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Angiotensin I Converting Enzyme-Inhibitory Peptides from Commercial Wet- and Dry-Milled Corn Germ

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Bioprocesses were developed to enhance the value of proteins from deoiled corn germ. Proteins were hydrolyzed with trypsin, thermolysin, GC 106, or Flavourzyme to generate the bioactive peptide sequences. At an enzyme to substrate ratio of 1:100, protein hydrolysis of wet-milled germ was greatest using thermolysin followed by trypsin, GC 106, and Flavourzyme. For the dry-milled corn germ, protein hydrolysis was greatest for GC 106 and least for Flavourzyme. Electrophoretic patterns indicated that the hydrolysis conditions used were adequate for generating low molecular weight peptides for both germs. Unhydrolyzed dry- and wet-milled corn germ did not appear to contain angiotensin I converting enzyme (ACE)-inhibitory peptides. After hydrolysis with trypsin, thermolysin, and GC 106 but not Flavourzyme, ACE inhibition was observed. ACE inhibition was greatest for the GC 106 hydrolysate for both wet- and dry-milled corn germ. Denaturing the protein with urea before hydrolysis, in general, increased the amount of ACE-inhibitory peptides found in the hydrolysate. Membrane fractionations of both the wet- and dry-milled hydrolysates indicated that most of the ACE-inhibitory peptides were in the <1 kDa fraction. Examination of the control total protein extracts (before treatment with proteases) from wet- and dry-milled germ revealed that neither had ACE-inhibitory properties. However, when both total corn germ control protein extracts were fractionated, the <1 kDa fraction of wet-milled corn germ proteins exhibited ACE inhibition, whereas the comparable low molecular weight fraction from dry-milled corn germ did not.

KEYWORDS: Corn germ; commercial wet-milled; dry-milled; angiotensin I converting enzyme-inhibitory activity; bioactive peptides

INTRODUCTION

The value of dietary proteins traditionally has been assessed by evaluating nutritional quality, specifically the availability of essential and nonessential amino acids and the absence of non-nutritional compounds that can restrict protein digestibility. More recently, there have been reviews of food proteins that have been valued for the potential availability of biologically active peptides (*1–3*). Numerous bioactive peptides have been identified, and most are derived from milk and dairy products. Bioactive peptides are most commonly generated using heat, acid and alkaline conditions, enzymatic hydrolysis, or microbial fermentation of proteins (*4*). The most common approach, however, has been enzymatic hydrolysis followed by ultrafiltration to enrich specific peptide fractions. A group of milk tripeptides was reported to inhibit the effect of angiotensin I converting enzyme (ACE) and perhaps has applications for lowering blood pressure (*5, 6*). ACE, a dipeptidylcarboxypeptidase, controls blood pressure by regulating the conversion of a protein, angiotensin I, to its active form, angiotensin II, resulting in narrowing of small blood vessels and an increase in blood pressure. ACE inhibitors slow the activity of the enzyme, thereby reducing constriction of the blood vessels and lowering blood pressure. Several types of corn peptides have been reported to have biological activity. Miyoshi et al. have shown a reduction in the systolic blood pressure of spontaneously hypertensive rats after intraperitoneal injection of α-zein hydrolysate (*7*). Fourteen ACE-inhibiting peptides, formed after the hydrolysis of corn zein with thermolysin and fractionated by reversed phase HPLC, have been isolated. The three strongest inhibitors all have similar structures and a proline residue (*8*). Recently there has been considerable interest in nutraceuticals or engineered foods containing bioactive compounds with health-promoting or disease-preventing properties. It is therefore important to identify bioactive compounds from food protein in cases when an important benefit is likely to occur. In this study, the enzymatic conditions leading to the formation and

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degradation of ACE inhibitory peptides from deoiled wet- and dry-milled corn germ were investigated.

MATERIALS AND METHODS

Materials. The protein assay kit used was a Bio-Rad DC protein assay (Hercules, CA). Dialysis tubing, nominal MW cutoff of 3500 Da, was from (Spectra-Por Biotech, Rancho Dominguez, CA). A centrifugal filter device, MW cutoff of 10000 and 5000 Da was from Millipore Corp. (Bedford, MA), and Microsep 1K omega, MW cutoff of 1000 Da, was from Pall Life Sciences (Ann Arbor, MI). Commercial corn zein F-4000, 90% protein, 1% lipid, and 0% starch (dry basis), was obtained from Freeman Inc. (Tuckahoe, NY). Angiotensin converting enzyme (ACE) from rabbit lung (EC 3.4.15.1), ACE inhibitor (ACEI) (pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro), captopril, trypsin from bovine pancreas, Flavourzyme, *Aspergillus* sp., and *N*-hippuryl-His-Leu (HHL) tetrahydrate were from Sigma (St. Louis, MO). Thermolysin of A grade was from Calbiochem (San Diego, CA), and GC 106 was from Genencor International, Inc. (Rochester, NY).

Wet- and Dry-Milled Corn Germ. Samples of wet- and dry-milled corn germ were obtained from commercial sources. All germ samples were stored in sealed containers at 4 °C until used.

Defatted Commercial Corn Germ. For wet- and dry-milled corn germ, 100 g was ground to a size of <3 mm and extracted twice with 500 mL of hexane at room temperature for 30 min. The oil content, 42.50 and 19.25% for the wet- and dry-milled corn germ, respectively, was determined after the combined supernatants were decanted and dried under nitrogen.

Preparation of Germ Protein Hydrolysates. Protein hydrolysis was performed on the defatted germ according to a modification of previously reported methods (*9, 10*). Enzymatic hydrolysis of germ proteins was determined for trypsin, thermolysin, GC 106, or Flavourzyme. The enzyme was added to a 5% (w/v) aqueous suspension of defatted germ at an E:S of 1:100. Samples were vortexed continuously at 45 °C for 4 h, and the mixture was quenched at 100 °C for 10 min and then centrifuged at 8000*g* for 10 min; the supernatant was assayed for ACE-inhibitory peptides. Alternatively, germ samples were denatured as follows. Ten milliliters of 1 M urea in water was added separately to 500 mg of wet- and dry-milled corn germ and vortexed at 45 °C for 4 h. The samples were hydrolyzed, quenched, and centrifuged as described above and the supernatants dialyzed overnight at 0 °C against 0.2 M phosphate plus 0.3 M NaCl, pH 8.3, buffer with three changes; the dried retentates were assayed for ACE-inhibitory peptides.

Fractionation of Germ Protein Hydrolysates. After protein hydrolysis of the germ protein, the quenched supernatant was passed through a 10 kDa molecular weight cutoff membrane. Portions of the solution were removed immediately, and the filtrates were passed through 5, 3, and 1 kDa molecular weight cutoff membranes so that fractions were >10, 5–10, <5, <3, and <1 kDa. The protein content and angiotensin I converting enzyme inhibition (ACEI) assay of all permeates were measured.

Protein Analysis. The total protein content of the lyophilized corn germ was determined in duplicate using the Bio-Rad DC protein assay. Trichloroacetic acid (TCA) precipitated protein and nonprotein nitrogen (NPN) of clear supernatants were determined after treatment of samples with a TCA solution to a final concentration of 6% (w/v), shaken for 20 min, and then centrifuged 12000*g* for 10 min at 4 °C (*11*). The lowest value of NPN occurred below 20% TCA. Above this concentration, germ proteins are soluble and the NPN assay was invalid. The degree of proteolysis was defined by the ratio of the NPN to the total protein.

Assay of Angiotensin Converting Enzyme-Catalyzed Reactions. The assay used to measure ACE inhibitory activity of acid- or enzyme-hydrolyzed defatted corn germ was a modification of the standardized method reported by Hernandez-Ledesma et al. (*12*). The total reaction volume was 210 μL made up of 150 μL of 10.0 mM HHL, dissolved in 0.2 M phosphate plus 0.3 M NaCl buffer, pH 8.3, 30 μL of ACE solution in 0.25 unit/mL 50% glycerol water, and 30 μL of different concentrations of hydrolysate incubated for 80 min at 37 °C with continuous agitation. The reaction was terminated by the addition of 250 μL of 1 M HCl followed by 1.5 mL of ethyl acetate shaken vigorously for 2 min at room temperature and centrifuged for 2 min at 14000*g*. One milliliter of the clear upper layer was placed in vials and dried in a vacuum oven that was operated at 15 in. of Hg at 95 °C. After the ethyl acetate was evaporated, 1 mL of water was added to dissolve the residue prior to measurement of the absorbance at 228 nm. Four replicates were run and ACE inhibition was calculated: ACEI $= 100(1 - X/C)$, where X and C are absorbances of the sample and the control, respectively.

Gel Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) of lyophilized corn germ proteins was carried out on a Phast System (Amersham GE, Piscataway, NJ) with a Phast homogeneous gel of 20% acrylamide. Hydrolyzed and unhydrolyzed protein samples from defatted wet- and dry-milled corn germ were solubilized in 200 μL of loading buffer (0.44 M Tris buffer containing 1 mM EDTA and 10% SDS, pH 8.0) plus 40 μL of 2-mercaptoethanol (ME), and the mixtures were heated at 100 °C for 10 min. Gels were stained with 0.2% (w/v) Coomassie R350 dye. Molecular weight standards (See Blue Plus2 Pre-Stained Standards, Invitrogen Corp.) were 98 kDa (phosphorylase), 62 kDa (BSA), 49 kDa (glutamic dehydrogenase), 38 kDa (alcohol dehydrogenase), 28 kDa (carbonic anhydrase), 17 kDa (myoglobin red), 14 kDa (lysozyme), 6 kDa (aprotinin), and 3 kDa (insulin, B chain).

Table 1. Total Protein, Precipitated Protein, and Nonprotein Nitrogen in Commercial Wet- and Dry-Milled Corn Germ

germ	wt %		
	total protein	TCA ppt protein	NPN
wet-milled	12.55 (12.71)[a]	5.53	7.18
dry-milled[b]	14.74 (13.81)[a]	10.64	3.17

[a] Values in parentheses are the sums of TCA ppt protein + NPN. [b] Values are from ref *13* with permission.

RESULTS AND DISCUSSION

Biologically active peptides are usually specific protein fragments that have a positive impact on body function (*2*). As part of a project on the aqueous enzymatic extraction of oil from corn germ, the objective of this research was to develop processes to enhance the value of proteins from the defatted germ. Previously obtained gel electrophoretic patterns of wet- and dry-milled corn germ samples indicated a greater number of protein bands for the dry- compared to wet-milled corn germ samples after extraction with Tris, SDS buffer containing 2-ME at 100 °C for 10 min (*13*). Even though the total protein contents of both wet- and dry-milled samples were similar (**Table 1**), approximately half the amount of TCA precipitated protein is present in commercial wet-milled germ compared to dry-milled corn germ. In addition, more than twice the amount of nonprotein nitrogen (NPN) was found in the wet- compared to dry-milled corn germ (7.18 vs 3.17%).

It is generally recognized that some hydrolysis of the proteins is required to generate the bioactive peptide sequences. In this study the enzymes used for the hydrolysis of the deffated corn germ were trypsin, GC 106, and Flavourzyme, which were chosen to produce hydrolysates considered to be safe in food applications and have been extensively studied. Thermolysin was included because it has been shown to be effective in the hydrolysis of prolamines such as zein (*7, 8*).

The degree of proteolysis was determined by the ratio of the NPN to total protein (*11*) and expressed as percent hydrolysis. For all four enzymes, extent of proteolysis varied for both wet- and dry-milled corn germ samples and the enzymes used (**Figures 1**). At an enzyme to substrate ratio of 1:100, protein hydrolysis of wet-milled germ was greatest using thermolysin followed by trypsin, GC 106, and Flavourzyme (**Figure 1a**).

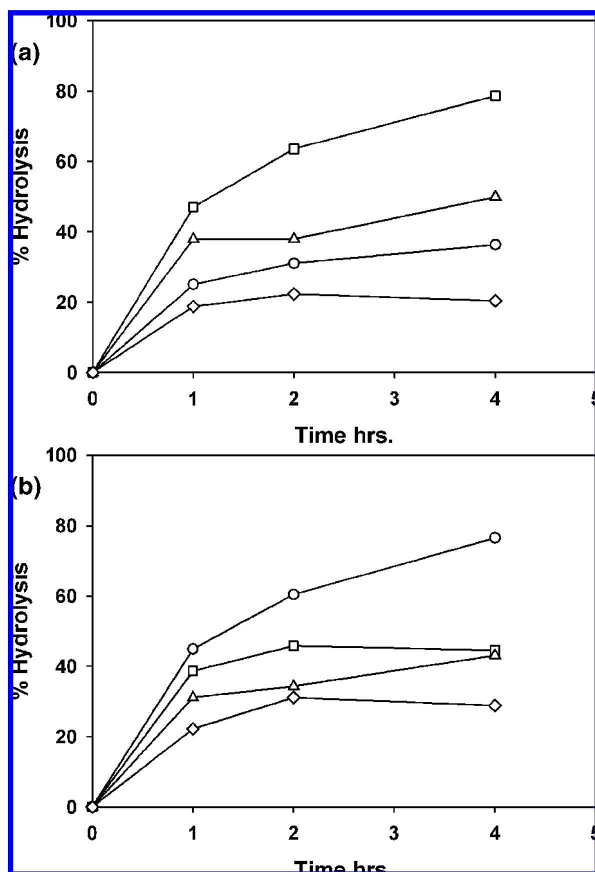

Figure 1. Hydrolysis of (**a**) defatted wet-milled corn germ and (**b**) defatted dry-milled corn germ using thermolysin (□), trypsin (△), GC 106 (○), and Flavourzyme (◇).

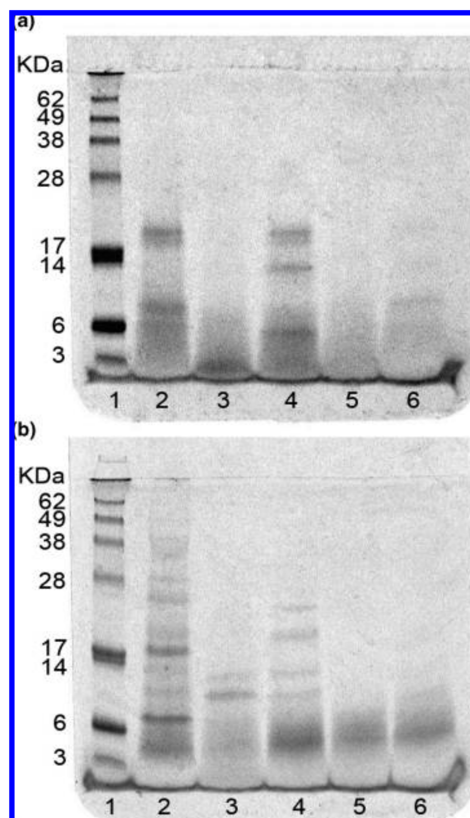

Figure 2. SDS-polyacrylamide gel electrophoresis of (**a**) wet-milled corn germ and (**b**) dry-milled corn germ hydrolyzed at 37 °C for 1.5 h: lane 1, molecular weight standard; lane 2, corn germ; lanes 3–6, corn germ hydrolyzed with trypsin, thermolysin, GC 106, and Flavourzyme, respectively.

For the dry-milled corn germ protein, hydrolysis was greatest for GC 106, least for Flavourzyme, and intermediate for thermolysin and trypsin, which were equivalent after 4 h (**Figure 1b**).

Electrophoretic profiles of the heat-quenched unhydrolyzed wet-milled corn germ indicated that α-zeins (19 and 22 kDa) were the only proteins present with molecular masses greater than 10 kDa (**Figure 2a**, lane 2), whereas a number of higher molecular weight protein bands were present in the corresponding dry-milled corn germ sample (**Figure 2b**, lane 2). Except for wet- and dry-milled corn germ hydrolyzed with thermolysin (**Figure 2**, lane 4) the majority of protein bands were smaller than 10 kDa. For all four enzymes, hydrolysis at 45 °C for 4 h appeared to be adequate for generating low molecular weight peptides from both defatted wet- and dry-milled corn germ (**Figure 2**) and was therefore the hydrolysis condition used to prepare ACE inhibitory peptides.

To determine the reliability of our method to quantify ACEI peptides, we used the same ACE inhibition assay conditions to determine the IC_{50} values for the standard and captopril, which were 14 and 81 nM, respectively (*14*). Unhydrolyzed dry- and wet-milled corn germ did not appear to contain ACEI peptides (**Table 2**). Only after hydrolysis with trypsin, thermolysin, or GC 106, but not Flavourzyme, was ACE inhibition observed. Less protein hydrolysis was observed using Flavourzyme for both wet- and dry-milled germ (20 and 30%, respectively, after 4 h) compared to the other enzymes tested (**Figure 1**). More ACEI peptides were present in hydrolyzed wet-milled corn germ compared to hydrolyzed dry-milled germ (**Table 2**). ACE inhibition was greatest for the GC 106 hydrolysate from both wet- and dry-milled corn germ compared to other enzymes

Table 2. ACE-Inhibitory Activity of Dry- and Wet-Milled Corn Germ Hydrolysate

commercial	enzyme[a]	ACEI[b] (%)	SD (ACEI)[c] (%)
dry-milled	none	0	
	Ty	22.85	6.53
	Th	15.23	6.71
	GC 106	25.09	10.24
	Flav	0[d]	5.77
wet-milled	none	0	
	Ty	26.08	6.07
	Th	35.92	5.38
	GC 106	40.75	6.86
	Flav	0[d]	6.16

[a] Ty, trypsin; Th, thermolysin; Flav, Flavourzyme. [b] ACEI activity expressed as percent inhibition. [c] Standard deviation (SD) expressed as percent ($n = 4$). [d] Absorbance for samples exceeded absorbance for the control.

tested. Alternatively, germ proteins were denatured with urea to increase formation of ACEI peptides. Denaturing unhydrolyzed dry- or wet-milled corn germ did not appear to result in the formation of ACEI peptides (**Table 3**). Denaturing the protein before hydrolysis in general increased the amount of ACEI peptides found in the hydrolysate, particularly for the dry-milled germ proteins compared with wet-milled germ proteins. Denatured Flavourzyme-hydrolyzed dry-milled corn germ contained a significantly greater amount of ACEI peptides than its wet-milled counterpart. Hydrolysis of the denatured germ protein with GC 106 still generated the greatest amount of ACEI peptides, as was the case for the undenatured corn germ (**Table 2**). IC_{50} values for dry- and wet-milled corn germ protein hydrolysates ranged from 3.26 to 7.77 mg/mL compared to soy

Table 3. ACE-Inhibitory Activity of Urea-Denatured Dry- and Wet-Milled Corn Germ Hydrolysate

commercial	enzyme[a]	ACEI[b] (%)	SD (ACEI)[c] (%)
dry-milled	none		
	Ty	15.35	8.25
	Th	45.57	5.70
	GC 106	53.83	6.04
	Flav	40.82	3.83
wet-milled	none		
	Ty	0[d]	11.54
	Th	36.44	3.21
	GC 106	40.55	8.41
	Flav	2.42	7.53

[a] Ty, trypsin; Th, thermolysin; Flav, Flavourzyme. [b] ACEI activity expressed as percent inhibition. [c] Standard deviation (SD) expressed as percent ($n = 4$). [d] Absorbance for samples exceeded absorbance for the control.

Table 4. ACE-Inhibitory Activity of Fractionated[a] Dry- and Wet-Milled Corn Germ Hydrolysate

commercial	enzyme[b]	ACEI[c] (%)	SD (ACEI)[d] (%)	ACEI (%)/mg of protein
dry-milled	none	0		0
	Ty	23.73	5.91	249.53
	Th	22.28	8.40	193.40
	GC106	41.26	4.44	230.76
	Flav	23.88	11.01	454.85
wet-milled	none	26.63	7.30	507.24
	Ty	34.49	7.89	246.71
	Th	43.56	5.88	186.87
	GC 106	45.98	5.31	470.14
	Flav	35.45	4.31	492.36

[a] <1 kDa. [b] Ty, trypsin; Th, thermolysin; Flav, Flavourzyme. [c] ACEI activity expressed as percent inhibition. [d] Standard deviation (SD) expressed as percent ($n = 4$).

protein hydrolysates having IC_{50} values ranging from 0.126 to 0.340 mg/mL (*15*). The amount of ACEI peptides present in denatured germ samples hydrolyzed with trypsin was significantly less than that in the undenatured samples (compare **Tables 2** and **3**).

Membrane fractionation of the hydrolyzed corn germs indicated that the ACEI activity was greatest for fractions of <1 kDa. Some activity was present in 5–10 kDa fractions, but no activity was observed in fractions of >10 kDa. For the <1 kDa fraction (**Table 4**), the most significant finding was that the Flavourzyme hydrolysate for both the dry- and wet-milled germs exhibited ACEI activity that was not observed previously for the same hydrolysates (compare **Tables 2** and **4**). In addition, ACEI activity was observed in the fractionated, unhydrolyzed wet-milled corn germ but not the dry-milled corn germ treated the same way (**Table 4**). Because biologically active peptides, which exhibit ACE inhibitory properties, were found in unhydrolyzed wet-milled corn germ but only in the <1 kDa fraction, it appears that the commercial steeping process (0.2% SO_2 at 52 °C for 24–36 h) used for the preparation of wet-milled corn germ generated ACEI peptides but in poor yield.

In conclusion, future work will be directed to developing a better understanding of the formation of ACE-inhibitory peptides as a result of acid steeping and enzymatic hydrolytic processes to improve the purity and yield of these peptide fractions.

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LITERATURE CITED

(1) Yamamoto, N. Antihypertensive peptides derived from food proteins. *Biopolymers* **1997**, *43*, 129–134.
(2) Kitts, D.; Weiler, K. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. *Curr. Pharm. Des.* **2003**, *9*, 1309–1323.
(3) Ondetti, M. A.; Cushman, D. W. Angiotensin-converting enzyme inhibitors: biochemical properties and biological actions. *CRC Crit. Rev. Biochem.* **1984**, *16*, 381–411.
(4) Philanto-Leppala, A. Bioactive peptides derived from bovine whey proteins: opioid and ACE-inhibitory peptides. *Trends Food Sci. Technol.* **2001**, *11*, 347–356.
(5) Fitzgerald, R. J.; Meisel, H. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *Br. J. Nutr.* **2000**, *84*, S33–S37.
(6) Fitzgerald, R. J.; Murray, B. A.; Walsh, D. J. Hypotensive peptides from milk proteins. *J. Nutr.* **2004**, *134*, 980S–988S.
(7) Miyoshi, S.; Kaneko, T.; Yoshizawa, Y.; Fukui, F.; Tanaka, H.; Maruyama, S. Hypotensive activity of enzymatic α-zein hydrolysate. *Agric. Biol. Chem.* **1991**, *55* (5), 1407–1408.
(8) Miyoshi, S.; Kaneko, T.; Ishikawa, H.; Tanaka, H.; Maruyama, S. Production of bioactive peptides from corn endosperm proteins by some proteases. *Ann. N. Y. Acad. Sci.* **1995**, *750*, 429–431.
(9) Gobbetti, M.; Smacchi, E.; Corsetti, A.; Bellucci, M. Inhibition of proteolytic enzymes from *Pseudomonas fluorescens* ATCC 948 and angiotensin I-converting enzyme by peptides from zein, hordein, and gluten hydrolysates. *J. Food Prot.* **1997**, *60*, 499–504.
(10) Wu, J.; Aluko, R. E.; Muir, A. D. Improved method for direct high-performance liquid chromatography assay of angiotensin-converting enzyme-catalyzed reactions. *J. Chromatogr., A* **2002**, *950*, 125–130.
(11) Vermeirseen, V.; Van Camp, J.; Devos, L.; Verstaete, W. Release of angiotensin I converting enzyme (ACE) inhibitory activity during in vitro gastrointestinal digestion: from batch experiment to semicontinuous model. *J. Agric. Food Chem.* **2003**, *51*, 5680–5687.
(12) Hernandez-Ledesma, B.; Martin-Alvarez, P. J.; Pueyo, E. Assessment of the spectrophotometric method for determination of angiotensin-converting-enzyme activity: influence of the inhibition type. *J. Agric. Food Chem.* **2003**, *51*, 4175–4179.
(13) Parris, N.; Moreau, R. A.; Johnston, D. B.; Singh, V.; Dickey, L. C. Protein distribution in commercial wet- and dry-milled corn germ. *J. Agric. Food Chem.* **2006**, *54*, 4688–4872.
(14) Lo, W. M. Y.; Li-Chan, E. C. Y. Angiotensin I converting enzyme inhibitory peptides from in vitro pepsin-pancreatin digestion of soy protein. *J. Agric. Food Chem.* **2005**, *53*, 3369–3376.
(15) Kuba, M.; Tana, C.; Tawata, S.; Yasuda, M. Production of angiotensin I-converting enzyme inhibitory peptides from soybean protein with *Monascus purpureus* acid proteinase. *Process Biochem* **2005**, *40*, 2191–2196.

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