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Peptide with Angiotensin I-Converting Enzyme Inhibitory Activity from Hydrolyzed Corn Gluten Meal

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Corn gluten meal (CGM) was hydrolyzed by Alcalase after starch removal of CGM was applied as a pretreatment. A new inhibitory peptide for angiotensin I-converting enzyme (ACE) was isolated from the hydrolysate of CGM with the use of Bio-Rad P-2 gel filtration and followed by reverse-phase high-performance liquid chromatography (RP-HPLC). The sequence of the active peptide was determined to be Ala–Tyr after the application of amino acid analysis and HPLC/MS. The IC_{50} of the peptide was 14.2 μM, and it was not affected by preincubation with 30 mU of ACE at 37 °C for 3 h. Ala–Tyr also exerted antihypertensive effects after oral administration to spontaneously hypertensive rats. A maximal reduction of systolic blood pressure of 9.5 mmHg was observed 2 h after oral administration of Ala–Tyr at doses of 50 mg/kg.

KEYWORDS: Corn gluten meal; Alcalase; ACE inhibitory peptides; antihypertensive effect; spontaneously hypertensive rats

INTRODUCTION

Hypertension, which carries with it a high risk of cerebrovascular, cardiac, and renal complication, has become the most common serious chronic health problem over the world in recent years. The number of people considered to be suffering from hypertension depends on levels of "normality" for blood pressure given by different health organizations. Generally, 20–45% of a population and nearly 50–60% of elderly people have elevated blood pressure (*1–3*).

Angiotensin I-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) plays an important physiological role in the regulation of blood pressure by virtue of two different reactions which it catalyzes: conversion of the inactive decapeptide angiotensin I (DRVYIHPFHL) into a powerful vasoconstrictor and salt-retaining octapeptide, angiotensin II (DRVYIHPF), and inactivation of the vasodilator nonapeptide bradykinin, which is conducive to lowering blood pressure (*4*). Synthetical ACE inhibitors including Captopril, Enalapril, Lisinopril, and others have been developed and are effective for decreasing blood pressure (*5*). However some undesirable side effects have been reported, such as coughing, dizziness, headache, abnormal taste (metallic or salty taste), and kidney and liver problems. Therefore, it is better to develop natural ACE inhibitory peptides for the treatment and prevention of hypertension. Recently, many studies have focused on various ACE inhibitory peptides derived from plant (*6–11*), casein (*12–14*), and fish muscle proteins (*15–17*). The digest "Katsuo-bushi oligopeptide" was even approved as a food for specified health use in Japan on the basis of the research of Yokoyama et al. (*15*).

Corn gluten meal (CGM), a major byproduct of corn wet milling, contains 67–71% protein (w/w). At present, CGM is mainly used as forage. It may become a good source for preparation of ACE inhibitory peptides because of its high proportion of hydrophobic amino acid and proline (*18*).

The objective of this research was to isolate the ACE inhibitory peptide derived from CGM hydrolysate and to characterize the isolated peptide with respect to ACE inhibitory activity. The antihypertensive property through oral administration to spontaneously hypertensive rats (SHR) also was investigated.

MATERIALS AND METHODS

Materials. CGM was provided by Fuyuan Corn Company (Shandong, China), with a total protein content of 61.5% (w/w). Trypsin and angiotensin I-converting enzyme were purchased from Sigma (St. Louis, MO). The substrate peptide (hippuryl-histidyl-leucine; HHL) was obtained from thePeptide Institute. Protamex, Neutrase, and Alcalase were provided by Novo Co. (Nordisk Biochem, Inc. China).

Pretreatment of the CGM. Suspensions of 10% CGM were adjusted to pH 6.5 with 1.0 M NaOH and then incubated with α-amylase (30 U/g) at 70 °C for 30, 60, 90, 120, 150, and 180 min. The mixtures of the enzymatic reaction were filtered to remove hydrolysis products of starch through a PES membrane with a 5 kDa molecular weight cutoff (MWCO) and then washed three times with the same amount of water. Then, the hydrolysate was dried with a vacuum dryer. The residual contents of the starch in CGM were estimated by the phenol-sulfuric acid method (*19*).

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Enzymatic Hydrolysis of Corn Protein. The pretreated CGM (25 mg/mL) was suspended in 0.1% Na_2S0_3 with a pH of 10∼12 followed by heating at 90 °C for 15 min. Then, the CGM solution was adjusted to pH 8.0 with 1.0 M HCl and hydrolyzed with the protease at a ratio of protein to enzyme of 50:1 (w/w) for 5 h at 60 °C in a batch reactor. The pH was maintained at its optimal pH by the continuous addition of 1.0 M NaOH. After hydrolysis, the reaction was terminated by boiling for 10 min. The degree of hydrolysis (DH) was measured by the pH-stat method (*20*).

Purification of Peptides. The hydrolysate was filtrated through an ultrafiltration membrane with a 5 kDa molecular weight cutoff, followed by Bio-Rad P-2 gel filtration (700 × 15 mm i.d. column) at a flow rate of 0.25 mL/min, previously equilibrated with 2 mM phosphate buffer (pH 8.0). Each fraction was collected, and their ACE inhibitory activity was measured. The active fractions were pooled, ultrafiltrated, and lyophilized before purification using reverse-phase high-performance liquid chromatography (RP-HPLC). The separation was performed on a μ-bondapak C 18 column (300 × 7.8 mm i.d., Waters, Milford, MA) with a linear gradient of acetonitrile (0–40%, 1%/min), containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 3 mL/min. Absorbance was monitored at a wavelength of 220 nm for the detection of peptides.

Measurement of ACE Inhibitory Activity. ACE inhibitory activity was determined using the method of Cushman and Cheung (*21*) modified by Yamamoto et al. (*22*) and expressed in terms of IC_{50}: 80 μL of a 5 mM hippuryl-L-histidyl-L-leucine (HHL; Sigma Chemical Co.) borate buffer (containing 100 mM borate and 300 mM NaCl, pH 8.3) was preincubated at 37 °C for 3 min; 10 μL of ACE solution (containing 100 mM borate and 300 mM NaCl, pH 8.3) was added, and the mixture was incubated at 37 °C for 30 min. The borate buffer (100 mM, pH 8.3) was used instead of an ACE solution for bland determination. Then reaction was stopped by 0.2 mL of 1 M HCl. A total of 0.8 mL of ethyl acetate was added, mixed, and centrifuged (3500 rpm, 5 min). The ethyl acetate extraction solution was transferred to a test tube and dried at 80 °C for 45min. The hippuric acid was redissolved in 0.8 mL of deionized–distilled water. Absorbance was determined at a wavelength of 228 nm.

The IC_{50} value, defined as the concentration of a peptide that inhibits 50% of the ACE activity, was determined by measuring the ACE inhibitory activity and protein contents of each sample. The inhibition was shown as equal to $[(E_c - E_s)/(E_c - E_b)] \times 100$, where E_s is the absorbance with a test sample added to the reaction mixture, E_c is the absorbance with a buffer added (instead of the test sample), and E_b is the absorbance when the stop solution was added before the reaction occurred.

HPLC/MS Analysis of Peptide. The sequence of each purified peptide with ACE inhibition activity was measured by high-performance liquid chromatography coupled *online* to electrospray ionization mass spectrometry (HPLC/ESI-MS; ZMD 4000 LC/MS, Waters, Milford, MA), on a Lichrospher C18 column (150 × 2.1 mm i.d., Waters, Milford, MA) with a linear gradient of acetonitrile (1%/min), containing 0.1% formic acid at a flow rate of 0.3 mL/min. Absorbance was monitored at a wavelength of 220 nm for the detection of peptides, and mass spectrometry data were acquired in the scan mode (mass range *m*/*z* 150–1000).

Analysis of Amino Acids. The peptide sample was hydrolyzed with 6.0 M HCl, at 110 °C for 24 h. The analysis of amino acids using precolumn online derivatization with OPA was accomplished using fluorescence detection (Agilent 1100, Santa Clara, CA) after hydrolysate was filtrated.

Peptide Synthesis and Purification. Peptides were synthesized by a solid-phase method with a peptide synthesizer (Biosearch SAM TWO, San Rafael, CA); *t*-butyloxycarbonyl amino acids were successively coupled in the presence of *N*,*N*′-diisopropylcarbodiimide. Peptides were deprotected by the anisole/hydrogen fluoride method and purified by HPLC on a μ-Bondapak C18 column. The identity of peptides was confirmed by HPLC/ESI-MS, and the purity of peptides also was evaluated with HPLC/ESI-MS on a Lichrospher C18 column with a linear gradient of acetonitrile (0–40%, 1%/min), containing 0.1% formic acid at a flow rate of 0.3 mL/min.

Characterization of ACE Inhibitory Peptides. The ACE inhibition mode of peptides was evaluated through measuring IC_{50} values for ACE inhibition before and after preincubation of the individual peptides (0.1 mM) with 32 mU of ACE at 37 °C for 3 h. Additionally, the mixtures of enzymatic reaction were analyzed using RP-HPLC following preincubation with ACE to confirm the inhibition mode.

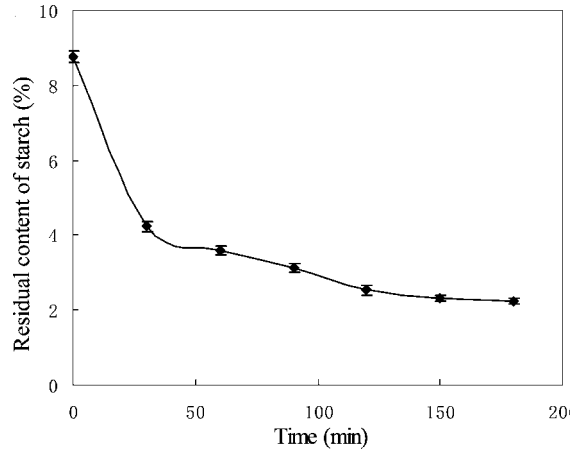

Figure 1. Effect of starch removal by amylase hydrolysis. Suspensions of 10% CGM were adjusted to pH 6.5 with 1.0 M NaOH and then incubated with α-amylase (30 U/g) at 70 °C for 30, 60, 90, 120, 150, and 180 min.

In Vivo Determination of Antihypertensive Effect of Peptides. Peptides dissolved in saline (1.0 mL) were administered orally to male SHR (16–24 weeks old) via a gastric metal zonde. Following oral administration of the peptide, the blood pressure was measured by the tail cuff method using the MK-2000 blood pressure meter (Muromachi Kikai, Japan) to ascertain antihypertensive activity, while only saline was administered, as a control.

Data Analysis. All results are expressed as means ± standard error of the mean (SEM). Statistical comparisons of the results between the two groups are made with Student's *t*-test.

RESULTS AND DISCUSSION

Pretreatment of CGM with α-Amylase. The CGM contains about 8.7% (w/w) starch. Because starch is bound tightly with corn protein, a pretreatment of a starch removal process by amylase hydrolysis is needed to prepare the corn gluten hydrolysate. The results of starch removal of the corn gluten with hydrolyzed by α-amylase are shown in **Figure 1**. The starch residue of corn gluten meal decreased dramatically within the initial 0.5 h. The content of the total reducing sugar in the corn gluten was 2.2% (w/w) after 3 h. The residue of the reducing sugar did not change obviously from 2–3 h, meaning that the starch in the corn gluten meal had been effectively removed. Meanwhile, the content of protein in CGM increased from 61.5 to 85.7%.

Hydrolysis of Corn Protein. Corn protein was suspended in 0.1% Na_2SO_3 at a pH of 10∼12, followed by heating at 90 °C for 15 min to increase its solubility before hydrolysis. To select suitable proteases for corn protein hydrolysis, corn protein was independently hydrolyzed with Protamex, Neutrase, Alcalase, and trypsin. The result of digestion with various proteolytic enzymes is shown in **Table 1**. Among them, the hydrolysates digested by Alcalase for 5 h revealed the highest DH value and most potent ACE inhibitory activity, showing IC_{50} values of 0.197 mg/mL (**Table 1**). These data suggest that extensive hydrolysis may release many low-molecular-weight peptides and results in high ACE inhibition. This seems to be consistent with the fact that most reported ACE inhibitory peptides are low-molecular-weight peptides (*6*, *8*, *15*). However, other studies concerning ACE inhibition of hydrolysates of a protein prepared with other enzymes showed that ACE inhibition may also decrease with prolonged hydrolysis. This result could be contributed to the fact that initially produced ACE

Table 1. ACE Inhibitory Activity[a] of CGM Hydrolysates with Various Enzymes

enzyme	ACE inhibition[a] (%)	DH[b] (%)
Protamex	13.94	0.85
Neutrase	47.43	2.48
Alcalase	85.26	16.96
trypsin	72.80	8.97

[a] ACE inhibition was determined with 10 μL of each hydrolysate solution, 10 mg/mL. [b] DH was measured by pH-stat method (*20*).

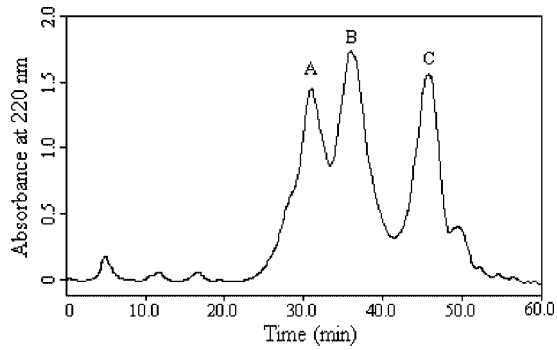

Figure 2. Gel filtration chromatography of the hydrolysate of corn protein on a Bio-Rad P-2 column. Separation was performed at a flow rate of 0.25 mL/min. All fractions were designated A, B, and C, which were used to measure ACE inhibitory activity.

inhibitory peptides were subsequently degraded (*23*). The hydrolysate of corn protein was ultrafiltrated by a 5 kDa membrane; then, the permeation solution was collected, since the molecular weight of the most active fractions is less than 1 kDa (data are not shown).

Isolation of ACE Inhibitory Peptides. ACE inhibitory peptides were purified by sequential chromatographic methods. Size exclusion chromatography on a Bio-Rad P-2 column was used to fractionate the ultrafiltrated hydrolysate first, and fractions A, B, and C were obtained (**Figure 2**). Among these different fractions, fraction C exhibited potent ACE inhibitory activity with an IC_{50} value of 0.085 mg/mL. Meanwhile, the other two fractions failed to show any ACE inhibitory activity (data are not shown). The active fraction C was further purified by RP-HPLC chromatography. The five main fractions were isolated from fraction C on a μ-Bondapak C 18 column, as shown in **Figure 3**. The fraction with the highest ACE inhibitory activity was isolated and designated fraction CIII (**Table 2**).

Table 2. ACE Inhibitiory Activity of Fractions Isolated by RP-HPLC

fractions	ACE inhibition[a] (%)
CI	13.54
CII	18.56
CIII	82.92
CIV	10.78
CV	21.38
CVI	12.36

[a] ACE inhibition was determined with 30 μL of each fraction.

Sequence Analysis of ACE Inhibitory Peptides. With the aim of identifying putative active peptides, fraction CIII was separated and further purified through the same RP-HPLC column with a different gradient. After the second chromatography, a single pure fraction was isolated and subjected to HPLC/ESI-MS. It has only one peak at 9.12 min retention time on the HPLC/ESI-MS chromatogram of fraction CIII, as shown in **Figure 4a**. Meanwhile, the total ion current (TIC) and mass spectrum of fraction CIII also was obtained. The retention time of the fragmentation is almost the same as that of fraction CIII, as shown in **Figure 4b**, and the fragmentation spectrum contained three major ions at *m*/*z* 165.2, 182.2, and 207.3, as shown in **Figure 4c**. The sequence of this peptide was identified as Ala–Tyr by ESI-MS on a Masslynx wokstation.

The sequence of the ACE inhibitory peptide was further confirmed with amino acid analysis since the ESI-MS spectrum usually matched ambiguously one sequence of the group of peptides selected by mass. The final result showed the peptide also is composed of an Ala and a Tyr, which is consistent with the above result obtained by the HPLC/ESI-MS system.

It was well known that ACE inhibitory peptides derived from protein hydrolysates were mainly affected by the amino acid sequences. Cheung et al. reported a series of peptides against ACE inhibitory activity, which indicated that aromatic amino acids at the C-terminal end and branched-chain aliphatic amino acids at the N-terminal end were suitable for a peptide binding to ACE as a competitive inhibitor (*24*), which is consistent with our results that two isolated peptides have an Ala and a Tyr at their N-terminal and C-terminal ends, respectively.

Classification of ACE Inhibitory Peptides. ACE inhibitory peptides can be classified into the following groups depending

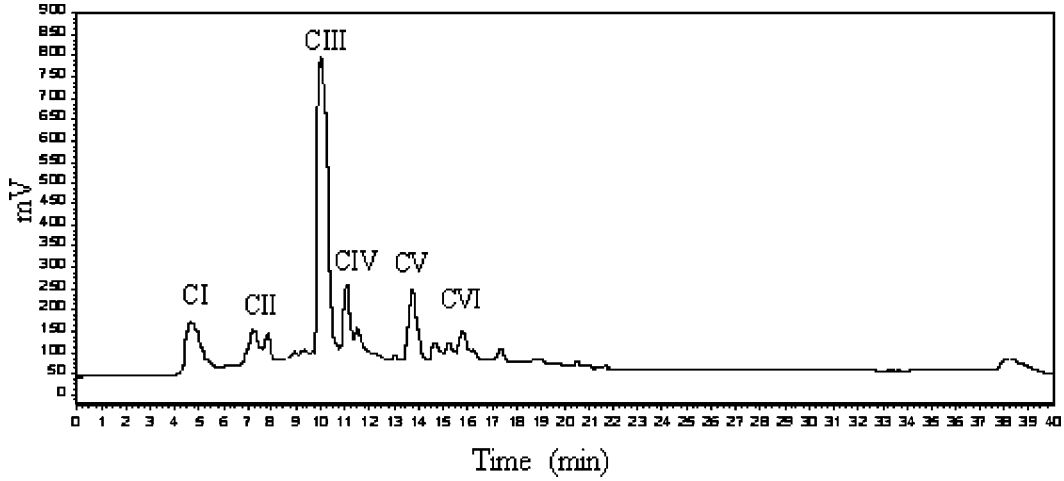

Figure 3. Separation of the peptide from the active fraction C by RP-HPLC. The separation was performed on a μ-Bondapak C 18 column (300 $\times$ 7.8 mm i.d., Waters, Milford, MA) with a linear gradient of acetonitrile (0–40%, 1%/min), containing 0.1% TFA at a flow rate of 3 mL/min. The fractions isolated from fraction C were designated CI–CVI and were used to measure ACE inhibitory activity.

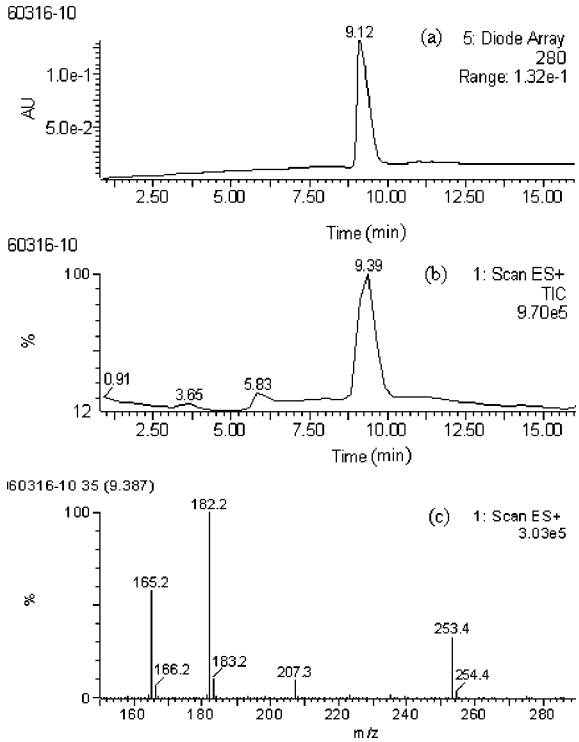

Figure 4. Chromatogram of fraction CIII on HPLC/MS (**a**), total ion current (TIC) of fraction CIII on HPLC/MS (**b**), and ESI-MS spectrum of fraction CIII on HPLC/MS (**c**). The separation was performed on a Lichrospher C18 column (150 × 2.1 mm i.d., Waters, Milford, MA) with a linear gradient of acetonitrile (1%/min), containing 0.1% formic acid at a flow rate of 0.3 mL/min. Absorbance at a wavelength of 220 nm was monitored for the detection of peptides; TIC and mass spectrometry data also were acquired in the scan mode (mass range *m/z* 150–1000).

on their interaction with ACE: (i) true inhibitors, which have high affinity with ACE but are resistant to cleavage by ACE and prevent the hydrolysis of the other substrates, such as angiotensin I; (ii) substrates for ACE, which are hydrolyzed into inactive fragments or fragments having a markedly lower activity by ACE; and (iii) "prodrugs", which are converted by ACE with the release of highly active fragments. Previous studies have proved that the real substrates, which show apparent ACE inhibitory activities in an assay used for screening, are inactive after being administered orally because they are hydrolyzed by ACE to inactive fragments (*26*, *27*).

In our previous studies, only true inhibitors and prodrugs have the ability to lower blood pressure (*6*, *8*). In order to discriminate the substrates from true inhibitors, peptides were preincubated with ACE prior to the measurement of ACE inhibitory activity. IC_{50} values of the true inhibitors remained at 14.2 μM, which is not affected by preincubation with ACE for 3 h, whereas substrates for ACE are altered by preincubation with ACE. The results showed AY could be considered as a true ACE inhibitor, because its IC_{50} value was unaltered at 14.2 μM by preincubation with ACE. Therefore, AY may have antihypertensive activity in vivo.

Antihypertensive Activities of Peptides in Vivo. To investigate whether isolated peptides can exert antihypertensive activity in vivo, Ala–Tyr was tested by oral administration to SHR. All the peptides exhibiting inhibition of the ACE activity were classified into three groups. Both inhibitor-type and prodrug-type peptides are characterized with long-lasting antihypertensive activities after oral administration in SHR; meanwhile, the substrates usually fail to exert any antihypertensive activity. Moreover, the prodrug-type ACE inhibitory peptides

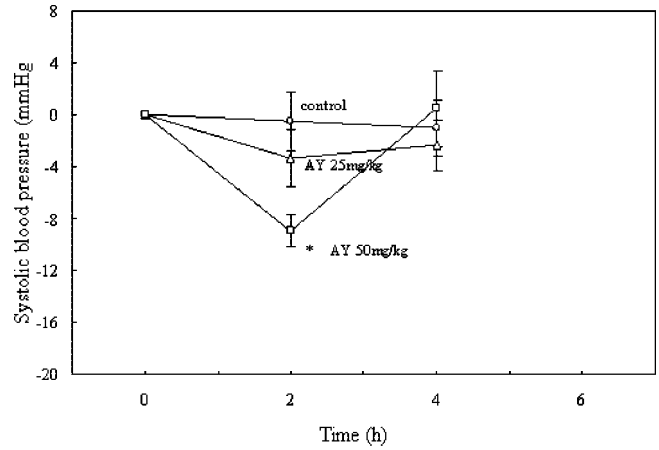

Figure 5. Antihypertensive activity of AY after oral administration in SHR. Peptides were administered as a solution in saline at doses of (○) 0 mg/kg (control), (△) 25 mg/kg, and (□) 50 mg/kg. Changes of systolic blood pressure from time zero were expressed with mean ± SEM. A * indicates significant differences against the control ($p < 0.05$), $n = 4$.

usually exert long-lasting antihypertensive activity (4 h, even longer) compared with true inhibitors (2 h) (*6*, *26*, *27*). This may be contributed to the greater time required for the longer peptide to be converted into a true inhibitor in vivo.

In the case of Ala–Tyr, a maximum reduction of the systolic blood pressure of 9.5 mm Hg was observed only 2 h after oral administration, with the minimum effective dose being 50 mg/kg, and failed to show effective antihypertensive activity after 4 h, as shown in **Figure 5**. So it is evident that Ala–Tyr does exert antihypertensive activity as a true inhibitor in vivo.

Conclusions. In summary, the obtained results prove that the enzymatic hydrolysis of CGM releases ACE inhibitory peptides having antihypertensive activity following oral administration to SHR. Therefore, CGM, as an abundant protein resource on earth, may therefore be important in preventing hypertension, which suggests that ACE inhibitory activities derived from CGM hydrolysate could be utilized to develop physiologically functional foods.

ABBREVIATIONS USED

CGM, corn gluten meal; ACE, angiotensin I-converting enzyme; TFA, trifluoroacetic acid; RP, reverse phase; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry; TIC, total ions current.

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