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Cationic peptides from enzymatic hydrolysates of soybean proteins exhibit LPS-neutralizing and angiogenic activities

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In this study, we prepared fractions containing multifunctional cationic peptides by separating the commercial soybean protein hydrolysate Hinute-AM into 20 fractions. These fractions contained peptides with various isoelectric points (pI), as indicated by ampholyte-free isoelectric focusing (autofocusing). Thus, we purified and identified the cationic peptides from fractions 19 and 20, which had pH values greater than 10, using reversed-phase high-performance liquid chromatography and matrix-assisted laser/desorption ionization–time-of-flight mass spectroscopy. Among 19 identified cationic peptides, NKNAKPPSPR, PGKKNAIV, KSGPGMSPR, NVSKPPRVV, RKVGAGGRKPLG, and LPCVIGGVPKRV had high pI values and were included as chemically synthesized peptides in assays of various functions, including lipopolysaccharide (LPS)-neutralizing and angiogenic activities. Chromogenic LPS-neutralizing assays using *Limulus* amebocyte lysates showed that 50% effective concentrations of these six peptides were between 1.63 and 2.65 μM, and were higher than that (0.12 μM) of polymyxin B. Moreover, in tube-formation assays in human umbilical vein endothelial cells, all of the six cationic peptides except LPCVIGGVPKRV exhibited angiogenic activities similar to those of the positive control LL-37. In addition, the six identified cationic peptides had no hemolytic activity at concentrations up to 500 μM in mammalian red blood cells. Our results demonstrate that five of the identified cationic peptides, excluding LPCVIGGVPKRV, have multiple functions and little or no hemolytic activity. These data indicate that fractions containing cationic peptides from Hinute-AM have the potential to be used as dietary supplements and functional ingredients in food products.

[**Key words:** Hydrolysates of soybean proteins; Cationic peptides; Lipopolysaccharide-neutralizing peptides; Angiogenic peptides; Non-toxic peptides]

Vegetable proteins have been increasingly considered as sources of bioactive peptides, including antioxidant peptides, immunomodulatory peptides, antimicrobial peptides (AMPs), hypocholesterolemic peptides, anticancer peptides, and inhibitors of various enzymes such as angiotensin I-converting enzyme (ACE), dipeptidyl peptidase IV (DPP-IV), and α-glucosidase (1–3). These peptides are encrypted within native sequences of proteins and are likely released during gastrointestinal digestion, fermentation, and food processing. Bioactive peptides are generated from proteins of multiple vegetables, including wheat (4), barley (5), rapeseed (6), rice (7,8), and soybean (9–11).

Increasing evidence indicates that peptides from soybean proteins have various functions with demonstrated antioxidant, cholesterol-lowering, antihypertensive, antidiabetic, anti-inflammatory, and anticancer activities. In particular, LLPHH from proteolytic digests of a soybean protein exhibited antioxidant activity by inhibiting the peroxidation of linoleic acid, and scavenged active oxygen and free radical species (12). Moreover, IAVPGEVA, IAVPTGVA, and LPYP from glycinin modulated cholesterol metabolism in HepG2 cells by activating the low density lipoprotein receptor-sterol regulatory element binding protein 2 pathway (13). In addition, DLP and DG from soybean protein hydrolysates (14) and VLIVP from glycinin (15) inhibited ACE activity, IAVPTGVA from glycinin inhibited DPP-IV with an IC_{50} value of 106 μM (16), and MITLAIPVN from β-conglycinin stimulated phagocytosis of human neutrophils (17). Additionally, the soybean hydrolysate peptide X-MLPSYSPY significantly affected cell cycle progression at the G2/M phase in a mouse monocyte-macrophage cell line (P388D1) (18). As described above, large numbers of bioactive peptides from soybean proteins have specific antioxidant, hypocholesterolemic, ACE-inhibitory, DPP-IV-inhibitory, immunostimulating, and anticancer activities. However, most bioactive peptides from soybean proteins comprise less than ten amino acids and are not always cationic and/or amphipathic. Few studies have investigated and characterized multifunctional peptides from soybean proteins. Among findings of these studies, TIIPLPV (positions 306–312 in the lipoxygenase-1 protein) from okara (a major by-product of soymilk industry) protein hydrolysates had significant antioxidant activities, with considerable reducing power and radical scavenging activity, and inhibited ACE (19). In addition, lunasin from 2S soybean albumin is a 43 amino acid 5.5 kDa peptide with antioxidant, anticancer, anti-inflammatory, and hypocholesterolemic activities, among others (20).

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Recent evidence indicates that peptides and peptide fractions from food proteins exhibit multifunctional effects, with superior antimicrobial, antioxidant, anti-inflammatory, enzyme-inhibitory, and growth-stimulating activities to those of other peptides with specific health-promoting agents (21–24). Among bioactive peptides, cationic peptides exhibit multifunctional effects and are generally small (12–50 amino acid residues), possess high isoelectric point (pI) values, and have amphipathic structures comprising approximately 50% hydrophobic residues (e.g., tryptophan, phenylalanine, leucine) (25–27).

Recently, we demonstrated that cationic peptide sequences of rice proteins and their amino acid-substituted analogs have antimicrobial, anti-inflammatory, lipopolysaccharide (LPS)-neutralizing, enzyme-inhibitory, and angiogenic activities (28–30). We also showed that cationic peptides from rice protein hydrolysates exhibited antimicrobial, LPS-neutralizing, and angiogenic activities (31,32). However, LPS-neutralizing and angiogenic activities have not been reported previously for multifunctional cationic peptides from soybean proteins. To introduce multifunctional peptides as widely used functional foods and supplements, enzymatic hydrolysates need to be derived from inexpensive protein sources, that are suitable for the production of natural bioactive components (33,34).

In this study, we selected soybean proteins as a source of multifunctional cationic peptides and used Hinute-AM (Fuji Oil Co., Ltd., Osaka, Japan), which is a commercial product of soybean protein hydrolysates. To separate cationic peptides from Hinute-AM, fractions containing peptides with varying pI values were prepared using ampholyte-free isoelectric focusing (autofocusing). Cationic peptides were then purified from fractions with pI values greater than 10 and were identified using matrix-assisted laser/desorption ionization–time-of-flight mass spectroscopy (MALDI-TOF MS). After identifying six peptides with high pI values, we synthesized them chemically and evaluated their biological effects, including LPS-neutralizing and angiogenic activities. We also determined hemolytic activities of these peptides against mammalian red blood cells (RBCs). Finally, we related the chemical properties of these peptides to their biological activities.

MATERIALS AND METHODS

Materials Hinute-AM was kindly provided by Fuji Oil Co., Ltd. We used smooth-type LPS from *E. coli* O55:B5 (List Biological Laboratories, CA, USA) as an endotoxin. All other reagents were of analytical grade and were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan).

Peptides used in this study Amino acid sequences and properties of the six peptides identified in this study are summarized in Table 1. Mean hydrophobicity values were calculated using hydrophobicity indices of amino acid residues as reported by Shang et al. (35). Six peptides were chemically synthesized and tested in assays of LPS-neutralizing and angiogenic activities. Chemically synthesized peptides, including LL-37, were obtained from Eurofins Genomics K. K. (Tokyo, Japan). Synthetic peptides were purified to >95% using reversed-phase high-performance liquid chromatography (RP-HPLC), and their molecular weights were confirmed using MALDI-TOF MS. Polymyxin B sulfate is a cationic cyclic AMP that is produced by *Paenibacillus polymyxa* and has been used for the treatment of infectious Gram-negative bacteria (36) and was used as a control, along with the bee venom component melittin, which is known to disrupt cell membranes (37). Polymyxin B sulfate and melittin were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

Fractionation of Hinute-AM using preparative isoelectric focusing Autofocusing is a preparative isoelectric focusing technique that is based on the ampholytic properties of peptide mixtures (38). Hinute-AM powder (200 mg) samples were dissolved in 50-mL aliquots of ultrapure water and were fractionated using ampholyte-free isoelectric focusing (autofocusing) with a Rotofor (Bio-Rad, Richmond, CA, USA) as described previously (31,32). Constant power and run times were adjusted to 12 W and 1.5 h, respectively. Samples were then collected into 20 fraction tubes and pH values and dry weights of each fraction were measured as described previously (31,32).

Purification and identification of cationic peptides Peptides in fractions 19 and 20 had pH values greater than 10 and were dissolved in 0.1% trifluoroacetic acid (TFA) in water and were then separated using RP-HPLC using a CAPCELL PAK C18 column (150 × 10 mm, 5 μm; Shiseido Co., Tokyo, Japan) as described previously (31,32) with a slight modification. Briefly, mobile phase A comprised 0.1% (v/v) TFA in water and mobile phase B contained 80% (v/v) acetonitrile and 0.1% (v/v) TFA. Peptide samples were eluted at a flow rate of 2.0 mL/min using the following gradient: 0–60 min (0–60% B) and 60–70 min (100% B). Fractions (1 mL) were collected and freeze-dried every 30 s and peptides in single peak fractions from the first RP-HPLC run were further purified in a second RP-HPLC elution using the same column. Fractions (1 mL) were collected and freeze-dried every 30 s and all chromatographic eluents were monitored at 220 nm using an SPD-10AVP detector (Shimadzu, Kyoto, Japan).

Peptides were identified using MALDI-TOF MS as reported previously (31,32). Briefly, molecular weights of isolated peptides were determined, and subsequent MS/MS analyses were performed using an autoflex III TOF/TOF instrument (Bruker, Billerica, MA, USA) as described previously (31,32). Amino acid sequences were identified using NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searches, and MS/MS ion searches were performed using the taxonomy (other green plants; GlycineMax_Pro) in the Mascot system (Matrix Science Ltd., London, UK).

LPS-neutralizing assays LPS binding and neutralizing activities of identified peptides were evaluated using chromogenic *Limulus* amebocyte lysate (LAL) assay kits (Endospecy ES-50M, Seikagaku Corporation, Tokyo, Japan) as described previously (29,31,32,39). Briefly, various concentrations of peptides were incubated with LPS at 0.5 endotoxin units (EU)/mL in microtubes at 37°C for 30 min to allow binding of peptides to LPS. Subsequently, 50-μL aliquots of these mixtures were added to equal volumes of LAL reagent containing the chromogenic substrate butyloxycarbonyl (Boc)-Leu-Gly-Arg-*p*-nitroanilide and were then incubated for 30 min. Changes in absorbance following cleavage of substrates were then monitored at 405 nm using a microplate reader (2030 ARVO X3; PerkinElmer, Waltham, MA, USA). Relative absorbances of peptide-containing reaction mixtures were expressed as ratios of A_{405} relative to those (100%) of reaction mixtures without peptide, and 50% effective (50% neutralizing) concentrations (EC_{50}) for each peptide were determined using plots of relative absorbance values vs. log peptide concentrations. In these experiments, polymyxin B sulfate was used as a positive control.

Angiogenic activity assays Human umbilical vein endothelial cells (HUVECs) migrate, attach to each other, and form tubular structures when cultured on reconstituted basement membrane gels. In the present study, HUVECs (Kurabo Industries, Osaka, Japan) were seeded in modified MCDB 131 medium (HuMedia-EG2, Kurabo Industries) and were incubated at 30°C in a humidified atmosphere containing 5% CO_2. After reaching 90%–95% confluence, cells were harvested and counted using a hemocytometer as described previously (31,32,39,40).

Tube formation assays were performed using Matrigel (Becton Dickinson and Company, Santa Clara, CA, USA) as described by the manufacturer (31,32,39,40). Briefly, solid gels were prepared on 96-well plates and HUVECs in HuMedia-EG2 containing peptides at varying concentrations were seeded onto solid Matrigel surfaces at 2×10^5 cells/mL. After 15 h incubation, tube formation was observed at 40 × magnification using an inverted light microscope (TS100F, Nikon Instruments Inc., Tokyo, Japan) and random phase contrast images in each of the five wells were recorded using a digital camera (Nikon Instruments Inc.). Tube-like structures were then analyzed using NIS-Elements BR Analysis software (Nikon Instruments Inc.) and average tube lengths per field were calculated. Relative tube lengths in the presence of peptides were

TABLE 1. Amino acid sequences and properties of cationic peptides identified from Nos. 19 and 20 fractions.

Fraction No.	Observed mass	Expected mass	Calculated mass	Peptide name	Sequence	Protein source	Accession number of gene identify	Position	MH[a]	pI	Net charge
19	1108.692	1107.6847	1107.649	SBH-NKN	NKNAKPPSPR	Uncharacterized protein	Glyma04g07100.1	107–117	3.60	11.17	+3
20	826.5331	825.5259	825.5072	SBH-PGK	PGKKNAIV	Uncharacterized protein	Glyma06g01280.1	66–73	6.25	10.02	+2
	916.5041	915.4968	915.4596	SBH-KSG	KSGPGMSPR	Uncharacterized protein	Glyma06g15230.1	10–18	4.12	11.00	+2
	995.6653	994.6580	994.5924	SBH-NVS	NVSKPPRVV	Kinesin-like protein	Glyma07g00730.1	371–379	6.84	11.00	+2
	1195.6498	1194.6425	1194.7309	SBH-RKV	RKVGAGGRKPLG	Uncharacterized protein	Glyma10g37630.1	34–45	4.61	12.02	+4
	1237.8533	1236.4860	1236.7377	SBH-LPC	LPCVIGGVPKRV	Uncharacterized protein	Glyma01g16380.1	31–42	9.49	9.51	+2

[a] The mean hydrophobicity (MH) values of the peptides were calculated using the hydrophobicity indices of amino acid residues reported by Shang et al. (35).

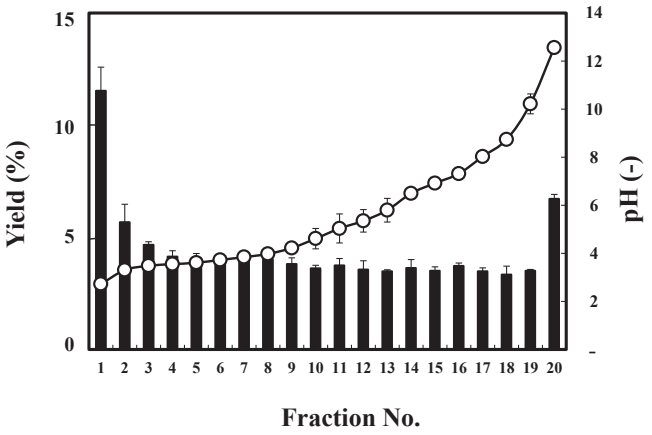

FIG. 1. Fractionation of Hinute-AM by autofocusing using a Rotofor. Runs were performed at a constant power of 12W for 1.5 h, and samples were then recovered into 20 fraction tubes. Yields (bars) and pH values (open circles) were shown for each fraction from Hinute-AM samples (100%, 200 mg dry weight). Data are expressed as the mean ± standard deviations (SD) of three independent experiments.

expressed as percentages of those (100%) in peptide free controls, and LL-37 was used as a positive control.

Hemolytic activities Hemolytic activities of peptides were measured as reported previously (28,31,32,39). Briefly, 50-μL aliquots of peptide solutions at various concentrations were added to 50-μL aliquots of 4% (v/v) suspensions of sheep RBC (Cosmo Bio Co. Ltd., Tokyo, Japan) in 96-well plates, and the resulting suspensions were incubated without agitation for 1 h at 37°C. Samples were then centrifuged at 2000 × *g* for 5 min, and hemoglobin release in supernatants was monitored by measuring absorbance at 405 nm. Percentage hemolysis was calculated using the following formula:

$$\text{Hemolysis}(\%) = \{(H_P - H_0)/(H_T - H_0)\} \times 100 \quad (1)$$

where H_P and H_T represent absorbance values of sRBC samples treated with peptide and 0.1% (w/v) Triton X-100, respectively, and H_0 is the absorbance of the sample without treatment. In these experiments, melittin was used as a positive control.

Statistical analysis All assays were performed three or five times and data from LPS-neutralizing, tube formation, and hemolysis assays were expressed as means ± standard deviations (SD) of three independent experiments. Differences were identified using Student's-*t*-test and were considered significant when $p < 0.05$.

RESULTS

Fractionation of Hinute-AM using preparative isoelectric focusing To prepare fractions containing cationic peptides, Hinute-AM was separated into 20 fractions using preparative isoelectric focusing. Dry weight yields and pH values were determined for each fraction from 200 mg-Hinute-AM samples (Fig. 1), and the 20 fractions formed a pH gradient between 3 and 12.5. In the Rotofor, volumes of fractions 1 and 20 were two-fold larger than those of the other fractions. These fractions contained more than 14 mg of dry sample (7% yield), whereas the dry weight yields of fractions 2–19 were 3.6%–6 %. The total yield of fractions 19 and 20 (with pH values greater than 10) was approximately 10.6%, and these fractions contained cationic peptides.

Purification and identification of cationic peptides In this study, we focused on cationic peptides in Hinute-AM samples based on our previous studies of the multifunctional properties of cationic peptides from rice protein hydrolysates (31,32). To purify and identify cationic peptides, autofocusing fractions 19 and 20 were separated using RP-HPLC on a CAPCELL PAK C18 column and a number of peaks were observed (data not shown). Peaks were then purified by subsequent re-RP-HPLC using the same column but under gradient elution conditions that differed from those in the first RP-HPLC.

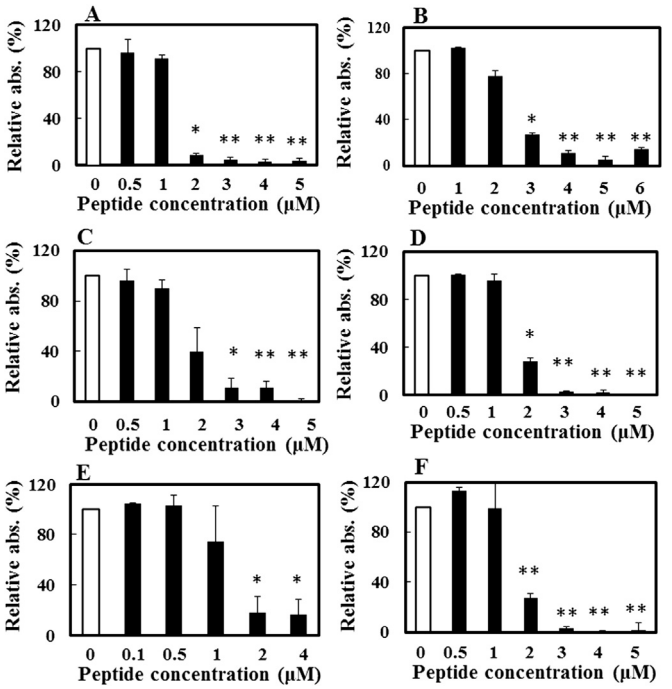

FIG. 2. Endotoxin-neutralizing abilities of the six peptides in LAL assays. (A–F) LAL values in the presence of SBH-NKN (A), SBH-PGK (B), SBH-KSG (C), SBH-NVS (D), SBH-RKV (E), and SBH-LPC (F). Absorbance was determined at 405 nm and values were expressed relative to those in the absence of peptide (100%). Data are presented as means ± SD of three independent experiments. *$p < 0.05$, ** $p < 0.01$ vs. control without peptide.

Molecular weights of peptides in the peaks from re-RP-HPLC were analyzed using MALDI-TOF MS and amino acid sequences were then determined in MS/MS analyses. Based on these MS/MS ion data, 19 cationic peptides were identified from fractions 19 and 20 (data not shown), and six of these had high pI values greater than 9.5 and net charges greater than +2. The peptide NKNAKPPSPR from fraction 19 and the five peptides PGKKNAIV, KSGPGMSPR, NVSKPPRVV, RKVGAGGRKPLG, and LPCVIGGVPKRV from fraction 20 were synthesized chemically. Table 1 shows the amino acid sequences (8–12 amino acid residues), protein sources, and some properties of these six cationic peptides, which were designated soybean hydrolysate (SBH)-NKN, SBH-PGK, SBH-KSG, SBH-NVS, SBH-RKV, and SBH-LPC, respectively, on the basis of their three N-terminal amino acid residues.

LPS-neutralizing activity of identified peptides Initially, we determined LPS-neutralizing effects of the six cationic peptides using the LAL assays (Fig. 2). All six cationic peptides inhibited the endotoxic activity of LPS in a concentration-dependent manner, with EC_{50} values of 1.63, 2.65, 1.88, 1.79, 2.00, and 1.85 μM for SBH-NKN, SBH-PGK, SBH-KSG, SBH-NVS, SBH-RKV, and SBH-LPC, respectively. As a control, the EC_{50} value of polymyxin B sulfate was 0.12 μM (data not shown), and was similar to that (0.11 μM) shown in our previous study (29), indicating that soybean hydrolysate peptides have lower LPS-neutralizing activities than polymyxin B sulfate.

Angiogenic activity of identified peptides To evaluate angiogenic properties of cationic peptides, we performed tube formation assays using Matrigel in HUVECs (Fig. 3). Similar to the positive control LL-37 (Supplementary Fig. S1), all cationic peptides apart from SBH-KGS significantly promoted tube formation in HUVECs. Fig. 4 shows the relative tube lengths in

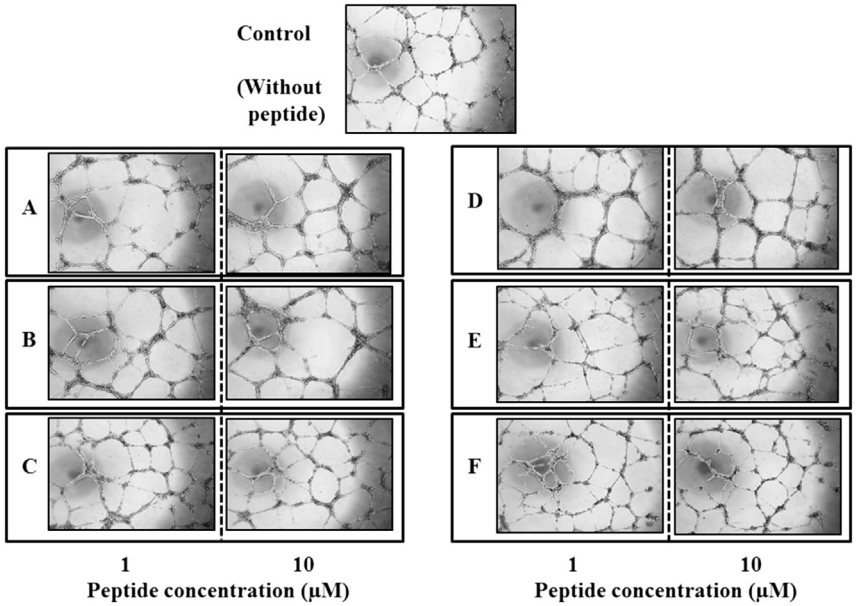

FIG. 3. Microscope photographs of HUVECs tube formation in the presence of the six peptides. The effects of various concentrations (1 and 10 μM) of peptides on tube formation are shown. (A–F) Results for SBH-NKN (A), SBH-PGK (B), SBH-KSG (C), SBH-NVS (D), SBH-RKV (E), and SBH-LPC (F). Microscope photographs of tube formation in Matrigel assays were obtained at 40 × magnification after incubation for 15 h. Random phase contrast images of five wells were taken and a representative picture is shown.

the presence of 1 and 10 μM, and indicates 7%–18% increases at 10 μM, as compared to control experiments.

Hemolytic activity of identified peptides To assess cytotoxic membrane disrupting activities of the six cationic peptides, we measured hemolysis of sheep RBCs as reported previously (Fig. 5) (28,31,32,39). These experiments showed no hemolytic activities of the six cationic peptides, with relative hemolytic ratios of 0.10%, 0.03%, 0.12%, 0.07%, 0.68, and 0.49% for SBH-NKN, SBH-PGK, SBH-KSG, SBH-NVS, SBH-RKV, and SBH-LPC, respectively, at 500 μM. In comparison, this ratio was 96% in the presence of positive control melittin at 20 μM, as shown in our previous study (94%) (28).

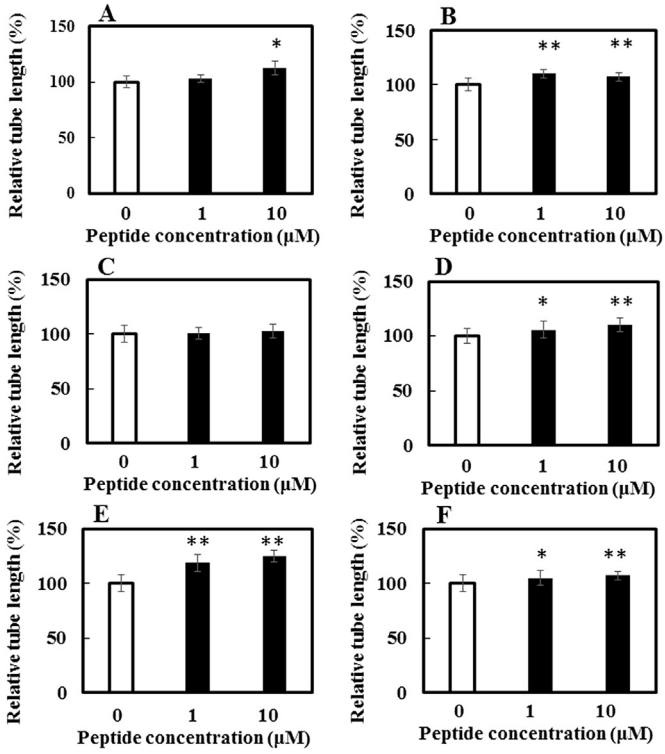

FIG. 4. Promotion of HUVECs tube formation by the six peptides. The effects of various concentrations (1 and 10 μM) of peptides on tube formation are shown. (A–F) Results for SBH-NKN (A), SBH-PGK (B), SBH-KSG (C), SBH-NVS (D), SBH-RKV (E), and SBH-LPC (F). Data are expressed as means ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control without peptide.

DISCUSSION

In our previous studies (28,29), AmyI-1-18 (HLNKRVQRELIGWLDWLK) was identified as a novel multifunctional cationic octadecapeptide from rice α-amylase (AmyI-1), and exhibited antimicrobial activity against human pathogens and inhibited LPS-induced production of NO in RAW264 macrophages by specifically binding to the lipid A moiety. AmyI-1-18 had angiogenic activities in tube formation assays in HUVECs and promoted cell migration in scratch migration assays (40). We also searched partial amino acid sequences of the major soybean proteins glycinin, β-conglycinin α-subunit (BCAS), and β-conglycinin β-subunit (BCBS), and identified the novel multifunctional cationic peptides Glycinin-17, BCAS-16, and BCBS-11, corresponding with residues 348–364, 208–223, and 52–62 of the parent proteins, respectively (39). These peptides had antimicrobial activities against *Porphyromonas gingivalis*, a periodontal disease-associated bacterium and *Candida albicans*, an opportunistic fungal pathogen. We also demonstrated high LPS-neutralizing activities of these cationic peptides in chromogenic LAL assays and angiogenic activities in tube-formation assays using HUVECs. Taken together, these data showed that cationic peptides from rice and soybean proteins have similar properties to the human multifunctional peptides LL-37 (41) and β-defensins (42), and can directly inhibit pathogens, neutralize endotoxins, and induce angiogenesis. Our previous findings demonstrated that cationic peptides from cereal proteins have potent multifunctional health-promoting effects (28,29,39,40). However, these cationic peptides (AmyI-1-18, G-17, BCAS-16, and BCBS-11) were not generated from enzymatic hydrolysates of food proteins, and were rather synthesized chemically according to partial sequences that are rich in

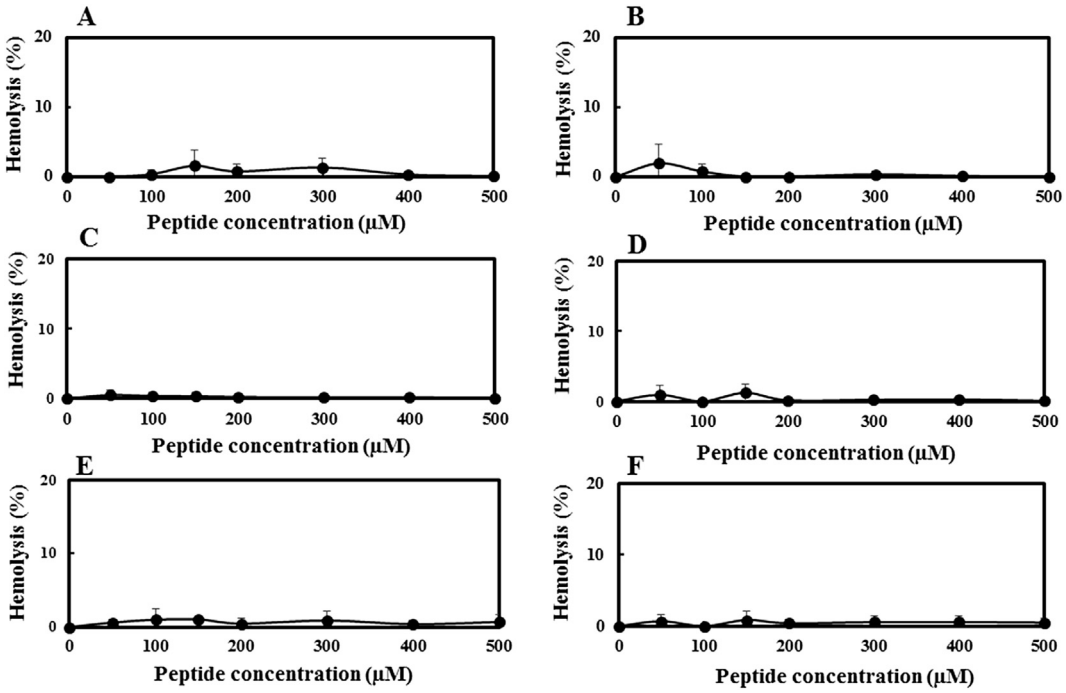

FIG. 5. Hemolytic activities of the six peptides. (A–F) Results for SBH-NKN (A), SBH-PGK (B), SBH-KSG (C), SBH-NVS (D), SBH-RKV (E), and SBH-LPC (F). Hemoglobin release into supernatants was monitored at A_{405}. Hemolysis induced by 0.1% (w/v) Triton X-100 was defined as 100%. Data are reported as means from three independent experiments.

cationic amino acids (lysine and arginine). Therefore, due to high production costs and safety concerns, these peptides have limited potential as functional food supplements.

To our knowledge, no previous reports show LPS-neutralizing and angiogenic functions of cationic peptides from soybean protein hydrolysates, although we identify multifunctional cationic peptide fractions from rice protein hydrolysates (31,32). Ampholyte-free isoelectric focusing (autofocusing) is considered a markedly superior method for fractionating cationic peptides from enzymatic hydrolysates of food proteins, and in previous study, cationic AMPs were successfully fractionated from casein hydrolysates by autofocusing (43). As shown in Fig. 1, we used autofocusing to obtain fractions containing cationic peptides from Hinute-AM. The total yield of fractions 19 and 20 at pH values greater than 10 was 10.6% of the total weight of Hinute-AM. These fractions 19 and 20 contained six cationic peptides with pI values greater than 9.5 and net charges greater than +2 (Table 1), and most of their synthetic counterparts had LPS-neutralizing and angiogenic activities but did not promote hemolysis. As shown in Table 2, SBH-KSG had LPS-neutralizing activity but was not angiogenic, whereas other five cationic peptides had LPS-neutralizing (Fig. 2) and angiogenic activities (Figs. 3 and 4), and negligible hemolytic activities (Fig. 5). In contrast with antimicrobial cationic peptides from rice protein hydrolysates (31,32), the present six cationic peptides from Hinute-AM had no antimicrobial activities (data not shown).

The six Hinute-AM cationic peptides of the present study contain multiple lysine and/or arginine residues (Table 1). Assessments of structure-activity relationships indicated various parameters that contribute functional properties of these peptides, including size, cationicity (net positive charge), charge distribution, hydrophobicity, amphipathicity, and secondary structure (25–27,44,45). LPS-neutralizing peptides with amphipathic propensities are known to bind directly to the lipid A moiety of LPS, which contains phosphate groups with negative charges and hydrophobic fatty acid chains (29). In our previous studies, we demonstrate pI values and mean hydrophobicity of peptides with high LPS-neutralizing activity (EC_{50}: 0.22–0.31 μM) of 9.99 and 11.30 for AmyI-1-18 (28), and 9.30 and 12.48 for BCBS-11, respectively (39). EC_{50} values of the present six cationic peptides were 10-fold higher than those of AmyI-1-18 and BCBS-11 (Table 2), and the comparatively lower LPS-neutralizing activities of these peptides may reflect low mean hydrophobicities (Table 1) and unsuitable hydrophilic (cationic)/hydrophobicity balances. Angiogenic activities of peptides can follow binding to vascular endothelial growth factor (VEGF) receptors and subsequent activation of relevant signal transduction pathways (40,46,47). Five cationic peptides from Hinute-AM significantly promoted tube-formation in HUVECs (Table 2), as shown in our previous studies of AmyI-1-18, G-17, BCAS-16, and BCBS-11 (39,40). We also demonstrated that AmyI-1-18 and its leucine-substituted analog induce angiogenesis in HUVECs by activating VEGF receptors (40). However, the angiogenic mechanisms of cationic peptides, including the five present cationic peptides from Hinute-AM, AmyI-1-18, and a leucine-substituted AmyI-1-18 analog, remain unclear, warranting further investigations of the angiogenic mechanisms of these cationic peptides. Antimicrobial activities of cationic peptides have been associated with binding of positively charged peptides to negatively charged microbial lipid membranes, and with hydrophobicity,

TABLE 2. Bioactivity of cationic peptides identified from hydrolysates of soybean proteins.

Peptide	LPS-neutralizing activity[a], EC_{50} (μM)	Angiogenic activity[b] (%)	Hemolytic activity[c] (%)
SBH-NKN	1.63	112	0.10
SBH-PGK	2.65	107	0.03
SBH-KSG	1.88	103	0.12
SBH-NVS	1.79	110	0.07
SBH-RKV	2.00	118	0.68
SBH-LPC	1.85	107	0.49

[a] EC_{50} values were calculated based on the results indicated in Fig. 2.
[b] Data indicate the relative angiogenic activity when each peptide was added at 10 μM (Fig. 4).
[c] Data indicate the relative hemolytic activity when each peptide was added at 500 μM (Fig. 5).

which facilitates incorporation into cell membranes and/or translocation into cells. Whereas pI values and mean hydrophobicities of the antimicrobial cationic peptides AmyI-1-18 and BCBS-11 were high (28,39), the present five cationic peptides, excluding SBH-LPC, had high pI values but mean hydrophobicities of less than 7 (Table 1), and no antimicrobial activities. These low mean hydrophobicities may reflect by negligible hemolytic activities of these cationic peptides, although amphipathicity has also been associated with these biological properties (28,31,32,39).

Taken together, data indicate that these cationic peptides from Hinute-AM are potent multifunctional peptide candidates without hemolytic activity. However, use as dietary supplements and functional ingredients in food products will require further evaluations of the functional properties of fractions containing multifunctional cationic peptides.

In conclusion, in this study, we selected the soybean protein hydrolysate Hinute-AM as a starting material for producing multifunctional cationic peptides. We successfully prepared 20 fractions containing peptides with varying pI values using autofocusing analyses with a Rotofor. Subsequently, we purified cationic peptides with pH values greater than 9.5 from fractions 19 and 20 and identified them using MALDI-TOF MS. Six identified cationic peptides were then chemically synthesized and designated SBH-NKN, SBH-PGK, SBH-KSG, SBH-NVS, SBH-RKV, and SBH-LPC, and LPS-neutralizing, angiogenic, and hemolytic activities were evaluated. Five of these peptides excluding SBH-KSG had multiple potential functions and negligible hemolytic activity. Taken together, our results demonstrate that fractions 19 and 20 from soybean protein hydrolysates contain non-toxic cationic peptides with multiple functions.

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jbiosc.2018.07.013.

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