JARQ 59 (1), 55-60 (2025) https://www.jircas.go.jp

Quantitative Analysis of Bovine and Caprine Alpha-S1-Caseins in Milk by Liquid Chromatography/Tandem Mass Spectrometry

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Abstract

Bovine milk is one of the leading causes of childhood food allergies. Alpha-S1-casein (aS1CN) is a significant allergen and the most abundant protein in bovine milk. In contrast, many Swedish Landrace goats have been reported to have a mutation that prevents the production of aS1CN in milk. In this study, quantitative analysis of bovine and caprine aS1CNs was performed using liquid chromatography/tandem mass spectrometry (LC/MS/MS), measuring a shared proteotypic peptide (YLGYLEQLLR) between these proteins. The concentration of caprine aS1CN in caprine milk was significantly (P < 0.01) lower than that of bovine aS1CN in bovine milk. Similarly, in comparison between bovine and caprine milk powders, caprine aS1CN showed a considerably lower content than bovine aS1CN. Although substantially lower than bovine aS1CN, caprine aS1CN was detected in all caprine milk and milk powders.

Discipline: Food
Additional key words: cow, goat, protein, proteotypic peptide, targeted proteomics

Introduction

Casein is a collective name defining a family of secreted calcium (phosphate) binding phosphoproteins in mammalian milk. These proteins account for 80% of total bovine milk protein and 2.75% of total milk ingredients. Caseins in bovine milk are composed of alpha-S1-casein (aS1CN), alpha-S2-casein, beta-casein, and kappa-casein (Głąb & Boratyński 2017), which have hydrophilic and hydrophobic regions. Because of their highly hydrophobic properties, these caseins are stabilized by forming a supramolecular casein micelle structure. These micelles heterogeneously contain four types of casein and calcium phosphate. Caseins are used as ingredients in diverse food products to control their physical properties, such as foaming, emulsification, and texture, and to enhance their nutritional value (Ranadheera et al. 2016, Sadiq et al. 2021). Caseins have been widely investigated due to their commercial significance and are considered as one of the best-known food protein systems (Fox & Brodkorb 2008).

aS1CN accounts for 40% of total caseins and is the most protein among the four types of caseins in bovine milk. This casein is composed of 199 amino acids, contains a large amount of proline residues, and lacks disulfide bonds (Geiselhart et al. 2021). The proteolytic activity of lactic acid bacteria on caseins produces small bactericidal and bacteriostatic peptides. Hydrolysis of aS1CN generates isracidin, which inhibits the growth of *Staphylococcus aureus*. Alpha-casozepine, a peptide derived from aS1CN, shows an anxiolytic effect (Pessione & Cirrincione 2016). Guesdon et al. (2006) highlighted the sleep-promoting effect of bovine aS1CN tryptic hydrolysate on rats under chronic stress. Moreover, bovine aS1CN tryptic hydrolysate improved sleep quality in 32 subjects aged between 25 and 40 (De Saint-Hilaire et al. 2009). Ingestion of bovine aS1CN tryptic hydrolysate and L-theanine has also been reported to increase sleepiness before bedtime and enhance sleep quality in middle-aged women (Matsuura et al. 2016). These reports indicate that products derived from bovine aS1CN have positively affected health.

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Received 19 February 2024; accepted 17 April 2024.

On the other hand, bovine milk is one of the major etiologies of childhood food allergies. More than 50% of the patients with bovine milk protein allergies are sensitized to four types of casein, beta-lactoglobulin, and alpha-lactalbumin (Calamelli et al. 2019). Interestingly, human milk does not contain aS1CN, which is a major allergen and the most abundant protein in bovine milk. Liu & Sathe (2018) presented that the numbers of T-cell and B-cell epitopes of bovine aS1CN archived in the Immune Epitope Database (IEDB, https://www.iedb.org/) were 39 and 204, respectively. It is common to enzymatically degrade allergenic proteins into peptides to produce hypoallergenic products. Fermented milk products such as yogurt and cheese benefit milk-allergic patients because allergenic proteins are decomposed (Geiselhart et al. 2021, Głąb & Boratyński 2017). If milk does not contain a particular allergenic protein, it would be appealing to individuals with an allergy to this protein.

Swedish Landrace goats are frequent carriers of a mutation that cannot produce aS1CN in milk. Several variants of the aS1CN gene (CSN1S1) relating to different content of aS1CN in caprine milk have been classified into high, medium, low, and non-expression (null or zero variant) types (Johansson et al. 2015). Caprine milk lacking aS1CN is expected to allow aS1CN-sensitized allergic patients to drink it. In the paper (Nakashima et al. 2020) about the comparison of the components in caprine and bovine milk obtained in Japan, the qualitative analysis of casein fractions using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the concentration of aS1CN in caprine milk was significantly lower than that in bovine milk. However, this study reported a qualitative comparison analysis between one sample of bovine milk and one sample of caprine milk and did not provide quantitative data for these milk samples.

In mass spectrometry (MS) proteomics, a proteotypic peptide is defined as a unique peptide that is generated from a targeted protein by trypsin and is easily detected by MS. Hence, the molar amount of the target protein in a sample can be obtained from the molar amount of the proteotypic peptide determined by liquid chromatography/tandem mass spectrometry (LC/MS/MS) (Anderson 2010). In this study, I developed a method for comparing bovine and caprine aS1CN content based on LC/MS/MS measuring a shared common proteotypic peptide between these aS1CNs. The amounts of aS1CNs in commercially available bovine and caprine milk in Japan were compared using this method.

Materials and methods

1. Materials

Ammonium bicarbonate, dithiothreitol, and trichloroacetic acid were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Guanidinium chloride and β-mercaptoethanol were purchased from Sigma-Aldrich (St. Louis, USA). Iodoacetamide was purchased from FUJIFILM Wako Pure Chemical Corporation and Sigma-Aldrich. Trypsin (MS grade) was purchased from Thermo Fisher Scientific (Waltham, USA). Acetonitrile (LC/MS grade), formic acid (LC/MS grade), and water prepared using a Milli-Q water purification system (Merck, Darmstadt, Germany) were used for LC/MS/MS measurements. The peptide YLGYLEQLLR was commercially synthesized by GenScript Biotech Corporation (Nanjing, China). Three commercial brands of bovine milk (Cows A, B, and C), three caprine milk (Goats D, E, and F), two bovine (Cows G and H) and two caprine (Goats J and K) whole milk powders, and one bovine (Cow I) and one caprine (Goat L) skimmed milk powders were purchased from local and online stores.

The amino acid sequences of bovine aS1CN (UniProt accession number P02662) and caprine aS1CN (UniProt accession number P18626) were obtained from UniProt (https://www.uniprot.org/). Trypsin digestion *in silico* of these proteins was conducted using the web-based software PeptideCutter (https://web.expasy.org/peptide_cutter/) to predict the cleavage probability of each potential cleavage site in the protein sequences.

2. Sample preparation

Milk proteins were extracted using a modified protocol from a previous study (Ippoushi et al. 2019). The milk (0.1 mL) and milk powder (20 mg) were suspended in 1 mL of 10% (w/v) trichloroacetic acid in acetone mixed with 2% (v/v) β-mercaptoethanol. The samples were preserved at −20°C overnight. They were centrifuged at 14,000g for 5 min at 4°C, and the supernatants were discarded. After adding cold acetone (1 mL, stored at −20°C) to the pellets, they were centrifuged at 14,000g for 5 min at 4°C, and their supernatants were discarded. This washing procedure was then repeated twice with cold acetone. After this, the pellets were mildly dried.

Milk protein extracts were re-suspended in 100 µL of a solution containing 50 mM ammonium bicarbonate and 6 M guanidinium chloride (Ippoushi et al. 2019). After adding 5 µL of 200 mM dithiothreitol in 50 mM ammonium bicarbonate, the samples were boiled for 10 min. Next, the samples were mixed with 4 µL of 1 M

iodoacetamide in 50 mM ammonium bicarbonate. These were then incubated in the dark for 1 h at room temperature. After iodoacetamide treatment, the samples were mixed with 40 µL of 200 mM dithiothreitol in 50 mM ammonium bicarbonate and incubated for 1 h at room temperature. After incubation, the samples were mixed with 50 mM ammonium bicarbonate (851 µL). Aliquots (50 µL) of these solutions were diluted with 450 µL of 50 mM ammonium bicarbonate. The diluted samples (50 µL) were mixed with 50 µL of 5 µg/mL trypsin in 50 mM ammonium bicarbonate to digest the milk proteins. After incubation for 24 h at 37°C, the samples were mixed with formic acid (0.5 µL) to terminate the trypsin enzymatic reaction. These were stored at −20°C until LC/MS/MS measurement.

3. LC/MS/MS measurement

An LC/MS/MS apparatus composed of an ACQUITY UPLC and XEVO TQD (Waters, Milford, USA) was utilized in this study. For the analysis of YLGYLEQLLR, the selection of multiple reaction monitoring (MRM) transitions calculated using Skyline software (https://skyline.ms/project/home/begin.view) and optimization of cone voltage and collision energy were performed through a direct infusion of the synthesized YLGYLEQLLR. The MS parameters and dwell times for the LC/MS/MS measurement of YLGYLEQLLR in the tryptic digests are shown in Table 1. Other MS parameters were set as follows: capillary voltage: 3 kV; cone gas flow: 50 L/h; desolvation gas flow: 1,000 L/h; desolvation temperature: 500°C; source temperature: 150°C.

The samples (5 µL) after tryptic digestion were injected into the ACQUITY UPLC BEH C18 Column (1.7 µm, 2.1 mm × 50 mm, Waters) at 30°C. The analytes were separated at a flow rate of 0.3 mL/min using a gradient mobile phase composed of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid): 30% B (0 min)–40% B (4 min)–98% B (4.1 min)–98% B (4.9 min)–30% B (5 min). Data acquisition and analysis were conducted using MassLynx (Waters) and Skyline software, respectively. Samples were quantitatively analyzed in triplicate for each milk and milk powder brand. An unpaired *t*-test was performed using Excel (Microsoft, Redmond, USA).

Results and discussion

1. Analytical method

The selection of a proteotypic peptide to comparatively quantify bovine and caprine aS1CNs was conducted in compliance with the following criteria: a unique representative of these aS1CNs within each proteome, a common shared tryptic peptide of both proteins, a peptide length between 6 and 16 amino acids (Kamiie et al. 2008, Uchida et al. 2013), and the prediction of cleavage probability. In this selection, YLGYLEQLLR was chosen as the proteotypic peptide among all tryptic peptides of these aS1CNs. This sequence exists in amino acids 106-115 of bovine and caprine aS1CNs (Fig. 1). YLGYLEQLLR is registered as a linear peptidic epitope of bovine aS1CN (epitope ID 74687) in IEDB (Xu et al. 2023).

The MRM transition was determined by the direct infusion of YLGYLEQLLR. Figure 2 shows the product ion spectrum of the doubly protonated precursor ion of YLGYLEQLLR in positive ion mode. Y-series ions derived from this precursor ion were detected in the spectrum. Because the single-charged y_8 ion had the largest MS signal among three y-series ions, the transition, *m/z* 634.4 to 991.6, and other transitions (Table 1) corresponding to y_8 ion and y_6 and y_5 ions were selected to quantify and identify YLGYLEQLLR, respectively. These transitions also fulfill the criterion in targeted proteomics that the product ion set for the MRM transition should possess a larger *m/z* than the precursor ion to intensify selectivity and remove any low-mass ion overlap (Yocum & Chinnaiyan 2009). The cone voltage and collision energy were optimized to improve the MS signal intensity of YLGYLEQLLR (Table 1).

The measurement of YLGYLEQLLR as a proteotypic peptide in the LC/MS/MS method has been reported to quantitatively determine the aS1CN content in bovine milk (Bär et al. 2019) and hypoallergenic infant formula (Hostetler et al. 2021). Nelis et al. (2023) also

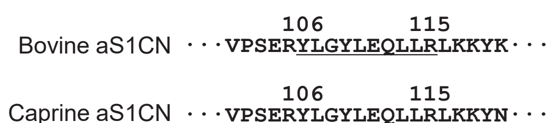

Fig. 1. Amino acid sequences of bovine and caprine aS1CNs
The proteotypic peptide used for the quantification of these aS1CNs is underlined.

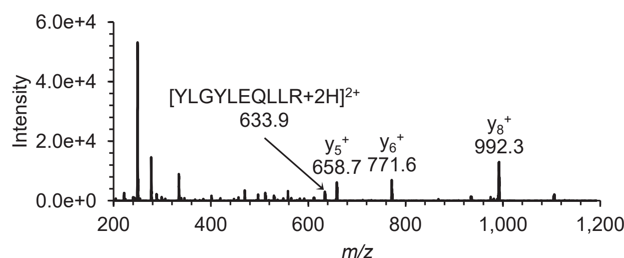

Fig. 2. Product ion spectrum of doubly protonated peptide YLGYLEQLLR

Table 1. MRM transition, cone voltage, collision energy, and dwell time for the measurement of YLGYLEQLLR

m/z		Cone voltage (V)	Collision energy (V)	Dwell time (ms)
Precursor ion (z = +2)	Product ion (z = +1)			
634.4	***991.6 (y_8)***	38	24	200
634.4	771.5 (y_6)	38	24	5
634.4	658.4 (y_5)	38	24	5

The *m/z* values of the precursor and product ions were calculated using Skyline software. Italicized transition in bold was used for the quantification of YLGYLEQLLR. Other transitions were used for identification.

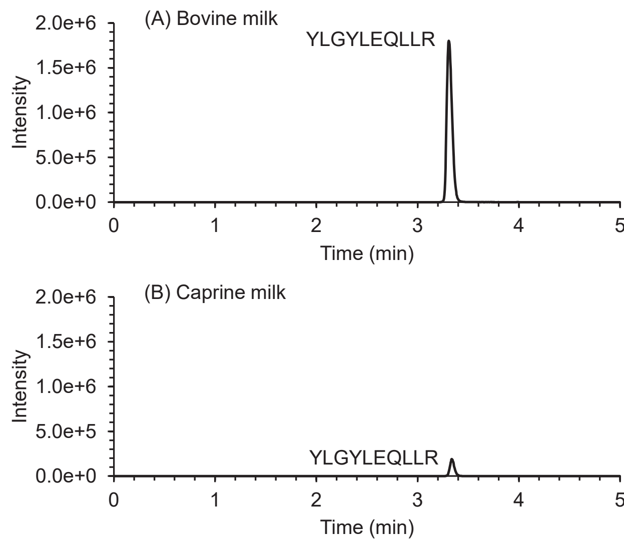

Fig. 3. MRM chromatograms of the trypsin-treated samples prepared from bovine (A) and caprine (B) milk
The lines depict the data obtained using the MRM transition (*m/z* 634.4 to 991.6, Table 1).

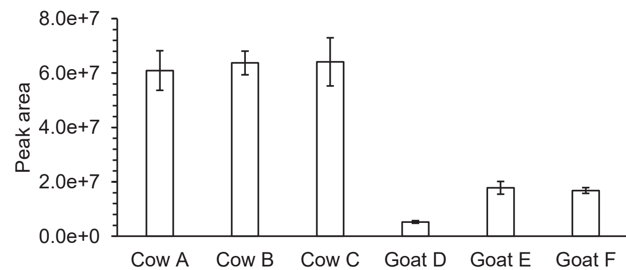

Fig. 4. Comparative quantification of bovine and caprine aS1CNs in milk
The data (mean ± standard deviation, n = 3) are shown as the peak area values per unit volume (mL of milk) of YLGYLEQLLR produced from aS1CNs.

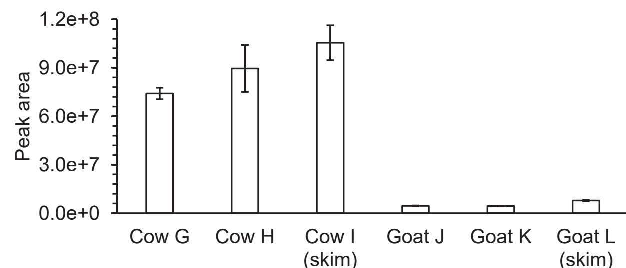

Fig. 5. Comparative quantification of bovine and caprine aS1CNs in milk powders
The data (mean ± standard deviation, n = 3) are shown as the peak area values per unit weight (g of milk powder) of YLGYLEQLLR produced from aS1CNs.

reported that YLGYLEQLLR was detected in the tryptic digests of caprine milk, feta made from a mixture of caprine and ovine milk, and feta made from bovine milk. Thus, YLGYLEQLLR was determined to be an adequate proteotypic peptide for the comparative quantification of bovine and caprine aS1CNs. MRM chromatograms of bovine (Fig. 3A) and caprine (Fig. 3B) milk protein digests were obtained by monitoring the transition (*m/z* 634.4 to 991.6, Table 1). In both chromatograms, the YLGYLEQLLR peak was observed at a retention time of 3.3 min, and no other main peak was observed. Therefore, comparative quantification of bovine and caprine aS1CNs was performed using the peak area values.

2. Content comparison of aS1CNs

The contents of aS1CNs in six brands of commercially available bovine and caprine milk were compared using the peak area values per unit volume (mL of milk) of YLGYLEQLLR (Fig. 4). The content of caprine aS1CN in three brands of caprine milk was significantly ($P < 0.01$) less than the content of bovine aS1CN in three brands of bovine milk. Its contents in Goats D, E, and F, and their average were 0.08-, 0.28-, and 0.27-, and 0.21-fold the average content in bovine milk, respectively. Quantification of aS1CNs in six brands of commercially available bovine and caprine milk powders is shown in Figure 5 and is expressed as the peak area values per unit weight (g of milk powder). In a comparison of whole milk powders, aS1CNs contents in Goats J and K, and their average were 0.06- and 0.05-, and 0.05-fold the average content in bovine whole milk

powders (Cows G and H), respectively. Similarly, in comparison between skimmed milk powders, aS1CN content in Goat L was 0.07-fold the content in Cow I.

Although considerably lower than the bovine aS1CN content, caprine aS1CN was detected in all six brands of caprine milk and milk powder (Figs. 4, 5). Zhang et al. (2022) reported that caprine aS1CN has lower allergenicity than bovine aS1CN in a mouse model. Similarly, if caprine aS1CN is less allergenic than bovine aS1CN in humans, caprine milk may be hypoallergenic to people with an allergy to aS1CN. Moreover, the caprine aS1CN content was considerably lower than bovine aS1CN. Although there are goats incapable of producing aS1CN (Swedish Landrace goats) (Johansson et al. 2015), if milk derived from goats producing aS1CN is mixed during the process of manufacturing caprine milk and milk powders, caprine aS1CN can be detected in these products.

In this study, a short column (50 mm) was used to reduce separation time (Fig. 3), increasing the number of analyses per day. Therefore, the constructed method is considered suitable for screening the presence or absence of aS1CN in many caprine milk samples. Similarly to Swedish Landrace goats, aS1CN is reported to be absent in caprine milk produced from Alpine and Saanen breeds in north-eastern Brazil by the analysis including SDS-PAGE (Da Costa et al. 2014). The method used in this study may contribute to identifying goats raised in Japan that cannot generate aS1CN, leading to the development of milk products for people with an allergy to aS1CN.

Acknowledgements

This work was partially supported by JSPS KAKENHI Grant Number JP20K02400 and Cross-ministerial Strategic Innovation Promotion Program (SIP, JPJ012287), "Building a sustainable food chain that provides abundant and nutritious food" (funding agency: Bio-oriented Technology Research Advancement Institution).

References

Anderson, N. L. (2010) Libraries of specific assays covering whole proteomes: From yeast to man. *Clin. Chem.*, **56**, 1521-1522.

Bär, C. et al. (2019) Protein profile of dairy products: Simultaneous quantification of twenty bovine milk proteins. *Int. Dairy J.*, **97**, 167-175.

Calamelli, E. et al. (2019) Component-resolved diagnosis in food allergies. *Medicina*, **55**, 498.

Da Costa, W. K. A. et al. (2014) Comparative protein composition analysis of goat milk produced by the Alpine and Saanen breeds in northeastern Brazil and related antibacterial activities. *PLoS One*, **9**, e93361.

De Saint-Hilaire, Z. et al. (2009) Effects of a bovine alpha S1-casein tryptic hydrolysate (CTH) on sleep disorder in Japanese general population. *The Open Sleep Journal*, **2**, 26-32.

Fox, P. F. & Brodkorb, A. (2008) The casein micelle: Historical aspects, current concepts and significance. *Int. Dairy J.*, **18**, 677-684.

Geiselhart, S. et al. (2021) Cow's milk processing—friend or foe in food allergy? *Foods*, **10**, 572.

Głąb, T. K. & Boratyński, J. (2017) Potential of casein as a carrier for biologically active agents. *Top. Curr. Chem.*, **375**, 71.

Guesdon, B. et al. (2006) A tryptic hydrolysate from bovine milk α_{S1}-casein improves sleep in rats subjected to chronic mild stress. *Peptides*, **27**, 1476-1482.

Hostetler, G. L. et al. (2021) Determination of casein allergens in extensively hydrolyzed casein infant formula by liquid chromatography-tandem mass spectrometry. *J. AOAC Int.*, **104**, 172-179.

Ippoushi, K. et al. (2019) Absolute quantification of the α, α', and β subunits of β-conglycinin from soybeans by liquid chromatography/tandem mass spectrometry using stable isotope-labelled peptides. *Food Res. Int.*, **116**, 1223-1228.

Johansson, M. et al. (2015) Relation between α_{S1}-casein content and coagulation properties of milk from Swedish dairy goats. *The Open Food Science Journal*, **9**, 1-4.

Kamiie, J. et al. (2008) Quantitative atlas of membrane transporter proteins: Development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel *in-silico* peptide selection criteria. *Pharm. Res.*, **25**, 1469-1483.

Liu, C. & Sathe, S. K. (2018) Food allergen epitope mapping. *J. Agric. Food Chem.*, **66**, 7238-7248.

Matsuura, N. et al. (2016) The effect of food product including bovine $alpha_{S1}$-casein tryptic hydrolysate and theanine on sleep in postmenopausal women with complaint of deteriorated sleep health. *J. Jp. Soc. Psychosom. Obstet. Gynecol.*, **21**, 114-120.

Nakashima, E. et al. (2020) *Journal of Warm Regional Society of Animal Science, Japan*, **63**, 101-111.

Nelis, J. L. D. et al. (2023) Safe food through better labelling; A robust method for the rapid determination of caprine and bovine milk allergens. *Food Chem.*, **417**, 135885.

Pessione, E. & Cirrincione, S. (2016) Bioactive molecules released in food by lactic acid bacteria: Encrypted peptides and biogenic amines. *Front. Microbiol.*, **7**, 876.

Ranadheera, C. S. et al. (2016) Utilizing unique properties of caseins and the casein micelle for delivery of sensitive food ingredients and bioactives. *Trends Food Sci. Technol.*, **57**, 178-187.

Sadiq, U. et al. (2021) Casein micelles as an emerging delivery system for bioactive food components. *Foods*, **10**, 1965.

Uchida, Y. et al. (2013) A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: Application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood–brain barrier in ddY, FVB, and C57BL/6J mice. *Fluids Barriers CNS*, **10**, 21.

Xu, Y. et al. (2023) Allergenicity evaluation of fermented milk

prepared by co-fermentation of *Lactobacillus plantarum* 7-2 and commercial starters after *in vitro* digestive. *Food Chem. X*, **20**, 100911.

Yocum, A. K. & Chinnaiyan, A. M. (2009) Current affairs in quantitative targeted proteomics: Multiple reaction monitoring-mass spectrometry. *Briefings in Functional Genomics and Proteomics*, **8**, 145-157.

Zhang, K. et al. (2022) Cow's milk α_{S1}-casein is more sensitizing than goat's milk α_{S1}-casein in a mouse model. *Food Funct.*, **13**, 6484-6497.