Analytica Chimica Acta 829 (2014) 33–39

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Determination of bovine lactoferrin in dairy products by ultra-high performance liquid chromatography–tandem mass spectrometry based on tryptic signature peptides employing an isotope-labeled winged peptide as internal standard

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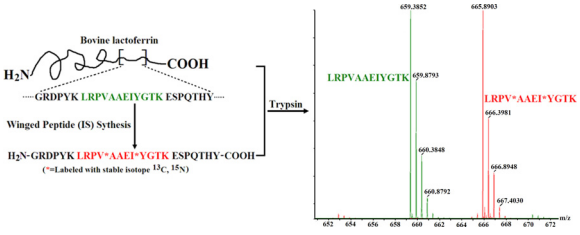
HIGHLIGHTS

- A UHPLC–MS/MS method for quantification of bovine lactoferrin was developed.
- Tryptic fragment LRPVAAEIYGTK was chosen as signature peptide of bovine lactoferrin.
- A winged peptide containing isotopically-labeled signature peptide was designed as internal standard.
- The method for determining lactoferrin does not discriminate between the different forms of lactoferrin.
- Meet the growing demand to quantify bovine lactoferrin in different dairy products.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form 9 April 2014
Accepted 11 April 2014
Available online 24 April 2014

Keywords:
Bovine lactoferrin
Ultra high-performance liquid chromatography–tandem mass spectrometry
Signature peptide
Tryptic digestion
Dairy products

ABSTRACT

A new and sensitive determination method was developed for bovine lactoferrin in dairy products including infant formulas based on the signature peptide by ultra high-performance liquid chromatography and triple-quadrupole tandem mass spectrometry under the multiple reaction monitoring mode. The simple pretreatment procedures included the addition of a winged peptide containing the isotope-labeled signature peptide as internal standard, followed by an enzymatic digestion with trypsin. The signature peptide was chosen and identified from the tryptic hydrolyzates of bovine lactoferrin by ultra high-performance liquid chromatography and quadrupole-time-of-flight tandem mass spectrometry based on sequence database search. Analytes were separated on an ACQUITY UPLC BEH 300 C18 column and monitored by MS/MS in seven minutes. Quantitative result bias due to matrix effect and tryptic efficiency was corrected through the use of synthetic isotope-labeled standards. The limit of detection and limit of quantification were 0.3 mg/100 g and 1.0 mg/100 g, respectively. Bovine lactoferrin within the concentration range of 10–1000 nmol L^{-1} showed a strong linear relationship with a linear correlation coefficient (r) of >0.998. The intra- and inter-day precision of the method were RSD < 6.5% and RSD < 7.1%, respectively. Excellent repeatability (RSD < 6.4%) substantially supported the application of this method for the determination of bovine lactoferrin in dairy samples. The present

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http://dx.doi.org/10.1016/j.aca.2014.04.025

method was successfully validated and applied to determination of bovine lactoferrin in dairy products including infant formulas.

1. Introduction

Lactoferrin is an iron-binding glycoprotein and classified as a member of transferrin family. It predominantly exists in mammalian milk including human and bovine milk [1]. The published literature indicates that lactoferrin plays multiple biological and pharmacological roles such as intestinal iron uptake and regulation, antibacterial, antiviral, antioxidant and anti-inflammatory activity, immune response, suppression of tumors growth and metastasis [2–7]. Lactoferrin content is considered as species and lactation period-dependent. Lactoferrin is present in human milk and colostrums with significantly higher levels compared to the bovine equivalent [1,8]. These factors stimulated an increasing trend of lactoferrin supplementation in foods for infants and an increasing commercial interest in exploiting the therapeutic value of lactoferrin throughout the world [9]. However, the efficacy of lactoferrin supplementation depends on the manufacturing process because thermal exposure may compromise protein structure and function [10]. These changes of protein structure may affect the applicability and accuracy of the traditional analytical methods. For nutritional assessment and quality control, it is necessary to establish reliable analytical methods for determination of lactoferrin at endogenous level in raw milk, at fortified level in lactoferrin-fortified products and at pharmaceutical level in milk protein isolates.

Currently, analytical techniques for the determination of lactoferrin are reported using the methods of immunochemical techniques [11,12], enzyme linked immunosorbent assay (ELISA) [13,14], reversed phase high-performance liquid chromatography (RP-HPLC) [15–17], surface plasmon resonance (SPR)-based immunosensors and capillary electrophoresis (CE). The immunodiffusion techniques have inherently poor precision and low sensitivity inspite of its easy operation and simple instrumentation. ELISA methods are more selective and sensitive, but their effectiveness depends on the quality of antigen and the antibodies. Furthermore, the potential modification of the target proteins as antigen during the manufacturing process of food and dairy may affect the binding affinity of the antigen and antibodies, which may further lead to false-negative or underestimated results in ELISA analysis. Recent developments in label-free, real-time optical biosensor techniques based on SPR-immunoassay have been used for the analysis of lactoferrin in milk and infant formulas [10,18–21]. However, SPR-based biosensor immunoassays are critically influenced by temperature, sample components and specificity of reversible interaction between antibody and antigen. RP-HPLC methods using a gradient elution has been reported for the determination of lactoferrin in goat milk, bovine whey samples and simulated gastrointestinal fluids, but they suffered from insufficient resolution and sensitivity for analyzing lactoferrin in low concentration. Furthermore, lactoferrin of three different forms (apo-, native- and holo-lactoferrin) because of the presence or absence of iron in different environmental conditions might affect their physicochemical properties [22], which in turn might affect their separation performance. Compared to other analytical techniques, CE is widely accepted to have advantages in protein analysis. It was employed to determining bovine lactoferrin in cheese whey concentrates and infant formulas [23,24]. Nevertheless, analysis of bovine lactoferrin using CE methods is very difficult to achieve because of poor reproducibility, low sample recovery and poor separation of lactoferrin from other whey proteins. To the best of our knowledge, thus far, neither official and confirmatory methods nor certified reference materials could be used to support a harmonized approach to the quantitative analysis of bovine lactoferrin in dairy products, especially in infant formulas.

In recent years, liquid chromatography–mass spectrometry (LC–MS) techniques, which combine high selectivity and sensitivity with accurate quantification, have been employed to characterize, identify, or quantify proteins on the basis of entire proteins or tryptic peptides. The analysis of major milk proteins including casein, β-lactoglobulin and α-lactalbumin has been investigated with LC–MS [25–27], but the quantitative determination of bovine lactoferrin was not reported.

In the present work, the aim was to develop and validate a simple, robust, sensitive and nonimmunological method for the rapid quantification of bovine lactoferrin based on specific peptide. The analytical procedure encompasses a simple enzymatic digestion of samples spiked with a winged peptide as internal standard to evaluate the digestion efficiency. Centrifugation and filtration are used to remove the insoluble residues after tryptic digestion. A signature peptide is selected from the tryptic lactoferrin solution as the representative of bovine lactoferrin protein. The isotopically-labeled signature peptide from the tryptic winged peptide is employed as the actual isotopically-labeled internal standard of the lactoferrin signature peptide during the quantitative analysis. Subsequent analysis is performed by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) in the multiple reaction monitoring (MRM) mode under the positive ionization mode. The contents of bovine lactoferrin in dairy products are calculated based on the equimolar relationship between lactoferrin protein and lactoferrin signature peptide. Recoveries, precision and measurement uncertainty were evaluated by replicate analysis and the satisfactory results were achieved. Finally, the validated LC–MS/MS method was applied to the determination of bovine lactoferrin contents in various dairy products including infant formulas and whey protein concentrates.

2. Materials and methods

2.1. Chemicals

Ammonium bicarbonate (NH_4HCO_3), dithiotheritol (DTT), iodoacetamide (IAA) and calcium chloride ($CaCl_2$) were analytical grade and purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid (FA) and acetonitrile (ACN) of HPLC grade were obtained from Merck (Darmstadt, Germany). Lactoferrin from bovine milk (≥85%), Fmoc-Val-OH-$^{13}C_5$,^{15}N, Fmoc-Ile-OH-$^{13}C_6$,^{15}N and Fmoc-Leu-OH-$^{13}C_6$,^{15}N (98% isotopic enrichment) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sequencing grade modified trypsin was from Worthington Biochemical Corporation (Freehold, NJ, USA). All chemical agents were prepared using 50 mM NH_4HCO_3 and without further purification. Ultrapure water was obtained by a Milli-Q Gradient A 10 water purification system (Millipore, Bedford, MA, USA) during all the experiments.

2.2. Synthetic peptide standards

The signature peptide LRPVAAEIYGTK, VDSALYLGSR (corresponding to amino acid residues 93–104 and 333–342 of bovine lactoferrin, respectively), stable isotope-labeled signature peptide LRPV*AAEI*YGTK (V*, Val-OH-$^{13}C_5$,^{15}N; I*, Ile-OH-$^{13}C_6$,^{15}N),