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Original Article

Purification and identification of anti-inflammatory peptides derived from simulated gastrointestinal digests of velvet antler protein (*Cervus elaphus* Linnaeus)

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ABSTRACT

The objective of this study was to identify anti-inflammatory peptides from simulated gastrointestinal digest (pepsin–pancreatin hydrolysate) of velvet antler protein. The hydrolysate was purified using ultrafiltration and consecutive chromatographic methods. The anti-inflammatory activity of the purified fraction was evaluated by the inhibition of NO production in lipopolysaccharide-induced RAW 264.7 macrophages. Four anti-inflammatory peptides, VH (Val–His), LAN (Leu–Ala–Asn), AL (Ala–Leu), and IA (Ile–Ala), were identified by liquid chromatography/tandem mass spectrometry. Each of these peptides demonstrated a U-shaped dose–effect relationship. VH, LAN, AL and IA showed the strongest anti-inflammatory activities at 200 μg/mL, that is, 15.5%, 13.0%, 16.0% and 11.2% inhibition of lipopolysaccharide-induced NO production, respectively. Additionally, the enhanced NO inhibitory activity was observed for the peptides mixture, indicating the possible synergistic effects. These results suggested that the peptides derived from velvet antler protein could potentially be used as a promising ingredient in functional foods or nutraceuticals against inflammatory diseases.

1. Introduction

Recently, it has been recognized that proteins provide a source of bioactive peptides with different health-promoting properties. These peptides can be produced from proteins during gastrointestinal digestion, fermentation or enzymatic hydrolysis. Several studies have shown that anti-inflammatory peptides can be obtained from protein hydrolysates [1–5]. Ahn et al isolated an anti-inflammatory

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tripeptide (PAY) from pepsin hydrolysate of salmon byproduct protein, which inhibited the production of NO, prostaglandin E2, tumor necrosis factor α, interleukin (IL)-6, and IL-1β, as well as the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 in lipopolysaccharides (LPS)-induced RAW 264.7 macrophages [1]. Oseguera-Toledo et al demonstrated that peptides from common bean inhibited inflammation through modulation of nuclear factor-κB pathways and blocking translocation of p65 subunit in LPS-stimulated RAW264.7 macrophages [2]. Peptides (LDAVNR and MMLDF) from enzymatic hydrolysate of the edible microalgae *Spirulina maxima* inhibit histamine release from mast cells and IL-8 generation from endothelial cells [3]. In addition, peptides from enzymatic hydrolysates of soy products [4] and *Ruditapes philippinarum* [5] also possess anti-inflammatory activities by inhibiting NO production in LPS-induced RAW 264.7 macrophages.

Velvet antler refers to the whole cartilaginous antler in a precalcified stage. It has been used for thousands of years in traditional Chinese medicine and functional food as an aid to help people maintain overall wellness. In recent decades, velvet antler has been reported to have pharmacological activity, such as immunomodulatory [6,7], anti-inflammatory [8,9], and wound-healing effects [10]. Velvet antler is a good source of protein, which may account for 60% (w/w) of dry matter [11]. However, velvet antler protein (VAP) has only received limited attention as a potential bioactive resource. Exploring bioactivities of protein from velvet antler is of significance to expand its applications in functional foods and nutraceuticals. Although an anti-inflammatory peptide with 68 amino acid residues (7.2 kDa) has been isolated from the antlers of *Cervus nippon* Temminck [9], there exists, to our knowledge, no further information about anti-inflammatory peptides derived from protein hydrolysates of velvet antler and the primary structure.

To evaluate the possibility of releasing bioactive peptides after consumption of food proteins, simulated gastrointestinal digestion has been used to mimic human digestion of proteins. The combination of pepsin–pancreatin has been used to simulate the gastrointestinal degradation of proteins in humans [7]. The objective of this study was to determine the effect of simulated gastrointestinal digest from VAP on the response of RAW 264.7 macrophages to inflammatory LPS *in vitro*. Peptides with molecular weight (MW) < 3 kDa were fractionated by gradient ethanol elution from a macroporous adsorption resin, and then separated on Sephadex G-15 and reverse-phase high performance liquid chromatography (RP-HPLC). Identification of the anti-inflammatory peptides was accomplished by liquid chromatography tandem mass spectrometry (LC-MS/MS).

2. Materials and methods

2.1. Materials

Velvet antlers (*Cervus elaphus* Linnaeus) were obtained from male red deer that were bred at the Daxing'an Mountain range (Heilongjiang, China). Samples were freeze-dried, homogenized, and stored at 4°C. Pepsin (EC 3.4.23.1, ≥ 2500 U/mg), pancreatin (8× USP specifications), LPS, protease inhibitors cocktail, Sephadex G-15, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and L-Tyr were purchased from Sigma–Aldrich (Shanghai, China). Murine macrophage cell line RAW 264.7 (ATCC TIB-71) was purchased from Cell Culture Center of Institute of Basic Medical Sciences Chinese Academy of Medical Sciences (Beijing, China). Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). Penicillin–streptomycin and NO Assay kit were purchased from Beyotime Biotechnology (Shanghai, China). HPLC-grade acetonitrile was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical grade unless otherwise mentioned.

2.2. Preparation of VAP

VAP was prepared according to Gao et al [12] with some modifications. Ten grams of velvet antler powder was incubated with 80 mL extract solution (100mM Tris + 6M guanidine–HCl + 20mM EDTA-2Na) at 4°C for 36 hours, followed by addition of 1% (v/v) protease inhibitors cocktail. After centrifugation at 5000 *g* for 30 minutes, the supernatant was brought to 70% saturation with solid ammonium sulfate. The protein pellet was recovered by centrifugation (5000 *g* for 30 minutes at 4°C), suspended in 20mM Tris–HCl buffer (pH 7.4) and dialyzed for 24 hours at 4°C against the same buffer. The dialysate was then lyophilized and the resulting protein extract was used for further investigations. The yield of VAP was 34.2% (w/w, in terms of dry weight of velvet antler).

2.3. Amino acid composition

The amino acid composition of VAP was determined using an L-8900 amino acid autoanalyzer (Hitachi, Tokyo, Japan) with an ion exchange column (4.6 mm × 60 mm). The sample was hydrolyzed in 6M HCl under vacuum at 110°C for 24 hours. Amino acids were detected and quantified at 570 nm for primary amines or at 440 nm for proline after a post-column reaction with ninhydrin at 135°C. Tryptophan content was determined after alkaline hydrolysis. The contents of individual amino acids were expressed as g/100 g of VAP.

2.4. Preparation of simulated gastrointestinal digests of VAP

In vitro simulated gastrointestinal digestion of VAP was carried out as reported previously [7] with slight modifications. VAP solution (5% w/v, in distilled water) was adjusted to pH 2.0 with 10M HCl, and pepsin (enzyme/substrate ratio of 1:100, w/w) was added. The solution was incubated at 37°C for 2 hours under constant stirring. After pepsin hydrolysis, the pH of the solution was adjusted to 6.8 with 10M NaOH, and pancreatin (enzyme/substrate ratio of 1:50, w/w) was added. The solution was incubated at 37°C for a further 4 hours under constant stirring. During the enzymatic hydrolysis, the pH of the solution was maintained constant by means of pH-stat technique using 1M HCl or 1M NaOH. After hydrolysis, enzymes were inactivated by heating in boiling water for 10 minutes. The hydrolysate was centrifuged at 10,000 *g* and 4°C for

20 minutes. The supernatant was fractionated using a stirred ultrafiltration cell (Model 8050; Millipore, Bedford, MA, USA) equipped with 3 kDa MW cut-off membrane to obtain the peptides fraction (SGD-VAP) with MW < 3 kDa. The resultant SGD-VAP solution was lyophilized and stored at −20°C for further use.

2.5. Determination of anti-inflammatory activity

2.5.1. Cell culture and cell viability

Murine macrophage cell line RAW 264.7 was cultured in Dulbecco's modified Eagle's medium supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability was estimated by the MTT assay as described previously [13]. Cells (3×10^4/well) were seeded in 96-well plates and treated with various concentrations of samples in the absence or presence of LPS (10 μg/mL). After incubation for 24 hours, the growth medium was replaced with 100 μL fresh growth medium. A 20-μL aliquot of MTT solution (5 mg/mL) was added to each well and incubated for a further 4 hours at 37°C. The purple formazan was diluted with 150 μL dimethyl sulfoxide. The absorbance was measured at 490 nm using a microplate reader (Spectra Max i3; Molecular Devices, Sunnyvale, CA, USA). The percentage of viable cells was calculated with respect to the cells treated with phosphate-buffered saline.

2.5.2. NO measurement in supernatant of RAW 264.7 macrophages

One hundred microliters of RAW 264.7 macrophages (3×10^5/mL) were seeded in 96-well plates and incubated with different concentrations of samples in the absence or presence of LPS (10 μg/mL). The plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 24 hours. After incubation, the supernatant was collected for NO production measurement. NO production was determined using an NO Assay Kit (Beyotime Institute of Biotechnology, Nantong, China). Fifty microliters of supernatant was mixed with 100 μL of the Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid) in a 96-well plate. After incubation at room temperature for 10 minutes, absorbance at 550 nm was determined in a microplate reader. Nitrite concentration was determined by using $NaNO_2$ as a standard.

2.6. Purification of anti-inflammatory peptides from SGD-VAP

Ten milliliters of SGD-VAP (30 mg/mL) was loaded onto a DA201-C (Suqing Chemistry Co., Jiangyin, China) column (26 mm × 400 mm), which was previously equilibrated with distilled water. Adsorbed peptides were eluted by gradient ethanol elution at a flow rate of 3 mL/min. The eluent was monitored by absorbance at 220 nm. Each of the fractions was pooled, concentrated by rotary evaporation (40°C), and freeze dried. The most potent anti-inflammatory fraction was loaded onto a Sephadex G-15 gel filtration column (16 mm × 700 mm) equilibrated with distilled water. Samples were eluted with distilled water at a flow rate of 1.5 mL/min. The eluent was monitored by absorbance at 220 nm. Fractions were pooled and freeze dried for an anti-inflammatory assay. The fraction showing the strongest anti-inflammatory activity was further purified using preparative RP-HPLC on an Eclipse XDB-C_{18} column (21.2 mm × 250 mm, 7 μm). Eluent A was 0.1% (v/v) trifluoroacetic acid in acetonitrile and Eluent B was 0.1% (v/v) trifluoroacetic acid in water. The gradient elution condition was 0–10 minutes, 1% eluent A; 10–60 minutes, 1–45% eluent A; 60–70 minutes, 45–100% eluent A; 70–85 minutes, 100% eluent A. The flow rate was 5 mL/min and temperature was 30°C. Elution peaks were monitored at 220 nm, and their anti-inflammatory activities were measured using the method previously described.

2.7. Identification and quantification of anti-inflammatory peptides

The MWs and amino acid sequences of purified peptides were determined using LC-MS/MS. The LC-MS/MS system consisted of an Agilent 1290 Infinity LC coupled to an Agilent G6500 quadrupole time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). LC separations were performed on an Agilent Zorbax Eclipse Plus C_{18} column (2.1 mm × 50 mm, 1.8 μm). Eluent A was 0.1% (v/v) formic acid in acetonitrile and Eluent B was 0.1% (v/v) formic acid in water. Gradient elution was carried out according to the following process: 0–2 minutes, 1% eluent A; 2–12 minutes, 1–50% eluent A; 12–15 minutes, 50–100% eluent A; 15–20 minutes, 100% eluent A at a flow rate of 0.2 mL/min. The temperature of the column was 30°C and the injection volume was 2 μL. Peptides eluted were analyzed online by MS and selected peptides were subjected to MS/MS sequencing. Spectra were recorded over the mass to charge (m/z) range of 50–3000. A precursor ion scan of the purified fraction was used to identify the unique peptide mass, which was then fragmented using a low collision energy (15 eV) induced dissociation to determine the peptide fragment for *de novo* sequencing. High-purity N_2 was used for drying (300°C, 5.0 L/min), and nebulization (15 psig). The voltage of the capillary was 3.5 kV. The peptide sequencing was performed by manual calculation.

The peptides were synthesized by GL Biochem (Shanghai, China) using a solid phase peptide synthesis method. The purity of the synthesized peptide was evaluated to be 95% by HPLC analysis, and its MW was determined using Voyager DE-Pro matrix-assisted laser desorption/ionization mass spectrometry (PE Biosystems, Framingham, MA). These synthetic peptides were used as standards for the quantification of the peptides in Fraction H3 using the LC-MS method under the same conditions. Quantification was carried out by integration of the extracted ion chromatogram peak areas using external standard calibration.

2.8. Statistical analysis

Each set of experiments was carried out with three replicates. Data were expressed as means ± standard deviation. The differences between two groups were assessed using one-way analysis of variance followed by Duncan's test. A value of $p < 0.05$ was considered statistically significant. The correlation was calculated under Pearson correlation coefficient (2-

tailed) in bivariate correlations. Analysis was done with SPSS version 13.0 (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Amino acid composition of VAP

The amino acid composition of VAP is summarized in Table 1. Glu (9.50 g/100 g) was determined to be the most abundant amino acid in VAP, followed by Gly, Asp, Leu, Pro and Ala. Trp was the amino acid present in the lowest amount. The amino acid composition of VAP was similar to that of velvet antler reported by Sunwoo et al [11]. VAP contained relatively high contents of hydrophobic amino acids and positively charged amino acids, which accounted for 39.1% and 18.1% of the total amino acids, respectively. Hydrophobic amino acids (such as Leu and Phe) and positively charged amino acids were reported to have a major influence on the anti-inflammatory activities of peptides [14,15]. In view of this, we speculated that anti-inflammatory peptides may be obtained from VAP by protease hydrolysis.

3.2. NO inhibitory activity of SGD-VAP

Pepsin–pancreatin hydrolysis was performed in order to simulate gastrointestinal digestion of VAP. The ultrafiltration fraction with MW < 3 kDa (SGD-VAP) accounted for 54.8% (w/w) of VAP hydrolysates (data not shown), indicating that VAP was mainly converted into low-MW peptides. Previous study has reported that low-MW peptides are less susceptible to proteolytic enzymes and could be easily absorbed by intestinal cells after oral administration [16]. Thus, SGD-VAP (MW < 3 kDa) from SGDs of VAP was selected for further study.

The cytotoxicity of SGD-VAP in RAW 264.7 macrophages was determined using the MTT assay prior to evaluating its anti-inflammatory activity. As shown in Fig. 1A, SGD-VAP did not exhibit any cytotoxic effect on RAW 264.7 macrophages at the tested concentrations (100–2000 μg/mL). SGD-VAP significantly ($p < 0.01$) enhanced the viability of LPS-induced macrophages at 200 μg/mL and 500 μg/mL, showing 24.3% and 31.7% increase in cell viability, respectively. Thus, the nontoxic concentrations (100–2000 μg/mL) of SGD-VAP were used to evaluate its NO inhibitory activities in LPS-induced RAW 264.7 macrophages.

LPS, a component of the Gram-negative cell wall, was used to activate macrophages. Overproduction of NO can be induced quantitatively in activated macrophages, which is related to inflammatory responses [7]. Therefore, inhibition of NO production in LPS-induced macrophages is one of the possible ways to screen anti-inflammatory components. To investigate the inhibitory effect of SGD-VAP on NO production, the accumulation of nitrite in the culture media was measured. As shown in Fig. 1B, treating RAW 264.7 macrophages with LPS resulted in 13.6-fold increased NO production compared to that in the blank group. SGD-VAP significantly inhibited LPS-induced NO production from RAW 264.7 macrophages in a dose-dependent manner ($r = -0.985$, $p < 0.01$).

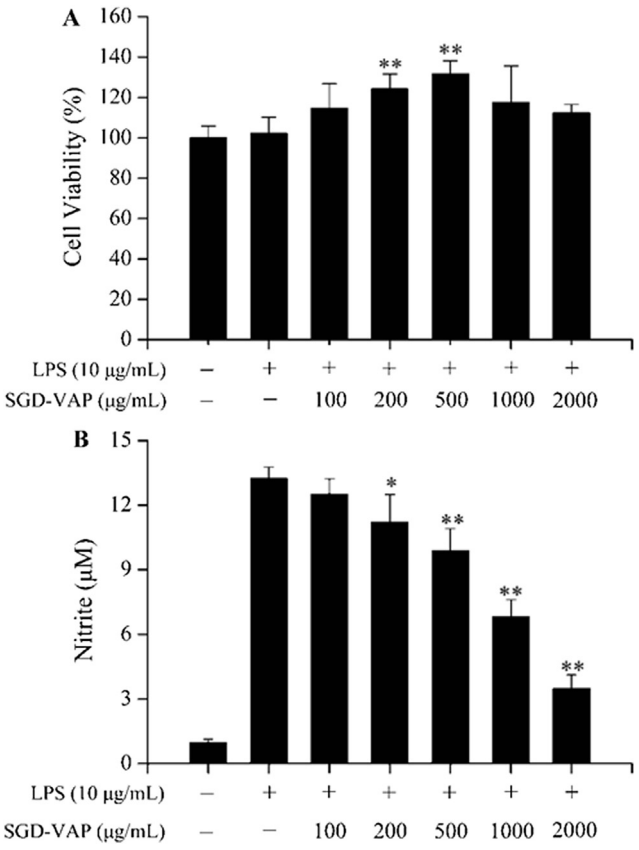

Fig. 1 – Effect of SGD-VAP on the cell viability (A) and NO production (B) in LPS-induced RAW 264.7 macrophages. SGD-VAP, peptides fraction (molecular weight < 3 kDa) obtained from simulated gastrointestinal digest of velvet antler protein by ultrafiltration. *$p < 0.05$, **$p < 0.01$ are significantly different compared with the group treated with only LPS. LPS = lipopolysaccharide; SGD = simulated gastrointestinal digest; VAP, velvet antler protein.

Table 1 – Amino acid compositions of velvet antler protein.

Amino acids	Content (g/100 g)	Amino acids	Content (g/100 g)
Thr	3.45 ± 0.20	Ser	3.81 ± 0.21
Val	3.91 ± 0.17	Glu	9.50 ± 0.29
Met	1.02 ± 0.06	Gly	8.29 ± 0.27
Ile	1.71 ± 0.18	Ala	5.87 ± 0.10
Leu	6.78 ± 0.22	Cys	1.41 ± 0.09
Phe	3.55 ± 0.15	Tyr	2.28 ± 0.08
Lys	5.73 ± 0.18	Arg	5.50 ± 0.26
Trp	0.65 ± 0.03	Pro	6.31 ± 0.25
Asp	7.27 ± 0.28	His	3.25 ± 0.15
HAAs[a]	31.34 ± 0.06	PCAAs[c]	14.49 ± 0.56
AAAs[b]	6.48 ± 0.10	Total	80.20 ± 0.76

Values are reported as the means ± standard deviation ($n = 3$).
[a] Hydrophobic amino acids (Ala, Val, Met, Ile, Leu, Phe, Pro and Tyr).
[b] Aromatic amino acids (Phe, Trp and Tyr).
[c] Positively charged amino acids (Arg, Lys and His).

The inhibitory effect of SGD-VAP became significant ($p < 0.05$) at 200 μg/mL (15.5% inhibition). At 2 mg/mL, SGD-VAP inhibited NO production by 73.7%. It was suggested that VAP contained some anti-inflammatory peptide fragments, which can be released during simulated gastrointestinal digestion. Purification and identification of these bioactive peptides from SGD-VAP could provide insights into the development of new anti-inflammatory products.

3.3. *Purification of anti-inflammatory peptide*

Sequential chromatography was used to purify anti-inflammatory peptides from SGD-VAP, including DA201-C macroporous adsorption resin, Sephadex G-15 gel filtration chromatography, and RP-HPLC. Fig. 2 illustrates the column chromatographic profiles of SGD-VAP and the anti-inflammatory activities of the fractions.

DA201-C macroporous adsorption resin is a nonpolar resin that adsorbs hydrophobic peptides. It has been used to separate peptides in protein hydrolysates by hydrophobicity using

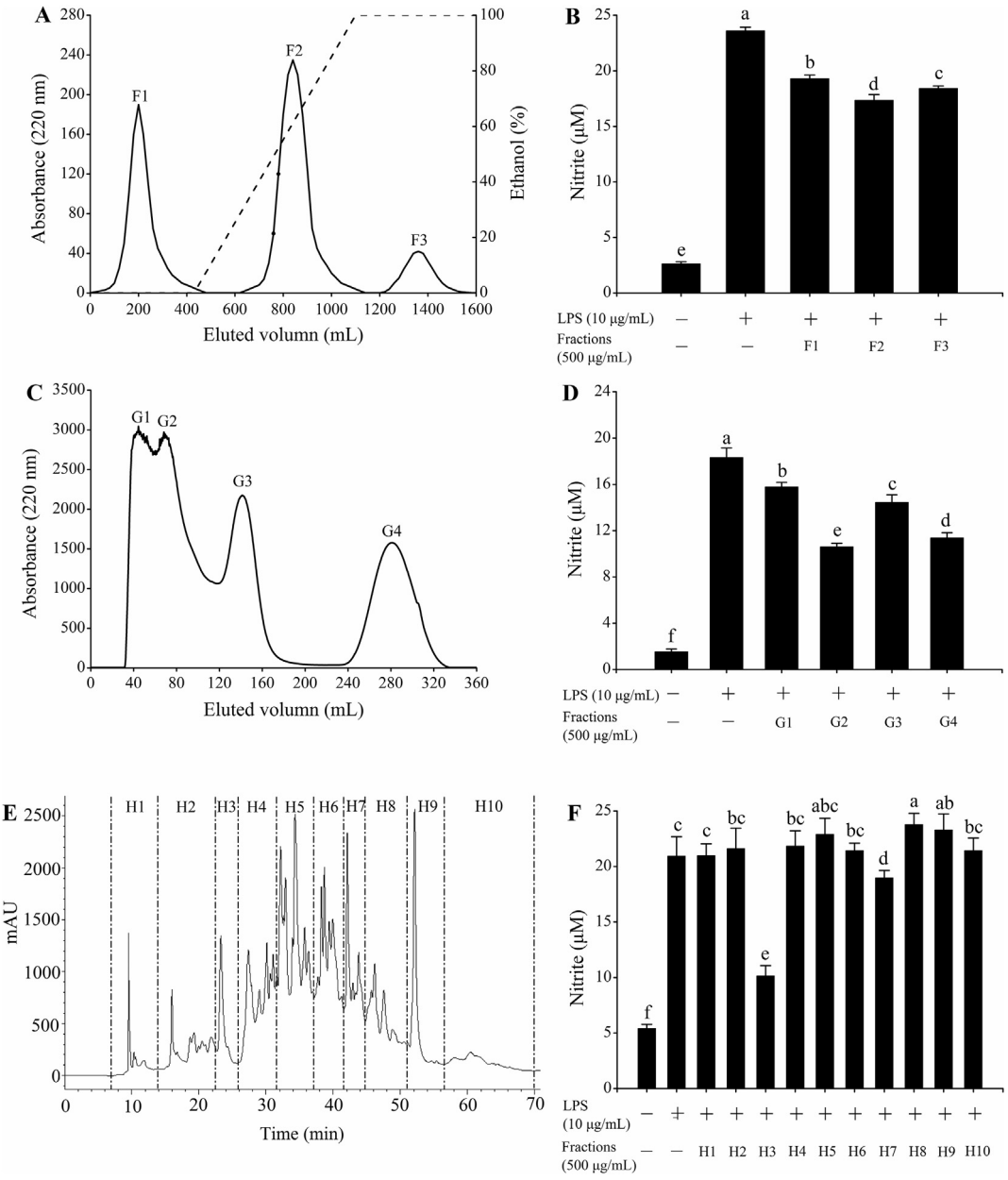

Fig. 2 – Purification of anti-inflammatory peptides from SGD-VAP. (A) Chromatogram of SGD-VAP separated by DA201-C macroporous adsorption resin. (C) Gel filtration chromatogram on Sephadex G-15 of Fraction F2 from (A). (E) Reverse-phase high-performance liquid chromatography of Fraction G2 from (C). (B, D, F) NO inhibitory activity of the separated fractions. SGD-VAP, peptides fraction (molecular weight < 3 kDa) obtained from simulated gastrointestinal digest of velvet antler protein by ultrafiltration. Means with different superscripts are significantly different ($p < 0.05$). SGD = simulated gastrointestinal digest; VAP, velvet antler protein.

an ethanol gradient elution [17]. SGD-VAP was initially separated into three fractions (F1–F3) on a DA201-C macroporous adsorption resin column by gradient ethanol elution (Fig. 2A). As shown in Fig. 2B, fraction F2 possessed the strongest NO inhibitory activity among the three fractions. At 500 μg/mL, fraction F2 inhibited LPS-induced NO production from RAW 264.7 macrophages by 26.4%. Thus, fraction F2 was subjected to Sephadex G-15 gel filtration column chromatography, and the active peaks were eluted using distilled water. The peptides in fraction F2 were further separated into four fractions (G1–G4) (Fig. 2C). Fraction G2 (500 μg/mL) inhibited LPS-induced NO production from RAW 264.7 macrophages by 42.2%, which was the strongest among the four fractions (Fig. 2D). Fraction G2 was further purified by RP-HPLC on a C_{18} column and separated into 10 additional fractions (Fig. 2E). Fractions eluted with higher concentrations of acetonitrile probably contained more hydrophobic compounds. Among these 10 fractions, H3 showed the strongest NO inhibitory activity. Fraction H3 (500 μg/mL) inhibited LPS-induced NO production from RAW 264.7 macrophages by 51.6%, which was about 22.3% higher than that of Fraction G2 from Sephadex G-15 separation.

3.4. *Peptide identification by LC-MS/MS analysis*

Fraction H3 with relatively high NO inhibitory activity as indicated above, was subject to LC-MS/MS analysis (Fig. 3). Using LC-MS, Fraction H3 was separated into six main peaks (Fig. 3A), which were then identified as nine main components (P1–P9). The MS/MS spectra are shown in Fig. 3B–3J, from which the amino acid sequences of peptides from Fraction H3 were identified. Various classical types of fragments (a-ion, b-ion, c-ion, x-ion, y-ion, and z-ion) were detected in the MS/MS spectra. Each ion has a weight depending on its components, according to the following criteria: a-ions, molecular mass of amino acid residue minus 27; b-ions, plus 1; c-ions, plus 18; x-ions, plus 45; y-ions, plus 19; and z-ions, plus 3 [18]. After the manual analysis, eight peptides (AHG, AHWK, VH, L/IAN, L/IA, AY, AL/I and TL/I) and Tyr from Fraction H3 were achieved, and none of them were more than four amino acid residues. Leu/Ile peptide isoforms presented nearly identical MS/MS profiles. In order to verify the amino sequences of P4, P6, P8 and P9, their retention times were compared with the corresponding Leu/Ile peptide isoforms by HPLC analysis. The retention times of P4 (1.522 minutes), P6 (2.268 minutes), P8 (3.819 minutes) and P9 (4.068 minutes) were comparable to those of LAN (1.501 minutes), IA (2.302 minutes), AL (3.825 minutes) and TL (3.986 minutes), respectively (data not shown). Thus, P4, P6, P8 and P9 were finally identified as LAN, IA, AL and TL. The content of each peptide in Fraction H3 was quantified based on calibration curves made from extracted ion chromatogram peak areas. The content of AHG, AHWK, VH, Y, LAN, IA, AY, AL and TL was 1.0%, 1.0%, 2.1%, 13.9%, 2.6%, 6.3%, 4.6%, 16.0% and 13.9%, respectively.

Several low-MW peptides were proved to have anti-inflammatory activities, such as VPY from soybean [19], PAY from salmon pectoral fin byproduct [1], LDAVNR and MMLDF from *Spirulina maxima* [3], and dipeptides LE, MW, MY and puroGlu-Leu [20,21]. High-MW peptides (e.g., lunasin) were also found to have potent anti-inflammatory activities [1]. Moreover, all of the identified peptides in our study were submitted for search in the BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep/) databases, which contain a list of biologically active and validated peptide sequences, to find out if any already established bioactive peptide was to be found. IA and AY have been reported to possess angiotensin-I-converting enzyme inhibitory activity. VH, IA, AY, AL and TL have been found to be dipeptidyl peptidase IV inhibitors, which are known to exert antidiabetic, cardioprotective and anti-inflammatory activities [22]. Thus, the anti-inflammatory activities of the eight identified peptides and Tyr from Fraction H3 in LPS-induced RAW 264.7 macrophages were further investigated.

3.5. *Anti-inflammatory activity of synthesized peptides*

Fig. 4 shows the cytotoxicity and anti-inflammatory activities of the eight synthesized peptides (AHG, AHWK, VH, LAN, IA, AY, AL and TL), Tyr, and peptides mixture (PepMix). PepMix was reconstituted with these eight peptides and Tyr according to their content ratio in Fraction H3. Fig. 4A illustrates that most of the samples showed no cytotoxic effect on RAW 264.7 macrophages at concentrations below 500 μg/mL. Treatment with AHWK (500 μg/mL), AY (500 μg/mL), and PepMix (100 μg/mL) led to a significant increase in cell viability. Although significant reduction in cell viability was observed in the cells treated with VH, LAN, TL and IA at 500 μg/mL, the viability remained > 88.0%. Thus, all samples were tested for their NO inhibitory activities at 50–500 μg/mL.

As shown in Fig. 4B, the inhibitory activity of AL against LPS-induced NO production in RAW 264.7 macrophages became significant at 100 μg/mL, while the inhibitory activities of VH, LAN, Y and IA became significant at 200 μg/mL. VH, LAN, Y, AL and IA showed the strongest NO inhibitory activities at 200 μg/mL, resulting in 15.5%, 13.0%, 12.1%, 16.0% and 11.2% inhibition of LPS-induced NO production, respectively. When the concentration increased to 500 μg/mL, VH, LAN, AL and IA showed no significant NO inhibitory activities. The dose–effect relationship of VH, LAN, Y, AL and IA followed a U-shaped curve, with inhibition most significant in the mid-range of tested concentrations and less inhibition at higher or lower concentrations. Similarly, marine oligopeptide preparation [23] and SGD from aqueous extract of velvet antler [7] have also been found to show bidirectional regulatory effects. LPS-induced RAW 264.7 macrophages incubated with PepMix (50–500 μg/mL) showed a dose-dependent inhibitory activity ($r = -0.957$, $p < 0.05$) against the NO production by macrophages. At 500 μg/mL, PepMix inhibited LPS-induced NO production in RAW 264.7 macrophages by 33.2% ($p < 0.01$). PepMix showed stronger NO inhibitory activity than any individual peptides at the same concentrations. The results suggested that the peptides in PepMix might demonstrate synergistic effects on NO inhibitory activity. Peptides have been reported to exert synergistic effect in many studies. Yan et al reported that RPNYTDA and TRTGDPFF showed synergistic antioxidant activity compared to each of them used alone [24]. Girgih et al and Raghavan and Kristinsson found that peptides acted synergistically to be more active when present together in the protein hydrolysate [25,26]. Fractionation of the peptides resulted in loss of this synergistic effect.

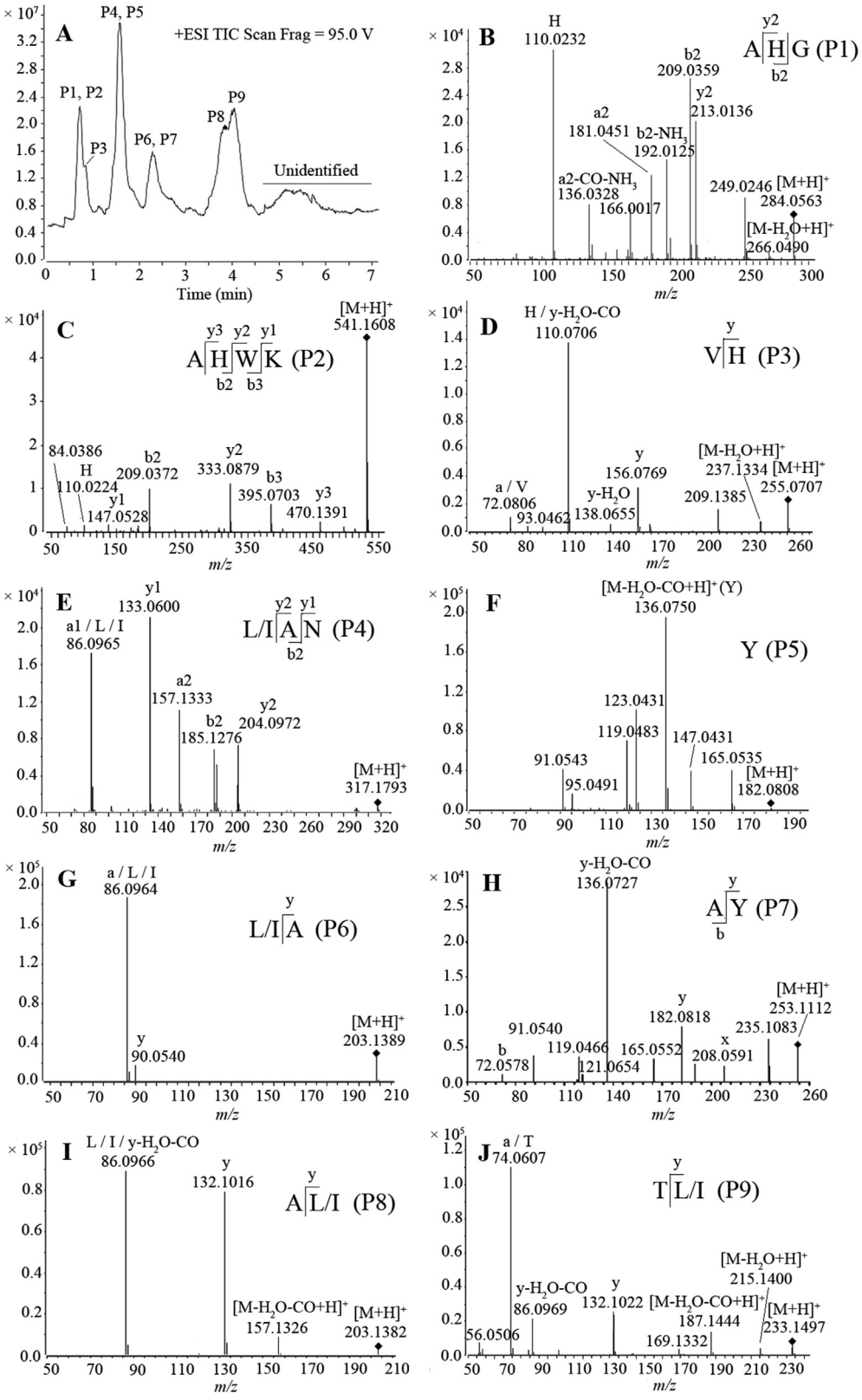

Fig. 3 – Identification of anti-inflammatory peptides from Fraction H3 by liquid chromatography tandem mass spectrometry. (A) Total ion current chromatogram of Fraction H3; (B) P1 (*m/z* 284.0563, RT 0.732 min); (C) P2 (*m/z* 541.1608, RT 0.741 min); (D) P3 (*m/z* 255.0707, RT 0.855 min); (E) P4 (*m/z* 317.1793, RT 1.522 min); (F) P5 (*m/z* 182.0808, RT 1.600 min); (G) P6 (*m/z* 203.1389, RT 2.268 min); (H) P7 (*m/z* 253.1112, RT 2.345 min); (I) P8 (*m/z* 203.1382, RT 3.819 min); (J) P9 (*m/z* 233.1497, RT 4.068 min). Following sequence interpretation, P1–P9 were identified as AHG, AHWK, VH, L/IAN, Y, L/IA, AY, AL/I and TL/I, respectively. RT, retention time.

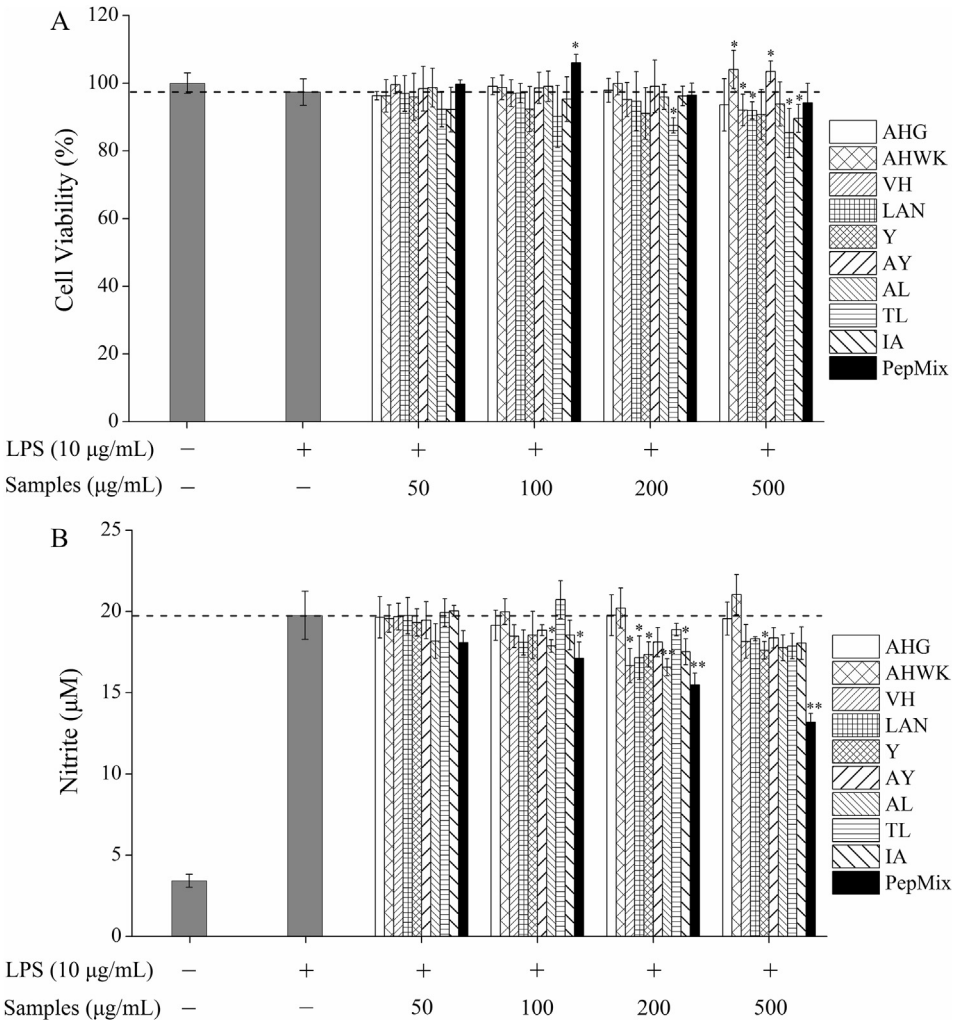

Fig. 4 – Effects of identified peptides from Fraction H3 and peptides mixture (PepMix) on the cell viability (A) and NO production (B) in LPS-induced RAW 264.7 macrophages. *$p < 0.05$, **$p < 0.01$ are significantly different compared with the group treated with only LPS. PepMix was reconstituted with 1.0% AHG, 1.0% AHWK, 2.1% VH, 13.9% Y, 2.6% LAN, 6.3% IA, 4.6% AY, 16.0% AL and 13.9% TL according to their content ratio in Fraction H3. LPS = lipopolysaccharide.

However, the inhibitory activity of PepMix was still 35.7% weaker than that of Fraction H3 from RP-HPLC separation. It was indicated that some other unidentified components in Fraction H3 might have NO inhibitory activity.

The structure–activity relationship of bioactive peptides (especially antioxidant and angiotensin-I-converting enzyme-inhibitory peptides) has been extensively studied. The activities of bioactive peptides depend on their structures, such as amino acid composition, type of amino acid in C and N termini, hydrophobicity/hydrophilicity, charge distribution, length of peptide chain, and spatial structure [27]. Jacquot et al studied the proliferative effects of 11 immunomodulatory peptides (MW 554.31–1728.97 Da) and found that longer hydrophobic peptides bearing 2–3 positive charges have greater potential to stimulate the proliferation of murine splenocytes [28]. Vogel et al suggest that the anti-inflammatory and immunomodulatory properties are more related to a positively charged region of the peptide, which may act as a chemokine [15]. Although some anti-inflammatory peptides have been prepared and identified, the effects of structural groups on their anti-inflammatory activities are not well understood. Further exploring the structure–activity relationship and revealing the mechanism of anti-inflammatory peptides will provide an important theoretical basis for the development of functional foods or nutraceuticals.

4. Conclusions

Our study demonstrated that SGD-VAP (MW < 3 kDa) from pepsin–pancreatin hydrolysate (SGD) of VAP has the potential to limit LPS-induced inflammation *in vitro*. The anti-inflammatory peptides were purified from SGD-VAP by consecutive chromatographic methods. Four anti-inflammatory peptides VH, LAN, AL and IA were identified from SGD-VAP using MS/MS. Each of these peptides

demonstrated a U-shaped dose–effect relationship. In addition, the anti-inflammatory activity of PepMix might be related to the synergistic effect of different peptides present in the mixture. To unravel the potential anti-inflammatory activity of SGD-VAP, other unidentified active components should be clarified. More research is needed to confirm the potential health benefits of SGD-VAP or its isolated peptides under inflammatory stress *in vivo*.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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