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Improved RP-HPLC method for determination of bovine lactoferrin and its proteolytic degradation in simulated gastrointestinal fluids

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ABSTRACT: The objective of this study was to qualitatively and quantitatively evaluate bovine lactoferrin (bLf) and its stability using a rapid RP-HPLC method. bLf could be rapidly detected within 20 min and quantitated at levels down to 5 μg/mL, and the equation of linearity was $y = 86.10x + 178.31$ with the correlation coefficient (r^2) 0.9997. Quantitative data obtained in the present study proved the improved RP-HPLC method to be a sensitive and accurate analytical tool for bLf determination. The proteolytic cleavage of bLf in simulated human gastrointestinal fluids was further analyzed by RP-HPLC, and found to follow pseudo-first-order kinetics. The typical equation obtained by pepsin was $\log_{10}[A_t]/[A_0] = -0.03x$ ($r^2 = 0.85$), and $\log_{10}[A_t]/[A_0] = -0.01x$ ($r^2 = 0.81$) for trypsin and chymotrypsin combination. Pepsinolysis of bLf in simulated gastric fluid was relatively fast with the half-life $t_{1/2}$ 23.1 min. The digestion of bLf in simulated intestinal fluid was slower with about a 3-fold increase in half-life (69.3 min). After the complete proteolysis of bLf, small cleaved peptide fragments were fully separated and identified by RP-HPLC. The proteolytic study indicated that this validated RP-HPLC was able to evaluate bLf stability though monitoring the derivatization products. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: bovine lactoferrin (bLf); RP-HPLC; proteolysis; pseudo-first-order kinetics; half-life $t_{1/2}$

Introduction

Lactoferrin (Lf) is a ~80 kDa member of the transferrin family of iron binding glycoprotein (Metz-Boutique *et al.*, 1984). Lf is predominantly present in the milk of many species, including human, pig, horse, cow, buffalo, sheep, camel, mouse, goat, elephant and alpaca (Baker, 2005). Large-scale manufacturing of bovine lactoferrin (bLf) at high purity was established over 30 years ago, using bovine skim milk and whey as the source. Many studies have already reported that Lf features in a variety of biological roles such as anti-bacterial (Takakura *et al.*, 2003), anti-viral (Murphy *et al.*, 2001), anti-oxidative (Sandomirsky *et al.*, 2003), anti-tumor (Tsuda *et al.*, 2002), anti-inflammatory (Hayashida *et al.*, 2004) and immunomodulatory (Yamauchi *et al.*, 1998). Furthermore, a study carried out by Cornish *et al.* (2006) reported the first evidence that oral administration of bLf exerts combined anabolic effects on osteoblast and inhibitory effects on osteoporosis. According to these biological functions, oral administration of Lf is considered to be of benefit to human and animal health.

The enzyme linked immunosorbent assay can be used to determine Lf concentration at ng/mL levels (Yoshise *et al.*, 2007). However, this very sensitive technique suffers from high cost. Therefore for routine use an easy and low cost detection method is required to determine Lf concentration in biological fluid or preparations. High-performance liquid chromatography (HPLC) in its various modes has become the pivotal technique in the isolation and characterization of peptides and proteins (Aguilar, 2004). In particular, reverse-phase high-performance liquid chromatography (RP-HPLC) has been utilized to identify casein variants and whey protein variants in bovine milk (Visser *et al.*, 1991).

Lipid-based carriers can be used as oral delivery systems to protect free proteins from proteolysis in the gastrointestinal tract and thus enhance their therapeutic action. Liposomalization of Lf has been shown to improve its pharmacological effects through oral administration (Roseanu *et al.*, 2010). In addition, solid lipid nanoparticles (SLNs) have been successfully developed to load with other proteins such as calcitonin (García-Fuentes *et al.*, 2005) and insulin (Zhang *et al.*, 2006). However, the encapsulating systems must ensure the delivery of Lf in its native folding state to sustain its biological activity. Entrapment efficiency of molecules in such potential carriers is determined based on separation of free molecules from molecule-loaded carriers by centrifugation and subsequent quantification using an appropriate HPLC

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Abbreviations used: bLf, bovine lactoferrin; DCM, dichloromethane; Lf, lactoferrin; PBS, phosphate-buffered saline; SLN, solid lipid nanoparticles; TFA, trifluoroacetic acid.

assay (Venkateswarlu and Manjunath, 2004). For protein molecules, HPLC also has been widely employed to evaluate their stability, since it allows the identification of the peptide fragments derived from protein transformation (Emami *et al.*, 2009). However, the challenge of analyzing protein samples using RP-HPLC is the selection of the initial separation conditions and subsequent optimization of the appropriate experimental parameters.

In the present work, the aim was to improve and validate the existed RP-HPLC method for a rapid determination of bLf. In particular, blank liposomes and blank SLNs were spiked with bLf to investigate their influences on bLf separation as well as peak integrity. This kind of assay specificity would provide an indicator for bLf encapsulated delivery systems in our further study. Finally, various proteolytic enzymes were applied with bLf to investigate their influences on the stability of bLf by the validated RP-HPLC method.

Materials and methods

Materials

Standard bLf from bovine colostrums, trifluoroacetic acid (TFA; Puriss p.a., for HPLC, ≥99% GC), L-α-phosphatidylcholine from soybean, cholesterol (Sigma, ≥99%), stearic acid (grade I, ≥99% GC), pepsin, trypsin and α-chymotrypsin were purchased from Sigma Aldrich (USA). HPLC-grade acetonitrile and Tween® 80 for synthesis were purchased from Merck (USA). Soybean lecithin was purchased from BDH (UK). Poloxamer 188 (Lutrol F68) was purchased from BASF (Germany). All other reagents and chemicals were of analytical grade.

Methods

Instrumental conditions. RP-HPLC analysis was performed using an Agilent 1100 HPLC system (Germany) with a diode array UV detector G1315B. Data acquisition was provided through the Agilent ChemStation software (revision A.10.02). Absorbance was measured at the wavelength 210 nm. Separation was performed on a C_{18} HPLC column (Jupiter 5u C_{18} 300R, 250 × 4.6 mm, 5 μm, Pheonomenex) fitted with a C_{18} guard column (10 × 3.0 mm).

A constant flow rate of 0.5 mL/min was used. The injection volume was 50 μL and the column temperature was maintained at 37°C. Mobile phase A consisted of 0.1% TFA in water and acetonitrile (95:5 v/v). Mobile phase B consisted of 0.1% TFA in water and acetonitrile (5:95 v/v). Elution started with an isocratic elution from 35% mobile phase B for 1 min followed by a linear gradient to 60% mobile phase B for 19 min, and a final 10 min (post time) for equilibration.

Sample preparation. A 2 mg aliquot of bLf was accurately weighted into 10 mL volumetric flask to which distilled water (5 mL) was added followed by sonication (Bandelin sonorex, Germany) for 5 min. Distilled water was added to a 10 mL volume to give a final stock solution concentration of 0.2 mg/mL bLf. A series of 5, 15, 25, 50, 75 and 100 μg/mL bLf standards were prepared by transferring 25, 75, 125, 250, 375 and 500 μL, respectively, of the stock solution into separate graduated microtubes and diluting to 1 mL with mobile phase A. Standard solutions of bLf were stored at 4°C for no more than 1 h before being discarded.

Assay validation

Specificity. The specificity was demonstrated by comparing the peak area of standard bLf, bLf-spiked blank liposomes and bLf-spiked blank SLNs. The HPLC chromatograms were visually inspected for interfering peaks from endogenous substances. Blank liposomes were prepared by reverse-phase evaporation method as described by Szoka and Papahadjopoulos (1978) with modifications. Briefly, L-α-phosphatidylcholine (32 mg) and cholesterol (8 mg) were accurately weighed into a round-bottom flask and dissolved into chloroform–methanol mixture (4:1 v/v; 10 mL). A thin lipid film was formed on the inner side of the flask by evaporating the organic solvents under vacuum at 40°C, using a rotary evaporator (R215 rotavapor, Buchi, Switzerland) followed by nitrogen flushing to remove residue solvents. The lipid film was redissolved in diethyl ether (10 mL), in which the reversed-phase vesicles were formed. Then 5 mL of phosphate-buffered saline (PBS; 0.1 M, pH 7.4) containing Tween 80 (10 mg) was mixed with the organic phase. The w/o emulsion was sonicated using a probe sonicator (UP200S, Hielscher, Canada) for 5 min at frequency of 0.5 cycles and 50% amplitude in an ice bath. The w/o emulsion was then placed on the rotary evaporator again, and a gel was obtained following evaporation of diethyl ether under atmospheric pressure at 40°C. The resultant gel was broken to form the liposomes by further vigorous rotary evaporation. Finally, another 5 mL of PBS (0.1 M, pH 7.4) was added with gentle vortex and the remaining fumes of diethyl ether were evacuated by nitrogen flushing. A final liposomes suspension aliquot of ~10 mL was stored at 4°C in a refrigerator.

Blank SLNs were prepared by emulsion-solvent evaporation method as described by Sjostrom and Bergenstahl (1992). In brief, the organic phase was formed by dissolving 32 mg stearic acid and 8 mg lecithin in 10 mL of mixture of acetone–dichloromethane (DCM) (1:4 v/v). Then 5 mL of PBS (0.1 M, pH 7.4) was added slowly to the organic phase in a bath sonicator. The mixture was sonicated by probe sonicator for 5 min at frequency of 0.5 cycles and 50% amplitude in an ice bath. The formed w/o primary emulsion was immediately poured onto 25 mL of PBS buffer (0.1 M, pH 7.4) containing 10 mg poloxamer 188 continuously stirred at 500 rpm and DCM allowed to evaporate for ~6 h. An SLN suspension aliquot of ~30 mL was collected and stored at 4°C in a refrigerator.

Prior to HPLC determination, blank liposome and blank SLNs were passed three times through a 0.45 μm syringe filter (Sartorius, Germany). The obtained solution was then diluted approximately 4.5-fold with mobile phase A for liposomes and 2-fold for SLNs. The bLf was accurately weighed and dissolved into diluted liposome solution and diluted SLN solution to give a final concentration of 50 μg/mL bLf.

Linearity. A standard curve was obtained by plotting the peak area against six concentrations of the standard bLf solution ($n = 3$) in the range of 5–100 μg/mL. The slope, *y*-intercept and linearity of the curve were evaluated by linear regression analysis.

Sensitivity. Sensitivity was determined by evaluation of limit of detection (LOD) and limit of quantification (LOQ). The LOD was defined as a signal-to-noise ratio of 3 (3 S/N), and the LOQ was defined as a signal-to-noise ratio (S/N) of 10.

Repeatability. Repeatability was evaluated by calculating the percentage of relative standard deviation (RSD) for results of five

sample solutions of bLf at low, medium and high concentration levels (15, 50 and 75 μg/mL) on the same day.

Recovery. Recovery of bLf was calculated by comparing the results for the directly injected bLf standards (10, 60 and 100 μg/mL) with the concentration of bLf in the spiked samples at 10, 60 and 100 μg/mL.

Accuracy and precision. Intra-day accuracy and precision were determined by three replicate analyses of 10, 60 and 100 μg/mL standard solutions ($n = 3$) on the same day. Inter-day accuracy and precision were determined by the analysis of the same three standard solutions on three consecutive days. Precision was calculated in terms of RSD of the results. Percentage accuracy was calculated by dividing the means of measured concentrations by their true concentrations.

Proteolysis of lactoferrin

The protein substrate was prepared by dissolving bLf into PBS buffer (0.1 M, pH 7.4; 2 mg/mL). bLf cleavages with pepsin, trypsin and α-chymotrypsin were conducted to simulate the human gastrointestinal digestion process. Proteolysis of bLf in simulated gastric fluid was simulated by adding pepsin at pH 2 (adjusted with 1 M HCl). Trypsin and α-chymotrypsin (1:1) at pH 7.4 (adjusted with 1 M NaOH) simulated the intestinal fluid. The enzyme–substrate mixture was incubated at 37°C with in a ratio of 1/20,000 (w/w). Samples were taken at various times (0, 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 min). Subsequently, the samples were placed in a boiling water bath for 5 min to inactive enzyme activity, and centrifuged at 13,400 rpm for 5 min at 4°C. The supernatants were applied to RP-HPLC system for separation of proteolytic fragments under step gradient conditions as described above.

Results

Chromatography and specificity

Specificity was assessed by comparing the chromatograms of the standard bLf with the corresponding spiked samples (Fig. 1). The standard bLf exhibited a sharp and symmetric peak at 11.600 min, well separated from the solvent front. The spiked bLf samples (with blank liposomes and blank SLNs) maintained the peak corresponding to bLf at the retention times of 11.596 and 11.552 min, respectively. Under these chromatographic conditions, interferences from the matrix components on the retention time of standard bLf were not observed.

Linearity, sensitivity and repeatability

The assay exhibited linearity between the mean peak area (y) and the corresponding concentration of bLf (x), over 5–100 μg/mL. The typical equation obtained was $y = 86.10x + 178.31$ ($n = 3$). The results of linear regression analysis showed that the correlation coefficient (r^2) of the standard curve was 0.9997. The LOD (found at 3 S/N) and LOQ (found at 10 S/N) were 1 and 5 μg/mL, respectively. The repeatability was obtained from the results from replicate measurements ($n = 5$) of each sample and expressed as the RSD. The mean RSD was 2.05%, and demonstrated that the developed RP-HPLC assay was reproducible.

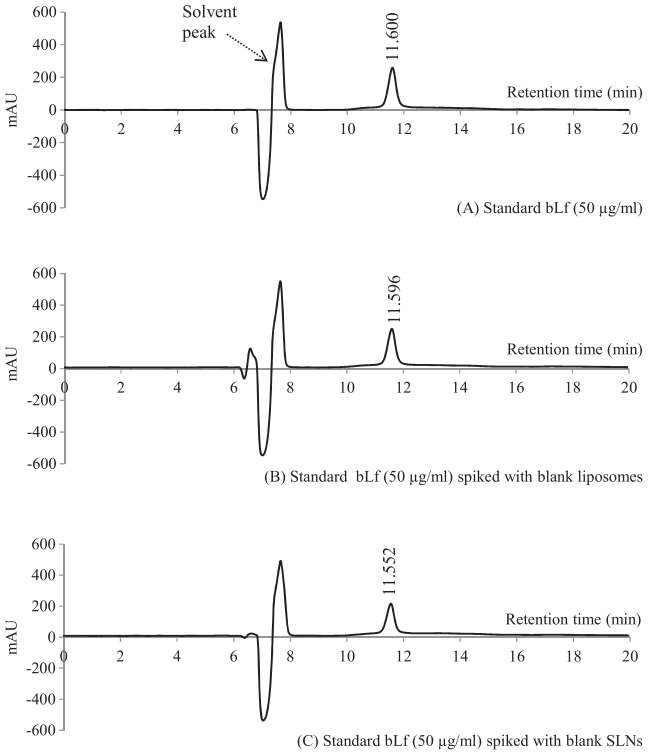

Figure 1. Chromatograms of standard bovine lactoferrin (bLf; 50 μg/mL) (A), and the bLf samples which were spiked with blank liposomes (B) and blank solid lipid nanoparticles (C) at 210 nm.

Recovery, accuracy and precision

The intra-day and inter-day accuracy and precision of the assay were determined by three replicate measurements of each sample at low, medium and high concentration. Results are summarized in Table 1. In this assay, the mean intra-day and inter-day precision were 2.11 and 1.84% respectively. The mean intra-day and inter-day accuracies were 101.3 and 100.9% respectively, which demonstrated that the measured values agreed well with the true values. In all cases, the mean recovery of bLf at each concentration spiked with blank liposomes and SLNs was >95%. As evidenced by the results above, the RP-HPLC assay for bLf determination was reliable, accurate and precise.

Lactoferrin degradation

The effect of proteolytic enzymes on the integrity of bLf and the corresponding peptide mapping was determined by RP-HPLC. Pepsinolysis of bLf was shown in Fig. 2, which illustrates an accumulation of degraded bLf fragments. The peak corresponding to bLf decreased by approximately 89% after 5 min of incubation. Complete cleavage of bLf with pepsin occurred after 90 min of incubation at pH 2.0. As a result, several derivation products were generated. Such digestion products separated into four major peaks (Fig. 2) and identified by RP-HPLC. Similarly, trypsin/chymotrypsin digested bLf efficiently (Fig. 3), leaving only 8.4% intact bLf after 15 min of incubation. However, the complete degradation of bLf with trypsin/chymotrypsin was observed after 180 min, with the consequence of three major peptides peak generated. Owing to the high sensitivity of the developed RP-HPLC method for this study, it was able to isolate each peptide fragment from bLf during its proteolysis.

Table 1. Intra- and inter-day precision and accuracy of HPLC assay for determination of bovine lactoferrin ($n = 3$ at each concentration)

Concentration (μg/mL)	Average calculated concentration (μg/mL)	Accuracy (%)	Precision (%), RSD
Intra-day			
10	9.89 ± 0.10	98.9	1.01
60	60.84 ± 1.76	101.6	2.89
100	103.25 ± 2.51	103.3	2.43
		Mean 101.3	2.11
Inter-day			
10	10.05 ± 0.41	100.5	4.08
60	60.04 ± 0.74	100.1	1.23
100	101.99 ± 0.21	102.0	0.21
		Mean 100.9	1.84

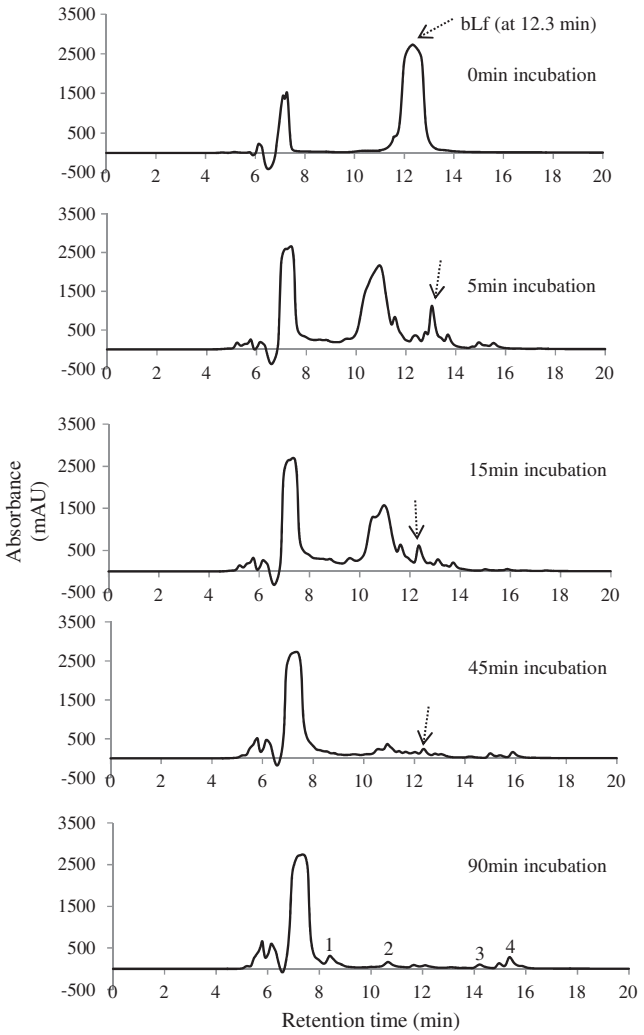

Figure 2. RP-HPLC chromatograms of bLf (initial concentration, 2 mg/mL) at retention time of 12.3 min after 0, 5, 15, 45 and 90 min of pepsin digestion incubated at 37°C. Major fractions of peaks 1–4 were observed after the incubation of 90 min.

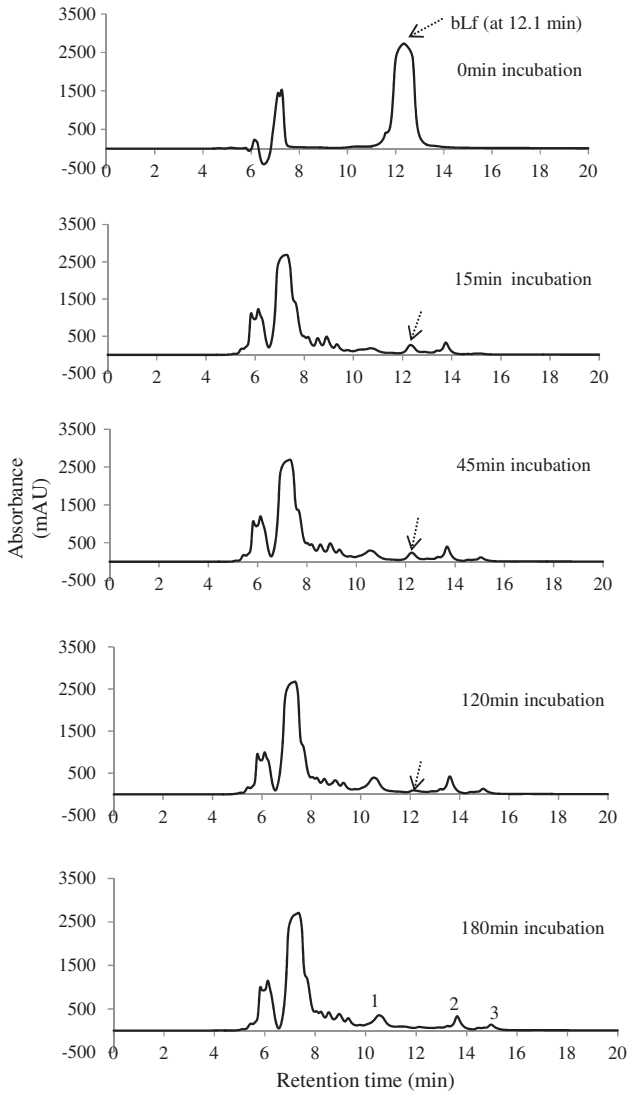

Figure 3. RP-HPLC chromatograms of bLf (initial concentration, 2 mg/mL) at a retention time of 12.1 min after 0, 15, 45, 120 and 180 min of trypsin/chymotrypsin digestion incubated at 37°C. Major fractions of peaks 1–3 were observed after an incubation of 180 min.

The kinetics of bLf proteolysis was described by HPLC analysis by following the decrease in the concentration of the residual intact bLf as a function of the incubation time. The resulting kinetics (Fig. 4) followed a pseudo-first-order rate, described by the following equation: $\log_{10}[A_t]/[A_0] = -k.t$, where A_t is the concentration of the intact bLf (μg/mL) at the incubation time t (min), A_0 is the initial concentration of bLf, and k is the pseudo-first-order rate constant. The typical equation obtained

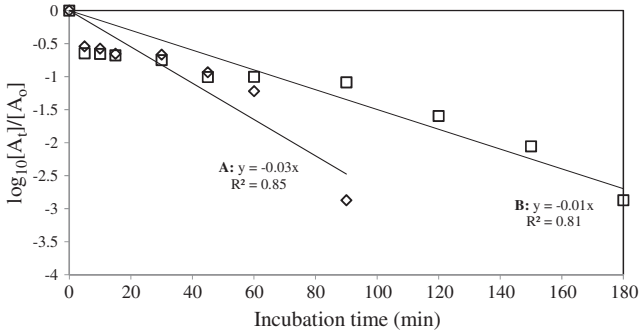

Figure 4. Kinetic analysis of proteolytic digestion of bLf incubated with (A) pepsin and (B) trypsin: α-chymotrypsin (1:1) at 37°C ($n = 3$).

by pepsin is $\log_{10}[A_t]/[A_0] = -0.03x$ ($r^2 = 0.85$), and $\log_{10}[A_t]/[A_0] = -0.01x$ ($r^2 = 0.81$) for trypsin and chymotrypsin combination. As the rate constant k was found, the half-life $t_{1/2}$ was calculated from the equation: $t_{1/2} = \ln 2/k$. The $t_{1/2}$ with pepsin digestion equalled 23.1 min, while $t_{1/2}$ incubated with trypsin and chymotrypsin was 69.3 min. In simulated gastric fluid, acid hydrolysis at pH 2 conjugated with pepsin cleavage had a significant effect on the proteolysis of bLf, resulting in rapid degradation. In comparison, only proteolytic enzymes contributed to the predominant degradation of bLf in simulated intestinal conditions.

Discussion

The RP-HPLC in this study was designed to present the protein profile with high resolution and sensitivity and allow bLf to be eluted quickly from the column. The separation of bLf was based on a C_{18} column. However, the optimum column for protein separation had C_3–C_8 packing (Boyes and Alpert, 1997). Many significant studies have reported utilizing RP-HPLC with a C_4 or C_8 column to determine Lf derived from various sources, such as rabbit tears, bovine milk and goat milk (Zhou *et al*., 2003; Wedholm *et al*., 2006; Drackova *et al*., 2009). Recently, a C_{18} column has been applied for bLf determination (Krol *et al*., 2010). Our RP-HPLC method using C_{18} column showed high sensitivity, accuracy and reproducibility, which is in good agreement with these reports. However, bLf was eluted quickly at ~12 min, which is a shorter run time than that reported by Zhou *et al*. (2003) (~28.5 min) and Hoek *et al*. (1997) (~20 min). The improved RP-HPLC should be an ideal analytical method for routine daily bLf determination.

The investigation of proteolysis of bLf in simulated gastrointestinal tract was based on the improved RP-HPLC method. bLf was proteolytically cleaved to generating small peptide fragments. These small peptide fragments were easily separated and identified by RP-HPLC. We observed that the solvent peak area was increasing slowly during the digestion process (Figs 2 and 3). That is because small hydrophilic peptides generating from bLf degradation eluted very early in the chromatogram, perhaps even as part of the solvent peak (Hojrup, 2004). Furthermore, many potential small molecule peptides derived from Lf during the degradation were successfully isolated and characterized by RP-HPLC with a C_{18} column (Hoek *et al*., 1997), and this implied that the evaluation of Lf stability can be achieved by monitoring the degradation products using RP-HPLC. As a result, the HPLC analysis in this work provided the necessary resolution to evaluate qualitative and quantitative changes in bLf.

The kinetics results showed that the nonprotected bLf had a short lifetime against proteolysis in simulated gastrointestinal fluids. In other words, bLf cannot avoid enzyme proteolysis and acid hydrolysis to maintain its actual protein structure, resulting in poor bioavailability in human gastrointestinal tract. Many reports have demonstrated that various therapeutic proteins carried by lipid-based systems can enhance their bioavailability by improving their stability, permeability and giving a target ability and time release (Bernkop-Schnurch and Schmitz, 2007; Tan *et al*., 2010). When bLf is encapsulated into a lipid-based delivery system, understanding the ability of bLf to interact with the lipid layer is essential. For SLNs, protein molecule insertion into the monolayers leads to increased surface pressure (Busquets *et al*., 2003). Similarly, proteins have the capacity to bind to phospholipid bilayers of liposomes, and penetrate the lipid matrix (Wickner, 1979). As a result, the binding and penetration of protein into the lipid layer can markedly alter the biophysical properties of these lipid-based delivery systems, such as expansion and fluidization of the lipid layer, alteration of the enthalpy and phase transition temperature, and increase in permeability to ions and small molecules (Busquets *et al*., 2003). In this study, there was no differences in peak shape and retention time between the chromatograms of standard bLf and bLf spiked with liposomes and SLNs (Fig. 1). This implied that the lipid matrix in liposomes or SLNs did not affect the chemical integrity of bLf. Moreover, the absence of additional peaks indicated that no degradation chemicals were formed during the process. Delivery systems that are not only able to enhance the bioavailability of bLf, but also allow the preservation of its integrity have been the subject of much attention.

Conclusion

In summary, the improved and validated C_{18}-RP-HPLC method is a rapid, sensitive, reproducible and specific assay for determination of bLf. bLf well maintained its integrity whether it was spiked with liposomes or SLNs. It is able to provide a quantitative analysis of bLf, especially by monitoring any peptides derived from bLf during the degradation. Free bLf is highly susceptible to proteolysis in simulated human gastrointestinal fluids. Protection of bLf against degradation is of great significance for its potential use in enhancing its absorption via oral administration. bLf, as a multifunctional therapeutic protein, combined with lipid-based delivery systems, needs to be further studied in future investigations.

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