Journal of Bioscience and Bioengineering
VOL. 127 No. 4, 472–478, 2019

www.elsevier.com/locate/jbiosc

Identification and characterization of multifunctional cationic peptides from traditional Japanese fermented soybean Natto extracts

Masayuki Taniguchi,[1,*] Ryousuke Aida,[1] Kazuki Saito,[1] Akihito Ochiai,[1] Satoshi Takesono,[2] Eiichi Saitoh,[2] and Takaaki Tanaka[1]

Department of Materials Science and Technology, Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan[1] and Graduate School of Technology, Niigata Institute of Technology, Niigata 945-1195, Japan[2]

Received 2 August 2018; accepted 24 September 2018
Available online 16 October 2018

In this study, we investigated the lipopolysaccharide (LPS)-neutralizing and angiogenic activities of cationic peptides derived from the traditional Japanese fermented product Natto, which is made by fermenting cooked soybeans using *Bacillus subtilis*. Initially, we prepared 20 fractions of Natto extracts with various isoelectric points (p*I*'s) using ampholyte-free isoelectric focusing (autofocusing). Cationic peptides were then purified from fractions 19 and 20, whose pH values were greater than 12, using reversed-phase high-performance liquid chromatography, and were identified using matrix-assisted laser/desorption ionization–time-of-flight mass spectroscopy. Among the 13 identified cationic peptides, seven (KFNKYGR, FPFPRPPHQK, GQSSRPQDRHQK, QRFDQRSPQ, ERQFPFPRPPHQK, GEIPRPRPRPQHPE, and EQPRPIPFPRPQPR) had p*I*'s greater than 9.5, positive net charges, and differing molecular weights. These peptides were then chemically synthesized and applied to chromogenic LPS-neutralizing assays using *Limulus* amebocyte lysates, and 50% effective (neutralizing) concentrations of 2.6–5.5 μM were demonstrated. In addition, tube formation assays in human umbilical vein endothelial cells revealed angiogenic activities for all but one (GEIPRPRPRPQHPE) of these seven cationic peptides, with increases in relative tube lengths of 23–31% in the presence of peptides at 10 μM. Subsequent experiments showed negligible hemolytic activity of these peptides at concentrations of up to 500 μM in mammalian red blood cells. Collectively, these data demonstrate that six cationic peptides from Natto extracts, with the exception of GEIPRPRPRPQHPE, have LPS-neutralizing and angiogenic activities but do not induce hemolysis.

[**Key words:** Natto extracts; Cationic peptides; Lipopolysaccharide-neutralizing peptides; Angiogenic peptides; Non-toxic peptides]

Increasing evidence indicates that bioactive peptides from food proteins have various biological roles, including antioxidant, antihypertensive, immunomodulatory, hypocholesterolemic, antimicrobial, and anticancer agents (1–3). Bioactive peptides have potential as both dietary supplements and functional food ingredients. They are encrypted and inactive within the sequences of their parent protein molecules and are liberated upon digestion by gastrointestinal enzymes, fermentation by proteolytic bacterial and fungal strains, and hydrolysis by proteolytic enzymes from animals, plants, and microorganisms (4,5). In these ways, bioactive peptides are released from various food proteins, including those in wheat (6), rice (7), rapeseed (8), soybean (9), egg (10), milk (11), fish (12), and algae (13). The amino acid sequences of many bioactive peptides from food proteins have been determined in previous studies (6–13), and their physicochemical properties and biological functions have been characterized.

Recent studies have shown that multiple functional peptides have superior and specific activities as functional components in health-promoting food products (3,7,10). Among multifunctional peptides (peptides with multiple bioactivity), cationic peptides from cereal proteins have properties similar to the human multifunctional cationic peptides LL-37 (14) and β-defensins (15), which have antimicrobial, anti-inflammatory, endotoxin-neutralizing, and wound healing activities. Previous studies have shown that lipopolysaccharide (LPS) induces pro-inflammatory responses through Toll-like receptor 4 signaling, leading to pyrogenic responses and the destruction of tissues and cells (3,16,17). Angiogenesis is the process of the formation of new blood vessels and is a prerequisite for tissue repair and remodeling (18,19). Therefore, in addition to cationic peptides with antimicrobial activity, our laboratory has paid attention to those with LPS-neutralizing and angiogenic activities.

We previously searched partial amino acid sequences of the major soybean proteins glycinin (G), β-conglycinin α-subunit (BCAS), and β-conglycinin β-subunit (BCBS), and identified RKSREWRSKKTQPRRPR (G-17), KNQYGRIRVLQRFNQR (BCAS-16), and RIRLLQRFNKR (BCBS-11), respectively, as cationic peptides with antimicrobial, LPS-neutralizing, and angiogenic activities (20). We also showed that the three cationic peptides NKNAKPPSPR, NVSKPPRVV, and RKVGAGGRKPLG from enzymatic hydrolysates of soybean proteins have LPS-neutralizing and angiogenic activities and do not promote hemolysis (21). Our previous studies (20,21) suggested that most non-toxic cationic peptides from soybean proteins exhibit antimicrobial activity, neutralize endotoxins, and induce angiogenesis, as is the case for LL-37 and β-defensins.

* Corresponding author. Tel./fax: +81 25 262 6716.
E-mail address: mtanig@eng.niigata-u.ac.jp (M. Taniguchi).

1389-1723/$ – see front matter
https://doi.org/10.1016/j.jbiosc.2018.09.016

TABLE 1. Amino acid sequences and properties of cationic peptides identified from natto extracts.

Fraction no.	Observe mass $[M + H]^{+}$[a]	Calculated mass [M]	Peptide name	Sequence	Protein source	Glycine max Genome database ID	Position	MH[b]	pI	Net charge
19	912.4667	911.4977	FSB-KFN	KFNKYGR	Putative uncharacterized protein	Glyma08g21600.1	123–129	7.14	10.29	+3
	1250.6997	1249.6720	FSB-FPF	FPFPRPPHQK	Beta-conglycinin alpha subunit	Glyma20g28650.1	153–162	9.82	11.00	+2
	1423.6526	1422.7076	FSB-GQS	GQSSRPQDRHQK	Glycinin G1	Glyma03g32030.1	121–132	1.37	10.84	+2
20	1161.4742	1160.5687	FSB-QRF	QRFDQRSPQ	Uncharacterized protein	Glyma10g39170.1	215–223	4.74	9.60	+1
	1663.7780	1662.8743	FSB-ERQ	ERQFPFPRPPHQK	Beta-conglycinin alpha subunit	Glyma20g28650.1	150–162	7.86	10.84	+2
	1665.6509	1664.8859	FSB-GEI	GEIPRPRPRPQHPE	Beta-conglycinin alpha subunit	Glyma20g28650.1	71–84	5.31	9.52	+1
	1714.8907	1713.9427	FSB-EQP	EQPRPIPFPRPQPR	Beta-conglycinin alpha subunit	Glyma20g28650.1	99–112	7.88	11.70	+2

[a] $[M + H]^{+}$, protonated molecule.
[b] The mean hydrophobicity (MH) values of the peptides were calculated using the hydrophobicity indices of amino acid residues reported by Shang et al. (33).

Fermentation of foods by microorganisms and their enzymes leads to desirable biochemical changes and significant modifications of flavor and texture and to improved nutritional and functional properties (22,23). The bioactive peptides that are the end-products of fermentation are increasingly recognized as being promising health-promoting functional agents. Naturally formed bioactive peptides have been identified in fermented food products, including yogurt (24), cheese (25), Douchi (26), soy sauce (27), miso paste (28), and Natto (29). These peptides reportedly have antioxidant, antihypertensive, immuno-stimulatory, antimicrobial, and antidiabetic effects and have the potential to provide treatments for such diseases and inflections (22,23,30). To our knowledge, however, no reports have demonstrated LPS-neutralizing and angiogenic functions of cationic peptides from fermented soybean products.

We selected the Japanese traditional fermented soybean product Natto as a source of multifunctional cationic peptides. As a starting material, Natto extracts were separated into 20 fractions containing peptides with varying isoelectric points (p*I*'s) values using preparative isoelectric focusing. Subsequently, cationic peptides were identified using matrix-assisted laser/desorption ionization–time-of-flight mass spectroscopy (MALDI-TOF MS). Seven of these were then chemically synthesized for the assessment of LPS-neutralizing, angiogenic, and hemolytic activities. Here, we discuss the chemical properties and biological activities of these cationic peptides.

MATERIALS AND METHODS

Materials Hikiwari-type Natto was purchased from Dairiki Natto Co. Ltd. (Niigata, Japan), and a smooth-type LPS from *Escherichia coli* O55:B5 (List Biological Laboratories, Campbell, CA, USA) was used as an endotoxin. The bee venom component melittin, which is known to disrupt cell membranes (31), was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). The LPS-binding antimicrobial peptide polymyxin B sulfate (32), which has been used as a treatment for Gram-negative bacterial infections, was purchased from Sigma–Aldrich Co. All other reagents were of analytical grade and were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan).

Peptides 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 molecular weights were determined using MALDI-TOF MS. The amino acid sequences and properties of the seven peptides investigated in this study are summarized in Table 1. Hydrophobicity indices of amino acid residues were used to calculate the mean hydrophobicity values as reported by Shang et al. (33). We used ExPASY ProtParam software (https://web.expasy.org/compute_pi/) to estimate the p*I* values.

Preparation of Natto extracts Ninety grams of Natto with a water content of 60% were homogenized in 250-mL of ultrapure water using a Polytron homogenizer (Kinematica, Bohemia, NY, USA). Mixtures were then centrifuged at 12,000 × *g* for 30 min at 4°C and the supernatants were freeze-dried. The resulting crude Natto extract samples (3 g) were dissolved in 60-mL of ultrapure water and then 300-mL of cold ethanol was added to precipitate high molecular weight components. Suspensions were then centrifuged at 12,000 × *g* for 30 min at 4°C, and ethanol was removed from the supernatants using a *vacuum*-rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland). After freeze-drying, resulting samples (1 g) were dissolved in 20-mL of ultrapure water and then dialyzed against ultrapure water using Spectra/Por Dialysis Tubing (MWCO, 500–1000 Da; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) to remove components with low molecular weights. Dialysates were finally freeze-dried, termed Natto extract samples, and used in subsequent separation stages.

Fractionation of Natto extracts by autofocusing Ampholyte-free autofocusing is a preparative isoelectric focusing technique that exploits the ampholytic properties of peptide mixtures (34). Natto extract samples (200 mg) were dissolved in 50-mL aliquots of ultrapure water and fractionated using autofocusing with a Rotofor (Bio-Rad, Richmond, CA, USA) at 12 W constant power for 2 h. Samples were recovered in 20 fractions and the pH values and the dry weights of each fraction were determined as described previously (7,21).

Purification and identification of cationic peptides Fractions 19 and 20 had pH values of 12.2 and 12.6, respectively, and lyophilized samples were dissolved in ultrapure water containing 0.1% trifluoroacetic acid (TFA). Peptide solutions were then separated using RP-HPLC with a CAPCELL PAK C18 column (150 × 10 mm, 5 μm; Shiseido Co., Tokyo, Japan) as described previously (7,21), with a slight modification. Specifically, mobile phase A comprised 0.1% (v/v) TFA in ultrapure 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 1.0 mL/min using the following gradient: 0–10 min (100% A), 10–80 min (0–70% B), and 80–90 min (100% B). Fractions (1 mL) were collected and freeze-dried, and peptides in resultant peaks were again purified using RP-HPLC as described previously (7,21). Absorbance of chromatographic eluents was monitored at 220 nm using an SPD-10AVP detector (Shimadzu, Kyoto, Japan).

Peptides were identified using MALDI-TOF MS as described previously (7,21). Briefly, molecular weights of purified peptides were determined, and MS/MS analyzes were performed using an autoflex III TOF/TOF instrument (Bruker, Billerica, MA, USA). Amino acid sequences were identified in searches of the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and MS/MS ion searches were performed using the taxonomy (other green plants; GlycineMax_Pro) in the Mascot system (Matrix Science Ltd., London, UK).

Hemolytic assays Hemolytic activities of peptides were evaluated using melittin as a positive control. Briefly, 50-μL aliquots of peptide solutions at varying

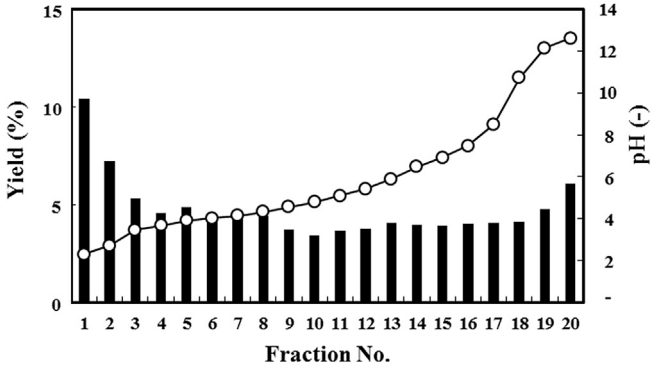

FIG. 1. Fractionation of Natto extracts by autofocusing using a Rotofor. Runs were performed at a constant power of 12 W for 2 h, and samples were recovered in 20 fractions. Yields (bar) and pH values (open circles) are shown for each fraction (100% = 200 mg dry weight). The volumes of fractions 1 and 20 were twice as large as the others. Data are expressed as mean ± standard deviation (SD) of three independent experiments.

concentrations were added to 50-μL aliquots of 4% (v/v) sheep red blood cell (RBC; Cosmo Bio Co. Ltd., Tokyo, Japan) suspensions in 96-well plates, and mixtures were incubated without shaking for 1 h at 37°C. Hemoglobin released from the blood cells was recovered by centrifugation (2000 × *g*, 5 min, 25°C), and quantified by measuring the absorbance of the supernatant at 405 nm as described previously (7,21).

LPS-neutralizing assays LPS binding and neutralizing activities of identified peptides were determined using chromogenic *Limulus* amebocyte lysate (LAL) assay kits (Endospecy ES-50M, Seikagaku Corporation, Tokyo, Japan) according to the manufacturer's instructions. Briefly, peptide solutions of various concentrations were incubated with LPS at 0.5 endotoxin units (EU)/mL in microtubes at 37°C for 30 min to allow binding to LPS. Equal volumes of LAL reagent containing the chromogenic substrates were added to 50-μL aliquots of these mixtures and incubated for 30 min at 25°C. We then monitored changes in absorbance at 405 nm (A_{405}) due to the cleavage of substrates (Boc-Leu-Gly-Arg-*p*-nitroaniline). Relative absorbances of peptide-containing reaction mixtures were expressed as ratios of A_{405} relative to those (100%) of reaction mixtures without peptide as described previously (7,21). Finally, we calculated 50% neutralizing (50% effective) concentrations (EC_{50}) for each peptide as described previously (7,21). Polymyxin B sulfate was used as a positive control.

Angiogenesis assays To assess the angiogenic properties of the identified peptides, we performed tube formation assays using Matrigel (Becton Dickinson and Company, Santa Clara, CA, USA) according to the manufacturer's instructions (7,21). Briefly, human umbilical vein endothelial cells (HUVECs; Kurabo Industries, Osaka, Japan) were seeded into modified MCDB 131 medium (HuMedia-EG2, Kurabo Industries) and incubated at 30°C in a humidified atmosphere containing 5% CO_2. After reaching 80–90% confluence, cells were recovered and counted using a hemocytometer. Solid Matrigels were then prepared in 96-well plates and HUVECs in HuMedia-EG2 medium containing peptides at varying concentrations were seeded onto the solid Matrigel surfaces at 2×10^5 cells/mL. After 15-h of incubation, tube formation was assessed using an inverted light microscope (TS100F, Nikon Instruments Inc., Tokyo, Japan) at 40 × magnification, and tube lengths were quantified as described previously (7,21) and compared to those generated in the presence of the positive control, LL-37. Relative tube lengths in the presence of peptides were expressed as percentages of controls without peptide (100%).

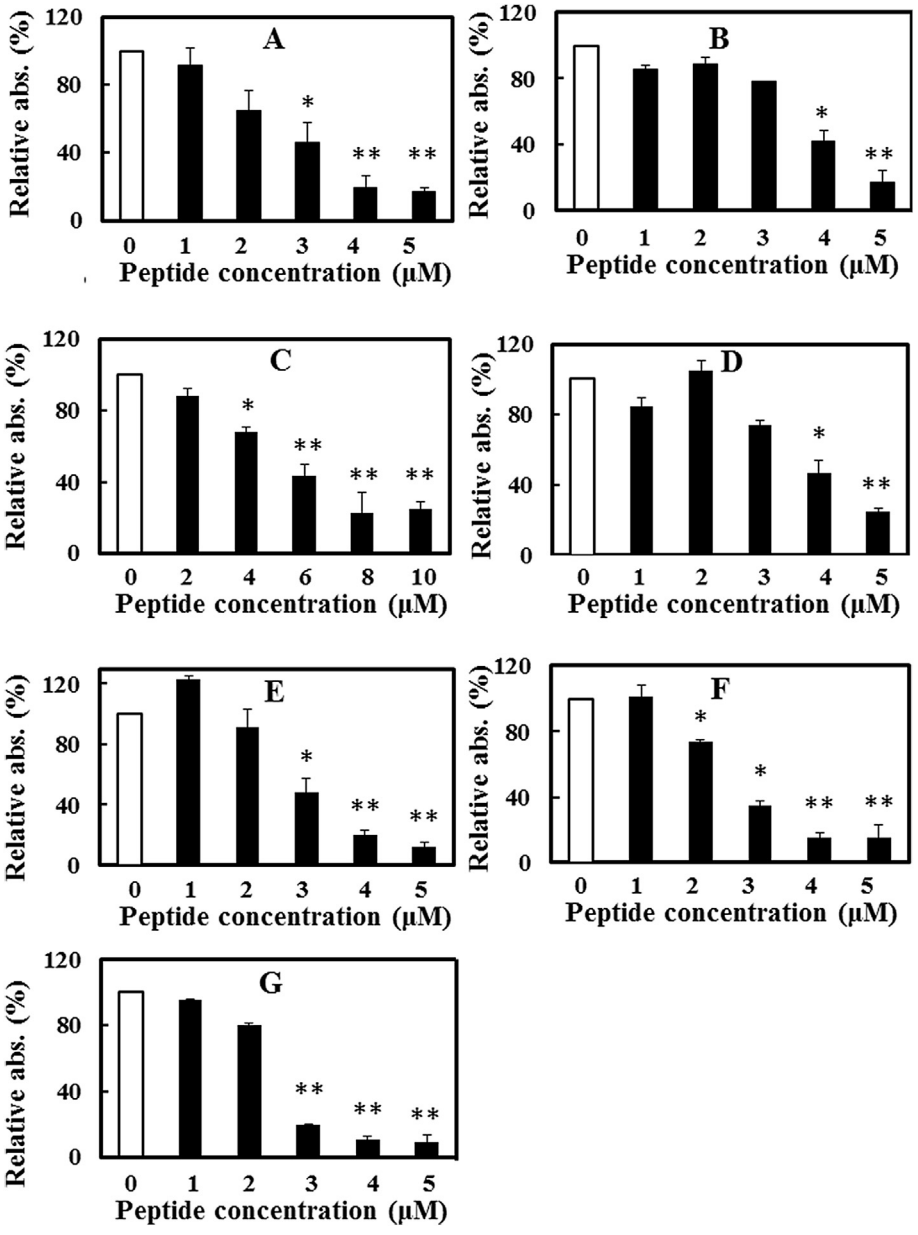

FIG. 2. Endotoxin-neutralizing effects of Natto derived cationic peptides in LAL assays. LAL values in the presence of FSB-KFN (A), FSB-FPF (B), FSB-GQS (C), FSB-QRF (D), FSB-ERQ (E), FSB-GEI (F), and SBH-EQP (G). Absorbance was determined at 405 nm and the values are expressed relative to those in the absence of peptide (100%). Data are presented as mean ± SD of three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$ vs. control without peptide.

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

RESULTS

Fractionation of Natto extracts using autofocusing After extraction with ultrapure water, 1.2–1.3 g of dry water-soluble components were obtained from 90 g of Natto sample with a water content of 60%. Precipitation with cold ethanol and subsequent dialysis of soluble material produced 0.36–0.39 g of dry Natto extract from 1.2 to 1.3 g of dry water-soluble components. We then separated 200 mg of Natto extract preparations into 20 fractions containing peptides with various p*I* values using the autofocusing procedures. The 20 fractions formed a pH gradient between 2.6 and 12.5, and the pH and dry weight yield of each fraction were determined (Fig. 1). Fractions 1 and 20 contained 21 and 12 mg of dry material (10.5% and 6% of the total recovered), respectively, and dry weights of fractions 2–19 ranged between 3.5% and 7.2% of the total. The combined recovery of fractions 19 and 20 was approximately 10.7% of the total dry weight, and these fractions contained the cationic peptides described below.

Purification and identification of cationic peptides The pH values of fractions 19 and 20 were greater than 12 after the autofocusing procedures, and their constituents were then separated by RP-HPLC. Numerous peaks were identified and purified by subsequent rechromatography using the same column. The molecular weights of the peptides in the peak fractions were determined by MALDI-TOF MS, and amino acid sequences were then determined in MS/MS analyzes. MS/MS ion data revealed the presence of eight and five cationic peptides in fractions 19 and 20, respectively (Supplementary Table S1), and seven of these had p*I*'s greater than 9.5, net positive charges, varied molecular weights, and were from different protein sources (Table 1). Thus, we synthesized the three peptides KFNKYGR, FPFPRPPHQK, and GQSSRPQDRHQK from fraction 19 and the four peptides QRFDQRSPQ, ERQFPFPRPPHQK, GEIPRPRPRPQHPE, and EQPRPIPFPRPQPR from fraction 20. Table 1 shows the amino acid sequences (7–14 amino acid residues), protein sources, and some properties of the seven cationic peptides from fermented soybean (FSB) product, and these were designated FSB-KFN, FSB-FPF, FSB-GQS, FSB-QRF, FSB-ERQ, FSB-GEI, and FSB-EQP on the basis of their three N-terminal amino acid residues.

Hemolytic activities of identified peptides To evaluate the cell membrane-disrupting activities of the identified cationic peptides, we measured hemolysis of sheep RBCs. These experiments showed little or no hemolytic activities, with relative hemolytic ratios of less than 0.24% at 500 μM for all the seven peptides (data not shown). In contrast, melittin was highly hemolytic at 20 μM (relative hemolytic ratio, 92%), which was consistent with the 94% reported in our previous studies (94%) (7,21).

LPS-neutralizing activities of the identified peptides In chromogenic LAL assays (Fig. 2), FSB-KFN, FSB-FPF, FSB-GQS, FSB-QRF, FSB-ERQ, FSB-GEI, and FSB-EQP exhibited concentration-dependent LPS-neutralizing activities, with EC_{50} values of 2.7, 3.8, 5.5, 3.9, 3.1, 2.7, and 2.6 μM, respectively. In agreement with our previous studies (7,21), the EC_{50} of the positive control, polymyxin B sulfate, was 0.12 μM, indicating a higher LPS-neutralizing activity than those of the cationic peptides.

Angiogenic activities of the identified peptides In tube formation assays using Matrigel and HUVECs (Fig. 3), we observed significant promotion of angiogenesis and tube formation with all peptides except FSB-GEL, and the effects of the peptides were comparable to that of the positive control, LL-37 (data not shown). Fig. 4 shows the relative lengths of the tubes formed in the presence of peptides at 1 and 10 μM, which indicate 23–31% increases at 10 μM relative to controls without any peptides.

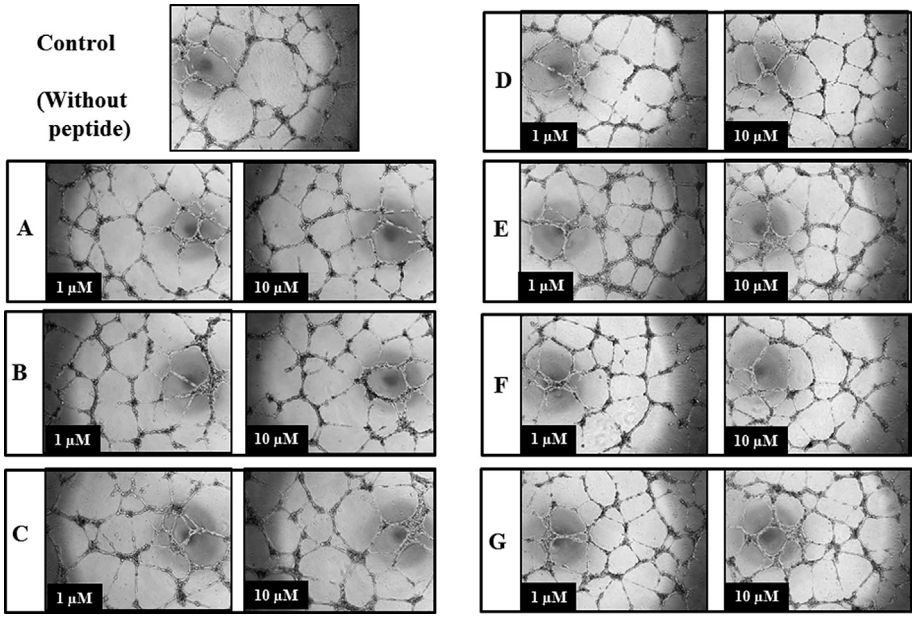

FIG. 3. Micrographs of tube formation in HUVECs in the presence of the seven peptides. The effects of peptides on tube formation are shown at concentrations of 1 and 10 μM. Tube formation in the presence of FSB-KFN (A), FSB-FPF (B), FSB-GQS (C), FSB-QRF (D), FSB-ERQ (E), FSB-GEI (F), and SBH-EQP (G). Micrographs of tube formation in Matrigel were obtained at 40 × magnification after incubation for 15 h. Random phase-contrast images of five wells were taken, and representative pictures are shown.

DISCUSSION

Numerous bioactive peptides have been isolated and characterized from enzymatic hydrolysates of food proteins (2,3,5,35). Fermentation of food proteins by microorganisms, including lactic acid bacteria (*Lactobacillus* and *Lactococcus* species), *Bacillus* species, and filamentous fungi (*Aspergillus* species), leads to the production of bioactive peptides through proteolysis (22,36,37). A few peptides that inhibit angiotensin I-converting enzyme (ACE) and have antioxidant and antimicrobial activities have been identified in fermented foods (4,5,25,38). Nakamura et al. (39) were the first to isolate, identify, and evaluate ACE-inhibitory activity of tripeptides (VPP and IPP) from skimmed milk that was fermented by *Lactobacillus heliveticus*. Gupta et al. (40) identified the antioxidant peptides VKEAMAPK and HIQKEDVPSER in cheddar cheese, which corresponded to residues 98–105 of β-casein and 80–90 of α_{s1}-casein, respectively. Cheng et al. (41) also isolated and characterized peptides from soybean meal fermented by *B. subtilis* and showed that they have antimicrobial activity against *Vibrio* species.

Along with soy sauce and miso paste, Natto is a traditional Japanese fermented food product produced from boiled soybeans. Although the antihypertensive, antithrombotic, and antioxidant functions of diary Natto are well known (22,23,29,30), only a few studies have identified the bioactive peptides that contribute to these effects. For example, five ACE-inhibitory peptides II, ID, IFY, LFY, and LYY (42) and an antimicrobial peptide of 45 amino acid residues (43) have been identified in Natto. In addition, two dipeptides KL and KR previously isolated from Natto inhibit dipeptidyl peptidase IV in a concentration-dependent manner (29). However, to our knowledge, no previous studies have reported the LPS-neutralizing and angiogenic functions of cationic peptides from Natto. We previously used autofocusing to separate water-soluble

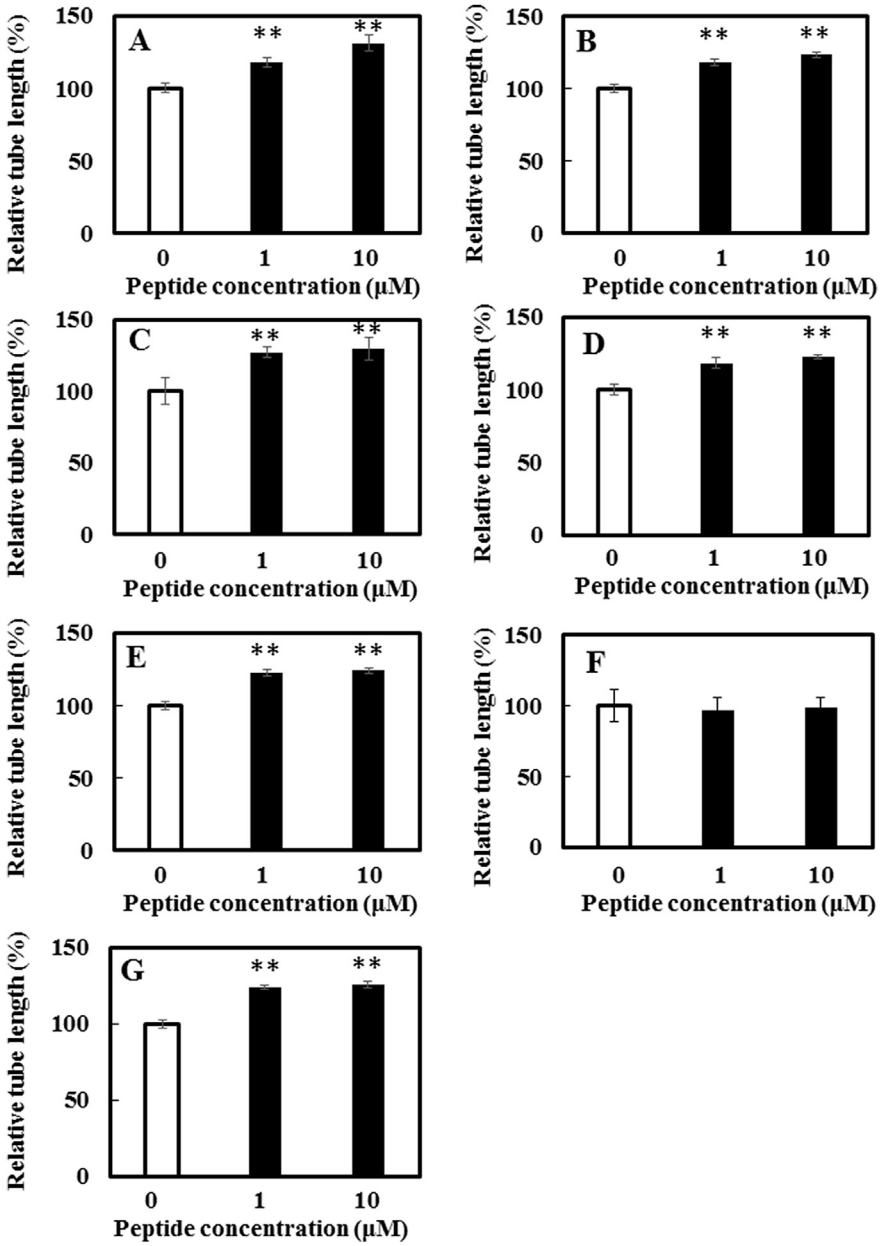

FIG. 4. Promotion of tube formation in HUVECs by Natto derived cationic peptides. The effects of peptides on tube formation are shown at concentrations of 1 and 10 μM. The relative tube lengths in the presence of FSB-KFN (A), FSB-FPF (B), FSB-GQS (C), FSB-QRF (D), FSB-ERQ (E), FSB-GEI (F), and SBH-EQP (G). Data are expressed as mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control without peptide.

fractions containing cationic peptides from rice and soybean hydrolysates (7,21). In this study, we employed the same approach with Natto (Fig. 1). The total cationic peptide yield from fractions 19 and 20, which had the pH values of 12.2 and 12.6, respectively, was 10.7% of the total sample weight. These fractions also contained seven cationic peptides from various protein sources. These peptides had varying numbers of amino acid residues, p*I* values greater than 9.5, and net positive charges (Table 1). Moreover, most synthetic versions of these peptides had LPS-neutralizing and angiogenic activities and were not hemolytic (Table 2). As shown in Table 1, FSB-GEI had the lowest p*I* (9.52) and a low mean hydrophobicity of 5.31, and exhibited LPS-neutralizing activity but did not induce angiogenesis. The lack of angiogenic activity of FSB-GEI may be attributable to the short distances between arginine residues, leading to weak interactions with targets on HUVECs. In contrast, the other six cationic peptides FSB-KFN, FSB-FPF, FSB-GQS, FSB-QRF, FSB-ERQ, and SBH-EQP, which have proper distributions of arginine and/or lysine residues, exhibited both LPS-neutralizing (Fig. 2) and angiogenic activities (Figs. 3 and 4) without significant hemolytic effects. Although cationic peptides from rice protein hydrolysates had antimicrobial activity (7), the present seven cationic peptides from Natto did not (data not shown), which was also the case with cationic peptides from soybean protein hydrolysates (21). The lack of, or low, antimicrobial activities of these soybean protein-derived cationic peptides may reflect their low mean hydrophobicities.

Peptides with LPS-neutralizing activity bind directly to the lipid A moiety of LPS, which contains hydrophobic fatty acid chains and phosphate groups with negative charges (44). In our hands, the peptide BCBS-11 (pI = 9.30, mean hydrophobicity = 12.48) exhibited high LPS-neutralizing activity (EC_{50}: 0.31 μM) (16). The EC_{50} values of the seven cationic peptides identified here were 8–18-fold higher than that of BCBS-11 (Table 2), and the lower LPS-neutralizing activities of these peptides may be attributable to suboptimal hydrophilic/hydrophobicity balances and low mean hydrophobicities (Table 1).

Previous studies have shown that angiogenesis is promoted in HUVECs following activation of the vascular endothelial growth factor (VEGF) receptor by peptides and VEGF (45–47). Hence, angiogenic activities of peptides may depend on interactions with the VEGF receptor and subsequent intracellular signaling. Six of the cationic peptides from Natto promoted tube-formation in HUVECs (Table 2), as were also observed with G-17, BCAS-16, BCBS-11 (16), and cationic peptides from soybean hydrolysates (21). However, the peptide receptors on HUVECs were not identified, warranting further studies of the mechanisms behind receptor interactions of these peptides. The contributions of amino acid residues to functional interactions of cationic peptides with LPS and VEGF receptors will be examined in the future studies.

The antimicrobial properties of cationic peptides have been associated with binding of positively charged peptides to negatively charged and hydrophobic microbial lipid membrane components, which facilitate interactions with cell membranes and/or translocation into cells (3,16). Although the antimicrobial cationic peptide BCBS-11 has a high p*I* of 9.3 and a mean hydrophobicity of 12.48 (16), all of the present cationic peptides, except FSB-FPF, had p*I* values greater than 9.5, but low mean hydrophobicities of less than 8 (Table 1). These low mean hydrophobicities may limit the antimicrobial and hemolytic activities of the peptides, although hydrophilic/hydrophobicity balances have also been associated with these biological activities (7,16,21,45–47). Collectively, the present data indicate that six cationic peptides from Natto have LPS-neutralizing and angiogenic activities but are not significantly hemolytic. Further investigations are also warranted to define the health benefits of multifunctional cationic peptides from other fermented food products.

In conclusion, in this study, we isolated multifunctional cationic peptides from Natto extracts. Seven of these peptides were chemically synthesized and their biological functions were examined. Six of them exhibited LPS-neutralizing and angiogenic activities, with negligible hemolytic effects. Taken together, our results demonstrate that basic fractions of Natto extracts contain non-hemolytic cationic peptides with multiple biological functions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.09.016.

TABLE 2. Bioactivity of cationic peptides identified from natto extracts.

Peptide name	LPS-neutralizing activity[a], EC_{50} (μM)	Angiogenic activity[b] (%)	Hemolytic activity (%)
KFN	2.7	131	<0.10
FPF	3.8	123	<0.10
GQS	5.5	129	<0.10
QRF	3.9	123	<0.10
ERQ	3.1	124	<0.10
GEI	2.7	98	<0.10
EQP	2.6	126	0.24

[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).

ACKNOWLEDGMENTS

This study was partially supported by a Grant-in-Aid for Scientific Research (JSPS KAKENHI grant number 16K06869) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a General Research Grant (grant number No. 13–2017) from Fuji Foundation for Protein Research.

References

1. **Cavazos, A. and de Mejia, E. G.:** Identification of bioactive peptides from cereal storage proteins and their potential role in prevention of chronic diseases, Compr. Rev. Food Sci. Food Saf., **12**, 364–380 (2013).
2. **Maestri, E., Marmiroli, M., and Marmiroli, N.:** Bioactive peptides in plant-derived foodstuffs, J. Proteomics, **147**, 140–155 (2016).
3. **Taniguchi, M. and Ochiai, A.:** Characterization and production of multifunctional cationic peptides derived from rice proteins, Biosci. Biotechol. Biochem., **81**, 634–650 (2017).
4. **Korhonen, H. and Pihlanto, A.:** Bioactive peptides: production and functionality, Int. Dairy J., **16**, 945–960 (2006).
5. **Rizzello, C. G., Tagliazucchi, D., Babini, E., Rutella, G. S., Saa, D. L. T., and Gianotti, A.:** Bioactive peptides from vegetable food matrices: research trends and novel biotechnologies for synthesis and recovery, J. Funct. Foods, **27**, 549–569 (2016).
6. **Egorov, T. A., Odintsova, T. I., Pukhalsky, V. A., and Grishin, E. V.:** Diversity of wheat anti-microbial peptides, Peptides, **26**, 2064–2073 (2005).
7. **Taniguchi, M., Kameda, M., Namae, T., Ochiai, A., Saitoh, E., and Tanaka, T.:** Identification and characterization of multifunctional cationic peptides derived from enzymatic hydrolysates of rice bran protein, J. Funct. Foods, **34**, 287–296 (2017).
8. **He, R., Alashi, A., Malomo, S. A., Girgih, A. T., Chao, D., Ju, X., and Aluko, R. E.:** Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates, Food Chem., **141**, 153–159 (2013).
9. **Singh, B. P., Vij, S., and Hati, S.:** Functional significance of bioactive peptides derived from soybean, Peptides, **54**, 171–179 (2014).
10. **Zambrowicz, A., Pokora, N., Setner, B., Dabrowwska, A., Szoltysik, M., Babij, K., Szewezuk, Z., Trziszka, T., Lubec, G., and Chrzanowska, J.:** Multifunctional peptides derived from an egg yolk protein hydrolysate: isolation and characterization, Amino Acids, **47**, 369–380 (2015).
11. **Korhonen, F.:** Milk-derived bioactive peptides: from science to applications, J. Funct. Foods, **1**, 177–187 (2009).
12. **Sila, A. and Bougatef, A.:** Antioxidant peptides from marine by-products: isolation, identification and application food systems. A review, J. Funct. Foods, **21**, 10–26 (2016).
13. **Fan, X., Bai, L., Zhu, L., Yang, L., and Zhang, X.:** Marine algae-derived bioactive peptides for human nutrition and health, J. Agric. Food Chem., **62**, 9211–9222 (2014).

14. **Xhindoli, D., Pacor, S., Benincasa, M., Scocchi, M., Gennaro, R., and Tossi, A.:** The human cathelicidin LL-37-A pore forming antibacterial peptide and host-cell modulator, Biochim. Biophys. Acta, **1858**, 546–566 (2016).
15. **Semple, F. and Dorin, J.:** β-Defensins: multifunctional modulators of infection, inflammation and more? J. Innate Immun., **4**, 337–348 (2012).
16. **Akira, S. and Takeda, K.:** Toll-like receptor signaling, Nat. Rev. Immunol., **4**, 499–511 (2004).
17. **Rosenfeld, Y., Papo, N., and Shai, Y.:** Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides, J. Biol. Chem., **281**, 1636–1643 (2006).
18. **Gurtner, G. C., Werner, S., Barrandon, Y., and Longaker, M. T.:** Wound repair and regeneration, Nature, **453**, 314–321 (2008).
19. **Sorg, H., Tilkorn, D. J., Hager, S., Hauser, J., and Mirastschijski, U.:** Skin wound healing: an update on the current knowledge and concepts, Eur. Surg. Res., **58**, 81–94 (2016).
20. **Taniguchi, M., Saito, K., Nomoto, T., Namae, T., Ochiai, A., Saitoh, E., and Tanaka, T.:** Identification and characterization of multifunctiona cationic and amphipathic peptides from soybean proteins, Peptide Sci, **108**, e23023 (2017).
21. **Taniguchi, M., Noda, Y., Aida, R., Saito, K., Ochiai, A., Saitoh, E., and Tanaka, T.:** Cationic peptides from enzymatic hydrolysates of soybean proteins exhibit LPS-neutralizing, and angiogenic activities, J. Biosci. Bioeng., **127**, 176–182 (2019).
22. **Gan, R.-Y., Gunaratne, A., Sui, Z.-Q., and Corke, H.:** Effects of fermented edible seeds and their products on human health: bioactive components and bioactivities, Compr. Rev. Food Sci. Food Saf., **16**, 489–531 (2017).
23. **Marco, M. L., Heeney, D., Binda, S., Gifelli, C. J., Cotter, P. D., Foligné, B., Gänzke, M., Kort, R., Pasin, G., Pihlanto, A., Smid, E. J., and Hutkins, R.:** Health benefits of fermented foods: microbiota and beyond, Curr. Opin. Biotechnol., **44**, 94–102 (2017).
24. **Hafeez, Z., Cakir-Kiefer, C., Roux, E., Perrin, C., Milco, L., and Dary-Mourot, A.:** Strategies of producing bioactive peptides from milk proteins to functionalize fermented milk products, Food Res. Int., **63**, 71–89 (2014).
25. **Egger, L. and Ménard, O.:** Update on bioactive peptides after milk and cheese digestion, Curr. Opin. Food Sci, **14**, 116–121 (2017).
26. **Fan, J., Zhang, Y., Chang, X., Saito, M., and Li, Z.:** Changes in the radical scavenging activity of bacterial-type Douchi, a traditional fermented soybean product, during the primary fermentation process, Biosci. Biotechol. Biochem., **73**, 2749–2753 (2009).
27. **Nakahara, T., Sano, A., Yamaguchi, H., Sugimoto, K., Chikata, H., Kinoshita, E., and Uchida, R.:** Antihypertensive effect of peptide-enriched soy sauce-like seasoning and identification of its angiotensin I-converting enzyme inhibitory substances, J. Agric. Food Chem., **58**, 821–827 (2010).
28. **Inoue, K., Gotou, T., Kitajima, H., Mizino, S., Nakazawa, T., and Yamamoto, N.:** Release of antihypertensive peptides in miso paste during its fermentation, by addition of casein, J. Biosci. Bioeng., **108**, 111–115 (2009).
29. **Sato, K., Miyasaka, S., Tsuji, A., and Tachi, H.:** Isolation and characterization of peptides with dipeptidyl peptidase IV (DPPIV) inhibitory activity from natto using DPPIV from *Aspergillus oryzae*, Food Chem., **261**, 51–56 (2016).
30. **Sanjukta, S. and Rai, A. K.:** Production of bioactive peptides during soybean fermentation and their potential health benefits, Trends Food Sci. Technol., **50**, 1–10 (2016).
31. **Dempsey, C. E.:** The actions of melittin on membranes, Biochim. Biophys. Acta, **1031**, 143–161 (1990).
32. **Zavascki, A. P., Goldani, L. Z., Li, J., and Nation, R. L.:** Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review, J. Antimicrob. Chemother., **60**, 1206–1215 (2017).
33. **Shang, D., Liang, H., Wei, A., Yan, X., Yang, Q., and Sun, Y.:** Effects of antimicrobial peptide L-K6, a temporin-1CEb analog on oral pathogen growth, *Streptococcus mutans* biofilm formation, and anti-inflammatory activity, Appl. Microbial. Biotechnol, **98**, 8685–8695 (2014).
34. **Park, E. Y., Imazu, H., Matsumura, Y., Nakamura, Y., and Sato, K.:** Effects of peptides fractions with different isoelectric points from wheat gluten hydrolysates on lipid oxidation in pork meat patties, J. Agric. Food Chem., **60**, 7483–7488 (2012).
35. **Saadi, S., Saari, N., Anwar, F., Hamid, A. A., and Ghazali, H. M.:** Recent advances in food biopeptides: production, biological functionalities and therapeutic applications, Biotechnol. Adv., **33**, 80–116 (2015).
36. **Choi, J., Sabikhi, L., Hassan, A., and Anand, S.:** Bioactive peptides in dairy products, Int. J. Dairy Technol., **65**, 1–12 (2012).
37. **Maesh, A. J., Hill, C., Ross, R. P., and Cotter, P. D.:** Fermented beverages with health-promoting potential: past and future perspective, Trends Food Sci. Technol., **38**, 113–124 (2014).
38. **Marcone, S., Belton, O., and Fitzgerald, D. J.:** Milk-derived bioactive peptides and their health promoting effects: a potential role in atherosclerosis, Br. J. Clin. Pharmacol., **83**, 152–162 (2017).
39. **Nakamura, Y., Yamamoto, N., Sakai, K., and Takano, T.:** Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin-I converting enzyme, J. Dairy Sci., **78**, 1253–1257 (1995).
40. **Gupta, A., Mann, B., Kumar, R., and Sangwan, R. B.:** Identification of antioxidant peptides in cheddar cheese made with adjunct culture *Lactobacillus casei* spp. *casei* 300, Milchwissenschaft, **65**, 396–399 (2010).
41. **Cheng, A.-C., Lin, H.-L., Shiu, Y.-L., Tyan, Y.-C., and Liu, C.-H.:** Isolation and characterization of antimicrobial peptides derived from *Bacillus subtilis* E20-fermented soybean meal and its use for preventing *Vibrio* infection in shrimp aquaculture, Fish Shellfish Immunol., **67**, 270–279 (2017).
42. **Shimakage, A., Shinbo, M., and Yamada, S.:** ACE inhibitory substances derived from soy foods, J. Biol. Macromol, **12**, 72–80 (2012).
43. **Kitagawa, M., Shiraishi, T., Yamamoto, S., Kutomi, R., Ohkoshi, Y., Sato, T., Wakui, H., Itoh, H., Miyamoto, A., and Yokota, S.:** Novel antimicrobial activities of a peptide derived from a Japanese fermented food, Natto, against *Streptococcus pneumoniae* and *Bacillus subtilis* group strains, AMB Express, **7**, 127 (2017).
44. **Taniguchi, M., Toyada, R., Sato, T., Ochiai, A., Saitoh, E., Kato, T., and Tanaka, T.:** Effects of arginine- and leucine-substitutions on anti-endotoxic activity and mechanisms of action of a cationic and amphipathic antimicrobial octadecapeptide from rice α-amylase, J. Pept. Sci., **23**, 252–260 (2017).
45. **Taniguchi, M., Ochiai, A., Namae, T., Saito, K., Saitoh, E., Kato, T., and Tanaka, T.:** Antimicrobial and anti-endotoxic Amyl-1-18 from rice α-amlyase and its [N3L] analog induce angiogenesis and cell migration, Peptides, **104**, 78–84 (2018).
46. **Lee, C. H., Lee, M.-S., Kim, S. J., Je, Y. T., Ryu, S. H., and Lee, T.:** Identification of novel synthetic peptide showing angiogenic activity in human endothelial cells, Peptides, **30**, 409–418 (2009).
47. **Finetti, F., Basile, A., Capasso, D., Gaetano, S. D., Stasi, R. D., Pascale, M., Turco, C. M., Ziche, M., Morbidelli, L., and D'Andrea, L. D.:** Functional and pharmacological characterization of a VEGF mimetic peptide on preparative angiogenesis, Biochem. Pharmacol., **84**, 303–311 (2012).