International Dairy Journal 91 (2019) 119–128

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Positively charged peptides from casein hydrolysate show strong inhibitory effects on LDL oxidation and cellular lipid accumulation in Raw264.7 cells

Mengshi Pan [a], Yanjiao Huo [a], Chengtao Wang [b], Yanchun Zhang [c], Zhiyong Dai [c], Bo Li [a, *]

[a] College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China
[b] Beijing Engineering and Technology Research Center of Food Additives, Beijing Technology & Business University (BTBU), Beijing 100048, China
[c] Food and Nutritional Institute of Hu'nan Ausnutria, Changsha, Hu'nan 410200, China

ARTICLE INFO

Article history:
Received 30 April 2018
Received in revised form
1 September 2018
Accepted 23 September 2018
Available online 24 October 2018

ABSTRACT

Inhibiting low-density lipoprotein (LDL) oxidation and cellular lipid accumulation to reduce foam cell formation plays a key role in preventing atherosclerosis. Casein hydrolysate (66% < 1 kDa) was separated into four different charged fractions. The inhibitory effect of peptide fractions on LDL oxidation and cellular lipid accumulation was analysed using a $CuSO_4$ cell-free system and a Cu^{2+}-mediated and ox-LDL-induced Raw264.7 macrophage cell-based system. Casein peptide fractions not only significantly inhibited LDL oxidation but also prevented cellular lipid accumulation. Positively charged fractions exhibited stronger inhibitory effects than negatively charged fractions. Seven peptides with different charge properties were synthesised. With the increase in net positive charge, the ability of peptides to inhibit LDL oxidation was enhanced. Peptides containing lysine presented better inhibition than those that contained histidine. This study suggests that casein hydrolysate, especially positively charged peptide fractions, could be used as a natural antioxidant in functional foods to prevent atherosclerosis.

1. Introduction

Atherosclerosis (AS) is a crucial underlying cause of cardiovascular diseases (CVDs) that affect numerous individuals and leads to high morbidity and mortality worldwide (Agudelo, Gauthier, Pouliot, Marin, & Savoie, 2004). It is well recognised that AS is a chronic inflammatory disease that develops through a series of events, including damage to intima of arteries, recruitment of inflammatory cells, accumulation of lipids, foam cell formation, eventual calcification and plaque rupture.

Plasma low-density lipoprotein (LDL) oxidation by metal ions or free radicals is closely related to AS development. Oxidised LDL (ox-LDL) can cause a series of pathological processes, such as endothelial cell dysfunction, smooth muscle cell migration and proliferation, and platelet activation (Aluganti Narasimhulu, Selvarajan, Brown, & Parthasarathy, 2014). Inhibiting LDL oxidation and decreasing the damage induced by ox-LDL are important for the prevention and treatment of AS.

Uptake of ox-LDL by macrophages is known to result in cellular lipid accumulation and the formation of foam cells (Halliwell, 1997). ox-LDL stimulates the expression of adhesion molecules and induces the adhesion of monocytes to endothelium mediated by monocyte chemotactic protein-1 (MCP-1), and secreted macrophage colony stimulating factor (M-CSF), which promotes monocyte transition into macrophages (Jialal & Devaraj, 1996). Macrophage activation is also a characteristic of AS. Macrophages can quickly take up ox-LDL through scavenger receptors, such as type A scavenger receptor (SR-A), cluster of differentiation 36 (CD36) and lectin type ox-LDL receptor (LOX-1), a process that is not regulated by the intracellular accumulation of lipids (Rios, Gidlund, & Jancar, 2011). Consequently, macrophages continue the uptake of ox-LDL, and excessive lipids accumulate, leading to foam cell formation, which is a key event in early AS. It is well known that the risk of AS is increased by elevated ox-LDL levels in cells (Chandrasekara & Shahidi, 2011). Some studies have reported that a diet full of antioxidants can prevent and decrease the risk of AS (Jialal & Devaraj, 1996; Kaliora, Dedoussis, & Schmidt, 2006).

Many antioxidant peptides that can scavenge free radicals, quench oxygen and act as metal-ion chelators have been identified from the hydrolysates of various proteins (Pihlanto, 2006). Casein,

* Corresponding author. Tel.: +86 10 6273 7669.
E-mail address: libo@cau.edu.cn (B. Li).

https://doi.org/10.1016/j.idairyj.2018.09.011
0958-6946/

the main milk protein, is a good source of antioxidant peptides. Studies have shown that casein phosphopeptides (CPPs) and casein hydrolysates exhibited high antioxidant activities in trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays (Diaz & Decker, 2004). Yak casein hydrolysate was not only able to scavenge free radicals, but it also decreased the production of NO and pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (Mao, Cheng, Wang, & Wu, 2011). In our previous study, the positively charged peptide fractions of casein hydrolysate exhibited stronger inhibition effects on LDL oxidation than negatively charged fractions (Wang, Wang, Huo, & Li, 2016). Although previous studies have found using chemical experiments that the casein basic peptide fraction has a strong inhibitory effect on LDL oxidation, the inhibition effects on LDL oxidation in relation to macrophages and cellular lipid accumulation to prevent foam cell formation have not been investigated.

The aim of this study was to evaluate the inhibitory effects of differently charged peptide fractions from casein hydrolysate on LDL oxidation and cellular lipid accumulation in Raw264.7 cells. The four peptide fractions from casein hydrolysate were separated based on different charge properties. Cell models involving Cu^{2+}-treated Raw 264.7 macrophages and ox-LDL-induced Raw264.7 macrophages were used to study the inhibitory abilities of peptides on LDL oxidation and foam cell formation. Furthermore, seven peptides with different charges identified from casein peptide fractions were synthesised and analysed to explore the relationship between peptide charge properties and the effect on LDL oxidation inhibition.

2. Materials and methods

2.1. Materials

Casein (product number: C3400), alcalase (P4860, ≥2.4 U g^{-1}), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS), fluorescein, LDL, ox-LDL, glutathione (GSH), 2,2′-azobis(2-methylpropionamidine)-dihydrochloride (AAPH), picrylsulfonic acid solution (P2297, TNBS), phenyl isothiocyanate (PITC), and triethylamine (TEA) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), Hank's balanced salts solution (HBSS), and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were from HyClone (Waltham, MA, USA). The seven peptides, the Cholesterol Assay and Free Cholesterol Assay kits were purchased from Beijing Applygen Technologies Co. (Beijing, China). All other reagents were of analytical grade and purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China).

2.2. Preparation of casein hydrolysate

Casein hydrolysate was prepared according to the method reported by Xie, Wang, Ao, and Li (2013). Briefly, aliquots of casein solution (5%, w/v, pH 8.0) were hydrolysed with alcalase at 60 °C for 4 h in a constant temperature water bath. The ratio of enzyme to substrate was 2% (w/w). The hydrolysate was heated in boiling water for 10 min to inactive the alcalase and then cooled to room temperature. Subsequently, the pH of the hydrolysate was adjusted to 7.0 with 0.01 M HCl, and the hydrolysate was centrifuged at 8000× *g* for 15 min. The supernatant was lyophilised for further study.

2.3. Separation of casein hydrolysate using a SP Sephadex C-25 column

The method described by Wang, Wang, Huo, and Li (2016) was used to separate casein hydrolysate on a SP Sephadex C-25 cation exchange column (Ø 2.6 × 25 cm; GE Healthcare Life Science) equilibrated with buffer A (20 mM sodium acetate buffer, pH 4.0). Briefly, the column was eluted with buffer A from 0 to 120 min and with buffer B (0.5 M NaCl in buffer A) from 120 to 300 min. Peptide fractions were monitored at 220 nm, and the flow rate was controlled at 2.0 mL min^{-1}. Four fractions, named F1 to F4, were collected. The NaCl in the collected peptide fractions was removed using dialysis bags (Beijing Chemical Reagent Co., Ltd.) with a cut-off of 500 Da.

2.4. Determination of molecular mass distribution

The molecular mass distribution of casein peptides was analysed according to the method described by Xie, Liu, Wang, and Li (2014). Briefly, 10 μL samples (2 mg mL^{-1}) were filtered with a 0.22-μm microfiltration membrane and loaded onto the column (TSK gel G2000 SWXL, 7.8 mm i.d. × 300 mm); 45% (v/v) acetonitrile containing 0.1% (v/v) TFA was used as the buffer. The absorbance was monitored at 214 nm.

2.5. Amino acid composition analysis

The amino acid composition was analysed according to the method reported by Wang, Wang, and Li (2016). Briefly, 6 M HCl was used to hydrolyse the fractions at 110 °C for 24 h. After pre-column derivatisation with PITC and TEA, the amino acid analysis was conducted on a Shimadzu LC-15C HPLC system using an HPLC column (ZORBAX SB-C18, 4.6 mm i.d. × 250 mm, 5 μm, Agilent Technologies, Waltham, MA, USA) with a linear gradient mixture composed of solvent A (10 mM phosphate buffer, pH 6.9) and solvent B (ACN).

2.6. Antioxidant activities of peptide fractions

2.6.1. TEAC assay

The antioxidant activities of casein hydrolysate and the four peptide fractions were measured using the ABTS-radical cation decolorisation method described by Wang, Li, Wang, and Xie (2015). GSH was used as a positive control, and the activities of samples were interpolated to calculate the concentration in Trolox equivalents (TEs).

2.6.2. ORAC assay

The method reported by Wang et al. (2015) was applied. Fluorescein was used as a probe. The ORAC values of the samples are presented as TEs.

2.6.3. Measurement of malondialdehyde

The lipid peroxide content of LDL (purchased from Sigma–Aldrich Chemical Co.) is expressed as malondialdehyde (MDA) equivalents. An aliquot of 0.2 mL of LDL (400 μg mL^{-1}) was mixed with 0.2 mL of samples (2 mg mL^{-1}), and the mixture was incubated for 1 h. Oxidation was initiated by addition of 4 μL of $CuSO_4$ (1 mM). After a 2 h reaction at 37 °C, 10 μL of 1 mM EDTA was added to end the oxidation. MDA levels were evaluated with an MDA Kit. LDL alone was used as the control. The model group contained Cu^{2+} and LDL without samples.

2.7. Cell culture

Raw 264.7 macrophages were obtained from Sigma–Aldrich (Shanghai, China) and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified 37 °C incubator with 5% CO_2. The medium was changed each day. Cells were

subcultured when they reached 70% confluency. The cells were used at passages 6–8.

2.7.1. Cell treatment of Cu^{2+}-treated Raw 264.7 macrophages

Raw 264.7 cells were seeded into 96-well plates at a density of 5×10^4 cells/well and allowed to grow for 12 h. Then, the culture medium was replaced. The treatment concentration of LDL (100 μg mL^{-1}) was chosen according to MTT assay results showing cell viability above 80% at this concentration. The cells were treated with 5 μM $CuSO_4$ and 100 μg mL^{-1} of LDL with and without samples (1 mg mL^{-1}, H and F1–F4) for 24 h. Cells cultured in medium alone were used as controls in all experiments. Cells induced by $CuSO_4$ with LDL without samples were used as model groups. MDA levels were evaluated with an MDA Kit.

2.7.2. Cell treatment of ox-LDL-induced Raw 264.7 macrophages

Raw 264.7 cells were seeded into 96-well plates at a density of 5×10^4 cells per well and allowed to grow for 12 h. Then, the culture medium was replaced. The treatment concentration of ox-LDL (50 μg mL^{-1}) was chosen based on MTT assay results showing cell viability above 80% at this concentration. The cells were treated with ox-LDL (50 μg mL^{-1}) with and without samples (1 mg mL^{-1}, H and F1–F4) for 24 h. Cells incubated with ox-LDL without samples were taken as model groups. MDA levels were evaluated with an MDA Kit.

2.8. MTT assay

To investigate the potential cell toxicity of samples, cell viability was measured via MTT assays according to the method reported by Gentile et al. (2012) with some modifications. Raw 264.7 cells were plated in 96-well plates at a density of 5×10^4 cells per well and allowed to grow for 12 h. Subsequently, the culture medium was replaced. Cells were exposed to 5 μM Cu^{2+} and 100 μg mL^{-1} LDL or 50 μg mL^{-1} ox-LDL with and without samples. After 24 h, 100 μL of medium containing 5 mg mL^{-1} MTT (20 μL) was added. The medium was discarded after 4 h incubation at 37 °C, and 100 μL of DMSO was added and mixed for 10 min. At the end of this period, the optical density (OD) was read at 500 nm in a microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA).

2.9. Measurement of cholesterol

The cells were rinsed twice with HBSS after incubation for 24 h. Then, 0.1 mL of cell lysate was added to each well and mixed completely for 10 min. Cell lysates were collected and centrifuged at 10,800× *g* for 10 min at room temperature. Then, the supernatant was collected and analysed. The total cholesterol (TC) and free cholesterol (FC) content was measured with a Cholesterol Assay kit and a Free Cholesterol Assay kit. The optical density was read at 550 nm in a microtitre plate reader. Cholesteryl ester (CE) was calculated as the difference between TC and FC.

2.10. Peptide synthesis

To investigate the effect of the charge properties of casein peptides on LDL oxidation inhibition and foam cell formation, seven peptides (PVEPF, EMPFPK, MPFPK, KEMPFPK, HKEMPFPK, QPH, and HQPH; purity >95%, acetate salt, purchased from Shanghai Qiangyao Biotechnology Co., Ltd., Shanghai, China) with different charges that had been identified as intact absorbed peptide sequences from F3 and F4 by simulated gastrointestinal digestion and absorption (Wang et al., 2016), were synthesised, and the inhibition of LDL oxidation and lipid accumulation were analysed as described above.

2.11. Statistical analysis

All the experiments were conducted in triplicate. The results are presented as the means ± standard errors. Statistical analysis was performed using SPSS v 17.0 (SPSS, Chicago, IL, USA). Differences between means were compared with a Duncan assay. At $p < 0.05$, differences were considered statistically significant.

3. Results

3.1. Separation and analysis of casein hydrolysate

Four peptide fractions (F1–F4) of casein hydrolysate (H) were separated through an SP Sephadex C-25 cation exchange column (Fig. 1). F1 and F2 were eluted first using acetic acid buffer (pH 4.0) and showed negatively charged fractions. F3 and F4 were then eluted with acetic acid buffer (pH 4.0) plus 0.5 mol L^{-1} NaCl and presented positively charged fractions.

The amino acid compositions of H and F1–F4 are shown in Supplementary material Table S1. All samples were rich in glutamic acid, which is consistent with the reported high glutamic acid composition of casein (Agudelo et al., 2004). F1 had the highest percentage of acidic amino acids (42.73%), followed by F2 (39.96%). F3 and F4 contained a high amount of basic amino acids (17.49% and 24.75%, respectively). The results suggested that F1 and F2 presented a high number of negative charges, and F3 and F4 were rich in positive charges, which were consistent with the results of separation.

From Table 1, the fraction with a molecular mass lower than 1000 Da in casein hydrolysate was 66.3%. Thus, H was mainly composed of low molecular mass peptides. The molecular mass distribution of F1–F4, similar in all fractions, was mostly located 1000 to 500 Da.

3.2. Antioxidant activities of casein peptide fractions

Among the methods for estimating antioxidant capacity, ORAC and TEAC assays have been proposed for standardisation (Prior, Wu, & Schaich, 2005). The antioxidant activities of both H and F1–F4 were measured using TEAC and ORAC assays, as shown in Fig. 2. GSH (the positive control) had a higher TEAC value (0.37 mM TE) and ORAC value (2.29 mM TE) than H and F1–F4. The ORAC value of H was significantly higher ($p < 0.05$) than the F1–F4 values, and its TEAC value was significantly increased ($p < 0.05$) compared with those of F1, F2 and F3 but not F4, which differed from previous studies (Rival, Fornaroli, Boeriu, & Wichers, 2000; Suetsuna, Ukeda, & Ochi, 2000). This may be due to the loss of small molecule mass peptides during the separation and desalting process (Wang et al., 2015). Positively charged fractions (F3 and F4) had higher TEAC and ORAC values than negatively charged fractions (F1 and F2) ($p < 0.05$). It has been reported that His, Tyr and Met show high radical scavenging activity in ORAC assays. Tyr may act as a chain-breaking antioxidant to quench oxygen radicals (Dávalos, Miguel, Bartolomé, & Fandio, 2004). In the TEAC assay, Tyr, Trp, and especially Cys were the active amino acids (Clausen, Sktbsted, & Stagsted, 2009). F3 and F4 contained more basic amino acids (His and Lys) and aromatic amino acids (Tyr and Phe) than F1 and F2, which likely contributed to their high antioxidant activities.

3.3. Effect of casein peptide fractions on Cu^{2+}-induced LDL oxidation in a cell-free system

The inhibitory effects of casein hydrolysate (H) and casein peptide fractions (F1–F4) on LDL oxidation induced by Cu^{2+} are shown in Table 2. Cu^{2+}-treatment has been widely used to

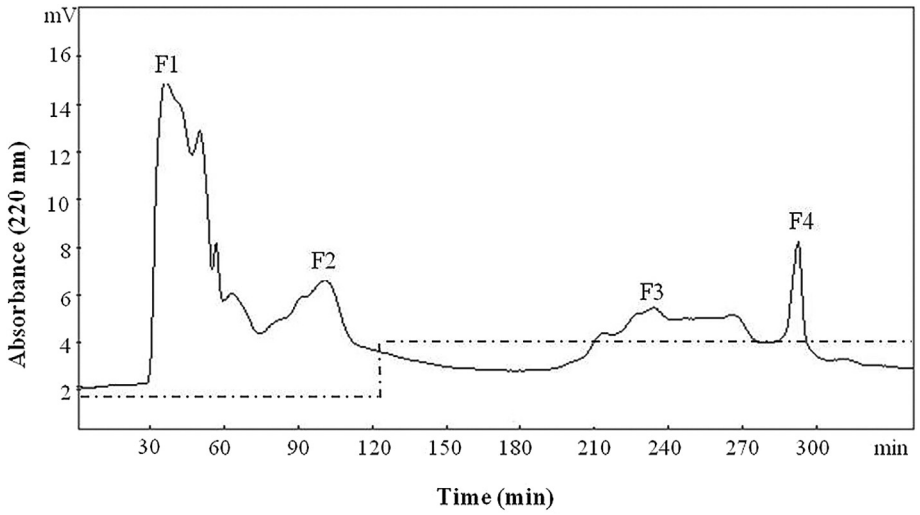

Fig. 1. Chromatographic profile obtained by fractionation of casein hydrolysate on a SP Sephadex C-25 column. The casein hydrolysate was separated into four fractions (F1–F4). From 0 to 120 min elution was with 20 mM acetate buffer, pH 4.0 (buffer A); from 120 to 300 min elution was with buffer A containing 0.5 M NaCl.

Table 1
Molecular mass distribution percentages of the casein hydrolysates (H) and the casein peptide fractions (F1–F4).

Fraction	Relative distribution (%)			
	4500–3000 Da	3000–1000 Da	1000–500 Da	<500 Da
Hydrolysate	1.69 ± 0.07	31.94 ± 0.06	43.16 ± 0.15	23.21 ± 0.08
F1	0.76 ± 0.01	29.32 ± 0.14	45.64 ± 0.07	24.28 ± 0.17
F2	1.12 ± 0.02	34.52 ± 0.22	40.98 ± 0.16	23.38 ± 0.11
F3	2.01 ± 0.12	36.27 ± 0.20	37.23 ± 0.09	24.49 ± 0.15
F4	3.81 ± 0.03	46.82 ± 0.11	35.16 ± 0.17	14.21 ± 0.08

stimulate lipid peroxidation using in vitro models. Under Cu^{2+} catalysis, polyunsaturated fatty acids (PUFAs) located on the lipid molecules in LDLs are easily oxidised and turn into lipid hydroperoxides, which produce a variety of aldehydes, such as MDA, in secondary cleavage reactions. Therefore, the MDA content reveals the extent of LDL peroxidation (Itabe, 2009). Table 2 indicates that the MDA content induced by Cu^{2+} in the model group was as high as 4.44 nmol mL^{-1} and was increased significantly compared with that in the control group ($p < 0.05$). H and F1–F4 significantly decreased the MDA levels compared with those in the model group ($p < 0.05$). The MDA concentrations in the F3 and F4 treatment groups were 1.60 nmol mL^{-1} and 1.64 nmol mL^{-1}, respectively, which were significantly lower than those in the H, F1 and F2 groups ($p < 0.05$). The positively charged peptide fractions F3 and F4 had the higher rates of MDA level inhibition than the negatively charged peptide fractions (F1 and F2). This suggested that the positively charged fractions have a stronger effect on LDL oxidation inhibition than negatively charged fractions. The results are consistent with a previous study by Wang et al. (2016), who

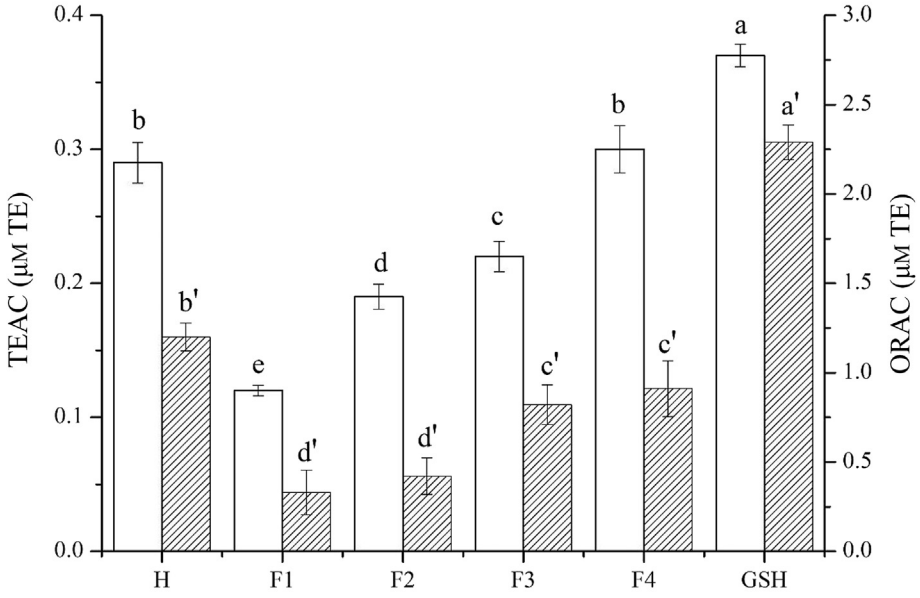

Fig. 2. TEAC assay and ORAC assay of casein hydrolysate and casein peptide fractions (F1–F4) with GSH as a positive control; the TEAC values (□) and the ORAC values (▨) of samples (1 mg mL^{-1}) are expressed as Trolox equivalents (TEs). Values are the means ± standard deviation (n = 3); columns with different lowercase letters above within the tests (a–e, TEAC; a'–d', ORAC) are significantly different ($p < 0.05$).

Table 2
The inhibitory effect of casein hydrolysate (H) and casein peptide fractions (F1–F4) on LDL oxidation induced by Cu^{2+} and by Cu^{2+}-treated Raw 264.7 macrophages.[a]

Group	LDL oxidation induced by			
	Cu^{2+}		Cu^{2+}-treated Raw 264.7 macrophages	
	MDA content	Inhibition rate (%)	MDA content	Inhibition rate (%)
Control	1.07 ± 0.22^{e}	–	1.11 ± 0.17^{d}	–
Model	4.44 ± 0.23^{a}	–	6.00 ± 0.39^{a}	–
H	2.31 ± 0.17^{c}	47.98	2.49 ± 0.17^{c}	58.50
F1	2.62 ± 0.17^{bc}	41.00	3.02 ± 0.17^{bc}	49.67
F2	2.89 ± 0.17^{b}	34.91	3.24 ± 0.23^{b}	46.00
F3	1.60 ± 0.22^{d}	63.97	2.67 ± 0.29^{c}	55.50
F4	1.64 ± 0.23^{d}	63.06	2.53 ± 0.11^{c}	57.83

[a] Controls were: LDL alone for LDL oxidation induced by Cu^{2+}; Raw 264.7 macrophages exposed to culture medium for LDL oxidation induced by Cu^{2+}-treated Raw 264.7 macrophages; models were LDL incubated with 1 mM $CuSO_4$ at 37 °C for 2 h and Raw 264.7 macrophages incubated with 5 μM Cu^{2+} and 100 μg mL^{-1} of LDL at 37 °C for 24 h, respectively. The inhibitory effect on LDL oxidation was evaluated by measuring MDA content with an MDA Kit; the values presented (in nmol mL^{-1}) are the means ± standard deviation (n = 3). Values with different superscript lowercase letters in the test are significantly different within the same column ($p < 0.05$).

reported that casein peptides with positive charges showed high LDL oxidation inhibition. Aluganti Narasimhulu et al. (2014) also reported that cationic peptides can inhibit Cu^{2+}-mediated LDL oxidation in a lysine-dependent manner.

3.4. Effect of casein peptide fractions on Raw 264.7 cell viability

The effect of peptides on cell viability was measured with MTT assays. As shown in Fig. 3, Cu^{2+} was used to induce LDL oxidation. This exerted a cytotoxic effect on Raw 264.7 cells, and cell viability declined to 87%. Interestingly, H and F1–F4 significantly increased cell viability ($p < 0.05$), which indicated an antioxidant effect on LDL. ox-LDL has been confirmed to be toxic to many cells. The viability of Raw 264.7 macrophages induced by ox-LDL declined to 82.5%. However, supplementation with casein hydrolysates (H and F1–F4) were not able to improve the cell viability significantly ($p > 0.05$).

3.5. Effect of casein peptide fractions on LDL oxidation inhibition and cellular lipid accumulation

Raw 264.7 cells were used as an established macrophage model. Cu^{2+} was added to produce an oxidative environment to enhance LDL oxidation. A recent study that measured the formation of thiobarbituric acid reactive substances (TBARS) and conjugated dienes reported that in the absence of LDL, Cu^{2+} alone did not induce macrophage lipid peroxidation, even at a concentration of 10 μM. In addition, Cu^{2+} exhibited no toxicity towards cells in the absence of LDL (Kang et al., 2002). As presented in Table 2, Raw 264.7 cells plus LDL exposed to Cu^{2+} (5 μmol L^{-1}) in the model group had the highest MDA content at 6.00 nmol mL^{-1}, which was significantly increased compared with that in the control group ($p < 0.05$). Following treatment with H and F1–F4, MDA levels were significantly decreased compared with those in model cells ($p < 0.05$). H showed the highest rate of MDA production inhibition at 58.50%. The rates of F3- and F4-mediated inhibition of MDA production were 55.50% and 57.83%, respectively, which were higher than those of F1 and F2. The MDA levels in cells treated with the positively charged peptide fractions (F3 and F4) were significantly decreased compared with those in cells treated with the negatively charged peptide fractions (F1 and F2) ($p < 0.05$).

After incubation with Cu^{2+}, when LDL is oxidised and modified, it will turn into ox-LDL, which is easily distinguished by macrophages. Macrophages take up ox-LDL immediately, which leads to cholesterol and lipid accumulation in cells (Guan, Wang, Li, Guan, & Fang, 2010). Therefore, the degree of LDL peroxidation can be expressed indirectly by lipid content. The levels of TC, FC and CE were significantly increased in Raw 264.7 cells induced by Cu^{2+} with LDL ($p < 0.01$) compared with the levels in the control group (Table 3). H and F1–F4 significantly decreased the levels of TC, FC and CE ($p < 0.05$). The degree of cytoplasmic lipid droplet

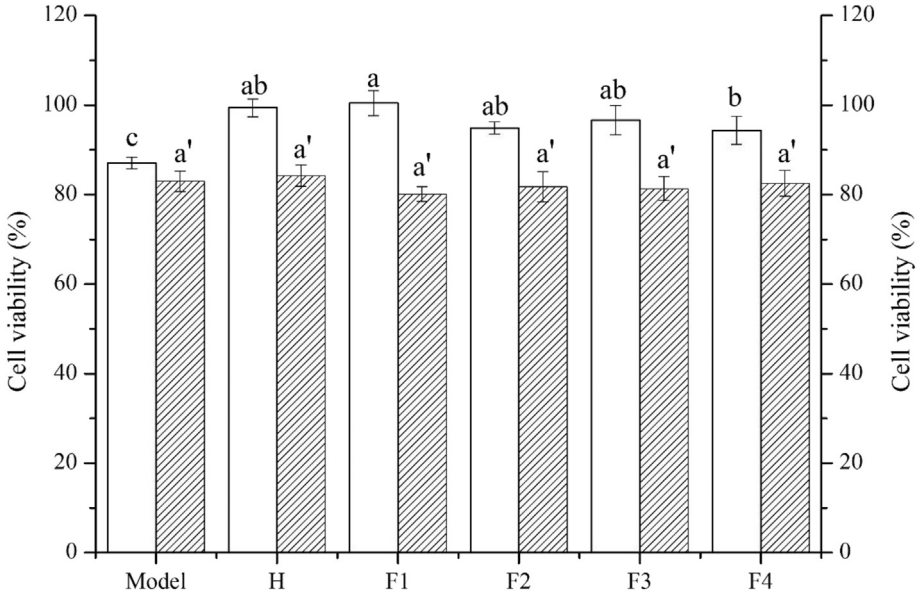

Fig. 3. Effect of casein hydrolysate and casein peptide fractions (F1–F4) on Raw 264.7 cell viability in Cu^{2+}-induced macrophages (□) and ox-LDL-induced macrophages (▨). In Cu^{2+}-induced macrophages systems, the cells were incubated with 5 μM Cu^{2+} and 100 μg mL^{-1} LDL with and without 1 mg mL^{-1}H and F1–F4 at 37 °C for 24 h. In ox-LDL-induced macrophages systems, the cells were exposed to 50 μg mL^{-1} ox-LDL for 24 h with and without 1 mg mL^{-1}H and F1–F4. The values are presented as the means ± standard deviation (n = 3); columns for Raw 264.7 cell viability in Cu^{2+}-induced macrophages with different lowercase letters above within the tests are significantly different ($p < 0.05$); no significant differences were found for the ox-LDL-induced macrophages system.

Table 3
The inhibitory effect of casein hydrolysate (H) and four casein fractions (F1–F4) on lipid accumulation induced by Cu^{2+}-LDL-treated Raw 264.7 macrophages and by ox-LDL-treated Raw 264.7 macrophages.[a]

Group	TC (mg g^{-1})	FC (mg g^{-1})	CE (mg g^{-1})	CE/TC (%)
Lipid accumulation induced by Cu^{2+}-LDL-treated Raw 264.7 macrophages				
Control	98.7 ± 7.6	62.0 ± 6.6	36.8 ± 7.6	37.4% ± 2.7
Model	267.5 ± 8.2	112.7 ± 10.8	154.8 ± 8.2	57.8% ± 1.8*
H	182.4 ± 8.2	99.1 ± 10.9	83.4 ± 8.2	45.8% ± 2.1$^{\#\#}$
F1	156.1 ± 11.8	79.7 ± 9.0	76.3 ± 11.8	49.2% ± 3.7$^{\#}$
F2	177.8 ± 12.3	90.7 ± 3.6	87.1 ± 12.3	49.1% ± 3.4$^{\#}$
F3	119.1 ± 7.5	64.0 ± 3.8	55.0 ± 7.5	46.4% ± 3.1$^{\#\#}$
F4	108.8 ± 7.9	64.8 ± 6.9	44.0 ± 7.9	40.7% ± 3.1$^{\#\#\#}$
Lipid accumulation induced by ox-LDL-treated Raw 264.7 macrophages				
Control	96.3 ± 4.9	60.1 ± 3.9	36.3 ± 4.9	37.7% ± 2.0
Model	365.6 ± 5.2	159.5 ± 2.7	206.1 ± 5.2	56.4% ± 0.8*
H	250.9 ± 10.1	128.3 ± 12.0	122.6 ± 10.1	48.9% ± 1.9$^{\#\#}$
F1	276.6 ± 6.5	134.9 ± 7.1	142.7 ± 6.5	51.3% ± 1.2$^{\#\#}$
F2	232.1 ± 9.1	119.1 ± 16.1	113.0 ± 9.1	48.8% ± 1.9$^{\#\#\#}$
F3	228.5 ± 8.4	127.3 ± 11.2	101.1 ± 8.4	44.3% ± 1.7$^{\#\#\#}$
F4	172.4 ± 6.8	96.7 ± 2.8	75.7 ± 6.8	44.0% ± 1.8$^{\#\#\#}$

[a] Abbreviations are: TC, total cholesterol (measured with a Cholesterol Assay Kit); FC, free cholesterol (measured with a Free Cholesterol Assay Kit); CE, cholesteryl ester (calculated as the difference between TC and FC). Control was Raw 264.7 macrophages cells exposed to culture medium at 37 °C for 24 h. Models were Raw 264.7 macrophages incubated with either 5 μM Cu^{2+} and 100 μg mL^{-1} LDL or 50 μg mL^{-1} ox-LDL at 37 °C for 24 h for lipid accumulation induced by Cu^{2+}-LDL-treated and ox-LDL-treated Raw 264.7 macrophages, respectively. Values are means ± standard deviation (n = 3); statistical differences are $^{*}p < 0.01$ compared with the control group and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with the model group.

accumulation was determined by calculating the ratio of CE and TC. Under treatment with F1–F4, with positive charges increasing, the CE/TC (%) was significantly decreased ($p < 0.05$). F4 showed the strongest lipid accumulation inhibition, and the level of CE/TC (%) was lowered to 40.7%.

Incubation of macrophages with ox-LDL leads macrophages to take up ox-LDL and results in cellular lipid accumulation, which may contribute to foam cell formation. For LDL that has been oxidised to ox-LDL, do casein peptides have an inhibitory effect on cellular lipid accumulation? Another cellular model was established with ox-LDL-induced Raw 264.7 macrophages for 24 h. As shown in Table 3, normal cells without ox-LDL stimulation had the lowest level of CE/TC (37.7%). Compared with those in the native cells, the amounts of TC, FC and CE in cells incubated with ox-LDL were significantly increased ($p < 0.01$), and the ratio of CE/TC was increased to 56.4% ($p < 0.01$). The lipid accumulation was significantly attenuated by applying H and F1–F4 ($p < 0.01$). The F3 and F4 groups had the lowest levels of CE/TC at 44.3% and 44.0%, respectively, which showed the strongest lipid accumulation inhibition. This result revealed that casein peptides, especially positively charged peptides, can reduce ox-LDL uptake by macrophages and inhibit cellular lipid accumulation.

3.6. Effect of single casein peptide charges on LDL oxidation inhibition and cellular lipid accumulation

Based on the above results, the charge properties of casein peptides may be responsible for their differences in antioxidant activity. Seven peptides with different charges and basic amino acids (PVEPF, EMPFPK. MPFPK, KEMPFPK, QPH, HQPH, and HKEMPFPK), which had been identified from F3 and F4 of casein hydrolysate as intact absorbed peptide sequences (Wang et al., 2016), were synthesised, and their antioxidant activities and effects on LDL oxidation, lipid accumulation and foam cell formation were investigated. The TEAC and ORAC values of the seven peptides were measured as an assessment of antioxidant activities. The TEAC and ORAC values of MPFPK were the highest. However, the negatively charged peptide PVEPF had the lowest TEAC and ORAC values. In the TEAC assay, MPFPK showed the highest TEAC value, followed by KEMPFPK, EMPFPK, HKEMPFPK, QPH, HQPH and PVEPF. In the ORAC assay, MPFPK also showed the highest value, followed by HKEMPFPK, EMPFPK, KEMPFPK, HQPH, QPH and PVEPF (Fig. 4).

Both in the cell-free system and in the Cu^{2+}-induced Raw 264.7 macrophage cell model (Table 4), these seven peptides decreased the MDA content significantly ($p < 0.05$), except for QPH in the Cu^{2+}-LDL-mediated cell system. The peptides were divided into 2 groups based on the types of basic amino acids. From EMPFPK to KEMPFPK (with Lys (K) as the main basic amino acid), as net charges increased, peptides showed a stronger inhibitory effect on MDA production ($p < 0.05$). Meanwhile, from QPH to HKEMPFPK (with His (H) as the main basic amino acid), the MDA levels were significantly decreased as net charges increased ($p < 0.05$), showing the same trend.

The inhibitory effects of peptides with the same net charge but a different number of basic amino acids were also compared.

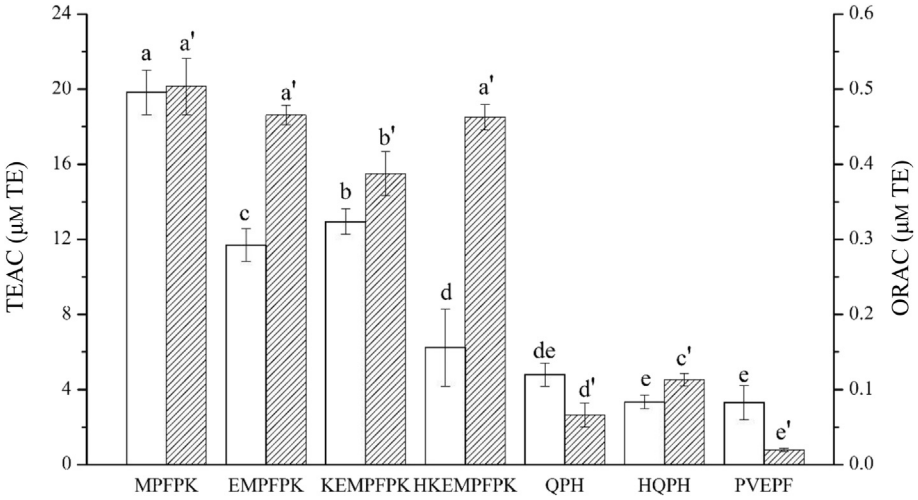

Fig. 4. TEAC assay and ORAC assay of 7 synthesised peptides. The TEAC values (□) and ORAC values (▨) of peptides (1 mg mL^{-1}) are expressed as Trolox equivalents (TEs). Values are the means ± standard deviation (n = 3); columns with different lowercase letters above within the tests (a–e, TEAC; a'–e', ORAC) are significantly different ($p < 0.05$).

Table 4
The inhibitory effect of seven synthesised peptides on LDL oxidation induced by Cu^{2+} and by Cu^{2+}-treated Raw 264.7 macrophages.[a]

Group	Net charge	LDL oxidation induced by			
		Cu^{2+}		Cu^{2+}-treated Raw 264.7 macrophages	
		MDA content (nmol mL^{-1})	Inhibition rate (%)	MDA content (nmol mL^{-1})	Inhibition rate (%)
Model		7.70 ± 0.20[a]	–	7.03 ± 0.31[a]	–
PVEPF	−1	7.16 ± 0.11[b]	7.01	6.23 ± 0.15[b]	11.33
EMPFPK	0	6.30 ± 0.03[d]	18.18	5.07 ± 0.20[c]	27.92
MPFPK	1	4.95 ± 0.13[e]	35.71	2.14 ± 0.43[e]	69.57
KEMPFPK	1	4.90 ± 0.12[e]	36.36	4.01 ± 0.27[d]	43.01
QPH	1	7.12 ± 0.19[bc]	7.53	6.51 ± 0.26[ab]	7.43
HQPH	2	6.83 ± 0.19[c]	11.3	6.14 ± 0.16[b]	12.67
HKEMPFPK	2	6.44 ± 0.10[d]	16.36	3.95 ± 0.21[d]	43.77

[a] Models were LDL incubated with 1 mM $CuSO_4$ at 37 °C for 2 h for LDL oxidation and Raw 264.7 macrophages incubated with 5 μM Cu^{2+} and 100 μg mL^{-1} LDL without synthesised peptides at 37 °C for 24 h for LDL oxidation induced by Cu^{2+}-treated Raw 264.7 macrophages. Values are the means ± standard deviation (n = 3); values with different superscript lowercase letters significantly different within the same column ($p < 0.05$).

MPFPK and KEMPFPK, which had 1 or 2 basic amino acids, respectively, showed no significant difference in decreasing MDA content ($p > 0.05$) in the cell-free model but exhibited a significant difference in the cell-based model ($p < 0.05$). KEMPFPK, despite having 2 basic amino acids, showed lower inhibitory effects on MDA content than MPFPK in the Cu^{2+}-LDL-induced Raw 264.7 macrophage cell model. These results indicated that it is not the number of basic amino acids but the net charge that is the key factor.

The inhibitory effects of peptides with the same net charge but different types of basic amino acids were also compared (MPFPK versus QPH; HKEMPFPK versus HQPH). For both test models, MPFPK showed a higher ability to prevent LDL oxidation than QPH, and HKEMPFPK showed a higher effect than HQPH ($p < 0.05$). This result suggested that Lys (K) had a stronger inhibitory effect on lipid oxidation than His (H). Among the seven peptides, MPFPK showed the best LDL oxidation inhibition, followed by KEMPFPK and HKEMPFPK, and both of them contained Lys.

The levels of TC, FC and CE accumulation in Raw 264.7 macrophages induced by Cu^{2+}-LDL treatment or ox-LDL treatment were measured and are shown in Table 5. The model cells incubated with Cu^{2+} showed the highest TC, FC and CE content, and the CE/EC was increased 50.8% ($p < 0.01$) compared with that in the control group. All peptides containing Lys (EMPFPK, MPFPK, KEMPFPK and HKEMPFPK) decreased the CE/EC ratio significantly ($p < 0.05$) compared with that in the model group, while peptides containing His (PVEPF, QPH and HQPH) showed no significant difference in reducing lipid accumulation ($p > 0.05$). As the net positive charge increased, the peptides containing Lys showed higher inhibitory effects on lipid accumulation (Table 5).

In the ox-LDL-induced model, compared with those in the control group, the levels of TC, FC and CE in ox-LDL-induced cells were significantly increased (Table 5, $p < 0.01$). However, only two positively charged peptides containing Lys (MPFPK and KEMPFPK) decreased the CE/TC ratio significantly ($p < 0.05$). Therefore, these results also suggested that positively charged peptides containing Lys have strong inhibitory effects on lipid accumulation and cellular lipid accumulation. Previously, it was reported that Lys could neutralise the ox-LDL charge and prevent macrophage uptake (Aluganti Narasimhulu et al., 2014).

Table 5
The inhibitory effect of seven synthesised peptides on lipid accumulation induced by Cu^{2+}-LDL-treated Raw 264.7 macrophages and by ox-LDL-treated Raw 264.7 macrophages.[a]

Group	Net charge	TC (mg g^{-1})	FC (mg g^{-1})	CE (mg g^{-1})	CE/TC (%)
Lipid accumulation induced by Cu^{2+}-LDL-treated Raw 264.7 macrophages					
Control	–	82.7 ± 4.4	55.9 ± 6.2	26.7 ± 4.4	32.4 ± 1.8
Model	–	236.4 ± 5.2	116.4 ± 2.7	112.0 ± 5.2	50.8 ± 1.1*
PVEPF	−1	137.0 ± 3.2	70.3 ± 4.1	67.0 ± 3.2	49.0 ± 1.2
EMPFPK	0	221.0 ± 13.4	120.3 ± 6.1	100.7 ± 13.4	45.8 ± 2.9#
MPFPK	1	155.3 ± 2.1	91.0 ± 4.4	64.3 ± 2.0	41.4 ± 0.5#
KEMPFPK	1	141.3 ± 7.7	85.3 ± 7.8	56.0 ± 7.7	39.7 ± 2.2#
QPH	1	220.7 ± 3.9	108.2 ± 5.6	112.4 ± 3.9	51.0 ± 0.9
HQPH	2	205.4 ± 9.4	109.2 ± 6.2	96.2 ± 9.4	46.9 ± 2.1
HKEMPFPK	2	152.1 ± 11.2	89.4 ± 10.4	62.7 ± 11.2	41.5 ± 3.0#
Lipid accumulation induced by ox-LDL-treated Raw 264.7 macrophages					
Control	–	86.8 ± 2.1	55.9 ± 4.2	30.9 ± 2.1	35.6 ± 0.8
Model	–	288.1 ± 7.0	116.4 ± 2.7	161.70 ± 7.0	56.2 ± 1.4*
PVEPF	−1	247.1 ± 8.6	108.3 ± 4.9	128.9 ± 8.5	52.6 ± 1.8
EMPFPK	0	225.8 ± 18.1	98.3 ± 5.0	117.5 ± 18.0	52.4 ± 4.2
MPFPK	1	168.0 ± 18.2	91.4 ± 7.4	76.7 ± 10.2	46.2 ± 4.9#
KEMPFPK	1	202.2 ± 11.4	97.3 ± 6.5	94.9 ± 11.4	47.1 ± 2.7#
QPH	1	269.1 ± 10.1	113.2 ± 7.2	145.8 ± 10.1	54.3 ± 2.1
HQPH	2	226.8 ± 4.8	92.1 ± 10.6	124.7 ± 4.8	55.0 ± 1.2
HKEMPFPK	2	247.1 ± 8.6	108.3 ± 4.9	128.9 ± 8.5	52.6 ± 1.8

[a] Abbreviations are: TC, total cholesterol (measured with a Cholesterol Assay Kit); FC, free cholesterol (measured with a Free Cholesterol Assay Kit); CE, cholesteryl ester (calculated as the difference between TC and FC). Control was Raw 264.7 macrophages cells exposed to culture medium at 37 °C for 24 h; models were Raw 264.7 macrophages incubated with either 5 μM Cu^{2+} and 100 μg mL^{-1} LDL or 50 μg mL^{-1} ox-LDL at 37 °C for 24 h for lipid accumulation induced by Cu^{2+}-LDL-treated and ox-LDL-treated Raw 264.7 macrophages, respectively. Values are the means ± standard deviation (n = 3); statistical differences are *$p < 0.01$ compared with the control group and #$p < 0.05$ compared with the model group.

4. Discussion

LDL particles rich in polyunsaturated fatty acids, phospholipids, triglycerides and 1 apolipoprotein B (apoB) containing 4356 amino acid residues were subjected to oxidation and indeed oxidised via oxidative stress due to free radicals and transition metals (Steinberg, 1997). In our previous study, casein peptides and their absorbates were found to have a strong ability to inhibit LDL oxidation (Wang et al., 2016), and thus, it was speculated that they may be able to prevent the development of AS. Although it is better to test this hypothesis using animal models or human clinical experiments, such methods are expensive and time-consuming. For initial antioxidant screening of foods and dietary supplements, cell culture models are cost-effective, fast, and address some issues of uptake, distribution and metabolism, which are biologically relevant to human metabolism (Liu & Finley, 2005). Thus, cell models were established.

There have been reports that LDL oxidation can be induced in cells, such as endothelial cells, smooth muscle cells and macrophages (Fernvik, Ketelhuth, Russo, & Gidlund, 2004; Henriksen, Mahoney, & Steinberg, 1982, 1983). This may be accounted for by production of reactive oxygen species and the activity of lipoxygenases in cells (Halliwell, 1997). However, Chu et al. (2003) reported that LDL oxidation showed a small increase in the present of macrophages in both TBARS and relative electrophoretic mobility (REM). It was also found that incubation of endothelial cells in DMEM was not able to induce LDL modification (Steinbrecher, Parthasarathy, Leake, Witztum, & Steinberg, 1984). Transition metals, such as copper and iron, can catalyse hydroxyl radicals and initiate lipid oxidation to stimulate a chain reaction of lipid peroxidation (Leake & Rankin, 1990). Studies have reported that high levels of Cu^{2+} in human serum were associated with increased LDL oxidation in vivo, which initiated AS progression (Salonen, Salonen, Seppanen, Kantola, & Suntioinen, 1991).

In our study, $CuSO_4$ was used to produce an oxidative environment to enhance LDL oxidation in a macrophage-based model (Steinbrecher et al., 1984). Both in the Cu^{2+}-induced cell-free system and Cu^{2+}-mediated Raw 264.7 macrophage system, casein peptides (H and F1–F4) significantly decreased the levels of MDA ($p < 0.05$) and inhibited LDL oxidation (Table 3). The inhibitory effects on LDL oxidation might be due to scavenging activity against free radicals. In our study, the higher TEAC or ORAC values the peptide fractions or synthesised peptides had, the stronger the inhibitory effects on LDL oxidation were. Sakanaka, Tachibana, Ishihara, and Juneja (2004) reported that casein calcium peptides showed scavenging activity against radicals such as superoxide and hydroxyl radicals. Casein and casein tryptic hydrolysates were proposed to act as free radical scavengers to inhibit lipoxygenase conversion of linoleic acid (Rival et al., 2000).

For Cu^{2+}-LDL-treated macrophages, the effects of F1 and F2 on the rates of LDL oxidation inhibition were enhanced by 49.7 and 46.0%, respectively, compared with measurements in the model group. The inhibition rates of F3 and F4 were 55.5 and 57.8% in the same system (Table 3). Positively charged peptides showed stronger LDL oxidation inhibition than negatively charged peptides, which may be associated with their active amino acids (basic amino acids and aromatic amino acids). Tyr can quench oxygen radicals to break chain reactions of lipid peroxidation. Dávalos et al. (2004) reported that His, Tyr and Met showed high radical scavenging activity in ORAC assays based on donation of H atoms. Histidine can react with peroxidising lipids or autoxidation products via free radical processes, which is the reason for its antioxidant activity (Yong & Karel, 1978). Antioxidant properties of peptides are also associated with their structure, molecular mass and sequence in addition to the type of amino acids (Li & Li, 2013; Orsini Delgado et al., 2016). In addition, chelating metals are also important for systems in which oxidation is induced by metals in addition to scavenging activity (Megias et al., 2008). The specific mechanism by which casein peptides inhibit LDL oxidation needs to be further investigated.

Native LDL is poorly recognised by cultured macrophages and taken up at relatively low rates by a limited number of receptors. Under oxidative stress, LDL is oxidised and its molecular structure altered, including apoB conformation and surface lipid packaging, which results in ox-LDL with increased electronegative charges that can be recognised quickly by scavenger receptors (Sánchez-Quesada, Villegas, & Ordonez-Llanos, 2012). Macrophages take up ox-LDL quickly and cellular lipids accumulate, which contributes to foam cell formation as a hallmark event of AS.

This study further investigated the effect of casein peptides on cellular lipid accumulation induced by ox-LDL. The content of CE/TC in ox-LDL-induced Raw 264.7 cells was increased to 56.4% ± 0.8% (Table 4). Casein peptides significantly decreased the levels of CE/TC ($p < 0.05$) in ox-LDL-induced Raw 264.7 cells (Table 4). These results indicated that casein peptides, especially positively charged peptides, not only inhibited production of ox-LDL but also blocked ox-LDL uptake by macrophages, which attenuated cellular lipid accumulation. Some studies have indicated that peptide protective effects against lipid accumulation may be associated with their interaction with scavenger receptors. Alvesab, Vasconcelosc, Bassinellod, de Mejiab, and Martinoa (2016) found that bioactive peptides derived from Carioca bean were able to reduce LOX-1 expression, which inhibited the AS process mediated by the LOX-1 signalling pathway. Another study indicated that N-acetylcysteine decreased macrophage scavenger receptor SR-A mRNA expression, which led to attenuated uptake of modified lipoproteins (Svensson, Norén, Lindmark, Ohlsson, & Hultén, 2002). Hao et al. (2015) reported that in ox-LDL-induced Raw 264.7 cells, Szeto-Schiller peptides could inhibit the expression of CD-36 and LOX-1 and decrease reactive oxygen species generation, which provided the beneficial effect of preventing foam cell formation. In further studies, it will be helpful to measure lipid accumulation with fluorescence staining (Schmitz & Farese, 2009).

According to the peptide fractions results, we speculated that the peptides with more positive charges showed higher inhibitory effects on LDL oxidation and lipid accumulation. Then, seven casein peptides with different charges, which had been identified from F3 and F4 in our previous study, were synthesised (Wang et al., 2016). In this study, the seven synthesised peptides were used to verify positive charged peptides showing strong inhibitory effects on LDL oxidation, and further analyse the effect of active amino acid amount. These 7 peptides form up to 2 groups with different basic amino acid residues but similar amino acid sequences.

Considering the peptide length varied greatly (from 3 to 8), function evaluation based on mass concentration was used to eliminate the difference of peptide length. In this case, the shorter peptides showed relatively higher bioactivities comparing with the results expressed on molar concentration basis, because of the higher molar concentration used. For example, the inhibition rate of HKEMPFPK was 2.17 times that of QPH (Table 4); however, it would be 5.79 times that of QPH based on the same molar concentration. Then, this trend would be more obvious that the inhibition rate of peptides increased with the increase of net positive charges.

The results not only confirmed the positive effects of the net positive charges of peptides but also found that the lysine-containing peptides were more effective than histidine-containing peptides (Tables 4 and 5). This may be attributed to the effective free radical scavenging abilities of positively charged peptides. Lys acted as a crucial amino acid in peptide sequences, showing efficient inhibition of LDL oxidation and foam cell

formation. Aluganti Narasimhulu et al. (2014) reported that cation peptides can inhibit Cu^{2+}-mediated LDL oxidation in a lysine-dependent manner. Studies have reported that Lys residues of the apoB polypeptides located on LDL particles interact with malondialdehyde generated from lipid oxidation, which affects the binding between modified LDL and scavenger receptors (Haberland, Fogelman, & Edwards, 1982). We speculated that lysine-containing peptides may act as a "victim" and react with malondialdehyde formed in oxidation and prevent LDL from oxidation. Moreover, CD36 (a type of scavenger receptor) binds ox-LDL at a site consisting of CD36 amino acids 118 to 182, of which the lysine residues 164/166 are indispensable for binding. Therefore, the lysine residues of peptides might act as competitors of CD36 to bind with ox-LDL, which could block scavenger receptor binding to ox-LDL and prevent foam cell formation (Kar, Ashraf, Valiyaveettil, & Podrez, 2008). However, the specific mechanism by which Lys residues inhibit these processes still needs to be investigated.

Casein is the main protein of milk, and casein-derived peptides are important dairy product components, especially as metabolites in many processed dairy products, such as various cheeses and fermented milk (Phelan, Aherne, FitzGerald, & O'Brien, 2009). It has been reported that peptides from casein have multifunctional biological activities, such as antioxidant, antimicrobial, opiate-like and osteoblast proliferation activity (Perpetuo, Juliano, & Lebrun, 2003; Reddi, Shanmugam, Kapila, & Kapila, 2016; Tang et al., 2015).

We have assessed the stability and antioxidant activity of casein peptide fractions during gastro-intestinal digestion and intestinal absorption. Caco-2 cell model was used to measure peptides absorption, in which the Caco-2 cell line forms monolayers with morphological and functional similarities to human small intestinal epithelium (Chen et al., 2016). The results showed that after Caco-2 cell absorption, TEAC and ORAC values of each fraction were reduced, but still exhibited higher values than those of GSH, which showed peptide fractions with effective bioactivity (Wang et al., 2016). Although antioxidants with scavenging activity are present in many kinds of food, our research indicated that casein peptide fractions not only significantly inhibited LDL oxidation but also attenuated cellular lipid accumulation to a certain extent, which is of great benefit for the prevention of AS. Positively charged peptides, especially those that contain Lys residues, may have great potential as natural antioxidants to prevent human AS.

Acknowledgements

The authors acknowledge financial support from the Natural Science Foundation of China (no. 31271846), the Program for New Century Excellent Talents in the University of the Ministry of Education of China (no. 2010JS078), and the fund of the Beijing Engineering and Technology Research Centre of Food Additives, Beijing Technology & Business University (BTBU).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.09.011.

References

Agudelo, R. A., Gauthier, S. F., Pouliot, Y., Marin, J., & Savoie, L. (2004). Kinetics of peptide fraction release during in vitro digestion of casein. *Journal of the Science of Food and Agriculture, 84*, 325–332.

Aluganti Narasimhulu, C., Selvarajan, K., Brown, M., & Parthasarathy, S. (2014). Cationic peptides neutralize ox-LDL, prevent its uptake by macrophages, and attenuate inflammatory response. *Atherosclerosis, 236*, 133–141.

Alvesab, N. E. G., Vasconcelosc, C. M., Bassinellod, P. Z., de Mejiab, E. G., & Martinoa, H. S. D. (2016). Digested protein isolate from fresh and stored Carioca beans reduced markers of atherosclerosis in oxidized LDL-induced THP-1 macrophages. *Journal of Functional Foods, 24*, 97–111.

Chandrasekara, A., & Shahidi, F. (2011). Bioactivities and antiradical properties of millet grains and hulls. *Journal of Agricultural and Food Chemistry, 59*, 9563–9571.

Chen, L., Lu, X., Liang, X., Hong, D., Guan, Z., Guan, Y., et al. (2016). Mechanistic studies of the transport of peimine in the Caco-2 cell mode. *Acta Pharmaceutica Sinica B, 6*, 125–131.

Chu, C. Y., Lee, M. J., Liao, C. L., Lin, W. L., Yin, Y. F., & Tseng, T. H. (2003). Inhibitory effect of hot-water extract from dried fruit of *Crataegus pinnatifida* on low-density lipoprotein (LDL) oxidation in cell and cell-free systems. *Journal of Agricultural and Food Chemistry, 51*, 7583–7588.

Clausen, M. R., Sktbsted, L. H., & Stagsted, J. (2009). Characterization of major radical scavenger species in bovine milk through size exclusion chromatography and functional assays. *Journal of Agricultural and Food Chemistry, 57*, 2912–2919.

Dávalos, A., Miguel, M., Bartolomé, B., & Fandio, R. L. (2004). Antioxidant activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection, 67*, 1939–1944.

Diaz, M., & Decker, E. A. (2004). Antioxidant mechanisms of casein-ophosphopeptides and casein hydrolysates and their application in ground beef. *Journal of Agricultural and Food Chemistry, 52*, 8208–8213.

Fernvik, E. C., Ketelhuth, D. F. J., Russo, M., & Gidlund, M. (2004). The autoantibody repertoire against copper- or macrophage-modified LDL differs in normolipidemics and hypercholesterolemic patients. *Journal of Clinical Immunology, 24*, 170–176.

Gentile, C., Allegra, M., Angileri, F., Pintaudi, A. M., Livrea, M. A., & Tesoriere, L. (2012). Polymeric proanthocyanidins from Sicilian pistachio (*Pistacia vera L.*) nut extract inhibit lipopolysaccharide-induced inflammatory response in RAW 264.7 cells. *European Journal of Nutrition, 51*, 353–363.

Guan, S., Wang, B., Li, W., Guan, J., & Fang, X. (2010). Effects of berberine on expression of LOX-1 and SR-BI in human macrophage-derived foam cells induced by ox-LDL. *American Journal of Chinese Medicine, 38*, 1161–1169.

Haberland, M. E., Fogelman, A. M., & Edwards, P. A. (1982). Specificity of receptor-mediated recognition of malondialdehyde-modified low density lipoproteins. *Proceedings of the National Academy of Sciences of the United States of America, 79*, 1712–1716.

Halliwell, B. (1997). Antioxidants and human disease: A general introduction. *Nutrition Reviews, 55*, 44–44.

Hao, S. Y., Ji, J. J., Zhao, H. T., Shang, L. C., Wu, J., Li, H. H., et al. (2015). Mitochondrion-targeted peptide SS-31 inhibited oxidized low-density lipoproteins-induced foam cell formation through both ROS scavenging and inhibition of cholesterol influx in RAW264.7 cells. *Molecules, 20*, 21287–21297.

Henriksen, T., Mahoney, E. M., & Steinberg, D. (1982). Interactions of plasma lipoproteins with endothelial cells. *Annals of the New York Academy of Sciences, 401*, 102–116.

Henriksen, T., Mahoney, E. M., & Steinberg, D. (1983). Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis, 3*, 149–169.

Itabe, H. (2009). Oxidative modification of LDL: Its pathological role in AS. *Clinical Reviews in Allergy and Immunology, 37*, 4–11.

Jialal, I., & Devaraj, S. (1996). The role of oxidized low density lipoprotein in atherogenesis. *Journal of Nutrition, 126*, 1053S–1057S.

Kaliora, A. C., Dedoussis, G. V. Z., & Schmidt, H. (2006). Dietary antioxidants in preventing atherogenesis. *Atherosclerosis, 187*, 1–17.

Kang, Y. H., Park, S. H., Lee, Y. J., Kang, J. S., Kang, I. J., Shin, H. K., et al. (2002). Antioxidant α-keto-carboxylate pyruvate protects low-density lipoprotein and atherogenic macrophages. *Free Radical Research, 36*, 905–914.

Kar, N. S., Ashraf, M. Z., Valiyaveettil, M., & Podrez, E. A. (2008). Mapping and characterization of the binding site for specific oxidized phospholipids and oxidized low density lipoprotein of scavenger receptor CD36. *Journal of Biological Chemistry, 283*, 8765–8771.

Leake, D. S., & Rankin, S. M. (1990). The oxidative modification of low-density lipoproteins by macrophages. *Biochemical Journal, 270*, 741–749.

Li, Y. W., & Li, B. (2013). Characterization of structure-antioxidant activity relationship of peptides in free radical systems using QSAR models: Key sequence positions and their amino acid properties. *Journal of Theoretical Biology, 318*, 29–43.

Liu, R. H., & Finley, J. (2005). Potential cell culture models for antioxidant research. *Journal of Agricultural and Food Chemistry, 53*, 4311–4314.

Mao, X. Y., Cheng, X., Wang, X., & Wu, S. J. (2011). Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis. *Food Chemistry, 126*, 484–490.

Megias, C., Pedroche, J., Yust, M. M., Giron-Calle, J., Alaiz, M., Millan, F., et al. (2008). Production of copper-chelating peptides after hydrolysis of sunflower proteins with pepsin and pancreatin. *Lebensmittel-Wissenschaft und-Technologie, 41*, 1973–1977.

Orsini Delgado, M. C., Nardo, A., Pavlovic, M., Rogniaux, H., Anón, M. C., & Tironi, V. A. (2016). Identification and characterization of antioxidant peptides obtained by gastrointestinal digestion of amaranth proteins. *Food Chemistry, 197*, 1160–1167.

Perpetuo, E. A., Juliano, L., & Lebrun, I. (2003). Biochemical and pharmacological aspects of two bradykinin-potentiating peptides obtained from tryptic hydrolysis of casein. *Journal of Protein Chemistry, 22*, 601–606.

Phelan, M., Aherne, A., FitzGerald, R. J., & O'Brien, N. M. (2009). Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. *International Dairy Journal, 11*, 643–654.

Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy Journal, 16*, 1306–1314.
Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry, 53*, 4290–4302.
Reddi, S., Shanmugam, V. P., Kapila, S., & Kapila, R. (2016). Identification of buffalo casein-derived bioactive peptides with osteoblast proliferation activity. *European Food Research and Technology, 242*, 2139–2146.
Rios, F. J. O., Gidlund, M., & Jancar, S. (2011). Pivotal role for platelet-activating factor receptor in CD36 expression and oxLDL uptake by human monocytes/macrophages. *Cellular Physiology and Biochemistry, 27*, 363–372.
Rival, S. G., Fornaroli, S., Boeriu, C. G., & Wichers, H. G. (2000). Caseins and casein hydrolysates. 1. Lipoxygenase inhibitory properties. *Journal of Agricultural and Food Chemistry, 49*, 287–294.
Sakanaka, S., Tachibana, Y., Ishihara, N., & Juneja, L. R. (2004). Antioxidant properties of casein calcium peptides and their effects on lipid oxidation in beef homogenates. *Journal of Agricultural and Food Chemistry, 53*, 464–468.
Salonen, J. T., Salonen, R., Seppanen, K., Kantola, M., & Suntioinen, S. (1991). Interactions of serum copper, selenium, and low density lipoprotein cholesterol in atherogenesis. *British Medical Journal, 302*, 756–756.
Sánchez-Quesada, J. L., Villegas, S., & Ordonez-Llanos, J. (2012). Electronegative low-density lipoprotein. A link between apolipoprotein B misfolding, lipoprotein aggregation and proteoglycan binding. *Current Opinion in Lipidology, 23*, 479–486.
Schmitz, G., & Farese, R. V. (2009). Lipid droplets as dynamic organelles of fat deposition and release. *Biochimica et Biophysic Acta, 1791*, 397–398.
Steinberg, D. (1997). Low density lipoprotein oxidation and its pathobiological significance. *Journal of Biological Chemistry, 272*, 20963–20966.
Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L., & Steinberg, D. (1984). Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proceedings of the National Academy of Science of the United States of America, 81*, 3883–3887.
Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *Journal of Nutritional Biochemistry, 11*, 128–131.
Svensson, L., Norén, K., Lindmark, H., Ohlsson, B., & Hultén, L. M. (2002). Inhibitory effects of N-acetylcysteine on scavenger receptor class A expression in human macrophages. *Journal of Internal Medicine, 251*, 437–446.
Tang, W. T., Yuan, H. N., Zhang, H., Wang, L., Qian, H. F., & Qi, X. G. (2015). An antimicrobial peptide screened from casein hydrolyzate by Saccharomyces cerevisiae cell membrane affinity method. *Food Control, 50*, 413–422.
Wang, B., Wang, C., Huo, Y. J., & Li, B. (2016a). The absorbates of positively charged peptides from casein show high inhibition ability of LDL oxidation in vitro: Identification of intact absorbed peptides. *Journal of Functional Foods, 20*, 380–393.
Wang, C., Li, B., Wang, B., & Xie, N. N. (2015). Degradation and antioxidant activities of peptides and zinc-peptide complexes during in vitro gastrointestinal digestion. *Food Chemistry, 173*, 733–740.
Wang, C., Wang, B., & Li, B. (2016b). Bioavailability of peptides from casein hydrolysate in vitro: Amino acid compositions of peptides affect the antioxidant efficacy and resistance to intestinal peptidases. *Food Research International, 81*, 188–196.
Xie, N. N., Wang, C., Ao, J., & Li, B. (2013). Non-gastrointestinal-hydrolysis enhances bioavailability and antioxidant efficacy of casein as compared with its in vitro gastrointestinal digest. *Food Research International, 51*, 114–122.
Xie, N. N., Liu, S. S., Wang, C., & Li, B. (2014). Stability of casein antioxidant peptide fractions during in vitro digestion/Caco-2 cell model: characteristics of the resistant peptides. *European Food Research and Technology, 239*, 577–586.
Yong, S. H., & Karel, M. (1978). Reaction of histidine with methyl linoleate: Characterization of the histidine degradation products. *Journal of the American Oil Chemists, 55*, 352–357.