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Antimicrobial and human cancer cell cytotoxic effect of synthetic angiotensin-converting enzyme (ACE) inhibitory peptides

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Abstract

Four peptides with high angiotensin-converting enzyme (ACE) inhibitory effect were separated from beef sarcoplasmic protein hydrolysates using commercial enzymes. They were identified as GFHI, DFHING, FHG, and GLSDGEWQ and their 50% inhibition concentration (IC_{50}) values against ACE were 117, 64.3, 52.9, and 50.5 μg/ml, respectively. These peptides were synthesised and further biological activities of these four peptides were measured, including antimicrobial, cytotoxic effect against cancer cells, and macrophage-stimulating effect. Peptide GLSDGEWQ showed growth inhibition on *Salmonella Typhimurium*, *Bacillus cereus, Escherichia coli, and Listeria monocytogenes* at a 100 ppm level but not on *Staphylococcus aureus and Pseudomonas aeruginosa*. Peptide GFHI showed higher inhibition activity on the growth of *E. coli* and *P. aeruginosa* at concentrations of 200 and 400 μg/ml. However, peptide FHG inhibited only *P. aeruginosa* at 200 and 400 μg/ml. The effect of separated peptides on breast cancer (MCF-7), lung cancer (A549), and stomach cancer (AGS) cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Peptide GFHI showed a slight decrease of MCF-7 cell viability in a dose dependent manner. When 400 μg/ml of peptide GFHI was applied to the AGS cell, its viability was decreased by 75%. However, peptide DFHINQ seemed to act as a nutrient to AGS cell because it increased its viability. None of the four peptides had a cytotoxic effect on A549 cells. Nitric oxide (NO) production of peptide GFHI by stimulation of macrophage was investigated at 100, 300, and 1000 μg/ml concentration. NO was not produced in all treatments. From these results it is expected that the ACE inhibitory peptides identified from beef sarcoplasmic protein hydrolysates have both antimicrobial and cancer cell cytotoxic effects.

Keywords: Antimicrobial; Cancer cell cytotoxicity; ACE-inhibition activity; Peptides

1. Introduction

The fact that peptides released from food proteins by enzyme hydrolysis may exhibit different biological activities is now generally accepted. Theses peptides are inactive within the sequence of the parent proteins but can be activated when released by the hydrolytic action of commercial enzymatic proteolysis (Gibbs, Zougman, Masse, & Mulligan, 2004) or gastro-intestinal digestion.

Peptides with anti-hypertensive, anticancer, antimicrobial, and immunomodulatory activity have been found (Fujita, Yokohama, & Yoshikawa, 2000; Jang & Lee, 2005). Among the different groups of bioactive peptides, ACE inhibitory peptides have been receiving special attention, due to their potential beneficial effects related to hypertension. The formation of ACE inhibitory peptides by enzymatic hydrolysis (Hernandez-Ledesma, Recio, Ramos, & Amigo, 2002; Pihlanto-Leppala, Koskinen, Piilola, Tupasela, & Korhonen, 2000), by milk fermentation (Leclerc, Gauthier, Bachelard, Santure, & Roy, 2002; Nakamura et al., 1995), and during cheese ripening (Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000) has been reported. Several compounds with anticarcinogenic activity are also found in relatively high concentrations in food protein (Kim, Pai, & Lee, 1998). Diets including soy

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protein isolates, fed to rats and mice, reduced the incidence and number of breast, skin, and colon tumours. Also, the hydrophobic peptides extracted from soy sauce exhibited cytotoxic activity on human colon carcinomas and mouse lymphoma cell lines (Kim et al., 1998).

Pellegrini, Hulsmeier, Hunziker, and Thomas (2004) suggested that peptides produced by the enzymatic digestion of ovalbumin, and their synthetic counterparts, were found to be strongly active against *Bacillus subtilis* and to a lesser extent against *Escherichia coli*, *Bordetella bronchiseptica*, *Pseudomonas aeruginosa*, and *Serratia marcescens*, as well as *Candida albicans*. Also, Xie, Huff, Huff, Balog, and Rath (2002) demonstrated that ovotransferrin can act as an immunomodulator, modulating macrophage and heterophil functions *in vitro* and the inhibition of proliferation of mouse spleen lymphocytes. Also, it has been suggested that the antibacterial activity of lysozyme from eggs may occur, *via* stimulation of the macrophage phagocytic function, and the hydrolysis products of peptidoglycan may act as an adjuvant or immunomodulator (Kovacs-Nolan & Mine, 2005).

In this respect, the aims of this study were to separate ACE inhibitory peptides from beef protein hydrolysates using seven commercial enzymes and to investigate the further biological activities of the synthesised peptides, including antimicrobial, cytotoxic activity against cancer cell, and macrophage-stimulating activity, in addition to ACE inhibition activity.

2. Materials and methods

2.1. Extraction of protein and hydrolysis by seven commercial enzymes

Beef sample (20 g) was extracted by the method of Jang and Lee (2005), which was slightly modified from Toldra's (1999). Sample extraction was performed with 200 ml of 0.02 M phosphate buffer (pH 7.4). Meat and buffer were homogenised in an Ultra-Turrax T 25 blender for 3 min and centrifuged at 15,770*g* for 20 min at 4 °C. The supernatant was sterilised by filtration through a 0.22 μm filter (Millipore filtration kit MA 01730, Millipore Co., Bedford, UK) in aseptic conditions and used as beef extract. The extracts were adjusted to optimal pH and temperature for each enzyme; thermolysin + proteinase A (pH 7.5/37 °C), trypsin (pH 7.6/25 °C), proteinase K (pH 7.5/37 °C), tyrosinase (pH 6.5/25 °C), pepsin (pH 3.0/37 °C), papain (pH 6.2/25 °C), and protease (pH 7.5/37 °C) and incubated with these enzymes for 8 h. The ratio of enzyme solution to protein isolate was 1:100 (v/v). The enzymatic hydrolysis was stopped by boiling for 5 min. All the enzymes were purchased form Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Protein concentration and SDS-PAGE

The protein concentration in the extracts was determined using a Protein Assay Kit (Sigma). Bovine serum albumin was used as a standard. The degree of hydrolysis (DH) of the hydrolysates was obtained by SDS polyacrylamide gel electrophoresis using 12% gels. Electrophoresis was carried out, as described by Laemmni (1970), with a constant current (20 mA/gel) for 2 h. The proteins were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Inc. Hercules CA, USA).

2.3. Ultrafiltration

Beef protein hydrolysates were separated into a large molecular weight fraction and a low molecular weight fraction by ultrafiltration at 4 °C by PM-10 membrane (MWCO: 10,000; Amicon Co., Beverly, MA, USA) and kept for use in gel filtration. Prior to use, the membrane was activated by spinning 10 ml of distilled water, and the remaining liquid was carefully removed.

2.4. Gel filtration

Ultrafiltrated extracts were again filtered through a Millipore membrane filter (0.45 μm) and applied to a column (2.6 cm × 1 m; Amersham Pharmacia Biotech, NJ, USA) saturated in Sephadex G-25 resin. The column was calibrated by injecting the following standards with known molecular mass: bovine serum albumin (68 kDa), chymotrypsinogen A (25 kDa), cytochrome C (12.5 kDa), aprotinin (6.5 kDa), ristocetin A sulfate (2.5 kDa), and pepstatin (686 Da). The eluent was 20 mM phosphate buffer (pH 7.4), containing 0.05% (w/v) sodium azide, and the flow rate was 1.6 ml/min. Fractions were collected at 5-min intervals with a fraction collector (G.E. Healthcare Biosciences AB, Uppsala, Sweden). Each fraction was assayed for ACE inhibition activity. Then the active ACE inhibition fractions were further purified by reverse-phase high-performance liquid chromatography (RP-HPLC).

2.5. RP-HPLC system

RP-HPLC is widely used to generate a peptide map from digested protein (Tossavaninen, Syvaoja, Tuominen, Heinanen, & Kalkkinen, 1997). The peptide mixture was separated by RP-HPLC on a Symmetry C18 column (25 cm × 0.46 cm; Waters, USA) under aseptic conditions and at a flow rate of 0.8 ml/min. The mobile phase was delivered by a Young-Lin M930 and M925 LC pump (Young-Lin Co., Ltd, Seoul, Korea).

Separation was made under linear gradient elution conditions, using acetonitrile as an organic modifier and trifluoroacetic acid (TFA) as a volatile buffer. Eluent A consisted of 0.1% TFA in Milli-Q water (v/v); eluent B of 0.07% TFA in acetonitrile. The solvent composition started at 100% solvent A. The gradient was as follows: 0–10 min, 0% B; 10–30 min, linear gradient from 0% to 65% B; 30–40 min, 100% B; 40–45 min, 0% B. The UV absorbance of the eluent was monitored at 214 nm. The fractions

containing ACE inhibition activity were successively rechromatographed under the same RP-HPLC conditions for further purification. The most active fraction was concentrated by lyophilisation and applied onto the peptide sequencer for amino acid structure.

2.6. Peptide sequence

The amino acid sequence of the purified ACE inhibitory peptide with the greatest activity was analysed by Edman degradation, using a peptide sequencer (Model 491, Perkin–Elmer, Waltham, MA, USA).

2.7. ACE inhibition activity assay

The determination of ACE inhibitory activity was performed using the spectrophotometric method described by Cushman and Cheung (1971). For each assay, 100 μl of protein hydrolysate and 100 μl of hippuryl-L-histidyl-L-leucine (HHL, 12.5 mM in 0.05 M sodium borate buffer) were incubated at 37 °C for 5 min. After incubation, 150 μl of ACE (peptidyldipeptide hydrolase, from rabbit lung acetone extract) was added and the mixture incubated for another hour. The enzymatic reaction was stopped by adding 250 μl of 0.5 N HCl. The hippuric acid, released by the action of the angiotensin-converting enzyme on HHL was extracted from the acidified solution into 1.5 ml ethyl acetate by vortexing for 15 s. This was centrifuged at 3290*g* for 10 min at 4 °C, and a 0.5 ml aliquot of each ethyl acetate layer was transferred to clean tubes and evaporated by heating at 120 °C for 20 min on a hot plate. The hippuric acid was redissolved in 3 ml of 1 M NaCl, and the amount formed was determined by its absorbance at 228 nm. The IC_{50} value, defined as the concentration of peptide that inhibits 50% of the ACE activity, was determined from the protein contents of each sample after regression analysis.

2.8. Peptide synthesis

The selected peptides, which have high ACE inhibitory activity, were identified from their peptide sequence as GFHI, FHG, DFHINQ, and GLSDGEWQ. Each peptide was synthesised in the solid phase by Peptron Co. (Daejeon, Korea), to determine further bioactivity. All of the synthetic peptides used in this study showed the same ACE inhibition activity as separated peptides from beef sarcoplasmic protein hydrolysates (data not shown).

2.9. Determination of antimicrobial effect

Antimicrobial activity was examined against six pathogens, *E. coli* (ATCC 43894), *Staphylococuus aureus* (KFRI 00188), *Salmonella Typhimurium* (KFRI 00251), *P. aeruginosa* (KFRI 00100), *Bacillus cereus* (KFRI 00181), and *Listeria monocytogenes* (KFRI 00719). Those pathogens were purchased from Korea Food Research Institute (Sungnam, Korea). *P. aeruginosa* and *L. monocytogens* were grown at 25 °C for 24 h in nutrient broth (Oxoid Ltd., Hampshire, UK.). The other bacteria were grown in the same medium at 35 °C for 24 h (Gokturk, Gulcan, & Osman, 2004). A bacterial suspension (250 μl) of the bacteria, adjusted to a final cell concentration of 10^6–10^7 cfu/ml were added to a petri dish (8 cm diameter) containing previously made nutrient agar, then spread evenly. The sensitivity of pathogens to the peptides was determined by the paper disc diffusion method. Sterilised filter paper discs (8 mm) were soaked with 100 μl of solutions (100, 200, and 400 μg/ml) of each peptide in dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany), and DMSO was used as a control. The soaked discs were put in the middle of plates and incubated at 37 °C for 12–24 h in the inverted position for *P. aeruginosa*. The other bacteria were grown in the same media at 35 °C for 12–24 h. Inhibition zones formed in the medium were measured in millimetres (mm). The diameters of the inhibition zones were designated as: (–) not detected, for diameters less than 8 mm; sensitive (+), for diameters 9–12 mm; and very sensitive (++), for diameters 13–18 mm. All the tests were made in triplicate.

2.10. Cancer cell cytotoxic effect

The cytotoxic effect of the peptides against MCF-7 (breast adenocarcinoma, human, ATCC HTB-22), AGS (stomach adenocarcinoma, human, ATCC CRL-1739), and A549 (lung carcinoma, human, ATCC CCL-185) was assayed by the MTT assay. The MCF-7, AGS, and A549 cells used were provided by Dr. Kang (College of Veterinary Medicine, Seoul National University, Seoul, Korea).

Cells were plated at 37 °C for 24 h on 96-well plates (Nunc, Roskilde, Denmark), at a density of 10^3–10^4 cells per well, with Dulbecco's Modified Eagle's Medium (DMEM): F-12 (1:1) with phenol red (Sigma) in a humidified atmosphere (5% CO_2). L-Glutamine (4 mM), penicillin (200 units/ml), streptomycin (200 g/ml), DMEM non-essential amino acids (100 mM) and 10% foetal bovine serum (FBS) were added to the medium. L-Glutamine, penicillin, streptomycin, DMEM non-essential amino acids, and foetal bovine serum were purchased from Invitrogen (Gibco™; Invitrogen, Carlsbad, CA, USA). After 24 h, the cells were treated with peptides in a medium containing 2% FBS (100 μl per well) and incubated for 36 h. Then, 50 μl of aqueous MTT solution (5 mg/ml) were added to each well (100 μl), and the mixture was incubated at 37 °C for 3 h. The MTT solution was carefully removed, and formazan was extracted from the cells with 100 μl of a 4:1 DMSO-ethanol mixture in each well. Colour (optical density, OD) was measured with a 96-well ELISA plate reader at 550 nm. All MTT assays were repeated three times. Percent cell viability of test samples was determined as

$$\% \text{ Cell viability} = (\text{average OD for test group}/\text{average OD for control group}) \times 100.$$

2.11. Macrophage cell (RAW 264.7) culture and nitric oxide formation

The murine macrophage cell line, RAW 264.7, was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Caspase-3 assay kits (Enzchek, E-13183) were purchased from Molecular Probes (Eugene, OR, USA).

Cells were cultured in DMEM containing 10% heat-inactivated foetal calf serum and maintained at 37 °C in a humidified incubator containing 5% CO_2 and 95% air for 48 h. Cells were removed from the dishes and resuspended at a concentration of 1.5×10^5. Cells were plated at 180 μl/well, allowed to adhere for 30 min, and then the medium was changed to the peptide GFHI solution with increasing concentration (100, 300, and 1000 μg/ml). After 48 h of incubation nitrite concentrations in the cell culture supernatant were measured by staining with Griess reagent and read by ELISA reader (Molecular Device, Sunnyvale, USA) at 540 nm. The nitrite was used as an index of the macrophage-stimulating activity because nitric oxide is unstable and easily changed to stable products, such as nitrite.

2.12. Statistical analysis

Statistical analysis was performed with the SAS program (2000) (SAS, Cary, NC). One-way ANOVA was performed and the mean and standard error were reported. When the significance was found, Duncan's multiple range test was carried out to analyse the significant differences between mean values at $p < 0.05$.

3. Results and discussion

3.1. SDS-PAGE electropherogram

In order to obtain beef sarcoplasmic protein hydrolysates, protein digestion was carried out with seven commercial enzymes. The degree of hydrolysis of the beef sarcoplasmic protein was shown in the SDS-PAGE electropherogram (Fig. 1). The hydrolysis patterns of thermolysin + proteinase A (lane 1) and trypsin digested protein (lane 2) were similar. However, low molecular bands were not clear in lane 2. Proteinase K and protease hydrolyzed almost the whole protein, and low molecular weight bands were shown at the bottom of the gel (lane 3 and lane 7). This result indicated that each enzyme hydrolysed beef sarcoplasmic protein at a different level.

Previously, ACE inhibitory water-insoluble protein fraction and myosin of porcine skeletal muscle were measured, after digestion with one of eight proteases (thermolysin, proteinase K, pronase E, ficin, papain, trypsin, α-chymotrypsin, and pepsin), and it was suggested that thermolysin was the most suitable for induction of high ACE inhibition activity (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001). Lee, Kwon, Shin, and Yang (1999) revealed that papain-catalysed hydrolysate had a high ACE inhibitory activity after 8 h of hydrolysis, compared to alcalase, bromelain, and α-chymotrypsin. However, pancreatin- and trypsin-catalysed hydrolysates were very poor for ACE inhibition. They also suggested that, in preparing from proteins specific peptides which had some bioactive functions, protease specificity was a more important factor to be considered than protease activity, for selecting the proteolytic enzyme.

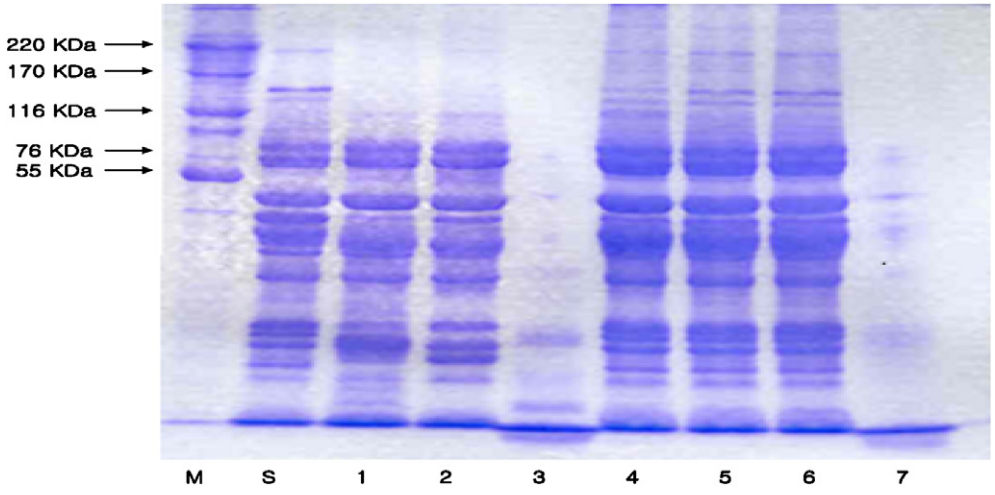

Fig. 1. Hydrolysis pattern of beef sarcoplasmic protein with various enzymes on SDS-PAGE profile (M: marker, S: un-hydrolyzed beef sarcoplasmic protein, lane 1–7 were hydrolyzed pattern by thermolysin + proteinase A (1), trypsin (2), proteinase K (3), tyrosinase (4), pepsin (5), papain (6), protease (7), respectively).

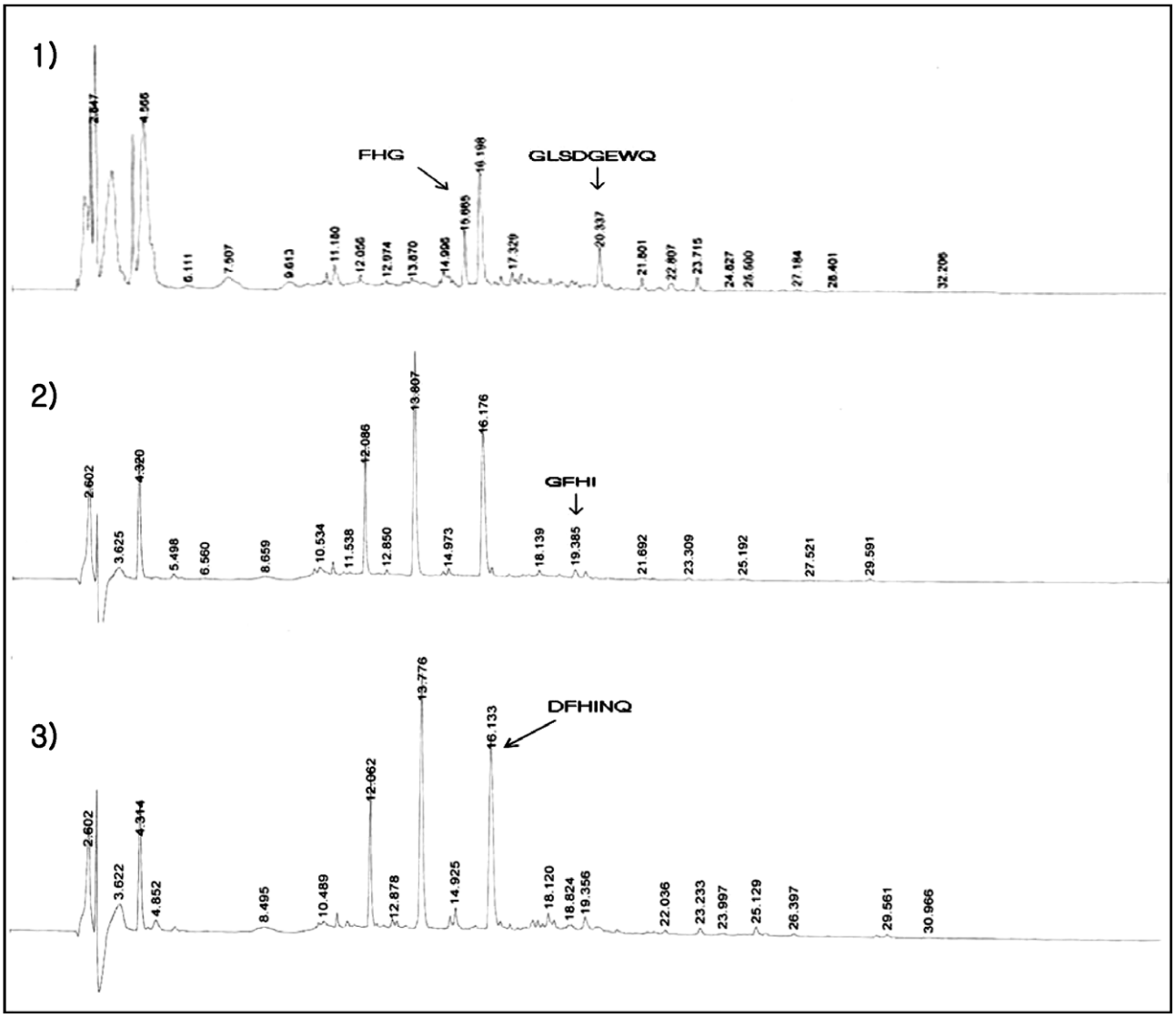

Fig. 2. HPLC chromatogram of ACE inhibitory peptides from beef sarcoplasmic protein hydrolysates by different commercial enzymes after gel filtration. (1) Chromatogram by thermolysin + proteinase A; (2) chromatogram by proteinase K; (3) chromatogram by protease (alcalase).

3.2. Peptide purification and determination of ACE inhibition activity

After ultrafiltration using a PM-10 membrane with molecular weight cut off (MWCF) at 10 kDa, the filtrates of all seven enzymes were loaded on a gel filtration column (Sephadex G-25), which has an optimal molecular weight range of 3 kDa, and separated with 20 mM phosphate buffer (data not shown). After gel filtration, the four ACE inhibitory peptide fractions with highest activity were collected and separated by RP-HPLC. Their chromatograms and the IC_{50} values of the peptides are shown in Fig. 2 and Table 1, respectively. The sample hydrolysed by thermolysin + proteinase A was divided into six peaks and the amino acid sequences of the high ACE inhibitory peaks were identified as FHG and GLSDGEWQ (Fig. 2 (1)) with IC_{50} values of 52.9 and 117 μg/ml, respectively. The amino acid sequence of the high ACE inhibitory peak from proteinase K hydrolysate was GFHI and its IC_{50} was 64.3 μg/ml (Fig. 2 (2)). The most active peak from the protease (alcalase) hydrolysate showed 50.5 μg/ml of inhibitory activity (IC_{50}) and was sequenced as DFHINQ (Fig. 2 (3)).

Table 1
ACE inhibition activity (50% inhibition concentration, IC_{50}) of separated peptides from beef sarcoplasmic hydrolysates

Peptide	GLSDGEWQ	GFHI	FHG	DFHINQ
IC_{50} (μg/ml)	117	64.3	52.9	50.5

Fujita et al. (2000) reported that a thermolysin digest of chicken muscle showed almost the same potent ACE inhibitory activity ($IC_{50} = 45$ μg/ml) as that of dried bonito ($IC_{50} = 29$ μg/ml). Also, IC_{50} values of the alcalase hydrolysate and peptic hydrolysate of sardine muscle were 250 and 620 μg/ml, respectively (Matsui et al., 1993).

ACE inhibitory peptides are generally reported to be dipeptides to decapeptides (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980; Matsui et al., 2002). ACE appears to prefer substrates or competitive inhibitors, containing hydrophobic amino acids at the three C-terminal positions (Kohmura et al., 1989). The most favourable C-terminal amino acids are aromatic amino acids and the imino acid, proline. Although the activity of di- or tri-peptides with ACE inhibitory activity cannot always be strictly extrapolated to larger peptides, the structural similarity of the C-terminal region may be predictive of similar activity (Hernandez-Ledesma et al., 2002). Kohmura et al. (1989) concluded that ACE is selective with regard to the C-terminal dipeptide sequence of its substrates and inhibitors, with aliphatic (V, I, A), basic and aromatic (Y, F) residues being preferred in the penultimate position. Aromatic (W, Y, F) proline and aliphatic (I, A, L, M) residues are preferred in the ultimate position.

Suetsuna (1998) reported that ACE inhibitory activity followed the order: Phe > Asn > Ser > Gly at the N-terminal; the dipeptide Phe-Tyr was the most potent inhibitor of ACE. Because the concentrations of the dipeptides required to inhibit ACE activity (IC_{50}) are rather high, it is possible that these peptides cause ACE inhibition by chelating zinc, which is required for ACE activity. They studied *in vivo* oral administration of seven isolated dipeptides and showed that blood pressure-reducing activity was qualitatively similar to that of captopril. Similar to the result of Suetsuna (1998), this present study showed that the identified peptides from beef sarcoplasmic protein hydrolysates were GLSDGEWQ, GFHI, and FHG, which possessed N-terminal amino acids G and F. However, no proline was identified in the sequence. This is supported by Maeno, Yamamoto, and Takano (1996) who reported that several ACE inhibitors have a proline residue in the C-terminal position, but this is neither sufficient nor essential to confer activity. On the other hand, Cheung et al. (1980) showed that among the N-terminal amino acids of dipeptides, the branched-chain aliphatic amino acids, valine and isoleucine, were the most effective in increasing peptide binding to the active site of ACE. Also, Jang and Lee (2005) identified a strong ACE inhibitory peptide, VLAQYK, from beef sarcoplasmic protein hydrolysates, which contained valine at its N-terminal. This peptide showed significant suppression of systolic blood pressure of spontaneously hypertensive rat when used as a supplement for up to 3 weeks (Jang et al., 2004). The four ACE inhibitory peptides, which we identified from beef sarcoplasmic protein hydrolysates were synthesised and characterised further, for the determination of biological activity, including antibacterial, cytotoxic effect against cancer cell lines, and macrophage-stimulating activity.

3.3. Antibacterial effect of synthesised peptides

A wide variety of organisms produce antimicrobial peptides as a primary innate immune strategy. Typically, peptides that are relatively short (less than 100 amino acids),

Table 2
Antimicrobial activity of the synthetic peptide GLSDGEWQ on six pathogens

Concentration (μg)	Microorganisms					
	S. Typhimurium	*B. cereus*	*E. coli*	*L. monoytogenes*	*S. aureus*	*P. aeruginosa*
100	+	+	+	+	–	–
200	+	+	+	+	–	–
400	+	+	+	+	–	–

+, Sensitive (9–12 mm inhibition); –, not detected.

Table 3
Antimicrobial activity of the synthetic peptide GFHI on six pathogens

Concentration (μg)	Microorganisms					
	S. Typhimurium	*B. cereus*	*E. coli*	*L. monoytogenes*	*S. aureus*	*P. aeruginosa*
100	–	–	+	–	–	++
200	–	–	++	–	–	++
400	–	–	++	–	–	++

++, Very sensitive (13–18 mm inhibition); +, sensitive (9–12 mm inhibition); –, not detected.

Table 4
Antimicrobial activity of the synthetic peptide FHG on six pathogens

Concentration (μg)	Microorganisms					
	S. Typhimurium	*B. cereus*	*E. coli*	*L. monoytogenes*	*S. aureus*	*P. aeruginosa*
100	–	–	–	–	–	+
200	–	–	–	–	–	++
400	–	–	–	–	–	++

++, Very sensitive (13–18 mm inhibition); +, sensitive (9–12 mm inhibition); –, not detected.

Table 5
Antimicrobial activity of the synthetic peptide DFHINQ on six pathogens

Concentration (μg)	Microorganisms					
	S. Typhimurium	*B. cereus*	*E. coli*	*L. monoytogenes*	*S. aureus*	*P. aeruginosa*
100	–	–	+	–	–	–
200	–	–	+	–	–	–
400	–	+	+	–	–	+

+, Sensitive (9–12 mm inhibition); –, not detected.

positively charged, and amphiphilic acid are reported to be active against bacteria, fungi, viruses and protozoa (Farnaud et al., 2004). Tables 2–5 show the effects of ACE inhibitory peptides on three Gram-positive (*B. cereus*, *L. monocytogens*, and *S. aureus*) and three Gram-negative (*S. Typhimurium*, *E. Coli*, and *P. aeruginosa*) pathogens. All the tested concentrations of peptide GLSDGEWQ resulted in a strong inhibition effect on *S. typhimurium, B. cereus, E. coli, and L. monocytogens* but not on *S. aureus and P. aeruginosa*. Peptide GFHI had a very strong antimicrobial effect, especially on *E. coli* and *P. aeruginosa* at 200 and 400 μg/ml (Table 3). However, only *P. aeruginosa* was inhibited by peptide FHG at 200 and 400 μg/ml (Table 4). Only *E. coli* was sensitive against peptide DFHINQ at all treated concentrations (Table 5). Also the small peptides (3–4 amino acids), FHG and GFHI, had a relatively high antimicrobial activity against *P. aeruginosa*.

Many scientists suggested that cationic peptides kill microorganisms by increasing cell membrane permeability. Although the present study did not identify whether these four peptides are cationic or anionic, it has been reported that antimicrobial peptides of frog skin are cationic due to the presence of multiple lysine residues, and contain at least 50% hydrophobic amino acids, of which leucine is usually the most abundant (Conlon, Al-Ghaferi, Abraham, & Leprince, 2007). Similarly, the peptides in the present study contained hydrophobic amino acids, such as Leu, Gly, Phe, and Ile.

Also, the C-terminal truncation of the three residues is critical for antibacterial activity against Gram-negative

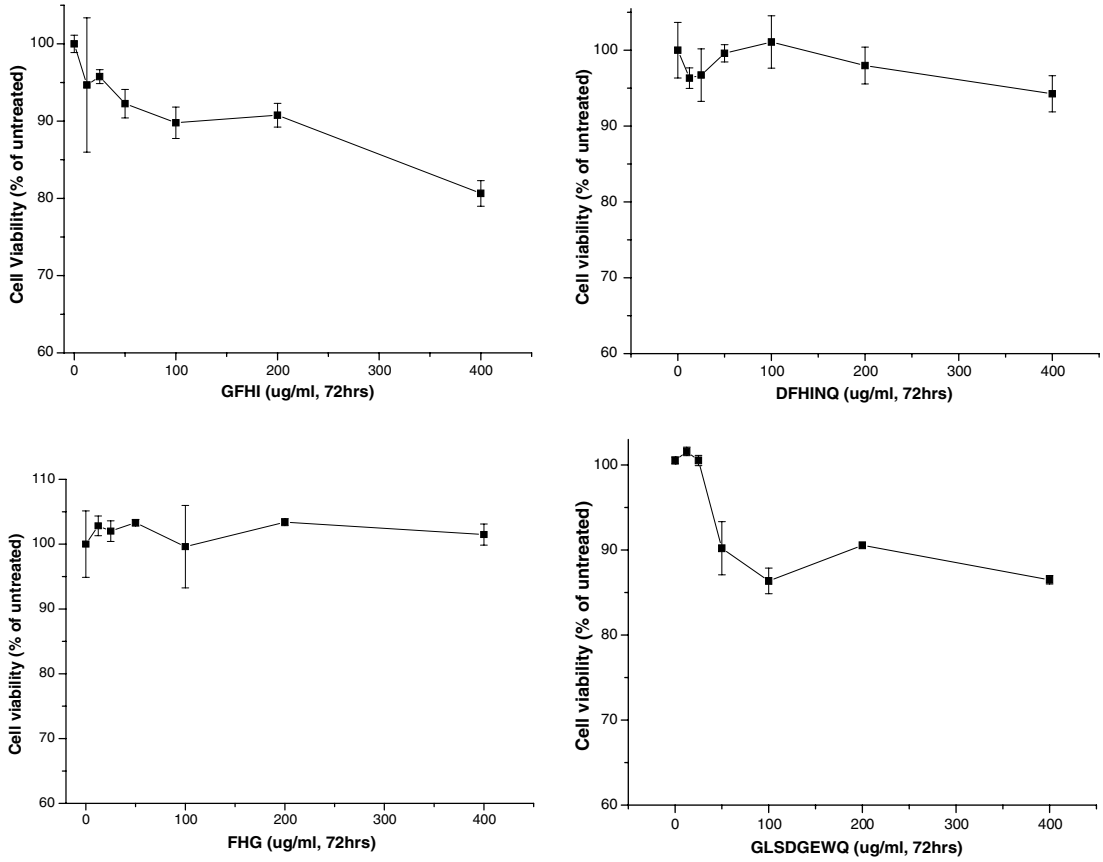

Fig. 3. Effect of the four synthetic peptides on cell viability of breast cancer cell line (MCF-7).

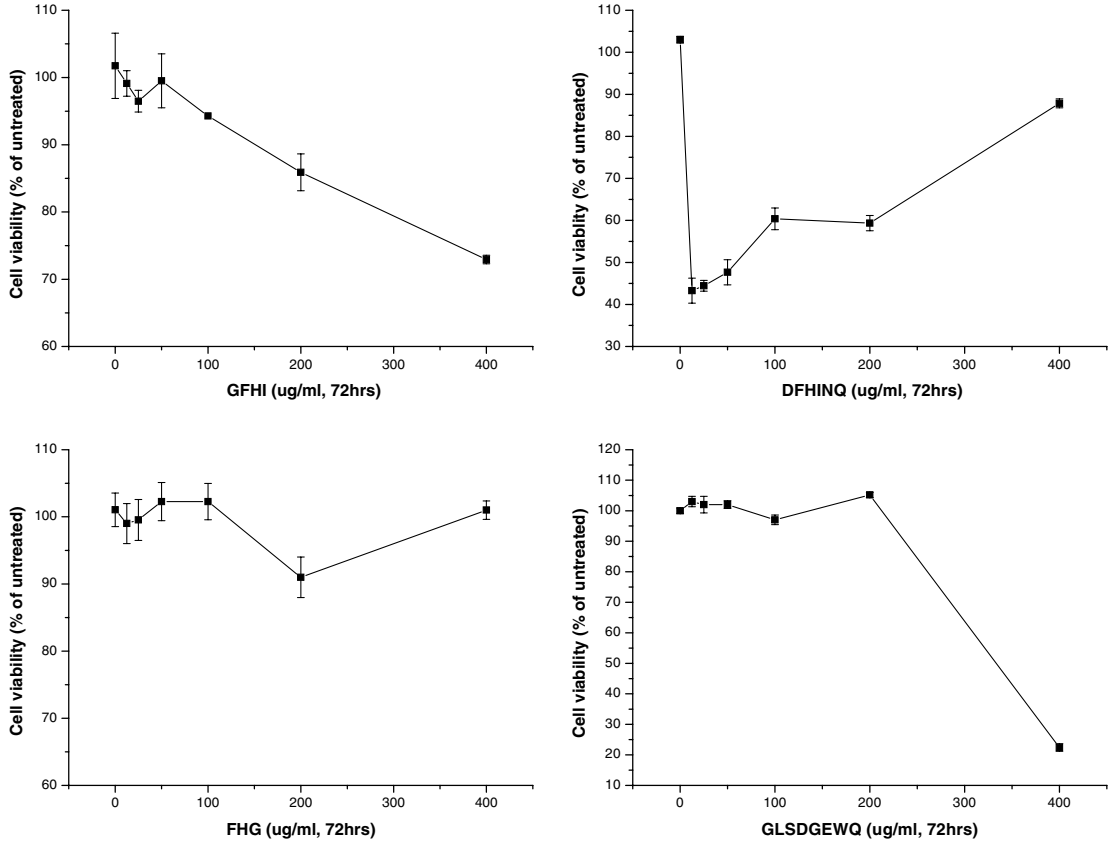

Fig. 4. Effect of the four synthetic peptides on cell viability of stomach cancer cell line (AGS).

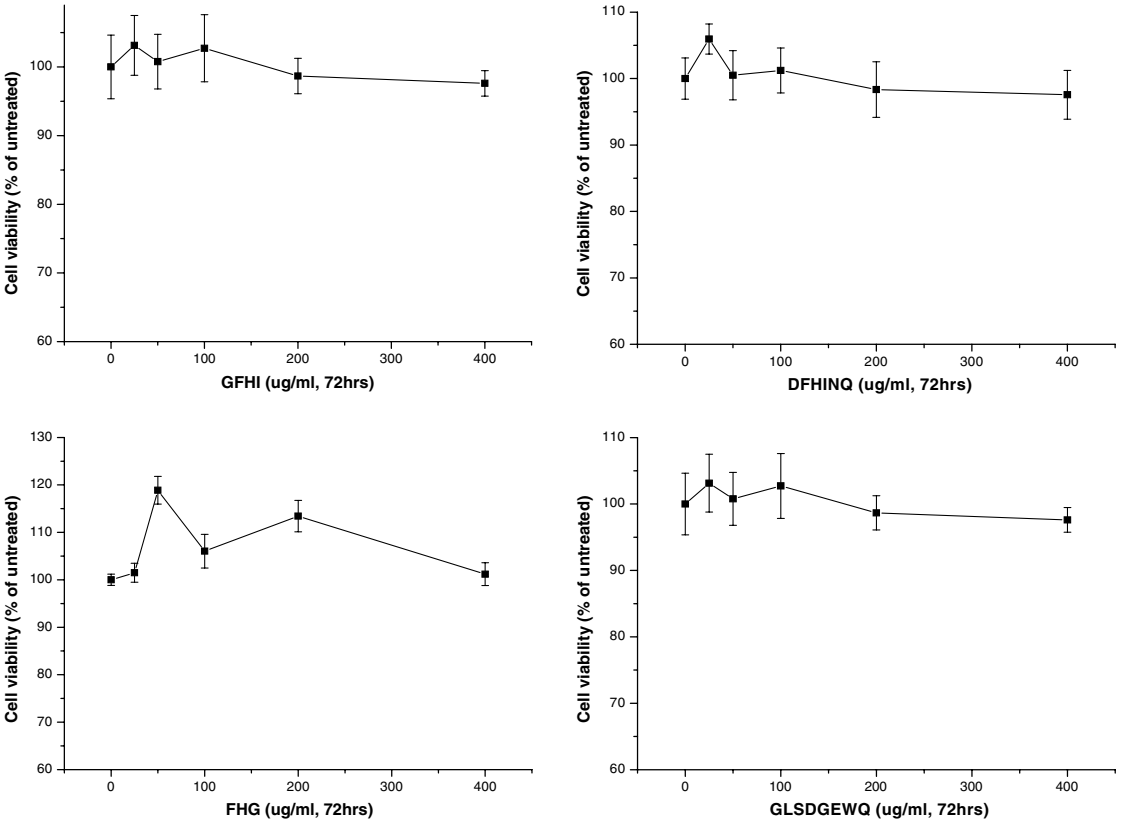

Fig. 5. Effect of the four synthetic peptides on cell viability of human lung cancer cell line (A549).

bacteria and some Gram-positive bacteria, but the N-terminal truncation of the three residues does not affect the antimicrobial activity (Lee, Cha, Lee, & Hahm, 2002).

3.4. Cytotoxic effect against cancer cells

Peptides from food proteins which have anti-carcinogenesis have been reported (Mills, Alcocer, & Morgan, 1992). The cytotoxic effects of separated peptides on breast, lung, and stomach cancer cells were measured by MTT assay. Fig. 3 shows that peptide DFHINQ slightly reduced the cell viability of MCF-7 cell lines. The most potent cytotoxic peptide on MCF-7 cells was GFHI in a dose dependent manner. In AGS cell, peptide GFHI caused a linear decrease of cell viability in a dose dependent manner (Fig. 4). Peptide GLSDGEWQ showed very strong inhibition of AGS proliferation by 80%, at a dose of 400 μg/ml. However, peptide DFHINQ seemed to act as a nutrient to AGS cell, increasing its viability. None of the peptides showed significant cytotoxic activity on A549 lung cancer cells (Fig. 5). Peptide GFHI showed only a 4% of reduction and there was no dose dependent effect.

González de Mejía, Visconez, de Lumen, and Nelson (2004) reported that nonapeptide, X-Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr, obtained by thermolase hydrolysis of defatted soy protein, showed an IC_{50} value of 0.16 mg/ml *in vitro* cytotoxicity on mouse monocyte macrophage cell line. A mechanism of cytotoxicity was proposed whereby the peptide selectively kills cells being transformed or newly transformed, by binding to deacetylated core histones exposed by the transformation event, disrupting the dynamics of histone acetylation-deacetylation and leading to cell death (De Mejia & De Lumen, 2006).

3.5. Macrophage-stimulating activity of synthesised peptides

Migliore-Samour and Jollés (1988) reported that immunomodulating casein peptides stimulated the proliferation of human lymphocytes and the phagocytic activities of macrophages. Nitric oxide (NO), is synthesised by nitric oxide synthase (NOS) and mediates diverse functions, including vasodilation, neuro-transmission, platelet aggregation inhibition, immunoresponse, and inflammation. Recent studies have demonstrated that NO serves as a factor of antitumour cell activity and a wide variety of physiological activities in the immune system (Kawakami, Minato, Imamura, Aizono, & Mizuno, 2003). Therefore, NO can be determined as a measure of the immunological activities of macrophages stimulated by peptide, GFHI.

To determine NO production from macrophage cells, 100, 300, and 1000 μg/ml of the synthetic peptide GFHI which showed high antimicrobial activity and cancer cell cytotoxicity, were added to the macrophage cells. However, no significant difference was found in nitric oxide production with peptide treatment (data not shown). This result may be related to the mild cancer cell cytotoxic effect of peptide GFHI. Miwa, Kong, Shinohara, and Watanabe (1990) examined if nitric oxide formation by macrophages was stimulated with water extracts from meats and offal. They also reported that a water extract of beef did not produce nitric oxide.

The relationships between the structure and activity and the mechanism of immunomodulatory effects of peptides from food origin are not yet defined. However, it has been suggested that an arginine residue, which is not included in peptide GFHI, at the N- or C-terminal region, may be the dominating entity recognised by specific surface membrane receptors of macrophages and lymphocytes (Paegelow & Werner, 1986).

From our results, the artificially synthesised ACE inhibitory peptides identified from beef sarcoplasmic protein hydrolysates have both antimicrobial and cancer cell cytotoxic effects. Therefore, the ACE inhibitory peptides can be expected to possess other biological functions and used as functional materials for food industry.

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