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# Identification of the human β-casein C-terminal fragments that specifically bind to purified antibodies to bovine β-lactoglobulin

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*The presence of foreign proteins in human milk after the ingestion of bovine dairy products is thought to be one of the possible causes of allergic sensitization in exclusively breast-fed predisposed infants. The immunologic determination of bovine β-lactoglobulin (LG) concentration in human milk has been reported by several researchers, but the results are conflicting. Moreover, a strong cross-reactivity between antibodies to bovine β-LG and human milk proteins and peptides was reported, throwing doubt on the reliability of radioimmunoassay and enzyme-linked immunosorbent assay detection and quantification assays for bovine β-LG in human milk. Thus, the goal of this study was to isolate human milk peptides with a molecular mass ≥ 1,000 Da cross-reactive with antibodies to bovine β-LG in order to identify possible common epitopes between human and bovine milk proteins. The proteins were first isolated by affinity chromatography with purified polyclonal antibodies to bovine β-LG, followed by gel filtration fast phase liquid chromatography and reverse phase-high performance liquid chromatography purification of the components specifically bound in the affinity separation step. Affinity-bound peptides were identified by determining their amino acid sequence. All the sequenced peptides belonged to the C-terminal part of human β-casein, which confirms the cross-reactivity of human milk proteins and peptides with antibodies to bovine β-LG and allows the identification of possible common epitopes between the two proteins. No bovine β-LG peptides with a molecular mass ≥ 1,000 Da were found in our milk samples from healthy mothers on a diet rich in bovine milk and dairy products.* (J. Nutr. Biochem. 11:332–337, 2000) 

**Keywords**: human milk; allergy; cross-reactivity; maternal diet

## Introduction

The presence of bovine β-lactoglobulin (β-LG) in human milk after the ingestion of bovine dairy products is thought to be one of the possible causes of allergic sensitization in exclusively breast-fed predisposed infants.[1–3] There are several reports of immunologic determination of bovine β-LG concentration in human milk, with conflicting results and considerable unexplained intra- and interindividual variability[1,4–6]; bovine β-LG concentrations showed no correlation with maternal daily cow's milk intake,[1] and not all milk samples tested gave a value of β-LG concentrations measurable by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).[1,4,6–10] In several studies, different kinetics of appearance/disappearance of immunoreactivity were observed after challenge with cow's milk,[1,5,6,11] with concentration peaks varying in the range 0.01 ng/mL[6] to 800 ng/mL[1] and occurring from 1 to 24 hours after challenge.[5,11]

It has been also suggested that the antigen may consist not only of intact β-LG molecules but also of peptides formed during digestion of β-LG in the mother's gut.[1] Moreover, a strong cross-reactivity between antibodies to bovine β-LG and human milk proteins and peptides (mainly those derived from casein hydrolysis) has been reported,

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throwing doubt on the reliability of RIA and ELISA detection and quantification assays for bovine β-LG in human milk.[12,13]

In this study chromatographic and amino acid sequencing techniques were used to isolate and characterize the peptides with molecular mass ≥ 1,000 Da from milk samples of mothers on a bovine milk rich diet reactive with antibodies to bovine β-LG.

## Methods and materials

### *Production and purification of polyclonal antibodies to bovine β-LG*

The polyclonal antibodies to bovine β-LG used were obtained in rabbits by immunization with three-times-crystallized bovine β-LG; the purity of the protein was tested by sodium docecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectrofocusing[12] and it did not cross-react with antibodies against other whey proteins, such as α-lactalbumin and serum albumin.[14] The antiserum was then purified by affinity chromatography on a Hi-Trap NHS-activated Sepharose 1 mL column (Pharmacia, Uppsala, Sweden) bound to 4 mg of purified bovine β-LG following the manufacturer's coupling protocol. Starting buffer was 0.15 M phosphate-buffered saline (PBS), pH 7.2, and elution buffer was 0.2 M glycine-HCl, pH 3.0; the flow rate was kept to 15 mL/hr. Fractions were neutralized with crystalline Tris and tested for immunoreactivity by ELISA. The specificity of the purified anti β-LG antibody was tested by SDS-PAGE and immunoblotting using bovine whey as a reference.[13]

### *SDS-PAGE and immunostaining*

Electrophoresis and Western blotting were performed on LKB-Pharmacia PhastSystem using SDS-PAGE 8-25 gradient gels and high density gels (Pharmacia; to detect high and low molecular weight components, respectively) and ProBlott (Applied Biosystems, Foster City, CA USA) as blotting membranes. The membranes were stained in 0.2% Coomassie brilliant blue R-250 dissolved in destaining solution (5:1:4 methanol:acetic acid: water).

Immunostaining was performed using antibody to bovine β-LG diluted 1:100 with 25 mM Tris/HCl in 0.5 M NaCl, pH 7.5, as the first antibody solution, and goat anti-rabbit:horseradish peroxidase conjugate (BioRad, Hercules, CA USA) diluted 1:3,000 as second antibody solution. 3,3′-4,4′-Biphenyltetramine (diaminobenzidine) 5 mg (Sigma Imm., Uno Chemicals, St. Louis, MO USA) in 30 mL of Tris buffer was used as substrate. Just before use, 10 μL of 30% hydrogen peroxide were added to the solution and the membrane was incubated at room temperature for 3 to 4 min for color development.

### *Affinity chromatography column*

Affinity purified anti-bovine β-LG immunoglobulins (IgG) 3 mg were coupled to 2.5 mL of Affigel-Hz hydrazide gel (BioRad), following the manufacturer's instructions.

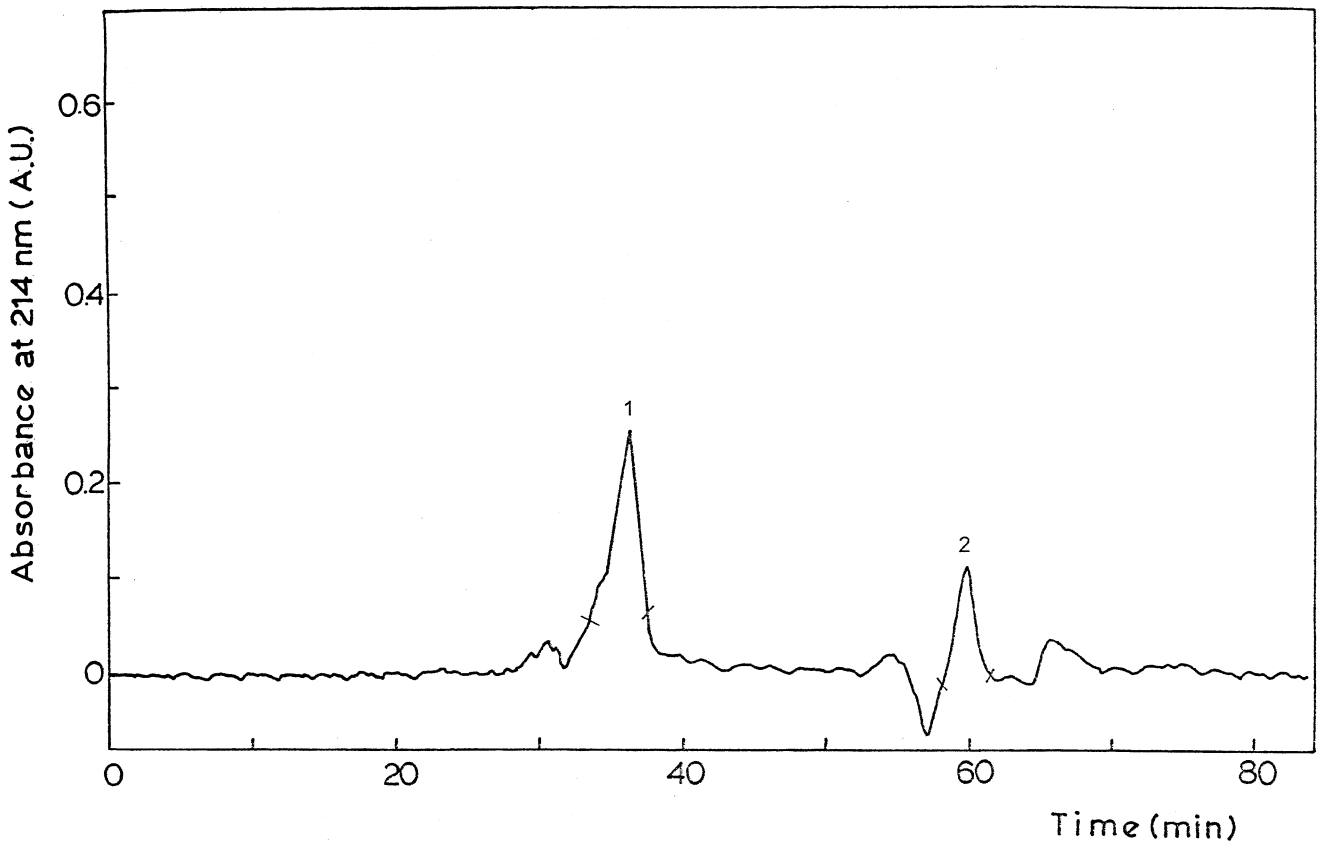


**Figure 1** Gel permeation-fast performance liquid chromatography fractionation on a Superdex Peptide HR 10/30 column of the human whey components specifically bound to the antibodies to bovine β-lactoglobulin. Fraction 1, which contained peptides with molecular masses <10 kDa, was further fractionated.

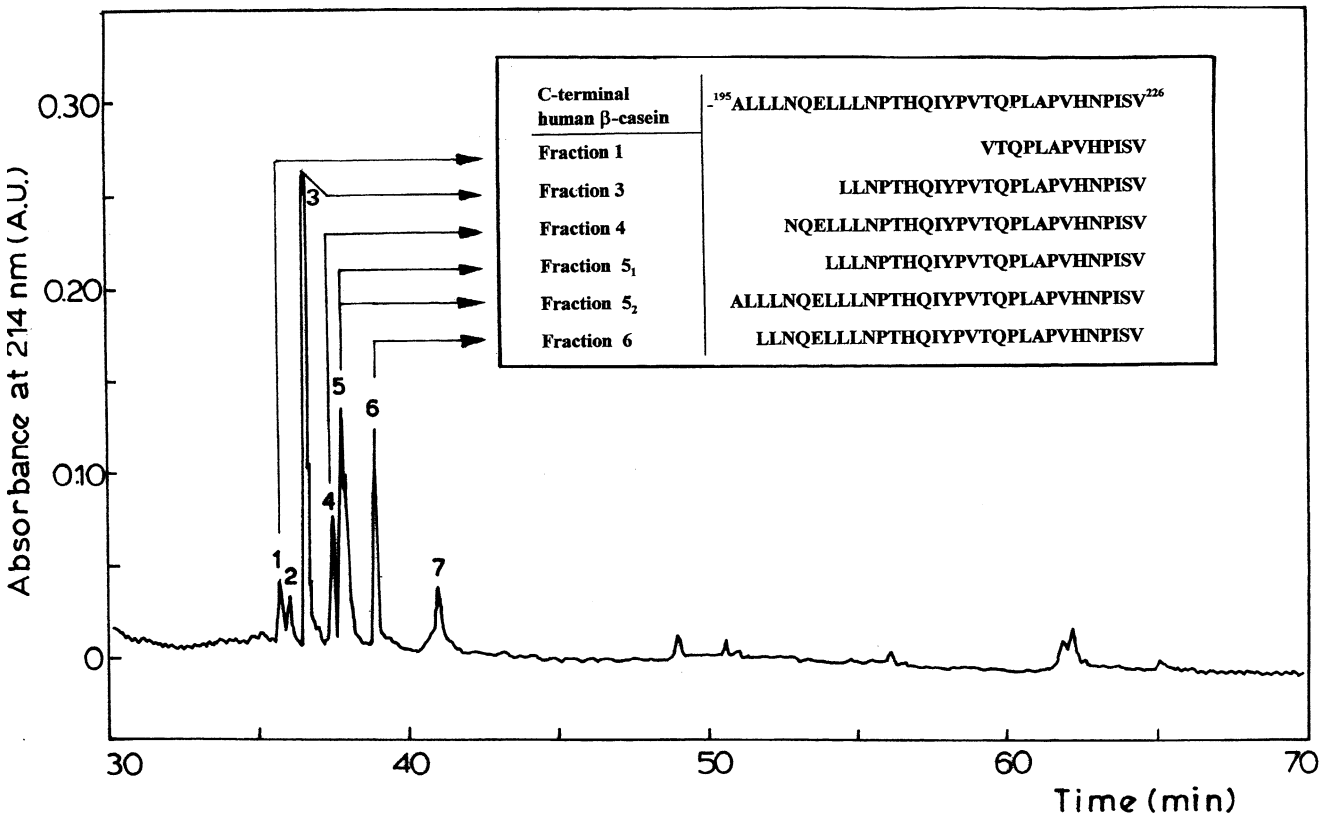


**Figure 2** Reverse phase-high performance liquid chromatography separation of the peptides contained in fraction 1 of *Figure 1*. Peaks 1, 3, 4, and 6 contained a single pure peptide each; peaks 2 and 7 did not contain any detectable peptides in the picomole range; peak 5, which showed two sequences, was further purified. The insert box shows the sequences obtained from each peak and their alignment with the C-terminal part of the human β-casein molecule.

### *Human milk samples*

Samples were collected from well-nourished, exclusively breast-feeding mothers, approximately 1 month after delivery, and immediately stored at −20°C. Mean age of the mothers was 26 years (range 24–29), and all delivered at term after normal pregnancy. None of the mothers or their offspring were affected by allergic or other diseases.

Mothers were on a bovine milk rich diet, ingesting more than 500 mL milk/day of pasteurized, homogenized bovine milk and unlimited amounts of dairy products. Mother's milk was always collected in the morning, approximately 2 hours after the last ingestion of bovine milk, in sterile tubes, by emptying one breast completely using an electric breast pump.

Milk samples from six mothers were pooled and skimmed by centrifugation at 1,500 g for 30 min at 4°C; the soluble proteins were obtained by ultracentrifugation at 189,000 g for 16 hours at 4°C, then dialyzed against a 0.9% NaCl solution, using a semi-permeable membrane with cut-off at 1,000 Da. The soluble fraction obtained by this method is comparable to the so-called whey obtained by acid precipitation: In any case variable amounts of casein subunits are present in the "whey" fraction.[15,16]

### *Affinity chromatography of milk whey samples*

Five milliliters of whey were chromatographed on the Affigel-Hz column described above using 0.9% NaCl as the starting buffer at a flow rate of 25 mL/hr. Aspecific ligands were eluted with 0.6 M NaCl, and specific ligands were eluted with 0.1 M $CH_3COOH$. The absorbance was monitored at 214 nm with a photodiode detector (Pharmacia LKB-VWM 2141).

### *Gel permeation-fast performance liquid chromatography*

Components specifically bound in the affinity chromatography described above were further purified on a gel permeation-fast performance liquid chromatography (GP-FPLC) Superdex Peptide HR 10/30 column (10 × 300 mm; Pharmacia) using a 30% $CH_3CN$, 0.1% trifluoroacetic acid (TFA) solution at a flow rate of 0.3 mL/min. The absorbance was monitored at 214 nm.

### *Reverse phase-high performance liquid chromatography*

Reverse phase-high performance liquid chromatography (RP-HPLC) was performed on a Varian 5040 Liquid Chromatograph using a C18 column (4 × 300 mm; Micropak SP, Varian, Palo Alto, CA USA). Fractions were separated in 0.1% TFA using a linear gradient of 0 to 70% $CH_3CN$ over 60 min, at a flow rate of 0.7 mL/min. The absorbance was monitored at 214 nm.

### *Automatic determination of the amino acid sequence*

Amino acid sequences were determined using a Perkin-Elmer Applied Biosystems 470A gas-phase sequencer (Foster City, CA

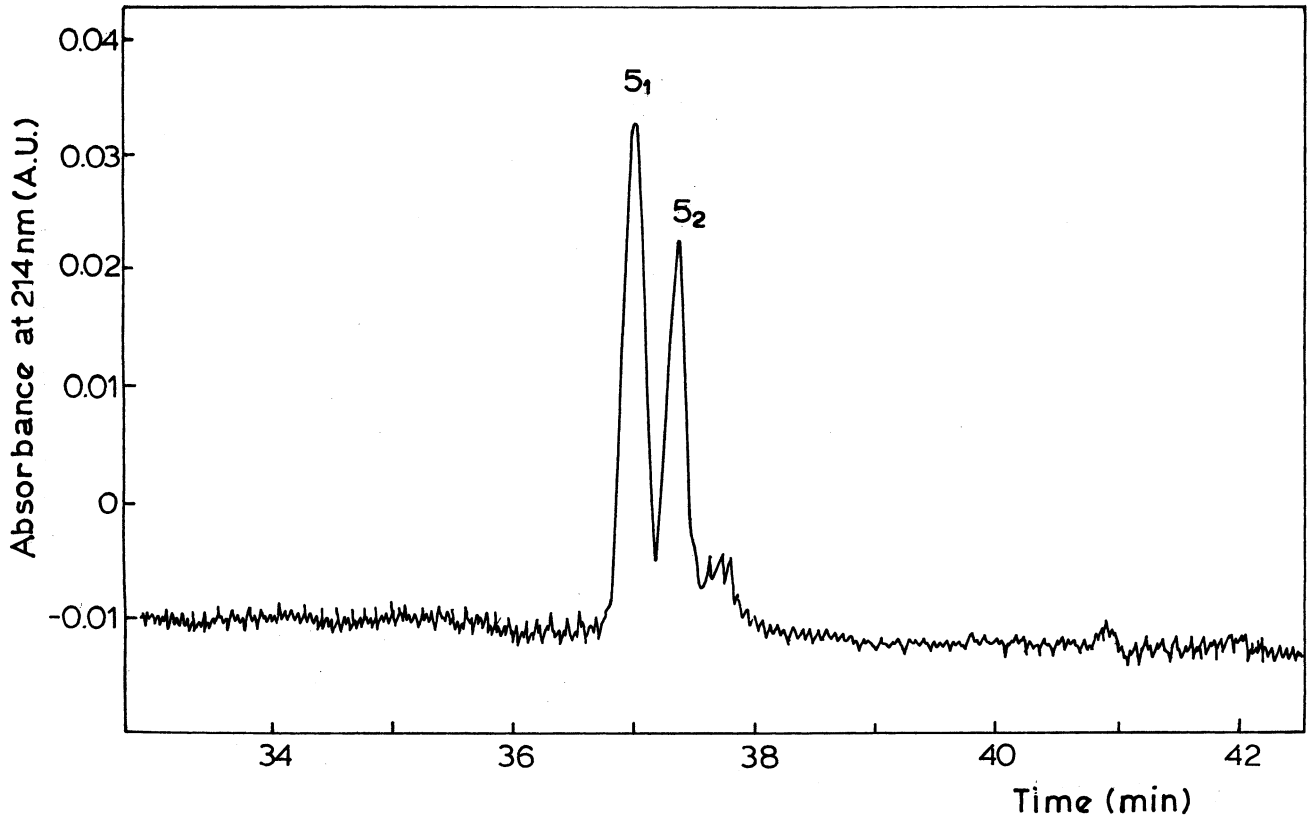


**Figure 3** Reverse phase-high performance liquid chromatography purification of the two peptides contained in fraction 5 of *Figure 2*. The amino acid sequences of the two peptides $5_1$ and $5_2$ are shown in the insert box of *Figure 2*.

USA) equipped with a 120A phenylthiohydantoin-amino acid derivative analyzer.

## Results

Western blotting after SDS-PAGE performed on bovine milk samples ingested by the mothers in all cases revealed a remarkable amount of immunoreactive β-LG by specific immunostaining.

The proteins and peptides contained in human milk, collected from subjects on a milk-rich diet, that specifically bound with antibodies to bovine β-LG in the affinity chromatography separation step, were first fractionated on a GP-FPLC column (*Figure 1*). The SDS-PAGE of the resulting fractions showed that peak 1 contained polypeptides with a molecular mass below 10 kDa, whereas peak 2 contained material undetectable by Coomassie staining. Fractions corresponding to peak 1 were pooled and rechromatographed on a C18 RP-HPLC column, and seven fractions were collected (*Figure 2*). Fraction 5 from the C18

**Table 1** Amino acid sequence comparison of the C-terminal part of the human and bovine β-casein molecules

| | | | | |
|---|---|---|---|---|
| Human β-casein | 120 KGRVMPVLKS | 130 PT IPFFDPQI | 140 PKLTDLENLH | 150 LPLPLLQP LM |
| Bovine β-casein | 129 KHKEMPFPKY<br>* ** * | 139 PVEPFTESQS<br>* ** * | 149 LTLTDVENLH<br>* * * * * * * | 159 LPLPLLQSWM<br>*** *** * * |
| Human β-casein | 160 QQVPQPIPQT | 170 LALPPQPLWS | 180 VPQPKVLPI P | 190 QQVVPYPQRA |
| Bovine β-casein | 169 HQPHQPLPPT<br>* ** * | 179 VMFPPQSVLS<br>** * * | 189 LSQSKVLPVP<br>* ** ** * | 199 QKAVPYPQRD<br>* ** * * * |
| Human β-casein | 200 VPVQALLLNQ | 210 ELLLNPTHQI | 220 YPVTQPLAPV | 226 HNPISV |
| Bovine β-casein | 209 MPI QAFLLYQ<br>* ** ** * | 219 EPVLGPVRGP<br>* * * | 224 FPIIV<br>* | |

**Table 2** Amino acid similarities between the C-terminal part of human β-casein and two different stretches of bovine β-lactoglobulin (LG)

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human β-casein 195-211 | **A** | **L** | L | L | **N** | Q | E | **L** | **L** | **L** | **N** | **P** | **T** | H | Q | I | **Y** |
| Bovine β-LG 102-118 | **A** | **L** | N | E | **N** | K | V | **L** | **V** | **L** | **D** | T | D | Y | K | K | **Y** |
| Bovine β-LG 158-170 | **A** | **L** | P | M | H | I | R | **L** | S | F | **N** | P | T | | | | |

RP-HPLC (*Figure 2*), the only fraction containing more than one component, was further purified using the same column and a narrower gradient and gave two fractions (fractions $5_1$ and $5_2$ in *Figure 3*). Fractions 1, 2, 3, 4, 6, and 7 from the first C18 RP-HPLC (*Figure 2*) and fractions $5_1$ and $5_2$ from the second C18 RP-HPLC (*Figure 3*) were sequenced.

Although fractions 2 and 7 did not give any readable sequence, all the other fractions were shown to contain a single peptide, differing from one another by a few N-terminal residues, as shown in the insert box in *Figure 2*. *Table 1* shows the alignment of the C-terminal half of human and bovine β-casein molecules; only the sequence of the human protein fits exactly with the peptide sequences determined, the bovine β-casein being quite different in that region of the molecule. The C-terminal part of the human β-casein molecule, comprising all the peptides sequenced (residues 195–226) shows a rather high degree of homology, with at least two stretches of the bovine β-LG molecule, with six identical amino acids in the β-LG regions 158 through 170 and 102 through 118 (46.2% identity in 13 residues overlap and 35.3% identity in 17 residues overlap, respectively), as shown in *Table 2*.

## Discussion

This study confirmed both the cross-reactivity of human milk polypeptides against purified antibodies to bovine β-LG (IgG) and doubts concerning the reliability of the immunologic dosage of this protein in human milk.[12,13] It is interesting to remark that neither bovine β-LG nor its peptides with molecular mass ≥ 1,000 Da were found in our milk samples, although these had been collected from mothers on a diet rich in bovine milk, containing large amounts of β-LG, whereas the fragments specifically bound to the antibody are all derived from the hydrolysis of human β-casein. Soluble β-casein fractions are present in the "whey"[17] regardless of the method of casein precipitation, whether acidic or by ultracentrifugation.[15,16] Intact, unphosphorylated human β-casein, called galactothermin, has also been reported in the human whey.[18] The fact that only relatively small fragments of the human β-casein, but not the whole protein, were bound to the antibodies to bovine β-LG does not imply an acidic precipitation of the protein in the affinity chromatography column, but is probably due to a higher affinity of the C-terminal fragments to the antibody, as already reported.[12]

The amount of soluble fragments of β-casein, either human or bovine, found in their respective wheys, probably because of the action of plasmin[19] or other proteolytic enymes, depends also on several parameters including time and conditions of storage and on the experimental processing of the milk samples. This could explain the conflicting results in the immunologic determination of bovine β-LG concentration in human milk.

Unlike previous studies, the structure of the immunoreactive peptides has now been determined, allowing identification of the possible common epitopes between bovine β-LG and human β-casein.

Although bovine casein has been reported to enter human milk,[8] the fact that no fragments of bovine β-casein were detected in this study may depend on the marked structural diversity between bovine and human β-caseins at the C-terminal zone (*Table 1*), which is the site of the possible epitope responsible for cross-reactivity with bovine anti β-LG serum.

On this basis it will be possible to design more specific antibodies to β-LG (both monoclonal and peptide antibodies) to employ in bovine β-LG detection and quantification in human milk.

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