This study sought to assess the cholesterol-lowering activity of peptides obtained from milk casein hydrolyzed with neutrase. The bioactive peptides were separated using a Sephadex G-10 chromatographic column after ultrafiltration using a 1-kDa molecular mass cutoff membrane. Via ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry, we determined that peptides Thr-Asp-Val-Glu-Asn [TDVEN; β-casein f(143–147)], Leu-Gln-Pro-Glu [LQPE; β-casein f(103–106)], Val-Ala-Pro-Phe-Pro-Glu [VAPFPE; αS1-casein f(40–45)], and Val-Leu-Pro-Val-Pro-Gln [VLPVPQ β-casein f(185–190)] reduced micellar cholesterol solubility. After Caco-2 cells were treated with LQPE, VLPVPQ, and VAPFPE, the Niemann-Pick C1-Like 1 (NPC1L1) protein levels decreased by (means ± SEM) 19.33 ± 2.47%, 52.1 ± 3.77%, and 23.09 ± 8.52%, respectively, compared with the control group. Treatment with each peptide induced significant upregulation of ATP binding cassette subfamily G member 8 antibody (ABCG8) mRNA expression by 398.1 ± 23.27%, 86.4 ± 27.07%, and 92.8 ± 8.49%, respectively. We found that VLPVPQ and LQPE significantly upregulated ATP-binding cassette transporter A1 (ABCA1) transcription by 203.9 ± 8.44% and 220.8 ± 36.42% respectively, whereas VLPVPQ significantly decreased mRNA expression of acetyl-CoA-acetyltransferase 2 (ACAT2) and microsomal triacylglycerols (MTP). The cholesterol-lowering action of milk-derived peptides may be induced by suppression of micellar cholesterol solubility and affects the expression of cholesterol absorption-related proteins and enzymes in intestinal epithelial cells. This research discovers new milk-derived peptides with decreasing cholesterol micellar solubility and provides a theoretical basis of in vitro cholesterol-lowering effects of peptides.

**Key words:** bioactive peptides, milk casein, cholesterol-lowering solubility of micellar, NPC1L1, Caco-2 cells

**INTRODUCTION**

Hyperlipidemia, arteriosclerosis, and other cardiovascular diseases caused by excessive cholesterol levels have major and widespread negative effects on human health (Musso et al., 2010; Gaudet et al., 2017). The homeostatic balance of cholesterol favorably influences blood fat levels (Xiao et al., 2016). Diet and lifestyle changes and pharmacological treatment have been proposed to deal with hypercholesterolemia in humans (Zhao et al., 2012). Functional foods may be able to play a preventive role for moderate hypercholesterolemia (Chen et al., 2011; Braithwaite et al., 2014). Many studies have reported hypolipidemic actions of plant peptides, such as soybean-derived peptide LPYPR (Zhang et al., 2013), WGAPSL (Zhang et al., 2013), and VAWWMY (Nagaoka et al., 2010). Milk-derived bioactive peptides can be regarded as potential functional food ingredients (Nagaoka et al., 2001; Korhonen, 2009; Beermann and Hartung, 2013; Martínez-Augustin et al., 2014; Suwal et al., 2019). Lactostatin (IIAEK) and β-lactotensin (HIRL) derived from milk lactoglobulin were identified as hypocholesterolemic peptides (Nagaoka et al., 2001; Yamauchi et al., 2003).

Mechanisms of hypolipidemic peptides on cholesterol-lowering activity include bile acid binding (Nagaoka et al., 2001; El-Tantawy and Temraz, 2019), inhibition of micellar solubility of cholesterol in vitro and cholesterol absorption in vivo (Nagaoka, 2019), inhibition of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoAR) activity (Boachie et al., 2018), and targeting of the expression of proteins involved in cholesterol metabolism (Duranti et al., 2004; Moriyama et al., 2004). Cholesterol homeostasis is maintained by a complex mechanism of sterol absorption, anabolism, catabolism,
and excretion (Nagaoka, 2018). Cholesterol, either from diet or from bile, is transported into enterocytes via intestinal Niemann-Pick C1-like 1 (NPC1L1). Intestinal acyl coenzyme A:cholesterol acyltransferase 2 (ACAT2) esterifies cholesterol to form cholesteryl ester, which is packed with microsomal triacylglycerols (MTP) into chylomicrons and transferred into blood through the lymphatic system. Adenosine triphosphate (ATP)-binding cassette transporters combined with transporter G superfamily member 8 antibodies (ABCG8) then return unabsorbed cholesterol to the lumen of the intestine for excretion (Chen et al., 2011). Caco-2 cell monolayers have routinely been used as an in vitro model to mimic substance absorption by the human intestinal epithelium (Picariello et al., 2013): NPC1L1 (Alrefai et al., 2007), ABCG8 (Ito et al., 2012), SREBP-2 (Field et al., 2001), ACAT2 (Song et al., 2006), and MTP (Mathur et al., 1997) are all expressed in the Caco-2 cell model. Nagaoka et al. (1997) reported that the suppression of cholesterol intestinal epithelia absorption by Caco-2 cell studies is part of the mechanism for the hypocholesterolemic action. Yang et al. (2018) explored the effects of 6 fatty acids on cholesterol uptake in human Caco-2 cells (enterocytes), and on the mRNA expression levels of the previously listed genes.

The purpose of this study was to purify the potential cholesterol-lowering peptides derived from hydrolyzed casein protein and characterize their amino acid sequences. The effects of Leu-Gln-Pro-Glu (LQPE), Val-Ala-Pro-Phe-Pro-Glu (VAPFPE), and Val-Leu-Pro-Val-Pro-Gln (VLPVPQ) on cholesterol absorption were analyzed using Caco-2 cell experiments, and expression of key genes that influence cholesterol absorption was studied. This study may promote the use of hydrolyzed peptides from casein protein as bioactive ingredients in functional foods.

MATERIALS AND METHODS

Chemicals and Reagents

Cells from the Caco-2 human colorectal adenocarcinoma cell line were purchased from the Institute of Biochemistry and Cell Biology (CAS; Shanghai, China). Fetal bovine serum and coverslips were purchased from Solarbio Science and Technology (Beijing, China). Cell culture medium, Dulbecco’s modified Eagle’s medium (DMEM), and trypsin were purchased from Hyclone Laboratories (Logan, UT). Hanks’ balanced salt solution (HBSS) and PBS were purchased from Gibco Invitrogen (Burlington, Canada). Water-soluble cholesterol was purchased from Sigma-Aldrich (Shanghai, China), and LQPE, VAPFPE, and VLPVPQ identified by ultra-high-performance liquid chromatography (UPLC)-MS were synthesized by Shanghai Bootech BioScience and Technology (Shanghai, China) with over 95% purity based on reverse-phase (RP) HPLC-MS/MS.

Production and Ultrafiltration of Casein Hydrolysates

An aqueous casein solution (5% wt/vol, protein basis) was adjusted to pH 7.0. The casein protein solution was hydrolyzed with neutrase (50 U/mg) at 48°C for 3.5 h. Hydrolysis pH was maintained at 7.0. After 3.5 h the reaction was stopped by heating the solution to 90°C for 20 min to inactivate the enzymes. After centrifugation at 7,000 × g for 20 min, supernatant was collected. Fractionation was carried out using hollow fiber ultrafiltration membrane (Shanghai Mosu Science Equipment Co. Ltd., Shanghai, China) at a flow rate of 7.5 mL/min and a membrane pressure of 0.25 MPa. The fraction below 1 kDa was lyophilized and stored at −20°C for subsequent purification.

Purification and Identification of Casein Peptides

The hydrolysate was applied to a Sephadex G-10 column (1.0 × 40 cm, Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with distilled water. Peptides were eluted with distilled water at a flow rate of 3.5 mL/min. Elution curves were obtained by measuring absorbance at 220 nm. The fractions were collected at 2-min intervals. The experiments were conducted at room temperature using MA99-2A Protein Chromatography Equipment (Shanghai Huxi Analysis Instrument Factory, Shanghai, China). The fractions were pooled and concentrated by freeze-drying. The protein concentration and inhibition activity of micellar solubility of cholesterol were then analyzed. The fraction with the highest activity was applied to UPLC-MS.

Inhibition Activity of Micellar Solubility of Cholesterol

Micellar cholesterol solubility was measured after adding 3 mg/mL of each peptide fraction to a suspension of intestinal micelles prepared in vitro. Cholesterol micelles were prepared according to the method of Zhang et al. (2012) with some modifications. Sodium phosphate buffer at pH 7.4 containing 6.6 mmol/L taurocholate and 132 mmol/L NaCl salts was mixed with 2 mmol/L cholesterol and 1 mmol/L linoleic acid. To form the micelles, the suspension was sonicated twice for 1 min using a Misonix 3000 sonicator (Misonix, ...
New York, NY) at 95% energy output (114 W). The supernatant fractions were filtered through a 0.22-μm Millex-GP filter (Millipore, Bedford, MA). The supernatant fractions (25 μL) were collected, and cholesterol concentrations were determined using an Amplex Red Cholesterol Assay Kit (Thermo Fisher Scientific, Waltham, MA) by measuring the absorbance at 510 nm. Cholesterol concentrations were determined from standard curves using cholesterol calibration standards. Cholesterol concentrations and inhibition ability were calculated using the following equations:

$$\text{Concentration of cholesterol (mmol/L)} = \frac{(\text{OD}_s/\text{OD}_c) \times \text{[concentration of calibrator (mmol/L)]}}{[1]}$$

where $\text{OD}_c$ is the calibrator’s absorbance measured by optical density and $\text{OD}_s$ is the sample’s absorbance; and

$$\text{Inhibition ability (\%)} = \left[\frac{C_o - C_s}{C_o}\right] \times 100\%$$

where $C_o$ is the cholesterol concentration of the original micelles and $C_s$ is the cholesterol concentration after the peptide fraction was added (Zhang et al., 2012; Marques et al., 2015a).

Identification of Amino Acid Sequences of Casein Peptides

The molecular mass of peptides purified by gel filtration with the highest inhibition micellar cholesterol solubility activity was determined using a Waters MALDI SYNAPT Q-TOF mass spectrometer (Milford, MA). The sequences were determined by secondary ion mass spectrometry performed with the same system. Samples were dissolved to a concentration of approximately 10 ppm in methanol and analyzed by infusion of the sample (8 μL) through the electrospray interface. The UPLC analysis was performed at 45°C using an Acquity UPLC BEH130 C18 column (2.1 × 150 mm, 1.7 μm; Waters).

Elution was performed with 100% acetonitrile (solvent A) and 0.1% methanoic acid in H2O (solvent B). Peptides were eluted with a linear gradient of solvent A going from 2 to 40% in 10 min, 40 to 100% in the next 5 min, remaining at 100% for 2 min, and then going to 2% in the next 5 min, and solvent B going from 98 to 60% in 10 min and 60 to 0% in the next 5 min, remaining there for 2 min, and then to 98% in the next 5 min. The flow rate was 0.3 mL/min. The variable wavelength absorbance detector was set at 204 nm, and an automatic injector was used. Mass data were obtained using an electrospray ionization in positive-ion mode and was calibrated using the lock-mass function with leucine encephalin. Conditions of MS were as follows: ion source and polarity were electrospray ionization; capillary voltages 3,500 V; sampling cone voltages 30 V; source temperature 100°C; desolvation temperature 300°C; desolvation gas flow 500 L/h; cone gas flow 50 L/h; and detecting voltage 1,600 to 1,700 V. Nitrogen was used as the sheath and auxiliary gas at pressures of 35 and 3 arbitrary units, respectively. Argon was used as the target gas (3 mTorr) for the collision. Collision energy was set at 6/35 V. Mass spectra were acquired over 100 to 1,500 m/z.

Data were collected and analyzed with Waters MassLynx v. 4.1 and BiopharmaLynx 1.3.3 software. Peptide identification involved the search for the masses in the NCBI database of bovine casein proteins and matching the MS/MS spectrum with the sequences selected by mass. We used GPMAW 8.0 software (Lighthouse Data, Odense, Denmark) and Mascot Server (Matrix Science Inc., Boston, MA) to identify peptide sequences.

Cell Culture Conditions

The Caco-2 cells were cultured in high-glucose DMEM with stable l-glutamine supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete growth medium) in a fully humidified atmosphere with 5% CO2 at 37°C. Cell culture medium was replaced every other day, and cells were passaged 1:2 after the confluent rate reached 70 to 80%.

MTT Assay

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The Caco-2 cells were seeded in a 96-well plate with DMEM at 7,500 cells/well and incubated for 24 h. After the old medium was discarded, the cells were incubated in the complete growth medium containing different concentrations (0.05, 0.1, 0.2, and 0.4 mg/mL) of 4 synthetic peptides (TDVEN,VLPVPQ, LQPE, and VAPFPE) or DMEM for 24 h. Then the treatment solvent was aspirated, and 100 μL/well of filtered MTT solution was added. After 2 h of incubation at 37°C in a 5% CO2 atmosphere, the 0.5 mg/mL solution was aspirated, and 100 μL/well of lysis buffer (8 mmol/L HCl + 0.5% NP-40 in dimethyl sulfoxide) was added. After 5 min of slow shaking, the absorbance at 570 nm was read on a Tecan PRO M200 microplate reader (Mannedorf, Switzerland). Cell viability (%) was expressed as OD ratio of the treatment to the control.
Effects of LQPE, VAPFPE, and VLPVPQ on Cholesterol Absorption in Caco-2 Cells in Vitro

Caco-2 cells (2 × 10^5 cells/cm² at passage 36) were seeded in Transwell permeable membrane (1.12-cm diameter, 0.4-μm pore size; Corning Life Sciences, Corning, NY). The DMEM culture medium (0.5 mL) containing the cells was added to the apical-side (AP) well and 1.5 mL of DMEM culture was added to the basolateral-side (BL) well. After inoculation, the fluid was changed every other day. The DMEM was changed every second day for 1 week and then changed every day until a monolayer formed. The integrity of the cell layer was tested by measuring the transepithelial electrical resistance (TEER; Guo et al., 2018) using a Millicell Voltohmometer (Millipore, Bedford, MA). Cell permeability measurements were conducted in triplicate. Only Caco-2 cell monolayers with TEER values greater than 500 Ω·cm² were used for the transport studies (Srinivasan et al., 2015). Alkaline phosphatase, which is the signature enzyme of the brush-border cells of the intestinal epithelium when cells begin to differentiate, was measured using a commercial AKP assay kit (Nanjing J inception Bioengineering Institute, Nanjing, China) in accordance with the manufacturer’s instructions.

The prepared Caco-2 cell monolayer were washed with PBS twice and nurtured with PBS in 5% CO₂ at 37°C for 30 min. The PBS was replaced with 0.5 mL of LQPE, VAPFPE and VLPVPQ (1 mmol/L) on the AP side and 1.5 mL fresh PBS on the BL side. The PBS was removed from the AP side and replaced with 0.5 mL of PBS control. All the plates were incubated at 37°C in 5% CO₂. After incubation for another 2 h, the peptide transportation in the BL side of each well were determined by UPLC-MS.

The permeability coefficients (P_app, cm/s) were used to evaluate permeability of peptides through Caco-2 cells, according to Eq. [3] (Sienkiewicz-Szlapka et al., 2009):

\[ P_{app} = \left( dQ \times dt^{-1} \right) \times \left( A \times C_0 \right)^{-1}, \]  

where \( dQ \times dt^{-1} \) is the function of the amount and time of the peptides present on the BL side (μmol/s), and the amounts of peptides were calculated using a peptide standard curve; and \( A \) (cm²) and \( C_0 \) (μmol/L) are the surface area of the monolayer and the initial peptide concentration on the AP side, respectively.

Water-soluble cholesterol was dissolved in the complete medium at 4 mg/mL. We dissolved LQPE, VLPVPQ, and VAPFPE in sterile PBS with the concentration adjusted to 0.05, 0.1, 0.2, and 0.4 mg/mL, and then sterilized them using a 0.22-μm microwell filter. The culture medium in the Transwell plate was removed and washed with 1 mL of PBS solution 3 times. We added 1 mL of cholesterol solution to the blank control group, and 900 μL of cholesterol solution and 100 μL of LQPE, VAPFPE, or VLPVPQ solution to the experimental group at different concentrations, respectively, on the AP side. We added DMEM culture medium (1.5 mL) to the BL side in all groups. After incubating at 37°C in 5% CO₂ for 24 h, the well solution was discarded and washed with 1 mL of PBS solution 3 times. The Caco-2 cells were digested by trypsin and centrifuged at 1,000 × g for 10 min at 4°C to collect cell pellets. Caco-2 cell pellets were washed twice with 0.1 mol/L phosphate buffer (pH 7.2) and centrifuged at 1,000 × g for 10 min at 4°C. The cells were resuspended in 0.3 mL of 0.1 mol/L phosphate buffer (pH 7.2) and sonicated in an ice-water bath (300 W, 5 s each at 30-s intervals for 5 repetitions). According to a Total Cholesterol kit (Nanjing J inception Bioengineering Institute, Nanjing, China), absorbance was measured at 510 nm, and the cholesterol content in the cells was calculated according to Equation [1] (Zhang et al., 2012).

Casein Peptides Regulating Cholesterol Absorption in Caco-2 cell model

The Caco-2 cell monolayers cultured in Transwell plate were rinsed with HBSS twice and incubated with HBSS for 30 min at 37°C in 5% CO₂ when the cell monolayer models were formed. Micellar cholesterol was prepared as described in the section “Inhibition Micellar Cholesterol Solubility Activity.” The HBSS was removed and replaced with micellar cholesterol, micellar cholesterol + 0.1 mg/mL LQPE, micellar cholesterol + 0.1 mg/mL VLPVPQ, or micellar cholesterol + 0.05 mg/mL VAPFPE on the AP side (amounts of peptides were according to MTT results) and 1.5 mL of fresh complete medium on the BL side. All of the plates were incubated at 37°C in 5% CO₂. After incubation for another 24 h, the cells were lysed to extract total RNA, nuclear protein, and cytoplasm protein to assay cholesterol absorption gene and protein expression.

RNA Extraction and qPCR Assay

Total RNA was extracted from Caco-2 cells using the RNeasy Mini kit following the manufacturer’s suggested protocol (Qiagen, Germantown, MD). RNA concentration was measured by spectrophotometry using a NanoDrop (NanoDrop Technologies, Wilmington, DE). We evaluated RNA integrity using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), and only
Table 1. Primer sequences used for real-time quantitative (RT-q) PCR analysis

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequences (5’-3’; F = forward, R = reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>F: 5′-CACGATGGAGGCGGGCCGACCTCATC-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-TAAAGACTACTTATGGCCACACGTT-3′</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>F: 5′-GCTCTCTGGGAGGGGCGGCT-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-TACCTCACTGCTTATGGCCT-3′</td>
</tr>
<tr>
<td>ABCG8</td>
<td>F: 5′-GCTCTCTGGGAGGGGCGGCT-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-TACCTCACTGCTTATGGCCT-3′</td>
</tr>
<tr>
<td>ACTB</td>
<td>F: 5′-GCCGACTCATAGTTCCCTT-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-ACTGGCTTGTCTACAGGATTTCT-3′</td>
</tr>
<tr>
<td>MTP</td>
<td>F: 5′-GGGCTCTTTGAACCCTTGAA-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GAGACCGACATGCTGTGAATT-3′</td>
</tr>
<tr>
<td>ABCA1</td>
<td>F: 5′-TGTGCTTTGGGTGTTGG-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-GAGACCGACATGCTGTGAATT-3′</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>F: 5′-ACCGTTTCTTGGTGATGG-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-AGTGCTCCGCTCTAAAGAGATGTTCC-3′</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>F: 5′-TTGGATGATGCCTAAGGCTCAGAA-3′</td>
</tr>
<tr>
<td></td>
<td>R: 5′-CCCACCTGCGACAGGGAAGT-3′</td>
</tr>
</tbody>
</table>

samples with an RNA integrity number ≥8 were used for the mRNA experiments. The cDNA was produced from 1 μg of total RNA by Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), and mRNA expression was measured by TaqMan quantitative PCR (qPCR) assay. We performed PCR amplification of cDNA as shown in Table 1.

The qPCR assays were carried out in 96-well plates using a 7500 Fast Real-Time (RT)-PCR system (Applied Biosystems, Waltham, MA). Amplification parameters were used for PCR as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of amplification at 95°C for 15 s and 60°C for 1 min. Eight reference genes were tested: ACTB, NPC1L1, ABCG8, ACT2, MTP, ABCA1, SREBP1, and SREBP2. Normalized mRNA expressions were calculated using the comparative cycle threshold (CT) method based on the 2−ΔΔCT formula (Livak and Schmittgen, 2001). Data are presented as the average of a minimum of 5 replicates.

Western Blotting of NPC1L1

For preparation of whole-cell extracts, cells were harvested using a cell scraper and lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease-inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany). The total cell lysates were then centrifuged at 10,000 × g for 20 min at 4°C to remove the insoluble materials. The protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL). Cytoplasmic and nuclear extracts were prepared with nuclear and cytoplasmic extraction reagents from Beyotime (Nanjing, Jiangsu, China) according to the manufacturer’s instructions. Forty micrograms of each protein extract were separated using 12% PAGE and electro-transferred to polyvinylidene difluoride membranes at 20 V for 60 min. The membranes were blocked with 5% skim milk in tris-buffered saline containing 0.1% Tween-20 (TBST). After washing with TBST, the membranes were incubated with target-protein-specific antibodies for 14 h at 4°C, followed by incubation with horseradish peroxidase–conjugated secondary antibodies for 1 h. Peroxidase activity was visualized using an enhanced chemiluminescence kit (Bio-Rad, Hercules, CA). Imaging was performed via G: BOX chemiXR5 (Syngene, Frederick, MD). Grayscale analysis of the results was performed using Gel-Pro32 software (Media Cybernetics, Rockville, MD). For equal loading and normalization purposes, β-actin was used as an internal control (Lammi et al., 2015).

Calculations and Statistics

Statistical analyses were carried out via one-way ANOVA (SPSS Statistics 20; IBM Corp., Armonk, NY) followed by Dunnett’s test. Results were expressed as mean ± SD. Differences were considered significant at P < 0.05 and extremely significant at P < 0.01.

RESULTS

Purification and Identification of Peptides from the Casein Protein Hydrolysate

After 3.5 h of hydrolysis with neutrase, casein fractions were fractionated through ultrafiltration membranes with 1 kDa cutoff values. The fractions with molecular weight <1 kDa were subjected to size-exclusion chromatography on a Sephadex G-10 column. Two subfractions (A and B) were obtained (Figure 1A). Fraction A displayed higher micellar cholesterol solubility activity than fraction B, both at concentrations of 100 μg/mL. The cholesterol micelle solubility inhibition rate of fraction A was 24.20 ± 0.24%, and that of fraction B was 4.30 ± 0.16% (Table 2). Fraction A was selected for further UPLC-MS analysis to analyze the amino acid sequences of the peptides. The chromatogram results for HBSS and fraction A are shown in Figure 1B and 1C. As analyzed with a protein and peptide editor program, 4 possible amino acid sequences for the casein-derived peptides were analyzed from fraction A (Table 3): TDVENC [β-casein f(143–147), Figure 2A], LQPE [β-casein f(103–106), Figure 2B], VLPVPQ [β-casein f(185–190), Figure 2C], and VAPFPE [αs1-casein f(40–45), Figure 2D].
Effects of TDVEN, LQPE, VAPFPE, and VLPVPQ Peptides on Micellar Cholesterol Solubility in Vitro

The TDVEN, LQPE, VAPFPE, and VLPVPQ peptides were synthesized at 95% purity. Cholesterol micelles with 4 peptides at concentrations of 0.2 mg/mL were configured. After TDVEN, LQPE, VAPFPE, and VLPVPQ were incubated with cholesterol micelles for 24 h, ultracentrifugation was used to determine the cholesterol content in the supernatant. As shown

Figure 1. (A) Elution profile of fractions A and B separated by gel filtration on Sephadex G-10 (Amersham Pharmacia Biotech, Uppsala, Sweden). A220 nm = absorbance at 220 nm. (B) Chromatogram of Hanks’ balanced salt solution by ultra-high-performance liquid chromatography (UPLC)-MS. (C) Chromatogram of sample A by UPLC-MS. TDVEN, LQPE, VLPVPQ, VAPFPE = milk-derived peptides.
in Table 2, the peptides had a certain inhibitory effect on the dissolution of cholesterol. The inhibition rate of cholesterol dissolution in cholesterol micelles by VLPVPQ reached 52.80%. The inhibitory effects of LQPE and TDVEN were obvious at 46.30% and 48.37%, respectively. The inhibition rate of VAPFPE was lower than those of the others, at 32.11%.

**Peptides Affect Cholesterol Absorption in Caco-2 Cells**

The integrity of the Caco-2 monolayer was checked consistently via TEER measurements (Figure 3B). The $P_{app}$ of LQPE was $2.75 \times 10^{-6}$ cm/s, VAPFPE was $1.73 \times 10^{-6}$ cm/s, and VLPVPQ was $6.68 \times 10^{-6}$ cm/s (Figure 3C). Figure 3D shows the effect of milk-derived peptides on the absorption of water-soluble cholesterol in Caco-2 cells. After 24 h of Caco-2 cell uptake experiments, the cholesterol concentration of the blank control group in the Caco-2 cells was 0.1136 mg/mL. At 0.05, 0.1, 0.2, and 0.4 mg/mL, LQPE and VLPVPQ significantly inhibited cholesterol uptake in the Caco-2 cells. At 0.4 mg/mL LQPE had the most obvious effect on decreasing cholesterol absorption, with an inhibition rate of 87.66%.

The VAPFPE peptide also inhibited the absorption of cholesterol by Caco-2 cells, and the absorbance was significantly lower than that of the blank control group ($P < 0.01$). The inhibitory effect of the 3 peptides was significantly different. At 0.05 mg/mL LQPE and VLPVPQ, the inhibition rate on Caco-2 cell monolayer cholesterol absorption was more than 50%, significantly higher than that of VAPFPE, which was 13.65% ($P < 0.01$). At 0.1 mg/mL, VLPVPQ inhibited cholesterol absorption in Caco-2 cells from monolayers significantly more than at 0.05 mg/mL ($P < 0.01$). The inhibitory effect of 0.2 and 0.4 mg/mL LQPE was not significantly different than that of 0.4 mg/mL VLPVPQ ($P > 0.05$). The results indicated dose-dependent effects of peptides concentration on the inhibitory effect on the cholesterol absorption of Caco-2 cells.

**Effects of LQPE, VAPFPE, and VLPVPQ on NPC1L1 Protein Expression**

To confirm the ability of LQPE, VAPFPE, and VLPVPQ to inhibit the catalytic activity of NPC1L1, an in vitro assay was performed using the purified casein peptides. Caco-2 cells were treated with 0.1 mg/mL LQPE, 0.1 mg/mL VLPVPQ, or 0.05 mg/mL VAPFPE with cholesterol micelles on the AP side. Each sample was investigated with immunoblotting experiments. As
Figure 4 shows, the LQPE, VAPFPE, and VLPVPQ all significantly reduced NPC1L1 protein expression in Caco-2 cells, by 19.33 ± 2.47% (P < 0.05), 23.09 ± 8.52% (P < 0.05), and 52.1 ± 3.77% (P < 0.01), respectively, at 0.1, 0.05, and 0.1 mg/mL, compared with the control.

Effects of LQPE, VAPFPE, and VLPVPQ on mRNA Expression of ABCG8, ACAT2, MTP, ABCA1, SREBP1, and SREBP2 Transporters in the Caco-2 Cell Line

According to the preliminary results, 3 peptides can be transported to the BL side of the Caco-2 cell transwell model. Caco-2 cells were treated with 0.1 mg/mL LQPE, 0.1 mg/mL VLPVPQ, 0.05 mg/mL VAPFPE, and cholesterol micelles on the AP side. Figure 5A shows that treatment with each peptide induced a significant upregulation of ABCG8 mRNA expression (P < 0.05). In particular, LQPE upregulated ABCG8 mRNA expression by 398.1 ± 23.27% (P < 0.001) at 0.1 mg/mL, VLPVPQ by 86.4 ± 27.07% (P < 0.05), and VAPFPE by 92.8 ± 8.49% (P < 0.05) compared with the control.

At 0.1 mg/mL, VLPVPQ significantly decreased mRNA expression of ACAT2 by 43.37 ± 5.47% (P < 0.05), whereas 0.1 mg/mL LQPE and 0.05 mg/mL VAPFPE had no significant effects on ACAT2 mRNA expression: 10.8 ± 18.39% (P > 0.05) and 14.4 ± 6.5% (P > 0.05), respectively, compared with the control (Figure 5B).

The mRNA expression of MTP was significantly inhibited by 0.1 mg/mL VLPVPQ (25.87 ± 9.29%, P < 0.05), whereas 0.1 mg/mL LQPE and 0.05 mg/mL VAPFPE had no significant effect on mRNA of MTP: 11.28 ± 1.52% (P > 0.05) and 17.97 ± 7.7% (P > 0.05), respectively, compared with the control (Figure 5C).

In the same experiments, the ABCA1, SREBP1, and SREBP2 mRNA expression level variations were measured via RT-PCR analysis. As shown in Figure 5D, 0.1 mg/mL VLPVPQ and LQPE significantly upregulated ABCA1 transcription by 203.9 ± 8.44% and 220.8 ± 36.42% (P < 0.01), respectively, whereas 0.05 mg/mL VAPFPE had no significant effect on the transcription of ABCA1 mRNA: 2.3 ± 5.32% (P > 0.05) compared with the control. No differences in the mRNA levels of SREBP1 or SREBP2 were detected among the 3 groups (Figure 5E–F).
DISCUSSION

The first objective of the present work is to prepare cholesterol-lowering peptides from casein protein. Milk casein was hydrolyzed with neutrase, purified using size-exclusion chromatography, and identified via UPLC-MS; TDVEN, LQPE, VLPVPQ, and VAPFPE were the first reported casein-derived peptides that have micellar cholesterol solubility inhibition activity.

The relationship between peptide properties and micellar cholesterol solubility inhibition activity is not yet clear. Table 2 shows the isoelectric point (pI) and GRAVY (grand average of hydropathicity) of identified casein-derived peptides that have micellar cholesterol solubility inhibition activity. The peptides TDVEN and LQPE are hydrophilic, whereas VLPVPQ and VAPFPE are hydrophobic. Hydrophilic peptides may bind with micellar hydrophilic bile salts and then reduce micellar cholesterol solubility (Lapphanichayakool et al., 2017). Conversely, hydrophobic peptides may interact with cholesterol, hindering cholesterol solubilization into the micelles (Marques et al., 2015a,b; Lapphanichayakool et al., 2017). The pI of TDVEN, LQPE, VLPVPQ, and VAPFPE are 3.01, 3.28, 7.00, and 3.28, respectively. However, Lapphanichayakool et al. (2017) reported that pI of bile acid–binding activity peptides are in the range of 4 to 12. The effects of pI on micellar cholesterol solubility inhibition activity are not clear and need further research.

In addition, we performed a preliminary study on the water-soluble cholesterol absorption of LQPE, VLPVPQ, and VAPFPE in Caco-2 cell monolayer. The P_{app} value can predict in vivo absorption of substances: P_{app} values <10^{-6} cm/s indicate malabsorption (0−20%); P_{app} values between 10^{-6} and 10^{-5} cm/s indicate moderate absorption of a substance (20−70%); and P_{app} values >10^{-5} cm/s indicate that a substance is easy to absorb (70−100%; Vieira et al., 2016). Peptides LQPE, VLPVPQ, and VAPFPE were bioaccessible and showed water-soluble cholesterol-lowering absorption activity, based on the inhibition of Caco-2 cell cholesterol absorption experiments (Figure 3D). We hypothesize that, in addition to lowering cholesterol micelle concentration, another explanation for the ability of peptides to lower cholesterol may be the effects of the expression of proteins and enzymes that regulate cholesterol absorption in intestinal epithelial cells.

A polyhedral transmembrane protein that is abundant on the AP surface of the small intestinal infiltrate (Alrefai et al., 2007), NPC1L1 mediates the passage of cholesterol through the brush border membrane of the intestinal epithelium and is critical for cholesterol absorption (Altmann et al., 2004). The function of intestinal NPC1L1 is to transport free cholesterol from...
the intestinal lumen into enterocytes. Reduction of cholesterol absorption via intestinal NPC1L1 inhibition is one of the ways by which some functional foods and nutraceuticals decrease plasma cholesterol levels (Zhou et al., 2017). Three peptides in this study significantly reduced NPC1L1 protein levels compared with the control (Figure 4), which may be one of the mechanisms by which the 3 milk-derived peptides inhibit cholesterol absorption.

Next we speculated that the cholesterol-lowering activity of peptides may be regulated by other cholesterol absorption–related genes. Therefore, we performed RT-PCR experiments with other genes. A heterodimer formed in the endoplasmic reticulum, ABCG8 is negatively correlated with cholesterol absorption. The unabsorbed cholesterol in intestinal epithelial cells can return to the intestinal lumen via the action of ABCG8 (Yu et al., 2014) or by ACAT-regulated cholesterol esterification into cholesteryl esters from the plasma membrane into the endoplasmic reticulum (Tang et al., 2009). Figure 5A shows that the addition of these peptides can promote transcription of the cholesterol efflux gene ABCG8 and the secretion of cholesterol from the cell to the outside, thereby inhibiting cell monolayer cholesterol absorption.

Additionally, ACAT2 plays a major role in facilitating cholesterol absorption. Following uptake of cholesterol into the enterocyte, the re-esterification of free cholesterol becomes a key step because cholesterol is secreted from the BL side in the form of esters (Wang et al., 2014). The ACAT2 gene is only expressed in small intestinal epithelial cells and hepatocytes. The main role of ACAT2 is to convert free cholesterol in the cell into cholesteryl ester (Castro-Torres et al., 2014), which has a positive effect on cholesterol absorption. Our results showed that VLPVPQ could downregulate ACAT2 transcription (Figure 5B), which suggests that VLPVPQ might be an ACAT2-specific inhibitor in enterocytes.

The microsomal triglyceride transfer protein (MTP) consists of 4 MTP α subunits and 4 MTP β subunits and is mainly involved in the secretion of apolipoprotein ApoB48 in the liver and small intestinal cells (Wang et al., 2015). This protein assembles the cholesteryl ester into chylomicrons, which are secreted through the BL membrane into the lymphatic system and then into the blood (Wang et al., 2015). Real-time PCR analysis demonstrated that LQPE and VAPFPE had no effect on MTP expression. However, VLPVPQ did downregulate MTP mRNA (Figure 5C).

The protein ABCA1 is a cell membrane transport carrier that plays an important role in mediating reverse cholesterol transport and high-density lipoprotein production. The first step in reverse cholesterol transport is ABCA1-mediated cholesterol export from peripheral cells (Qian et al., 2017). It was shown that LQPE and VLPVPQ increase transcription of ABCA1 and may promote the production of high-density lipoprotein, thereby reducing intracellular cholesterol levels (Figure 5D).

Sterol regulatory element binding protein is a conditional transcriptional activator involved in the
The regulation of cellular cholesterol metabolism in the endoplasmic reticulum (Horton et al., 1998). In human intestinal Caco-2 cells (Field et al., 2001), SREBP-2 is involved in an adaptive intestinal process of modulating the expression and activity of the intestinal apical sodium-dependent bile acid transporter in response to high cholesterol (Thomas et al., 2006). Alrefai et al. (2007) provided evidence for SREBP-2 involvement in the regulation of intestinal NPC1L1 expression. High cholesterol leads to suppression in the activity of intestinal SREBP-2 and a subsequent reduction in the expression of both NPC1L1 and apical sodium dependent bile acid transporter (ASBT), resulting in a concomitant decrease in cholesterol and bile acid absorption, favoring a decline in plasma cholesterol (Alrefai et al., 2007). High cholesterol inhibits the activity of intestinal SREBP-2, thereby reducing the expression of NPC1L1 and reducing intestinal cholesterol absorption (Alrefai et al., 2007). In this experiment, expression of SREBP2 in the 3 peptide-treated groups was not different from that of the control group (Figure 5E–F). This may be because cholesterol concentration is insufficient in the response of SREBP-2, and the specific mechanism needs to be further studied.

In conclusion, we found 3 new cholesterol-lowering peptides, LQPE, VLPVPQ, and VAPFPE, derived from milk casein hydrolysates in vitro. The cholesterol-lowering action was induced by inhibition of cholesterol absorption accompanying the suppression of micellar cholesterol solubility, and affects the expression of cholesterol absorption-related proteins and enzymes such as NPC1L1 in intestinal epithelial cells. This study included only in vitro experiments on the cholesterol-lowering activity of 3 peptides obtained after casein hydrolysis. However, more studies are needed to fully understand the biological activity of the hypolipidemic peptides and the specific molecular mechanisms involved. Therefore, we are conducting animal experiments to study the cholesterol-lowering activity of casein hydrolysate peptides.

Figure 5. Effects of adding milk-derived peptides LQPE, VAPFPE, and VLPVPQ on the expression of ABCG8, ACAT2, MTP, ABCA1, SREBP1, and SREBP2. mRNA levels of ABCG8, ACAT2, MTP, ABCA1, SREBP1, and SREBP2 were quantified using ImageLab software (Bio-Rad, Hercules, CA) and normalized via β-actin signals. Data are presented as average ± SD with 3 samples from each group. *P < 0.05, **P < 0.01, and ***P < 0.001 versus control (Dunnett test on one-way ANOVA; SPSS Statistics 20; IBM Corp., Armonk, NY).
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