Identification of four new angiotensin I-converting enzyme inhibitory peptides from fermented anchovy sauce

Hyun-Jin Kim1 · Seong-Gook Kang2 · Lily Jaiswal2 · Jinglei Li2 · Ju-Hee Choi2 · Sang-Mi Moon2 · Jeong-Yong Cho2 · Kyung-Sik Ham2

Received: 15 September 2015 / Accepted: 26 September 2015 / Published online: 15 January 2016
© The Korean Society for Applied Biological Chemistry 2016

Abstract The inhibitory activity of an angiotensin I-converting enzyme (ACE), a key regulatory enzyme of blood pressure from the fermented anchovy sauce, was evaluated, and ACE inhibitory peptides were purified. The ACE activity significantly increased with an increase in salt concentration. In addition, the ACE inhibitory activity of the fermented anchovy sauce containing high salt content (25 %) significantly increased with an increase in fermentation time. The maximum activity (96 %) was reached after 15 months of fermentation. Four ACE inhibitors [Pro-Lys (PK), Gly-Cys-Lys (GCK), Asn- His-Pro (NHP), and Asp-Gly-Gly-Pro (DGGP)] in the fermented anchovy sauce were purified through various chromatographic techniques and identified by electrospray ionization tandem mass spectrometer analysis. Four newly identified peptides were synthesized and analyzed for ACE inhibitory activity in order to confirm that the purified peptides were actually ACE inhibitors. The IC_{50} values for ACE inhibitory activities of synthesized peptides DGGP, GCK, NHP, and PK were 164, 178, 1172, and 4092 μM, respectively.

Keywords Angiotensin I-converting enzyme inhibitor · Fermentation · Fermented anchovy sauce · Hypertension · Peptide · Salt

Introduction

Hypertension, a major risk factor of cardiovascular diseases, is increasingly a global public health concern. The worldwide mortality prevalence of hypertension was estimated about 9.4 million every year (WHO 2008) and expected to rise to 1.56 billion by 2025 (Jao et al. 2012). A rennin–angiotensin–aldosterone system regulates blood pressure and fluid balance in the body. In particular, the angiotensin I-converting enzyme (ACE) plays an important role in controlling blood pressure (Wyvratt and Patchett 1985). That is, ACE converts angiotensin I to angiotensin II, a vasoconstrictor that releases aldosterone and increases blood pressure. Therefore, ACE inhibition is considered to be a major factor in preventing hypertension (Lau et al. 2013). Recently, the study has been focused on looking for safe and natural ACE inhibitors derived from foods and microorganisms because synthetic ACE inhibitors such as captopril, enalapril, lisinopril, and ramipril cause several side effects (Patchett et al. 1980).

ACE inhibitory peptides have been isolated from various food proteins such as fermented milk (Pihlanto et al. 2010), fish (Je et al. 2005), chicken breast muscle (Saiga et al. 2003), soybean (Wu and Ding 2001), and mushroom (Majumdar and Wu 2011). In particular, fermented foods contain a lot of low-molecular weight peptides that show ACE inhibitory activities (Shin et al. 2001; Gomez et al. 2002; Je et al. 2004; Robert et al. 2004).

Among the fermented foods, fermented anchovy sauce is prepared by fermenting a mixture of fresh anchovy and a
high salt concentration (20–25 %) for >12 months. The sauce is generally used as a seasoning agent in Korea and has various biological activities such as fibrinolytic, anticancer, ACE inhibitory, and antioxidant activities (Park et al. 1999; Lee et al. 2002; Ichimura et al. 2003; Park and Kim 2003). ACE inhibitory dipeptides including AP, KP, GP, EP, TP, VP GI, and DF from fermented anchovy sauce have been previously reported (Ichimura et al. 2003). In this case, fermented anchovy sauce was prepared with purified salt. However, the ACE inhibitory peptides of fermented anchovy sauce that was prepared with solar sea salt have not been investigated in detail yet.

The present investigation was aimed at the purification and identification of ACE inhibitory peptides from the fermented anchovy sauce using various chromatographic techniques and an electrospray ionization tandem mass spectrometer (ESI–MS), with guided purification.

Materials and methods

Chemicals and materials

The fermented anchovy sauce was prepared by fermenting anchovies with solar sea salt (25 % of total anchovy weight) at 10 °C for 12–24 months in Korea. The samples obtained during fermentation were immediately stored at −70 °C until use to determine ACE inhibitory activity and purify the compounds. Rabbit rung acetone powder, N-hippuryl-L-histidyl-L-leucine (HHL), hippuric acid, and captopril were purchased from Sigma Chemical Co. (USA). Solvents used for analyses were of high-performance liquid chromatography (HPLC) grade. All other chemicals were of reagent grade.

Determination of ACE inhibitory activity

The ACE inhibitory activity of the fermented anchovy sauces, the fractions purified from the chromatographies, and active peptides was measured by methods of Cushman and Cheung (1971), with slight modifications. The sample (50 μL) was mixed with 100 μL of a 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl and 50 μL of 5 mM HHL solution as a substrate. The mixture was pre-incubated at 37 °C for 10 min. After pre-incubation, the mixture was added to a 100 μL of ACE extract from lung acetone powder of a rabbit and further incubated at 37 °C for 1 h. The reaction was stopped by the addition of 0.1 mL of 1 N HCl and partitioned with 1 mL of ethyl acetate. The ethyl acetate layer (0.7 mL) was completely concentrated at 90 °C using nitrogen gas. The concentrate containing hippuric acid was dissolved in 1 mL of 1 M NaCl solution, and the absorbance was determined at 228 nm. The ACE inhibitory activities of the samples were determined when there was a percentage decrease in absorbance than a control test. The concentration of an ACE inhibitor required to inhibit 50 % of the ACE activity under same reaction condition was expressed as IC50.

The effect of salt concentration on ACE activity

Various salt concentrations (50 μL) were mixed with 100 μL of ACE solution, 50 μL of 5 mM HHL solution, and 100 μL of 0.1 M sodium borate buffer (pH 8.3). Final salt concentrations in the reaction solution were 0, 8.3, 16.6, 25, 33.3, 41.6, and 50 mg/mL. The reaction mixture was incubated as described above, and the absorbance was measured at 228 nm. The reaction mixture without salt was taken as a control.

Separation of ACE inhibitors from fermented anchovy sauce

The fermented anchovy sauce was filtered through ultrafiltration using a YM-3 membrane (MWCO: 3 kDa, Bio-Rad, Sweden). To remove the salts and separate ACE inhibitory compounds from the fermented anchovy sauce, Sephadex G-10 column chromatography was used. Briefly, the fermented anchovy sauce (6 mL) was separated on the column (2.5 × 100 cm) packed with Sephadex G-10 resin (Amersham Biosciences, Sweden) and eluted with distilled water (450, 5 mL/each fraction) at a flow rate of 4 mL/min. The absorbance of all fractions was monitored at 280 nm. The active fractions purified from Sephadex G-10 column chromatography were collected and concentrated. The concentrate was precipitated with methanol, and the supernatant was further separated on a DEAE Sephadex A-25 column (Amersham Biosciences). The column (2.5 × 20 cm) was previously equilibrated with a 20 mM Tris–HCl buffer (pH 8.0, solvent A). The active fraction (5 mL) was injected, and an elution was performed using a linear gradient to 0.5 M NaCl solution (solvent B): started with 100 % A for 55 min, increased to 100 % B for 120 min, and held at 130 min at a flow rate of 4 mL/min and monitored at 280 nm. Fractions of 6 mL were collected. The active fractions, which were purified by DEAE Sephadex A-25 column chromatography, were fractionated on a Sephadex LH-20 column (2.5 × 100 cm). Elution was done with 20 % methanol at a flow rate of 4 mL/min. Fractions of 6 mL were collected. Further separation of peptides from fractions showing ACE inhibitory activities was performed by reverse-phase (RP) HPLC equipped with a YMC™ ODS-AQ reverse-phase C18 column (250 × 4.6 mm, 5 μm; Waters, USA) and a Shimadzu SPD-M10V photodiode array detector (Japan). The column was initially equilibrated with water containing 0.05 %
trifluoroacetic acid (TFA). The active fraction (50 μL) was injected and eluted with a linear gradient to 30 % acetonitrile for 30 min at a flow rate of 1 mL/min. The compounds were scanned at 200–500 nm, and the peaks were collected. The pooled peaks were concentrated and applied to Superdex peptide 10/300 GL gel–filtration column (Amersham Biosciences) equilibrated with distilled water. Samples were eluted with distilled water at a flow rate of 0.4 mL/min, and the absorbance of the eluant was scanned at 200–500 nm.

**Identification of ACE inhibitor by Q-TOF-ESI–MS/MS**

De novo peptide sequencing on a single charged peptide and molecular peptide mass was carried out by ESI–MS/MS (Waters Corp.) using the quadrupole time-of-flight (Q-TOF) micro-mass spectrometer with capillary liquid chromatography using Masslynx software (V4.0, Waters Corp.). Each peptide sample was directly injected into the positive electrospray ion source and eluted for 10 min using 2 % aqueous acetonitrile containing 0.1 % formic acid at a flow rate of 1.5 μL/min. The mass scanning range was 300–1500 msu at 1 s with a 0.1 s inter-scan delay in continuum mode. Glu-fibrinopeptide was used in MS and MS/MS mode and infused through the nanoLockspray (Waters Corp.) for single-point real-time external mass calibration. Raw spectra were processed using Masslynx software and pacified by using the Savitzky and Golay method. A precursor ion scan of a purified fraction was used to identify unique peptide mass, which was then fragmented using low-energy collision-induced dissociation to reveal the peptide fragment. Manual sequence validation was completed using Biolynx software (V4.0).

**Peptide synthesis**

Purified peptides were synthesized by the solid phase, fluorenylmethoxycarbonyl chemistry, at New England Peptide, Inc. (USA). After the synthesis process, all peptides were purified by YMC C₁₈-HPLC eluting with a liner gradient of water containing 0.1 % TFA and acetonitrile containing 0.08 % TFA, and then, their molecular masses were determined by MALDI-TOF.

**Statistical analysis**

The experimental data pertaining to ACE inhibitory activities of synthesized peptides, fermentation time, and the effect of salt on ACE activity were subjected to analysis of variance (ANOVA) and analyzed by nonlinear regressions (SAS 9.1, SAS Inst. Inc., USA). The significant differences were determined using Duncan’s multiple range tests ($p < 0.05$).

**Results and discussion**

**Change in ACE inhibitory activity of fermented anchovy source during fermentation**

The ACE inhibitor activity of the fermented anchovy sauce significantly increased with an increase in fermentation time (Fig. 1). That is, the ACE inhibitory activity of the fermented anchovy sauce at 12 months of fermentation was 67 %; the inhibitory activity increased to 96 % at 15 months of fermentation and then saturated. Our data were similar to the previous results that ACE inhibitory activity increased during fermentation of anchovy sauce (Park and Kim 2003). This result also implicated that the commercial anchovy sauce fermented for 18–24 months exhibited high ACE inhibitory activity. The increase of ACE inhibitory activity in anchovy sauce might be at least partially due to the production of ACE inhibitory peptides as a result of protein hydrolysis by enzymes during fermentation. To verify this, the anchovy sauce fermented for 24 months was used to separate and identify ACE inhibitor peptides.

The fermented anchovy sauce was prepared by fermenting the mixture of fresh anchovies and high salt concentration (25 %). High salt intake has been reported to stimulate ACE activity and lead to an increase in blood pressure (Denton et al. 1995; He and Macgregor 2003). Therefore, it was necessary to know how ACE activity was

---

**Fig. 1** Changes in ACE inhibitory activity of fermented anchovy sauce during fermentation. The reaction solution containing 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, 5 mM HHL solution as a substrate, and rabbit rung acetone powder solution as an ACE source was incubated at 37 °C for 1 h. The absorbance of released hippuric acid was determined at 228 nm.
affected by various salt concentrations before purification of ACE inhibitors from the fermented anchovy sauce that contained high salt concentration. The ACE activity significantly increased with an increase in salt content and reached the maximum at 25 mg salt/mL (Fig. 2). The maximum ACE activity was 40 % higher compared to that of the reaction solution without salt. In this study, also it was confirmed that salt stimulated ACE activity. However, the fermented anchovy sauce had high ACE inhibitory activity in the presence of high salt concentration, which strongly suggested that ACE inhibitory peptides might be contained in this sauce.

**Separation of ACE inhibitors**

To separate low molecular ACE inhibitors from the fermented anchovy sauce, the anchovy sauce that had been fermenting for 24 months was filtered using an ultrafiltration membrane with low molecular weight (3 kDa) cutoff membrane; all sauce was filtered through the membrane (data not shown). This implicated that all high molecular compounds were hydrolyzed to low molecular compounds (less than 3 kDa) by various enzymes released from microorganisms during the fermentation. However, the filtrate still contained high amounts of salt, which could obstruct separation of ACE inhibitors. The filtrate was fractionated on a Sephadex G-10 open column chromatography to remove the salt and purify ACE inhibitors. The fractions (No. 20-48) having ACE inhibitory activity that were eluted before half of the column bed volumes were obtained (Fig. 3A). The fractions did not contain the salt because very small compounds like NaCl are eluted at around one bed volume of the gel filtration column. The active fractions were very broad, suggesting that the mixture of various compounds including ACE inhibitors was present. The fractions were collected and concentrated. The concentrate was precipitated with methanol. The supernatant exhibited high ACE inhibitory activity (92 %), whereas the precipitate dissolved in water showed only 52 % (data not shown). For further separation, the supernatant was subjected to DEAE Sephadex A-25 anion exchange open column chromatography and two active fractions (I and II) were obtained. Fraction I showing 60 % of ACE inhibitory activity did not bind with the DEAE anion exchange resin at pH 8.0, while fraction II containing 40 % of the activity bound with the resin was eluted by 0.2 M NaCl (Fig. 3B). This result indicated that the compounds in fraction I have a positive charge or no charge at pH 8.0. To remove the salt used in the ion exchange column and further purify ACE inhibitors, fractions I and II
were subjected to Sephadex LH-20 open column chromatography. However, the separations of ACE inhibitors from both the fractions were not pronounced (data not shown). Fraction I having high ACE inhibitory activity was further purified by RP-HPLC to obtain 15 peaks. The ACE inhibitory activities of peaks 2, 3, and 4 were 42, 64, and 51 %, respectively, while other peaks showed less than 30 % of inhibitory activity (Fig. 4). These three peaks (I-2, I-3, and I-4) were further purified by gel filtration chromatography (supplemental data), and identification of three main active peaks designated as fractions I-2, I-3, and I-4 was studied.

Identification of ACE inhibitors

Three active fractions (I-2, I-3, and I-4) obtained after separation of fraction I on RP-HPLC were subjected to an ESI–MS/MS (positive ion) analysis. In these experiments, one dipeptide PK (Pro-Lys) in peak I-2 and three peptides GCK (Gly-Cys-Lys), NHP (Asn- His-Pro), and DGGP (Asp-Gly-Gly-Pro) in peak I-3 were detected (Fig. 5). These compounds were confirmed by amino acid sequencing analysis. In addition, the ESI–MS/MS data of these compounds were assignable to those of the corresponding synthesized peptides (PK, GCK, NHP, and DGGP). However, no promising compound in peak I-4 could be detected. Consequently, the peptides that were identified in the fermented anchovy sauce were PK, GCK, NHP, and DGGP.

Interestingly, the peptides purified in this study were different from previously reported ACE inhibitory dipeptides (AP, KP, GP, EP, TP, VP GI, and DF) of fermented anchovy sauce that was prepared with purified salt (Ichimura et al. 2003). Therefore, these observations suggested that the fermentation conditions such as types of salt (purified salt and solar sea salt) and fermentation period could affect the concentration and types of low molecular weight peptides in the fermented anchovy sauce.

ACE inhibitory activities of peptides identified from fermented anchovy sauce

Four peptides (PK, GCK, NHP, and DGGP) were identified as a mixture from fermented anchovy sauce. Therefore, four synthesized peptides (PK, GCK, NHP, and DGGP) were used to determine in vitro ACE inhibitory activity. These peptides showed the ACE inhibitory activity, although their activities were very low compared to captopril, a well-known ACE inhibitor drug. DGGP and GCK had significantly higher ACE inhibitory activity than other peptides (PK and NHP). The IC50 values of DGGP with proline and GCK with lysine at C-terminal for ACE inhibitory activity were 164 and 178 µM, respectively (Table 1). It was already reported that the di- and tri-peptides having hydrophobic proline, aromatic amino acids such as tryptophan and phenylalanine or positively charged lysine, and arginine residues at C-terminal contributed substantially to ACE inhibitory potency (Li et al. 2004; Balti et al. 2010; Pihlanro et al. 2010; Majumdar and Wu 2003).
However, PK (IC$_{50}$ = 4092 $\mu$M) with lysine at the C-terminal and NPH (IC$_{50}$ = 1172 $\mu$M) had low inhibitory potency (Table 1). Therefore, these results indicate that all peptides having proline or lysine residues at C-terminal do not always possess strong ACE inhibitory activity, although these residues are important active factors in ACE inhibitory activity.

ACE inhibition has been proven to reduce blood pressure via the suppression of angiotensin II production. In this study, we demonstrated that ACE inhibitory activity of the fermented anchovy sauce increased with an increase in the fermentation period, although a high amount of salt that could have stimulated the ACE activity was present in the anchovy sauce. In addition, four ACE inhibitory peptides

![Fig. 5](image-url) ESI–MS/MS spectra of peptides identified from peak I-2 and I-3. Peptide sequencing on singly charged peptide was done on positive ion mode on both ESI-MS and MS/MS using the Q-TOF micro-mass spectrometer with capillary liquid chromatography capability and electrospray ion source using Masslynx software. Manual sequence validation was done using Biolynx software.
(GCK, DGGP, NPH, and PK) with low molecular weight from the fermented anchovy sauce were identified. The low molecular weight peptides of fermented foods and protein hydrolysates have been found to exert ACE inhibitory activities (Li et al. 2004; Balti et al. 2010; Wijesekara and Kim 2010). These observations indicate that the ACE inhibitory activity of the fermented anchovy sauce may be attributed to low molecular weight peptides including GCK, DGGP, NPH, and PK identified in this study. These results will provide useful information to improve the quality and antihypertensive activity of fermented anchovy sauce during fermentation. Further investigation on the preventive effect of fermented anchovy sauce on hypertension in vivo will be needed.

Acknowledgments This work was supported by Grant 20130290 to the Solar Salt Research Center of Mokpo National University from the Ministry of Oceans and Fisheries of Korea.

Table 1 ACE inhibitory activities of synthesized peptides

| Synthesized peptides | ACE inhibitory activity IC$_{50}$ (µM)$^1$ |
|----------------------|-----------------------------------------|
| PK                   | 4092.26 ± 139.28$^d$                   |
| NPH                  | 1172.43 ± 40.36$^c$                   |
| GCK                  | 177.75 ± 26.62$^b$                   |
| DGGP                 | 163.51 ± 8.45$^b$                   |
| Captopril$^2$        | 0.05 ± 0.00$^a$                       |

Values are expressed as mean ± SD (n = 3). Different letters indicate a significant difference among the compounds at p < 0.05

1 IC$_{50}$ value is defined as peptide concentration required to inhibit 50 % of the ACE activity

2 Captopril was used as positive control

References

Balti R, Arroume NN, Bougatef A, Guillochon D, Nasri M (2010) Three novel angiotensin I converting enzyme (ACE) inhibitory peptides from cuttlefish (Sepia officinalis) using digestive proteases. Food Res Int 43:1136–1143

Cushman D, Cheung H (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol 20:1637–1648

Denton D, Weisinger R, Mundy NI, Wickings EJ, Dixon A, Moisson P, Pingard AM, Sharde R, Carey D, Ardaillou R (1995) The effect of increased salt intake on blood pressure of chimpanzees. Nat Med 1:1009–1016

Gomez RJÁ, Ramos M, Recio I (2002) Angiotensin-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starter cultures. Int Dairy J 12:697–706

He FL, Macgregor GA (2003) How far should salt intake be reduced? Hypertension 42:1093–1099

Ichimura T, Hu J, Aita DQ, Maruyama S (2003) Angiotensin I-converting enzyme inhibitory activity and insulin secretion stimulative activity of fermented fish sauce. J Biosci Bioeng 96:496–499

Jao CL, Huang SL, Hsu KC (2012) Angiotensin I converting enzyme (ACE) inhibitory peptides: inhibition mode, bioavailability, and antihypertensive effects. Biomedicine 2:130–136

Je JY, Park PJ, Kwon JY, Kim SK (2004) A novel angiotensin I converting enzyme inhibitory peptide from Alaska pollack (Theragra chalcogramma) frame protein hydrolysate. J Agric Food Chem 52:7842–7845

Lau CC, Abdullah N, Shuib AS (2013) Novel angiotensin I converting enzyme (ACE) inhibitory peptides derived from an edible mushroom, Pleurotus cystidiosus O.K. miller identified by LC-MS/MS. BMC Complement Altern Med 13:313

Lee SS, Kim SM, Park UY, Kim HY, Shin IS (2002) Studies on proteolytic and fibrinolytic activity of Bacillus subtilis JM-3 isolated from anchovy sauce. Korean J Food Sci Technol 34:283–289

Li GH, Le GW, Shi YH, Shrestha S (2004) Angiotensin I–converting enzyme inhibitory peptides derived from fish proteins and their physiological and pharmacological effects. Nutr Res 24:469–486

Majumdar K, Wu J (2011) Purification and characterisation of angiotensin I converting enzyme (ACE) inhibitory peptides derived from enzymatic hydrolysate of ovotransferrin. Food Chem 126:1614–1619

Park JH, Kim SM (2003) Biofunctionality of peptides purified from naturally fermented anchovy sauce. J Korean Soc Food Sci Nutr 32:1120–1125

Park JO, Yoon MS, Cho EJ, Kim HS, Ryu BH (1999) Antioxidant effects of fermented anchovy. Korean J Food Sci Technol 21:1378–1385

Patchett AA, Harris E, Tristam EW, Wyvrratt MJ, Wu MT, Taux B, Peterson ER, Ikeler TJ, Ten Broeke J, Payne LG, Onuleyka DL, Thorsett ED, Greenlee WJ, Lohr NS, Hofsfommer RD, Joshua H, Rayle WV, Rotherock JW, Aster SD, Maycock AL, Robinson FM, Hirschnann R, Sweet CS, Ulm EH, Gross DM, Vassil TC, Stone CA (1980) A new class of angiotensin-converting enzyme inhibitors. Nature 298:280–283

Pihlanto A, Virtanen T, Korhonen H (2010) Angiotensin I converting enzyme (ACE) inhibitory peptides and antihypertensive effect of fermented milk. Int Dairy J 20:3–10

Robert MC, Razaname A, Mutter M, Juillerat MA (2004) Identification of angiotensin-I-converting enzyme inhibitory peptides derived from sodium caseinate hydrolysates produced by Lactobacillus helveticus NCC 2765. J Agric Food Chem 52:6923–6931

Saiga A, Okumura T, Makihara T, Katsuta S, Shimizu T, Yamada R, Nishimura T (2003) Angiotensin I-converting enzyme inhibitory peptides in a hydrolyzed chicken breast muscle extract. J Agric Food Chem 51:1741–1745

Shin ZI, Yu R, Park SA, Chung DK, Ahn CW, Nam HS, Kim KS, Lee HJ (2001) His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo. J Agric Food Chem 49:3004–3009

Wijesekara I, Kim SK (2010) Angiotensin-I-converting enzyme (ACE) from marine resources: prospects in pharmaceutical industry. Mar Drugs 8:1080–1093

World Health Organization (2008) Cause of death 2008: data sources and methods. World Health Organization. Geneva. http://www.who.int/healthinfo/global_burden_disease/cod_2008_sources_methods.pdf. Accessed 3 Aug 2015

Wu J, Ding X (2001) Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. J Agric Food Chem 49:501–506

Wyvrratt MJ, Patchett AA (1985) Recent developments in the design of angiotensin-converting enzyme inhibitors. Med Res Rev 5:483–531

© Springer