Amino acid analyses of Peak 1 and Peak 2 were performed in order to assay the composition of the peptides with ACE inhibitory activity. Data presented in Table 3 indicated that Peak 1 has a high content of Asx and Glx, along with all the amino acids except Met. The amino acid composition of Peak 2 showed that the peptide with ACE inhibitory activity was composed of Phe, Ile and Gly, with a content of 0.29 mmol/100 ml, 0.60 mmol/100 ml and 1.48 mmol/100 ml, respectively.

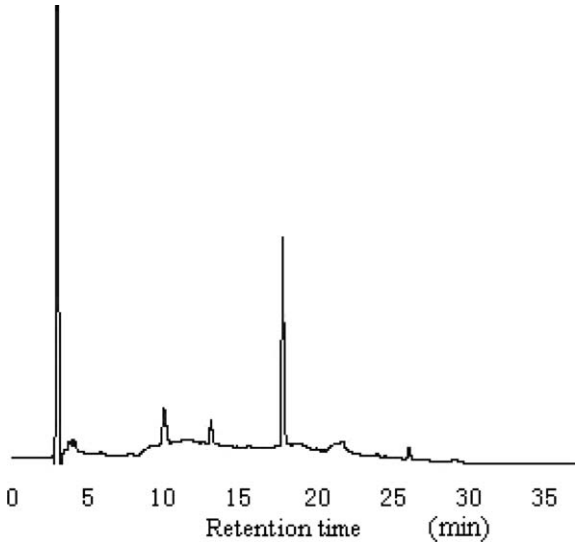

Fig. 5. HPLC chromatogram of Peak 1.

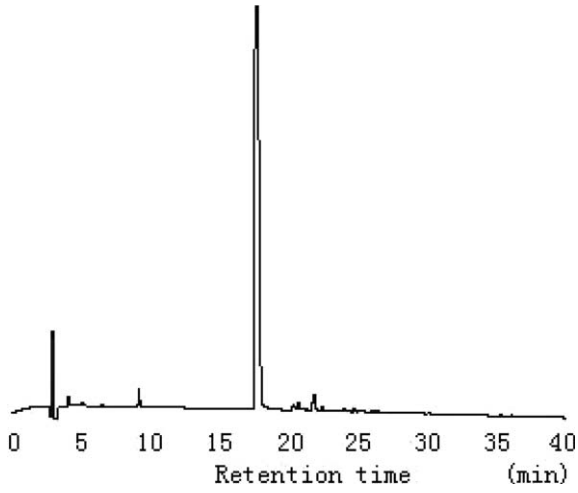

Fig. 6. HPLC chromatogram of Peak 2.

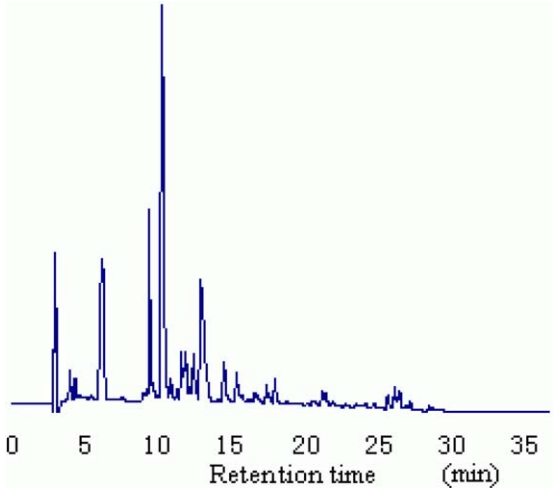

Fig. 7. HPLC chromatogram of Peak 3.

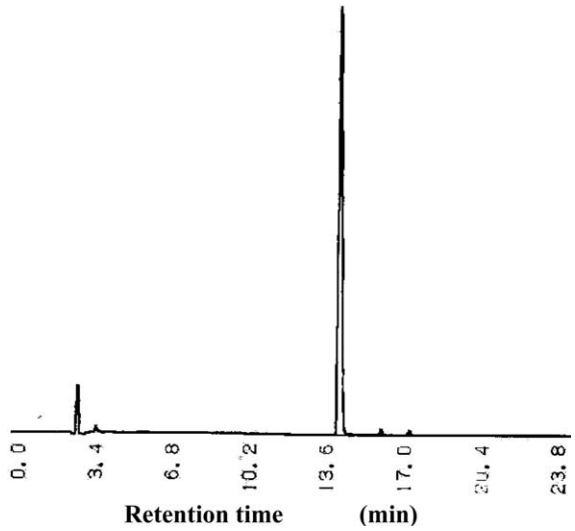

Fig. 8. HPLC chromatogram of Peak 4.

Table 3
Amino acid content of Peaks 1 and 2

Amino Acid	Concentration (mmol/100 ml)	
	Peak 1	Peak 2
Asx	4.95	/
Thr	0.31	/
Ser	1.10	/
Glx	8.99	/
Pro	1.30	/
Gly	1.69	0.29
Ala	0.72	/
Val	1.45	/
Ile	0.69	0.60
Leu	0.63	/
Tyr	0.79	/
Phe	1.27	1.48
Lys	1.10	/
His	0.50	/
Arg	0.72	/

4. Conclusion

Douchi qu, fermented by *A. egypticus*, showed clear ACE inhibitory activity, which was enhanced following primary and secondary fermentation. The ACE inhibitory activity of douchi qu was not affected by ACE preincubation, but improved greatly after digestion by gastrointestinal proteases. This indicates that the inhibitors were of the pro-drug type or a mixture of the pro-drug type and the true inhibitor type.

The elution profile of the 48 h-fermented douchi qu showed that the OD_{220} had 4 peaks, each demonstrating clear ACE inhibitory activity. The OD_{220} elution profiles of inhibitors digested by gastrointestinal proteases had only one peak, with an increased elution volume, indicating that the molecular weight of digested inhibitors was decreased.

The HPLC chromatogram of the four active peaks revealed that Peaks 2 and 4 had only one elution peak. The results of the amino acid analysis showed that the peptide with ACE inhibitory activity in Peak 2 was composed of Phe, Ile and Gly, with a ratio of approximately 1:2:5. Peaks 1 and 3 were more complex, as confirmed by amino acid analysis.

The ACE-inhibitory activity improved following primary and secondary fermentation. Inhibitors were of the pro-drug type or a mixture of the pro-drug type and the true inhibitor type, indicating that douchi may be an antihypertensive food.

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