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Isolation and Characterization of a Low Molecular Weight Peptide Contained in Sourdough

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To investigate a sourdough-specific peptide, low molecular weight peptides were extracted from sourdough. The peptide fraction was subjected to two kinds of chromatography to separate the peptides. Reverse-phase chromatography of the peptide fraction in the sourdough showed certain specific peptides. The specific peptide fraction was further separated by gel filtration chromatography. Liquid chromatography tandem mass spectrometry analysis identified one of the peptides as VPFGVG (six-mer). This sequence was estimated to occur at the 287−292 position of a low molecular weight glutenin subunit. The peptide (designed as SDP1) was produced by proteases derived from wheat flour. SDP1 showed angiotensin-converting enzyme (ACE) inhibitory activity, and the 50% inhibitory peptide concentration (IC_{50}) was 336 μM. It is possible that the SDP1 peptide partially confers ACE inhibitory activity in sourdough.

KEYWORDS: Sourdough; lactic acid bacteria; baker's yeast; ACE inhibitory peptide

INTRODUCTION

Sourdough is traditionally used in breadmaking and contains many kinds of compounds in comparison to bread dough, including organic acids, bacteriocins (*1*), antifungal compounds (*2*), and exopolysaccharides (*3*). Therefore, the use of sourdough in breadmaking affects bread properties such as aroma, taste, nutritive value, and shelf life (*4*). Sourdough fermentations are generally characterized by the association of lactic acid bacteria (LAB) and yeast (*4*, *5*). The nutritional and textural properties of sourdough depend on the metabolism and cooperative activities of the LAB and yeast. The utilization of sourdough fermentation increased proteolysis and amino acid liberation in the dough. This increased proteolysis is attributed to both the proteolytic activity of LAB and the enhanced proteolysis brought about by cereal enzymes under the acidic conditions of sourdough (*6*, *7*). The increased proteolysis also enhances the human tolerance to gluten (*8*, *9*).

The proteolytic activity of LAB, such as *Lactobacillus sanfranciscensis*, isolated from sourdough has been investigated and was found to include a proteinase, a dipeptidase, and an aminopeptidase (*10*). Certain *Lactobacillus* strains have the ability to hydrolyze the albumin, globulin, and gliadin fractions of wheat flour but not the glutenins (*8*, *9*). However, the primary proteolytic activity in wheat sourdough is the enhancement of cereal aspartic protease in acid aseptic doughs (*6*, *11*−*13*).

Sourdough fermentation is known to cause an increase in the total concentration of peptides and free amino acids as compared with bread dough (*14*−*17*). Peptides and amino acids play an essential role as a nitrogen source for microorganisms and as a flavor precursor of baked sourdough products. Specific amino acids and low molecular weight peptides (LMWPs) excreted from yeasts stimulate bacterial growth (*5*). A LMWP extracted from compressed yeast has been identified as a growth stimulant for *L. sanfranciscensis* (*18*). Although there has been a detailed analysis of the LMWPs of wheat dough (*19*), there has been little research conducted on the LMWPs of sourdough.

Whole grains used as ingredients in sourdough contain many bioactive compounds such as lignans, phenolic acids, phytosterols, tocopherols, and folates (*20*). Sourdough fermentation has been reported to increase folate content and decrease tocopherol content (*21*). It has been reported that the *Lactobacillus* strain decarbonizes glutamine and turns it into γ-aminobutyric acid (GABA) (*22*). GABA is considered to be effective in lowering blood pressure, in acting as a tranquilizer, and in promoting the activity of the visceral organs and brain (*23*, *24*). Although the amount of GABA may be increased in sourdough, there have been no investigations yet reported.

The objective of the present study was to clarify the LMWPs generated during sourdough fermentation. We determined the amino acid sequence of a sourdough-specific peptide. We found that the peptide shows angiotensin-converting enzyme (ACE) inhibitory activity. We also found that there was a higher amount of GABA in sourdough.

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Table 1. Dough Formulas

	control dough (g)	acid dough (g)	sourdough (g)
flour	100	100	100
NaCl	2	2	2
water	80	80	80
panettone sourdough starter	0	0	10
compressed yeast	0	0	2
lactic acid	0	0.5	0

MATERIALS AND METHODS

Microorganisms. Compressed yeast (regular type; Oriental Yeast Co., Ltd., Tokyo, Japan) used in breadmaking was used in this study. Panettone sourdough starter (a portion of ripe sourdough) (Panex, Gifu, Japan) containing LAB and yeast was used as a sourdough starter. The LAB were enumerated on de Man, Rogosa, and Sharpe agar (*25*) and sourdough bacteria agar (*26*) containing cycloheximide and sodium azide (10 mg/L) and were anaerobically incubated at 30 °C for 48h. Yeasts were enumerated on YPD plates. Individual isolates from the plates were randomly picked as representatives from all morphologically distinct colonies and were subcultured and purified.

Identification of LAB and Yeast. To identify the LAB in sourdough, the 16S rDNA partial region was amplified by polymerase chain reaction (PCR) using specific primers as described by Mori et al. (*27*). The sequences of the PCR products were determined directly with the primers as described above. Analyses of the DNA sequence reactions were performed with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The 16S rDNA sequence was compared with known sequences registered in the DNA Database of Japan (DDBJ). To identify the yeasts in sourdough, the 26S rDNA partial region was amplified by PCR using specific primers as described by Kurtzman and Robnett (*28*).

Dough Fermentation. A wholemeal wheat flour with 13% protein content (w/w dry basis), 0.5% ash content (w/w dry basis), and 13% moisture (w/w) was used in all tests. For prefermentation, the ingredients (50 g of flour, 50 g of water, and 50 g of panettone sourdough starter) were mixed and incubated at 30 °C for 24 h, and the same fermentation process was repeated. Preferments containing $1-10 \times 10^7$ cfu of LAB were used for sourdough making. The doughs were fermented with or without starters at 30 °C for 5 h (**Table 1**). After fermentation, the doughs were freeze-dried and milled to a powder using a coffee mill.

Extraction of Peptides. Peptide fractions were prepared using a modified method of sequential protein extraction from wheat flour (*29*). All centrifugation steps were carried out at 10000*g* for 20 min at 4 °C. Powders (2.5 g) of freeze-dried dough were extracted with 10 mL of distilled water for 1 h at 4 °C with inverting and centrifuged. After centrifugation, 5 mL of distilled water was added to the pellet for 30 min and the mixture was centrifuged. The two supernatants were combined (water-extractable fraction). The pellet remaining after extraction with water was further extracted first with 10 mL and then with 5 mL of 70% (v/v) ethanol for 1 h and 30 min, respectively, at 4 °C. Following centrifugation, the two supernatants were combined (ethanol-extractable fraction). Furthermore, the pellet was extracted with 10 mL of 0.05 M acetic acid for 1 h at 4 °C and centrifuged. The supernatant was defined as an acid-extractable fraction. All samples were filtered using Amicon Ultra-4 10K MWCO centrifugal devices (Millipore, Billerica, MA). Flow-through LMWP fractions (molecular mass under 10 kDa) were used for the high-performance liquid chromatography (HPLC) analysis. The peptide concentration was estimated using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Protease Assay. A protease assay was performed on the basis of the method described in Kruger (*30*). The water-extractable fraction described above or 0.1% pepsin was used as the enzyme solution. The azocasein substrate solution was prepared by dissolving 1 g of azocasein (Nacalai Tesque, Kyoto, Japan) in 50 mL of sodium citrate buffer (50 mM, pH 5.0). The reaction was started by adding the enzyme solution (0.5 mL) to the substrate solution (0.5 mL) and was incubated at 37 °C for 4 h. The reaction was terminated by the addition of 1 mL of 10% trichloroacetic acid. After 10 min at room temperature, the mixture was centrifuged at 10000*g* for 10 min. Sodium hydroxide (0.5 N, 0.5 mL) was added to 0.5 mL of the supernatant. After 20 min at room temperature, the absorbance of the solution was measured at 440 nm.

HPLC Analysis. The LMWP fractions were examined using an octadecyl silica (ODS) column (TSK-GEL ODS-80TS; Tosoh, Tokyo, Japan). The column was developed at a flow rate of 0.8 mL/min by a linear gradient of acetonitrile (0−30%/60 min) containing 0.05% trifluoroacetic acid. The ODS1 fraction was dried using a centrifugal concentrator. For size fractionation, the ODS1 fraction was applied to a gel filtration column (Shodex Asahipak GF-310 HQ; Showa Denko, Tokyo, Japan) and the column was eluted with 30% acetonitrile at a flow rate of 0.4 mL/min.

Mass Spectrometry and Amino Acid Sequencing. Nanoelectrospray tandem mass analysis was performed using an LCQ Advantage nanospray ionization ion trap mass spectrometer (Thermo Finnigan Co., San Jose, CA) combined with a Magic2002 HPLC system (Michrom BioResources, Inc., Auburn, CA). The peptide mixture sample was injected into the Magic2002 HPLC system, which was equipped with a MAGIC C18 column. Multiple tandem mass spectrometric (MS/MS) spectrum data were collected with the TurboSEQUEST program (Thermo Finnigan), merged as SEQUEST format files, and submitted to the Mascot Search program, available on the Matrix Science website (http://www.matrixscience.com/) for the MS/MS ion search.

In Vitro Assay for ACE Inhibitory Activity. The ACE inhibitory activity was determined following the method described by Cushman and Cheung (*31*) with slight modifications. Hippuryl-L-histidyl-L-leucine (Hip-His-Leu; Nacalai Tesque) was dissolved in 0.2 M sodium borate buffer (pH 8.3) containing 1 M NaCl. Next, 150 μL of 2 mg/mL Hip-His-Leu solution was mixed with 50 μL of peptide solution. The reaction was initiated by the addition of 50 μL of ACE (Sigma-Aldrich, St. Louis, MO) solution (0.05 U/mL), and the mixture was incubated for 1 h at 37 °C. The reaction was stopped by the addition of 250 μL of 1 N HCl. The hippuric acid liberated by ACE was extracted with 1.5 mL of ethyl acetate. After the removal of ethyl acetate by vacuum evaporation, the hippuric acid was dissolved by 1 mL of distilled water and measured at an optical density of 228 nm. The 50% inhibitory peptide concentration (IC_{50}) value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

Synthesis of Peptides. Two oligopeptides (SDP1, VPFGVG; and $CEI_{\beta 7}$, AVPYPQR) were synthesized by Tsukuba Oligo Service Co., Ltd. (Tsukuba, Japan). The purity of the synthesized peptides was above 95% in both cases.

Stability of SDP1 Peptide against Digestive Proteinases. The synthetic SDP1 peptide (50 μg) was incubated in 1 mL of 0.1% (w/v) pepsin (0.1 N HCl), at 37 °C for 2 h, and then, the reaction was neutralized with 1 N NaOH. The SDP1 peptide was also incubated in 1 mL of 0.1% (w/v) trypsin (0.2 M sodium borate buffer, pH 8.3, 5 mM $CaCl_2$) or 0.1% (w/v) chymotrypsin (0.2 M sodium borate buffer, pH 8.3) at 37 °C for 2 h. The reaction mixtures were filtered using Microcon YM-3 centrifugal devices (Millipore). Flow through (molecular mass under 3 kDa) was applied to an ODS column (TSK-GEL ODS-80TS; Tosoh) and then eluted with a linear gradient of 0−30% (v/v) of acetonitrile containing 0.05% TFA at a flow rate of 0.8 mL/min. The absorbance was monitored at 215 nm.

Measurement of Free Amino Acids Content. The content of the free amino acids was measured as described previously (*32*). Briefly, 0.2 g of freeze-dried dough was suspended to 1 mL of 75% (v/v) ethanol and vigorously stirred. After centrifugation, the supernatant was dried using a centrifugal concentrator. The residual substance was dissolved in 0.4 mL of the sample application buffer containing 5.3 mM trilithium citrate, 94 mM citric acid, 283 mM lithium chloride, 194 mM 2,2′-thiodiethanol, and 0.6 mM octanoic acid. The sample was injected into an Amino Acid Analyzer model LC-11A (Yanagimoto Mfg. Co., Ltd., Kyoto, Japan).

RESULTS AND DISCUSSION

Properties of Dough. Sourdough fermentation was carried out using a panettone sourdough starter and a commercial compressed yeast. In the panettone sourdough starter, the

Table 2. Physiological Properties of the Dough[a]

	cfu/g dough			
	yeast content	LAB content	pH (before fermentation)	protease activity (ΔOD_{440}/g dry dough)
control dough	<10	3.0×10^4	6.19 (6.32)	5.29 ± 0.29
acid dough	<10	2.7×10^4	5.17 (5.01)	6.02 ± 0.34
sourdough	5.2×10^6	4.2×10^6	5.52 (5.71)	6.11 ± 0.10

[a] Determinations were carried out in duplicate.

Table 3. Peptide Concentrations and ACE Inhibitory Activity of Dough Extracts[a]

	fraction	peptide yield (mg/mL) (before fermentation)	IC_{50} (mg peptide/mL)
control dough	water	18.46 (10.74)	2.99
	ethanol	3.10	ND
	acid	0.91	ND
acid dough	water	20.20 (12.57)	2.33
	ethanol	3.19	ND
	acid	0.90	ND
sourdough	water	8.01 (14.92)	2.10
	ethanol	2.09	ND
	acid	0.8	ND

[a] ND, not detected. Determinations were carried out in duplicate.

predominant LAB and yeast belonged to the species *L. sanfranciscensis* and *Candida humilis*, respectively (data not shown). The properties of the dough are shown in **Table 2**. We used three types of dough. The LAB of the flour was increased in the control and acid doughs. The pH values of the control dough and sourdough were decreased but that of the acid dough was increased. Acidified dough (acid dough and sourdough) slightly increased the protease activity. This observation was consistent with a previous report (*13*).

Peptide Extraction and Fractionation. After fermentation, the doughs were freeze-dried and milled to a powder. Peptides were extracted from the dough powder using distilled water, ethanol, and acetic acid. All fractions were subjected to ultrafiltration to acquire the LMWP fraction. **Table 3** shows the peptide concentration of each LMWP fraction. The concentration of LMWPs in the sourdough was decreased as compared with the control and acid doughs (**Table 3**). This decrease was caused by the fermentation of yeast and LAB. Benedito de Barber et al. (*17*) report that amino acid, peptides, and proteins decrease during fermentation. However, in the present study, the concentration of LMWPs in sourdough was found to be much higher than that in dough fermented by yeast only (data not shown).

Isolation of the Sourdough Peptide. To determine the sourdough-specific peptide, peptide fractions were applied to reverse-phase (RP) chromatography using an ODS column (**Figure 1**). Sourdough-specific peaks were detected in the water-extracted LMWP fraction, but no specific peaks were detected in the other fractions. One of the largest peak fractions (ODS1) in the water-extracted LMWP fraction was collected and subjected to further analysis. The ODS1 fraction from RP-HPLC was separated by gel filtration HPLC, and three major peaks were found at the fraction of molecular mass below 1000 Da (**Figure 2**). SDP1 was identified based on its amino acid sequence, but the other peaks were not identified. An analysis using liquid chromatography MS/MS (LC/MS/MS) showed that the SDP1 fraction was Val-Pro-Phe-Gly-Val-Gly (VPFGVG; six-mer, MW = 574.7). The SDP1 fraction was revealed to be below 300 Da by gel filtration HPLC because a hydrophobic

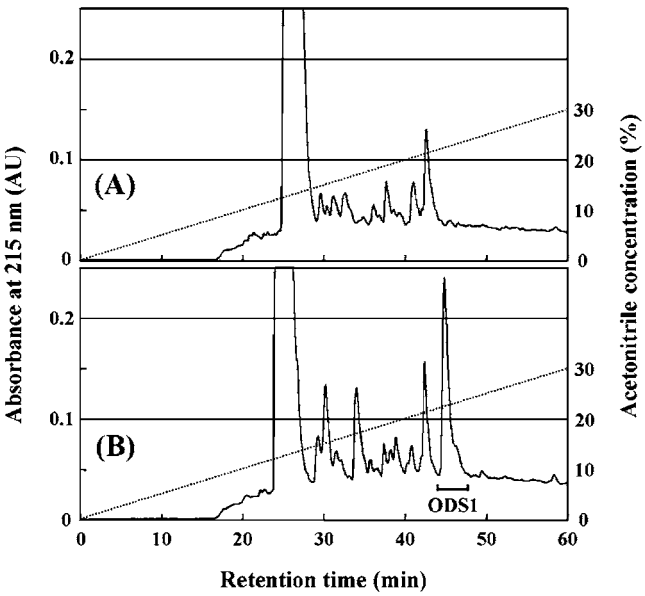

Figure 1. Reverse-phase chromatography of water extractable fractions of sourdough. Two milligrams of water extractable fractions of control dough (**A**) and sourdough (**B**) was injected and separated at a flow rate of 0.8 mL/min by a linear gradient of acetonitrile.

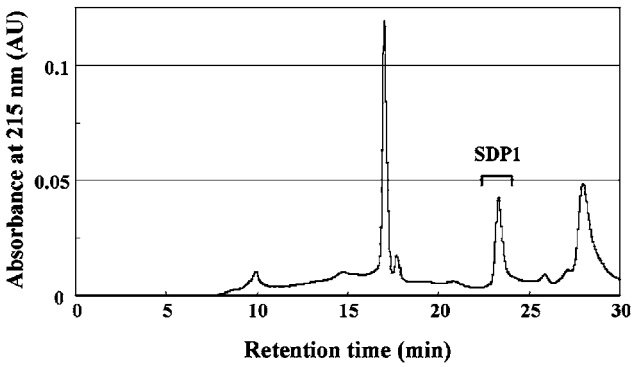

Figure 2. Gel filtration chromatography of the ODS1 fraction. The ODS1 fraction was loaded and eluted with 30% acetonitrile at a flow rate of 0.4 mL/min.

or hydrophilic interaction will affect the results obtained if the column is used. The amino acid sequence of the SDP1 peptide was estimated to be that at the 287−292 position of a low molecular weight glutenin subunit. The SDP1 peptide is possibly produced by proteases derived from wheat flour and/or LAB. To determine which protease is involved in the synthesis of the SDP1 peptide from low molecular weight glutenin subunits, we analyzed the chemically acidified dough (acid dough). As a result, the SDP1 peptide was presented in the acid dough (**Figure 3**). This result revealed that the production of SDP1 is mainly due to the activity of acid protease derived from wheat flour.

ACE Inhibitory Activity of Synthetic Peptides. SDP1 is similar to those of ACE inhibitory peptides (**Table 4**). SDP1 peptide was synthesized for the assay of ACE inhibitory activity. $CEI_{\beta 7}$ peptide was also synthesized as a control of ACE inhibitory peptide (*33*). Both the synthesized and the isolated SDP1 peptide showed the same elution profile on RP-HPLC and gel filtration HPLC. The IC_{50} of SDP1 was 336 μM. The SDP1 peptide showed relatively weak activity as compared with the $CEI_{\beta 7}$ peptide (IC_{50}, 22 μM). The SDP1 peptide content in sourdough was estimated at 5.8 μg/g dry weight by HPLC using

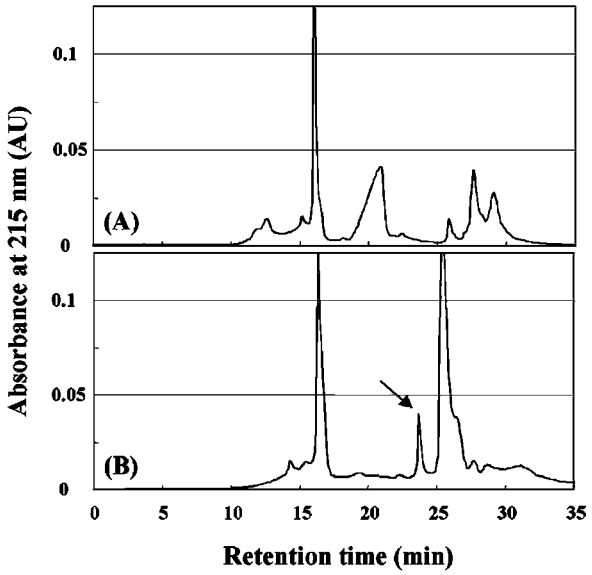

Figure 3. Detection of SDP1 peptide in the acid dough. The ODS1 fraction of the control dough (**A**) and acid dough (**B**) was loaded onto a gel filtration column and eluted with 30% acetonitrile at a flow rate of 0.4 mL/min. The arrow shows the peak of the SDP1 peptide.

the synthetic SDP1 peptide. It is possible that the SDP1 peptide contributes at least in part to the ACE inhibitory activity in sourdough.

Assay of ACE Inhibitory Activity in Sourdough. To determine the ACE inhibitory activity in sourdough, the ACE inhibitory activity of LMWP fractions was measured. The ACE inhibitory activity was detected in the water-extracted LMWP fraction (**Table 3**) but not in the other fractions. Although the control dough showed ACE inhibitory activity, the sourdough showed relatively strong activity, and the IC_{50} of the water-extracted LMWP fraction was 2.10 mg/mL. This activity may be the result of the digestion of wheat proteins by protease derived from wheat flour and/or LAB. The ACE inhibitory activity was increased in acid dough. Thus, the activity of acid proteases derived from wheat flour is important for the elevation of ACE inhibitory activity in the dough. We believe that the SDP1 peptide may function as an ACE inhibitory peptide in sourdough.

Table 4. Comparison of ACE Inhibitory Peptide with SDP1 Peptide

peptide	source	ref
VPFGVG	wheat	present study
AVPYPQR	β-casein	*33*
FFVAPFPEVFGK	αs1-casein	*34*
IYPFVEPI	h-β-casein	*35*
VGRPRHQG	tuna muscle	*36*

ACE inhibitory peptides derived from wheat have been previously reported (*37*−*39*). These peptides were isolated from wheat germ hydrolysate or wheat gliadin hydrolysate. SDP1 peptide is a novel peptide that can be isolated from sourdough. To be effective when eaten, the peptide must be resistant to digestive proteinases. The synthetic SDP1 peptide was digested with pepsin, trypsin, and chymotrypsin and was found to be resistant to these proteinases (**Figure 4**). Its relatively high resistance to digestive proteinases suggests that SDP1 might not be significantly digested after eating.

It was reported that free amino acids were decreased in sourdough by the fermentation of yeast and LAB (*16*). In **Figure 5**, the total free amino acids in sourdough were decreased as compared with the control dough. However, we found that GABA was slightly increased during fermentation in sourdough (**Figure 5**). GABA is known to be an amino acid that has an

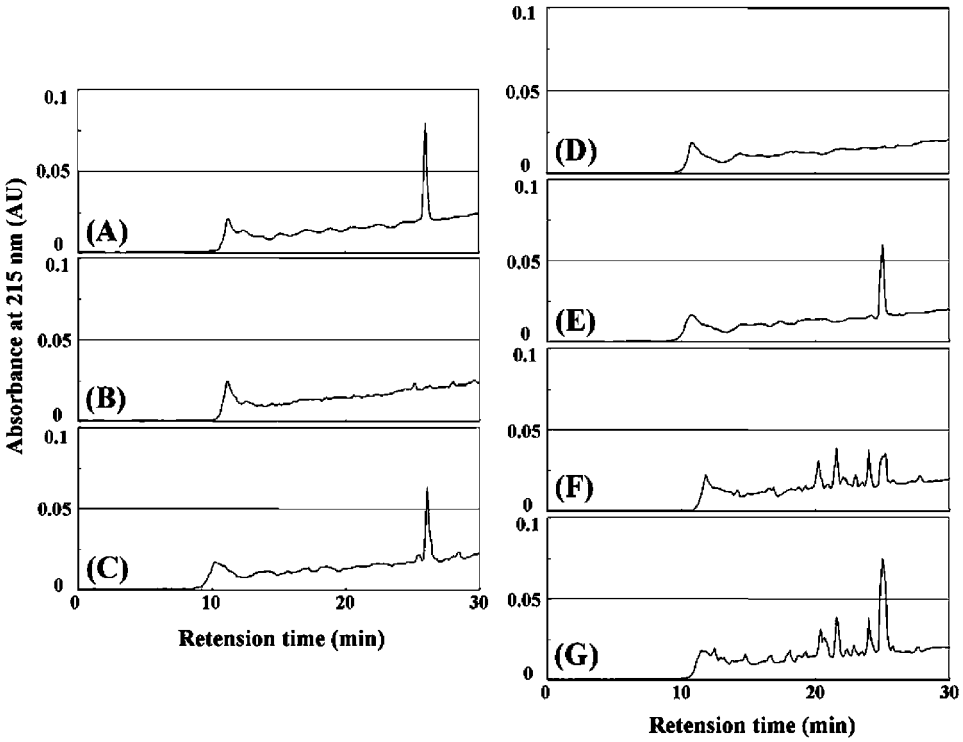

Figure 4. Digestion of the synthetic SDP1 peptide by pepsin, trypsin, and chymotrypsin. The synthetic SDP1 peptide was incubated in 0.1% proteinase solution at 37 °C for 2 h. The sample was injected and separated using a reverse-phase column at a flow rate of 0.8 mL/min by a linear gradient of acetonitrile. (**A**) SDP1 control, (**B**) pepsin control, (**C**) digested with pepsin, (**D**) trypsin control, (**E**) digested with trypsin, (**F**) chymotrypsin control, and (**G**) digested with chymotrypsin.

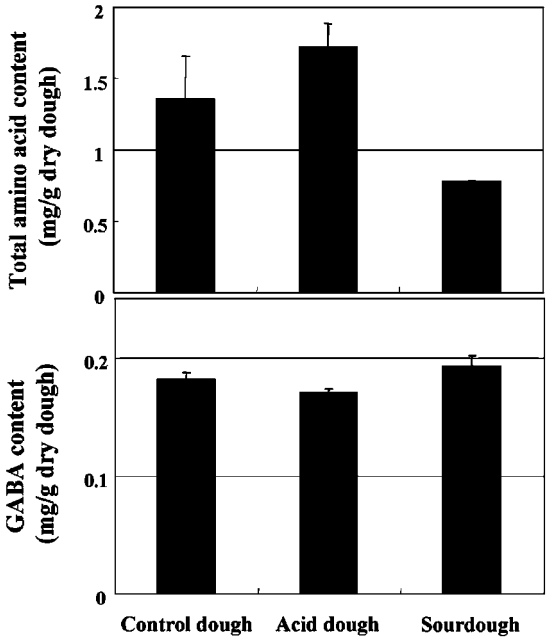

Figure 5. Concentrations of total free amino acids and GABA in doughs. Free amino acids were extracted from freeze-dried dough using 75% ethanol. Determinations were carried out in duplicate.

antihypertensive effect (*20*, *21*). Therefore, sourdough may have synergistic effects of antihypertension.

In summary, the present study successfully identified one of the sourdough-specific peptides. The peptide, named SDP1, was produced by acid protease activities derived from wheat flour as a consequence of a reduction in pH. Interestingly, SDP1 showed ACE inhibitory activity and was the first peptide isolated from sourdough. Our novel ACE inhibitory peptide shows weak activity but partially confers the ACE inhibitory activity observed in sourdough. However, considering that sourdough is baked after fermentation, it is unclear whether the peptide is present in sufficient amounts to have any antihypertensive function. Further research is needed to determine better conditions for sourdough fermentation and to characterize other LMWPs in sourdough.

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