Angiotensin I Converting Enzyme Inhibitory Peptides Produced by Autolysis Reactions from Wheat Bran

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The production of angiotensin I converting enzyme (ACE) inhibitory peptides by autolysis reactions from wheat milling byproducts was investigated. Milled whole grain, bran, shorts, and red dog acquired ACE inhibitory activity though water soaking treatment. Among the milled fractions, the preparation of shorts exhibited the strongest inhibitory activity ($IC_{50} = 0.08$ mg protein/mL) followed by that of bran, red dog, and whole grain in decreasing order. The production of ACE inhibitory peptides was almost completely inhibited by pepstatin A, indicating the contribution of aspartic proteases. The optimal pH for acquiring ACE inhibitory activity of the byproduct fraction (mixtures of bran and shorts) was 3.2. The level of inhibitory activity rose with increasing temperature up to 40 °C. The inhibitory activity reached a maximal level after a 12 h reaction time and maintained the same level up to 24 h at 40 °C, pH 3.2. From the hydrolysate of the byproduct fraction, six peptides were isolated by several steps of chromatography, and their amino acid sequences were Leu-Gln-Pro, Ile-Gln-Pro, Leu-Arg-Pro, Val-Tyr, Ile-Tyr, and Thr-Phe. Thus, wheat milling byproducts have the possibility to become an effective source for ACE inhibitory peptides.

KEYWORDS: Bran; byproduct; *Triticum aestivum*; autolysis; angiotensin converting enzyme

INTRODUCTION

The rennin−angiotensin−aldosterone system is considered to play a key role in blood pressure homeostasis (*1*). Angiotensin I converting enzyme (ACE, dipeptidyl carboxypeptidase I, EC 3.4.15.1) catalyzes both the production of angiotensin II and the inactivation of bradykinin. Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) constricts the vascular smooth muscle through binding to type 1 angiotensin receptors (*2*). In the adrenal cortex, their activation stimulates the release of aldosterone (*3*), thereby promoting sodium reabsorption in the mineralocorticoid-responsive segments of the distal nephron (*4*). Consequently, the critical role of this pathway in regulation of blood pressure is highlighted by the impressive efficacy of ACE inhibitors.

The outer layers of the kernel are recognized as byproducts in the wheat milling industry. These fractions account for about 30% of seed weight. Because most of these byproducts are usually used as feed for livestock, their effective utilization is of great interest for the wheat milling industry. Mature wheat seeds contain proteolytic enzymes, e.g., serine, aspartic, metalloproteases, and thiol proteases (*5, 6*), in parts of the aleurone, testa, and embryo. In addition, wheat bran has been reported to contain carboxypeptidases (*7*) and aspartic proteinase (*8*). It has been reported that proteinous amino acids and γ-aminobutyric acid are effectively produced from wheat byproduct fraction

by autolysis reactions (*9*). Therefore, this approach has a possibility to produce peptides with biological activities such as ACE inhibition. Usually, ACE inhibitory peptides are derived from food proteins, e.g., fish muscle (*10*), casein (*11*), soybean protein (*12*), and zein (*13*). Edible proteins in food industrial byproducts, such as wheat bran, may be substitutable for existing materials because of their lower supply cost. Moreover, the application of autolysis reactions has advantages of its simplicity and inexpensive costs.

The present study examined the acquisition of ACE inhibitory activity by autolysis in milled parts of wheat seed using whole grain, bran, shorts, red dog, 60% extracted flour, and byproduct fraction, and the optimum conditions for the reaction were explored. The isolation of ACE inhibitory peptides from the autolysate of byproduct fraction is also reported.

MATERIALS AND METHODS

Seed Materials and Reagents. Soft red type wheat, Fukusayaka, was grown and harvested at the National Agricultural Research Center for Western Region in Fukuyama, Hiroshima, Japan, in 2007 and 2008 and combined for the experiments. Immature seeds were harvested at 4 weeks after anthesis. For the preparation of germinated seeds, mature seeds were surface-sterilized in 1% (v/v) NaOCl for 20 min and washed twice with sterile water. Surface-sterilized grains were allowed to germinate on sterile filter paper soaked with water in the dark at 20 °C. After 3 days of germination, the seeds were blotted and air-dried. Dispo SPE C_{18} cartridges were purchased from YMC (Kyoto, Japan), LiChroprep RP-18 column was from Merck (Darmstadt, Germany), AG MP-1 resin

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was from Bio-Rad (Hercules, CA), Superdex 75HR column was from GE Healthcare (Uppsala, Sweden), and Jupiter C_4 and C_{18} columns were from Phenomenex (Torrance, CA). Purified rabbit lung ACE, Pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), PMSF, and EDTA were supplied from Sigma (St. Louis, MO).

Preparation of Seed Powder. The wheat samples were cleaned and conditioned to 14.5% moisture content and milled in a test mil (model MLU 202, Bühler, Uzwill, Switzerland) to obtain bran, shorts, and three break and three reduction milling fractions according to AACC approved method 26-31 (*14*). The mill was equipped with three break and three reduction rolls and a steel screen and a filter for each roll. Break (B1–B3) flour was recovered from the first mill roll after passing through a screen and a filter. Reduction (R1–R3) flour was recovered from the second mill roll in the same way. Bran was the unreduced fraction after the first mill roll and shorts sifted from the break and reduction flour fractions. The 60% extracted flour was a composite of break and reduction flour fractions combined in the order of B1, R1, B2, R2, B3, and R3 up to 60% of milling yield. Excess amount of flour over 60% yield was defined as red dog, which is normally included in R3 fraction. The byproduct fraction was prepared by mixing the bran and shorts fractions. Whole grain samples of immature, mature, and germinated seed and the bran and shorts fractions from Bühler test mill were disrupted into particles of less than 200 μm in size using a centrifugal mill (model ZM 200, Retsch, Germany) using a 0.5 mm screen. All samples were kept at −30 °C until use.

Autolysis Reaction and Preparation of Autolysate Sample. Portions (200 mg) of the milled samples were suspended in 4 mL of 50 mM citrate–phosphate buffer (pH 3.2) and autolyzed at 40 °C for 12 h. After being heated at 90 °C for 5 min, the mixture was centrifuged at 10000g for 20 min. The supernatant was recovered and applied to the Dispo SPE C_{18} cartridge (0.5 g) preconditioned by passing 4 mL of ethanol through and deionized water subsequently. The cartridge was washed with 4 mL of water, and ACE inhibitory peptides were eluted with 10% aqueous ethanol. The organic solvent was evaporated, and the rest was lyophilized and analyzed for inhibitory activity and protein content. Pepstatin A, E-64, PMSF, and EDTA were utilized at final concentrations of 1.46, 20, and 1000 μM, respectively. Each inhibitor was added to the reaction buffer, and the reaction was performed in the same manner as described above.

Assay for ACE Inhibitory Activity. ACE inhibitory activity was measured by applying a modified Lieberman's method (*15*). A mixture containing 50 μL of sample, 100 μL of ACE (25 mU/mL), and 100 μL of 12.5 mM hippuryl-L-histidyl-L-leucine in a borate buffer (pH 8.3) containing 1 M NaCl was reacted at 37 °C for 1 h. After the reaction was stopped by adding 250 μL of 0.5 M HCl, the hippuric acid was extracted with 1.5 mL of ethyl acetate. An aliquot (500 μL) from the upper layer was evaporated to dryness and then dissolved in 3 mL of 1 M NaCl. The concentration of the resulting hippuric acid was determined from its absorbance at 228 nm. The protein content was analyzed using a rapid N III (Elementar Analysensysteme GmbH, Donaustrasse, Germany). The IC_{50} value was defined as the concentration of peptide required to inhibit 50% of the ACE activity under the assayed condition.

Separation of ACE Inhibitory Peptides by Column Chromatographies. The byproduct fraction (100 g) was defatted three times using 500 mL of *n*-hexane, resuspended in 500 mL of water after drying, and subjected to autolysis at 40 °C for 12 h after the pH was adjusted to 3.2 with 0.1 M HCl. After being heated at 90 °C for 5 min, the mixture was filtered through filter paper. Then the filtrate was adjusted to pH 6.0 with 0.1 M NaOH and centrifuged at 10000g for 20 min. The supernatant was recovered and applied to a LiChroprep RP-18 low pressure column (2.5 cm × 25 cm). After the column was washed with 1 L of water, the retained compounds were eluted with 500 mL each of 10%, 20%, 50%, and 95% aqueous ethanol. The 10% aqueous ethanol fraction was concentrated under reduced pressure, the pH was adjusted to 9.0 by 1 M NH$_4$OH, and the eluate was applied to AG MP-1 anion exchange (OH$^-$) resin (3.0 cm × 20 cm). The compounds not adsorbed to the resin were collected and lyophilized.

Purification of ACE Inhibitory Peptides by HPLCs. ACE inhibitors from AG MP-1 fraction were purified by several steps of HPLC (Shimadzu LC-10AD system, Kyoto, Japan). For the first step of gel-permeation chromatography, an aliquot (100 μL) of the peptide preparation was put on a Superdex 75HR column (10 cm × 30 cm). The column was eluted using 30% acetonitrile (CH$_3$CN) containing

Table 1. ACE Inhibitory Activity of Autolysates from Various Part of Fukusayaka Seed after Dispo SPE C_{18} Separation

	IC_{50} (mg protein/mL)	protein (mg)
bran	0.14 ± 0.01[a]	6.0 ± 0.21[b]
shorts	0.08 ± 0.01	6.1 ± 0.24
red dog	0.24 ± 0.02	2.3 ± 0.13
60% extracted flour	2.1 ± 0.15	2.2 ± 0.09
whole grain	0.32 ± 0.02	3.3 ± 0.12
immature seed	0.74 ± 0.06	10.5 ± 0.68
germinated seed	0.30 ± 0.04	7.5 ± 0.51

[a] Mean of four replicates. [b] Protein content was determined using a rapid N III analyzer.

0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 mL/min with detection at 220 nm. Each fraction with ACE inhibitory activity was collected. A Jupiter C_4 reverse phase column (10 mm × 250 mm, 5 μm, 300 Å) was used as the second step of purification. The active fractions (50 μL) were injected into the column equilibrated with 0.1% TFA and eluted with a linear gradient of CH$_3$CN (0−35%, 30 min) in 0.1% TFA at a flow rate of 4.0 mL/min. The peptides of F3-1 to F3-3 were further purified using a Jupiter C_{18} reverse phase column (10 mm × 250 mm, 5 μm, 300 Å). The conditions of chromatography were the same as above.

Identification of ACE Inhibitory Peptides. The amino acid sequence was analyzed using a Shimadzu PPSQ-21 protein sequencer coupled with the HPLC identification of the resulting phenylthiohydantoin amino acids. The molecular weight analysis was performed using a Voyager-DE STR (Applied Biosystems, Foster City, CA) MALDI-TOF MS instrument using 2,5-dihydroxybenzoic acid as the matrix.

RESULTS AND DISCUSSION

Differences in ACE Inhibitory Activity of Milled Fractions. All seed parts of Fukusayaka except for 60% extracted flour substantially acquired ACE inhibitory activity after autolysis reactions for 12 h (**Table 1**). The inhibitory activity (IC_{50}) of the preparation of shorts, bran, red dog, whole grain, and 60% extracted flour were 0.08, 0.14, 0.24, 0.32, and 2.1 mg protein/mL, respectively. The difference in IC_{50} values between shorts and bran was attributable to that in components of these fractions. Bran consists of the outer covering of the kernel and part of the aleurone layer, while shorts is composed of fine particles of bran, the aleurone layer, and wheat germ in which the existence of an acid protease was reported (*16*). The protein content of shorts preparation was the same as that of bran, while those of red dog and 60% extracted flour were apparently lower than those of the outer seed parts. Gluten preparation has been reported to have protease activities (*17*), but the degradation of endosperm proteins was limited and derived peptides did not seem to have ACE inhibitory activity. As for whole grains with different maturities, the inhibitory activities of the mature seed (whole grain) and germinated seed were the same and that of immature seed was less than half. The larger content of protein from germinated seed autolysate compared with that of mature seed may be the result of protease activity, such as thiol protease (*18*) and carboxypeptidase (*19*) synthesized de novo in germinating seed that contribute to the degradation of storage proteins including gluten (*20*). As a result, shorts and bran were concluded to be effective sources for producing ACE inhibitory peptides among milling fractions.

Optimum pH and Temperature for Acquiring ACE Inhibitory Activity. The ACE inhibitory activity showed its maximum at pH from 3.1 to 3.2 with a shoulder of the activity at pH from 4.2 to 5.6 in the pH profile assayed over a pH range from 1.6 to 6.5 (**Figure 1**). The aspartic protease in wheat bran has been reported to have the pH optimum at 3.3 using globulin as a substrate (*8*). On the other hand, three isoforms of acid carboxypeptidase in dormant wheat seed have been reported to
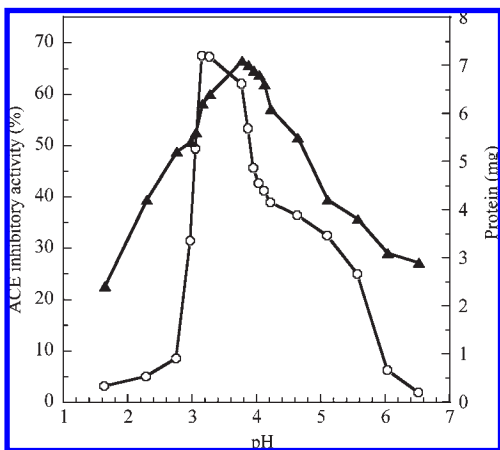
Figure 1. Effect of pH on acquiring ACE inhibitory activity by autolysis reaction: ACE inhibitory activity, ○; protein content, ▲ . Wheat byproduct fraction (200 mg) was incubated at 40 °C for 12 h. Partially purified autolysates by Dispo SPE C_{18} cartridge were examined for ACE inhibitory activity at 167 μg/mL and for protein content.
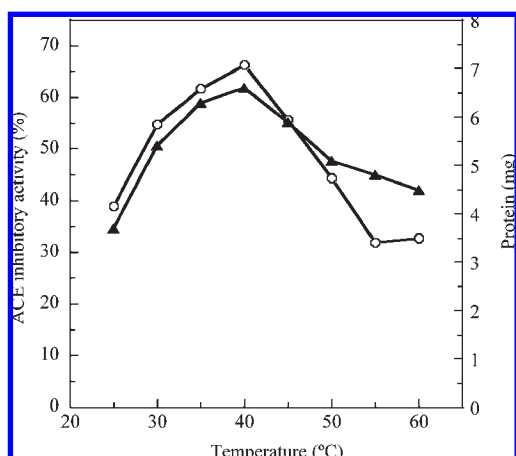

Figure 2. Effect of temperature on acquiring ACE inhibitory activity by autolysis reaction: ACE inhibitory activity, ○; protein content, ▲ . Wheat byproduct fraction (200 mg) was incubated at pH 3.2 for 12 h. The autolysates were examined as described in **Figure 1**.

have pH optima between 4.4 and 5.8, indicating that the acid carboxypeptidases are involved in the formation of the shoulder (*21*). The optimum pH of protein content was about pH 3.8 and formed a broad spectrum compared with that of ACE inhibitory activity. The pH optimum value of the protein content was supported by the report describing that mixtures of embryo proteases from mature seeds had the optimum pH at 4.0 (*6*). The effect of temperature on the acquisition of ACE inhibitory activity by autolysis reaction is shown in **Figure 2**. In this experiment, the optimal temperature of acquiring ACE inhibitory activity and protein content was 40 °C, indicating that proteases contributed to the release of ACE inhibitory peptides, and those participating in the degradation of proteins have optimal values close to each other. On the basis of these results, the condition of the autolysis reaction for producing ACE inhibitory peptides from the byproduct fraction was designated as pH 3.2 and 40 °C.

Effect of Protease Inhibitors on ACE Inhibitory Activity. The effect of potential protease inhibitors on the formation of ACE inhibitory activity of autolysate preparation from the byproduct fraction of Fukusayaka in a 12 h reaction is summarized in **Table 2**. The ACE inhibitory activity was almost completely

Table 2. Effect of Protease Inhibitors on the Formation of ACE Inhibitory Activity of Autolysate Preparation Obtained from the Byproduct Fraction of Fukusayaka

inhibitor	inhibition rate (%)[a]
Pepstatin A	95.7[b]
PMSF	14.6
EDTA	0.4
E-64	0.3

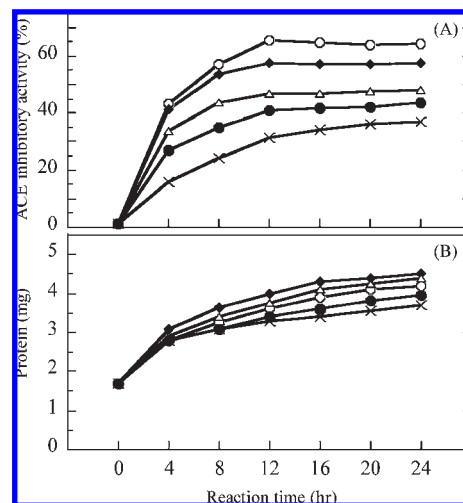[a] Inhibition value relative to that of the control. [b] Mean of three replicates.

Figure 3. Time dependent changes in acquiring ACE inhibitory activity (**A**) and protein content (**B**) reacted at various pH levels: pH 3.2, ○; pH 3.7, ◆; pH 4.2, △; pH 4.7, ●; pH 2.7; × . Wheat byproduct fraction (100 mg) was incubated at 40 °C. The autolysates were examined as described in **Figure 1**.

inhibited by pepstatin A, indicating the principal contribution of aspartic proteases, which is supported by the results of the optimum pH. The participation of serine proteases, such as carboxypeptidase, was also suggested by the result of 14.6% inhibition by PMSF (*22*). E-64, an inhibitor of thiolprotease, did not have any effect on ACE inhibitory activity as with the production of α-amino acids from the wheat byproduct fraction (*9*), although activity and quantity are different units. In amino acid production, the release of most kinds of amino acids was suppressed by EDTA in addition to pepstatin A and PMSF. Accordingly, it was suggested that proteases participating in the acquisition of ACE inhibitory activity were different partly from those in the production of amino acids.

Time Dependent Changes of ACE Inhibitory Activity. To understand changes of ACE inhibitory activity during a long reaction time, the byproduct fraction was autolyzed at 40 °C for 24 h at selected pHs. The increase in inhibitory activity at pH 3.2, 3.7, and 4.2 reached a maximum level in a 12 h reaction and maintained the same level until 24 h, as shown in **Figure 3A**. The protein content was not so much affected by the reaction pH as ACE inhibitory activity (**Figure 3B**). The inhibitory activities of samples at all pH values increased sharply in the first 4 h of reaction and increased gradually until 12 h. A longer reaction time than 24 h should result in increased amounts of proteins but may not always be related to the increase in ACE inhibitory activity. From these results, the reaction condition of the wheat byproduct fraction was designated as 40 °C, pH 3.2, and a 12 h reaction period.

Purification of ACE Inhibitory Peptides. The inhibitory activity and the yield of protein at each purification step are listed in

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Table 3. Summary of the Purification of ACE Inhibitory Peptides from the Autolysate of Byproduct Fraction of Fukusayaka

purification step	IC$_{50}$ (μg protein/mL)	yield (mg of protein)
reaction mixture	940	20250
LiChroprep RP-18	86	2100
AG MP-1	45	1200
Superdex 75HR		
F3	44	258
F7	76	87.4
Jupiter C$_{18}$		
F3-1	0.78	15.0
F3-2	1.35	15.1
F3-3	0.08	12.5
Jupiter C$_4$		
F7-1	5.90	19.2
F7-2	0.99	5.8
F7-3	4.81	3.1

Table 4. Amino Acid Sequence, Molecular Weight, and IC$_{50}$ Value of ACE Inhibitors Isolated from the Autolysate of Byproduct Fraction of Fukusayaka

peak id[a]	amino acid sequence	[M + H]$^+$ theoretical	[M + H]$^+$ experimental	IC$_{50}$ (μM)
F3-1	Leu-Gln-Pro	357.2140	357.2083	2.2 $\pm$ 0.08[b]
F3-2	Ile-Gln-Pro	357.2140	357.2083	3.8 $\pm$ 0.13
F3-3	Leu-Arg-Pro	385.2566	385.2420	0.21 $\pm$ 0.01
F7-1	Val-Tyr	281.1502	281.1447	21 $\pm$ 0.67
F7-2	Ile-Tyr	295.1659	295.1532	3.4 $\pm$ 0.11
F7-3	Thr-Phe	267.1346	267.1201	18 $\pm$ 0.62

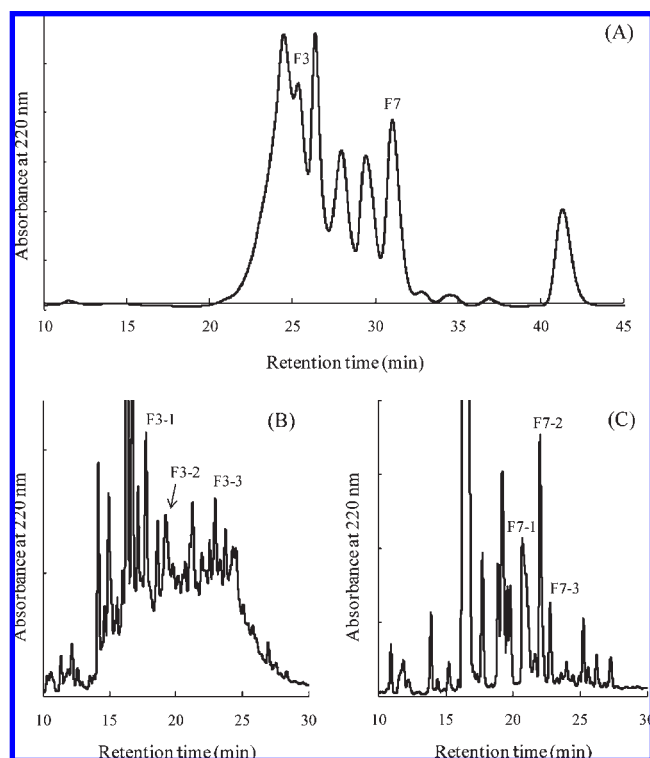[a] See **Figure 4** for the identification of peaks. [b] Mean of four replicates.

Figure 4. Purification of active fraction from an AG MP-1 column. The active fraction was separated into two peaks of F3 and F7 using a Superdex 75HR column (**A**). These peaks were separated on a C4 column, and the three peaks were obtained from F3 (**B**) and F7 (**C**).

Table 3. The inhibitory activity of crude autolysate was low (IC$_{50}$ = 940 μg protein/mL), but it was increased more than 10-fold by the first purification with ODS column chromatography. The IC$_{50}$ value (86 μg/mL) of this fraction was comparable to that of sardine peptides (83 μg/mL) derived from sardine muscle by alkaline protease hydrolysis and prepared by ODS column chromatography (*23*). Despite that the inhibitory peptides were not adsorbed to the AG MP-1 resin, the IC$_{50}$ value increased about 2-fold as a consequence of the adsorption of anionic peptides. In subsequent separation of the active fraction by high performance gel permeation chromatography, the elution of about 10 peaks was observed (**Figure 4A**). Fractions F3 (IC$_{50}$ = 44 μg/mL) and F7 (IC$_{50}$ = 76 μg/mL) that exhibited potent inhibitory activity among eluted peaks (data not shown) were collected (**Table 3**). By the following Jupiter C$_4$ reverse phase

column chromatography, three inhibitory peptides (F7-1, F7-2, and F7-3) in fraction F7 were purified (**Figure 4C**), while the purification of peptides from F3 fraction was unsuccessful (**Figure 4B**). Finally, each inhibitory peptide obtained from fraction F3 was further purified by Jupiter C$_{18}$ reverse phase column chromatography and isolated. Among isolated peptides, F3-3 had the most potent inhibitory activity (IC$_{50}$ = 0.08 μg/mL) and 12.5 mg was obtained from 100 g of byproduct fraction (**Table 3**).

Identification of ACE Inhibitory Peptides. Data of N-terminal amino acid sequence and molecular weight of the identified peptides are listed in **Table 4**. The difference between the observed and theoretical molecular weight, which were calculated from amino acid sequence data, was less than 0.015 for each peptide. From this result, peptides of F3-1, F3-2, F3-3, F7-1, F7-2, and F7-3 were determined to be Leu-Gln-Pro, Ile-Gln-Pro, Leu-Arg-Pro, Val-Tyr, Ile-Tyr, and Thr-Phe, respectively. To the best of our knowledge, Ile-Gln-Pro was novel as an ACE inhibitory peptide, while other peptides have been already reported. Leu-Gln-Pro has been isolated from the hydrolysate of α-zein (*24*), Leu-Arg-Pro from α-zein and bonito bowels (*25*), Val-Tyr from sardine muscle (*26*), and Ile-Tyr and Thr-Phe from wheat germ (*27*). The inhibitory potency of Ile-Gln-Pro (IC$_{50}$ = 3.8 μM) was similar to that of Leu-Gln-Pro (IC$_{50}$ = 2.2 μM) and Ile-Tyr (IC$_{50}$ = 3.4 μM), and its yield (15.1 mg) was relatively large among the isolated peptides. We wondered what kind of proteins these peptides were derived from. As a result of searching a database, all peptides were included in the sequence of either α/β-gliadin (*28, 29*) or γ-gliadin (*30*). Thus, these peptides were thought to be derived from gliadins in the byproduct fraction. Motoi et al. purified Ile-Ala-Pro as an ACE inhibitory peptide derived from wheat gliadin hydrolysate after hydrolysis by pepsin and protease M (*31*). Although this peptide is an internal sequence of α- and α/β-gliadin, it was not obtained in the present experiment. Possibly, different proteases may release different peptide fragments. Kawasaki et al. reported Val-Tyr to have an antihypertensive effect on mild hypertensive subjects via ACE inhibition (*26*). According to them, administration of a 100 mL drink containing 3 mg of the peptide twice a day significantly reduced systolic and diastolic blood pressure at the first week and kept the effect during the experimental period of 4 weeks. Viewed from the potency of Val-Tyr at such a low dose, the yield of Val-Tyr (19.2 mg/100 g) from the byproduct fraction seems to be practical for its utilization as a source of this peptide. All other peptides isolated in this study had stronger ACE inhibitory activity than Val-Tyr, while a potent inhibitor of ACE should not always exhibit a potent antihypertensive effect (*32*). Further studies are needed to estimate antihypertensive effects of each peptide.

The purpose of this study was to add values to wheat milling byproducts through finding of biologically active peptides. As a result, the byproduct fraction produced potent

ACE inhibitory peptides with good yields by autolysis reaction. The application of the autolysis reaction has the advantages of being a simple process and inexpensive costs. These findings would lead to effective utilization and a decrease of the excess amount of milling byproducts.

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