International Dairy Journal 21 (2011) 914–920

Contents lists available at ScienceDirect

International Dairy Journal

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

Chemical and structural characterization of bacterially-derived casein peptides that impair milk clotting

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ARTICLE INFO

Article history:
Received 1 April 2011
Received in revised form
16 May 2011
Accepted 31 May 2011

ABSTRACT

Milk-clotting parameters are highly affected by hydrolysis of casein. Previously, it was shown that products of the hydrolysis of casein impair milk clotting, affecting both clotting time and curd firmness. One of these fractions is of particular interest since it is produced exclusively by enzymes of *Streptococcus dysgalactiae*. The present study aims to further investigate the chemical and structural properties of this fraction in an attempt to understand its influence on milk clotting. Preparations of this fraction, obtained from either *S. dysgalactiae*-infected glands or ex vivo inoculations with the same bacteria, were found to be identical. Mass spectrometry and Edman degradation analyses indicate that it comprises primarily β-CN$^{83-209}$, generated by cleavage at a Val-Val peptide bond, presumably by bacterial thermolysin- or elastin-like proteases. A model offering a putative mechanism for interference with milk-clotting parameters through production of this fraction is presented.

1. Introduction

To a large extent, the quality of dairy products depends on that of the raw milk, which is, in turn, primarily affected by animal health, milking intervals, milk storage, and time until dairy processing (Leitner et al., 2008; O'Brien, Meaney, McDonagh, & Kelly, 2001; Roupas, 2001). It is commonly assumed that milk quality deteriorates to a similar extent in all cases of intramammary infection (IMI), regardless of its causative agent. Thus, somatic cell count (SCC) is used as a "gold standard" for milk payment schemes, because a high correlation between SCC and milk quality has been found in many studies (Auldist & Hubble, 1998; Barbano, Ma, & Santos, 2006; Barbano, Rasmussen, & Lynch, 1991; De Noni, Pellegrino, Cattaneo, & Resmini, 2007).

However, several studies in recent years have challenged this assumption by showing that milk from glands infected with different bacteria generates products of diverse qualities, despite having similar or small differences in somatic cell count (SCC; Forsbäck, Lindmark-Månsson, Andrén, Åkerstedt, & Svennersten-Sjaunja, 2009; Leitner, Krifucks, Merin, Lavi, & Silanikove, 2006; Merin et al., 2008; Okigbo, Richardson, Brown, & Ernstrom, 1985). The difference in milk-clotting parameters often correlates with the amount and composition of the casein (CN)-derived proteose–peptone (p–p) fraction of the milk. Leitner et al. (2006) conducted a comparative study of hydrolysis of CN and its effect on clotting parameters in milk of cows sub-clinically infected with four major udder pathogens (*Staphylococcus aureus*, *Escherichia coli*, *Streptococcus dysgalactiae* and *Staphylococcus chromogenes*); they found that milk from infected glands possessed inferior clotting parameters. Moreover, it was shown (Merin et al., 2008) that the addition of two p–p sub-fractions, labelled C and E, of milk obtained from *Str. dysgalactiae*-infected quarters to milk of bacteria-free glands increased rennet clotting time (RCT) and decreased curd firmness (CF). The bacterium-specific response raised again the question as to whether the increased hydrolysis of CN during IMI is a direct effect of secreted bacterial enzyme(s), or a bacteria-specific host innate immune response in the mammary gland, which is associated with a release of different leukocyte-associated proteases (Coulon et al., 2002; Leigh & Lincoln, 1997; Zavizion, White, & Bramley, 1997). This question may be resolved by analyzing the effect of bacteria on milk-clotting in ex vivo experiments. The present study attempts to further characterize the structure of fraction C (FC) mentioned above, and investigate the process leading to its formation and effect on milk-clotting parameters.

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doi:10.1016/j.idairyj.2011.05.012

2. Materials and methods

2.1. Cows, milk sampling, milk analysis and proteose–peptone preparation

Milk was collected from Israeli-Holstein dairy cows, in which one quarter or more were chronically sub-clinically infected by *Str. dysgalactiae* (5 cows) or *S. chromogenes* (4 cows). In addition, milk samples at the quarter level were taken from 7 cows, 30–60 days following recovery from clinical infection caused by *E. coli*. In cases of *Str. dysgalactiae* and *S. chromogenes*, the udder quarters of sampled cows were monitored for their bacteriological status and SCC for 2–3 months prior to the beginning of the study and the bacteria involved were identified by 3–5 samplings of foremilk. Sampling procedures during the pre-experimental period and on test-day were as follows: the first three squirts of milk from the sampled quarters were discarded and approximately 5 mL milk sample was taken in a sterile tube for bacteriological testing (Oliver, Gonzalez, Hogan, Jayarao, & Owens, 2004). Subsequently, the glands were milked into separate containers, mixed well and 1 L aliquots were taken for analysis of milk composition, measurement of coagulation parameters and separation and analysis of p–p fractions.

Statistical analysis was based on an animal model, in which milk properties from an uninfected quarter were compared with those taken from infected quarter of the same cow (Leitner et al., 2006). Each milk sample was analyzed for SCC using a Fossomatic 360 (Foss Electric, Hillerød, Denmark). The gross milk composition, protein, fat and lactose contents were determined with the Milkoscan FT6000 (Foss Electric) at the Israel Cattle Breeders' Association Laboratory (Caesarea, Israel). Casein was determined according to standard methods (Bradley et al., 1992) and whey proteins were estimated by difference between total protein and casein. Proteose–peptones from each milk sample were isolated according to Andrews (1983). The peptide/protein concentrations were determined according to the Bradford (1976) method adapted to ELISA plates (Stoscheck, 1990).

2.2. Ex vivo experiments

Milk samples (300 mL) from uninfected quarters and with low SCC ($\sim$50,000 cell mL^{-1}) were collected into sterile glass bottles. Each bottle was inoculated with 10^3 cfu of either *Str. dysgalactiae* (VL1989), *S. chromogenes* (M10) or *E. coli* (P4) in 100 μL phosphate buffered saline (PBS) or, as a control, a sample with 100 μL PBS added without bacteria being used. Milk was then incubated for 18 h at 37 °C. The identity of the bacteria in each of the inoculates was validated by spreading on blood agar. The bacteria were removed by centrifugation and the milk supernatants were collected. Proteose–peptones from the milk supernatants were harvested (Andrews, 1983). Each preparation was produced in triplicate, from three different cows.

2.3. Proteose–peptone fractionation

Size fractionation of the peptides by gel filtration was performed on a Superdex 75 (30 × 1.0 cm) column with an AKTA-FPLC System (Amersham, Uppsala, Sweden) eluted with 0.1 M ammonium bicarbonate buffer, pH 8.0, at a flow rate of 0.5 mL min^{-1}, loading 0.5 mL samples. Detection was by optical absorbance at 280 nm. The column was pre-calibrated with bovine serum albumin, ovalbumin, myoglobin, RNase A and aprotinin (67, 45, 17, 13.6, and 6.5 kDa, respectively). The chromatogram peaks were divided into fractions A–E according to their elution times, as previously described (Merin et al., 2008). Re-chromatography on a Superdex Peptide column (30 × 0.75 cm) was performed under similar conditions.

2.4. Milk coagulation properties

Coagulation properties of milk, i.e., RCT and CF, were determined with an Optigraph (Ysebaert, Frepillon, France) as previously described (Merin et al., 2008). The effect of the addition of p–p components on milk coagulation properties was analyzed by mixing the material to be tested at 0.5 mg mL^{-1} with uninfected milk before the Optigraph test was started. The assay was repeated three times, with milk from three different uninfected cows.

2.5. Polyacrylamide gel electrophoresis, mass spectrometry and Edman degradation

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 12.5% acrylamide gel according to the method of Laemmli (1970) using a Mini-Protein II cell (Bio Rad Laboratories, Hercules, CA). Protein/peptide bands were detected by Coomassie Blue staining.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) experiments were conducted using a matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectrometer at the Biological Services Department of the Weizmann Institute of Science, Rehovot, Israel. Bruker REFLEX™ reflector time-of-flight instrument with SCOUT™ multiprobe (384) inlet and gridless delayed extraction ion source. Samples were enzymatically cleaved in gel with either trypsin or chymotrypsin. N-terminal sequencing by the Edman degradation method (Edman, 1950) was performed after transfer of the proteins onto a polyvinylidene fluoride (PVDF) membrane (Pall Corporation, Port Washington, NY) using a Procise, ABI 490 Protein Sequencer (Carlsbad, CA) with an online 140C microgradient system and 785A programmable absorbance detector. Data was analyzed by the ABI software.

3. Results

3.1. Milk composition, proteose–peptone concentration and coagulation properties

In general, SCC in all the infected quarters were significantly higher than those in the uninfected ones (Table 1). Quarters infected with *Str. dysgalactiae* or *E. coli* had significantly higher SCC than those infected with *S. chromogenes*. No significant differences

Table 1
Somatic cell count (SCC), gross milk composition, p–p content and coagulation properties (rennet clotting time, RCT, and curd firmness, CF) of milk from uninfected quarters and quarters chronically infected with *Staphylococcus chromogenes*, *Streptococcus dysgalactiae* or *Escherichia coli*.[a]

Parameter	Uninfected	*S. chromogenes*	*Str. dysgalactiae*	*E. coli*
Number of quarters	10	4	5	7
Log SCC	4.22 ± 0.1^{c}	$5.35 \pm 0.0.1^{b}$	6.66 ± 0.2^{a}	6.56 ± 0.2^{a}
Fat (g L^{-1})	23.5 ± 2.4	26.5 ± 2.2	24.2 ± 2.6	29.1 ± 2.7
Protein (g L^{-1})	33.1 ± 1.5	33.1 ± 1.5	33.8 ± 1.5	37.2 ± 1.9
Casein (g L^{-1})	26.1 ± 1.2	25.4 ± 1.3	25.1 ± 1.4	26.2 ± 1.1
Whey protein (g L^{-1})	6.9 ± 0.3^{b}	7.7 ± 0.4^{b}	8.7 ± 0.1^{a}	11.0 ± 0.3^{a}
Proteose–peptone (g L^{-1})	0.16 ± 0.05	0.18 ± 0.07	0.32 ± 0.06	0.36 ± 0.04
Lactose (g L^{-1})	52.3 ± 0.6^{a}	50.5 ± 1.6^{a}	42.4 ± 2.4^{b}	42.9 ± 4.3^{b}
RCT (sec)	960 ± 27^{c}	1800 ± 100^{b}	4600 ± 670^{a}	3000 ± 460^{a}
CF (V)	11.3 ± 0.4^{a}	11.2 ± 0.5^{a}	2.3 ± 0.9^{b}	2.9 ± 0.3^{b}

[a] Values are means ± SE; values within row without a common superscript letter differ significantly ($P < 0.05$).

in fat, protein, or CN levels were found among the three bacterial species, whereas whey protein concentration was significantly higher in milk from glands infected with *Str. dysgalactiae* or *E. coli* than in milk from glands infected with *S. chromogenes*, or in uninfected glands. Lactose concentration showed an opposite response, being significantly lower in milk samples from cases of *Str. dysgalactiae* or *E. coli* than in glands infected with *S. chromogenes* or uninfected glands. The longest RCT and lowest CF were measured in samples taken from cows infected with *Str. dysgalactiae* or *E. coli* compared with those sampled from cases with *S. chromogenes* or uninfected glands. Although no significant difference in CF was observed between milk from a cow infected with *S. chromogenes* or uninfected milk, RCT was longer in the infected milk (Table 1).

3.2. Size fractionation of proteose–peptones

Size-dependent fractionation of the p–p samples showed a peak at 8–13 kDa (with a maximum at 12 kDa) in the p–p from milk of cows infected with *Str. dysgalactiae* but not in milk samples obtained from *E. coli*- or *S. chromogenes*-infected cows or from uninfected glands (Fig. 1). A similar pattern of proteolytic fragments was observed by SDS-PAGE analysis (Fig. 2A). The chromatogram revealed lower molecular mass bands (<15 kDa) only in samples obtained from *Str. dysgalactiae*-infected glands.

Fraction C (FC) from the *Str. dysgalactiae*-infected samples, obtained by gel filtration on Superdex G75, was pooled and rechromatographed on the same column. Further purification was achieved on a Superdex Peptide column (Fig. 1B, C). A single peak was obtained with an elution volume corresponding to 8–13 kDa. However, SDS-PAGE analysis of the pooled fraction showed two bands reacting with Coomassie stain, designated Band 1 and Band 2, with masses of ~12 and ~9.5 kDa, respectively (Fig. 2B).

3.3. Proteose–peptone fractions from milk samples inoculated ex vivo with bacteria

Similar to the results obtained in the in vivo experiments, inoculation of milk samples with *Str. dysgalactiae* or *E. coli* led to ~300% and ~240% increases in p–p concentrations in the milk, respectively (compared with control milk), while inoculation with *S. chromogenes* did not affect p–p concentrations (data not shown). SDS-PAGE analysis of the p–p fraction obtained from milk inoculated with *Str. dysgalactiae* ex vivo gave a pattern similar to that found in milk sampled from infected glands (Fig. 2A) showing two additional bands in the 9–12 kDa region. Size-distribution analysis on the Superdex 75 column also revealed a similar pattern to that obtained for the p–p samples from glands infected with bacteria, i.e., FC, in the ex vivo experiments, appeared exclusively in milk inoculated with *Str. dysgalactiae* (Fig. 3). However, when purified FC from ex vivo experiments were analyzed by SDS-PAGE, only a single band, corresponding to Band 1 of the in vivo preparation was observed (Fig. 2B).

3.4. Structural analysis of fraction C

The two bands obtained by the SDS-Page analysis for the in vivo FC and the single band obtained for the ex vivo FC (Fig. 2B) were excised from the gels. The bands in the in vivo gel were cut into two pieces. Each piece was cleaved with either trypsin or chymotrypsin and then subjected to MS/MS analysis; the band of the ex vivo sample was treated with chymotrypsin only. Bands 1 and 2 of the in vivo and the single band of the ex vivo FC yielded practically the same results, revealing mainly the C-terminal sequences of α_{S2}- and β-CN (Table 2). It is pertinent to note that MS/MS-detectable β-CN

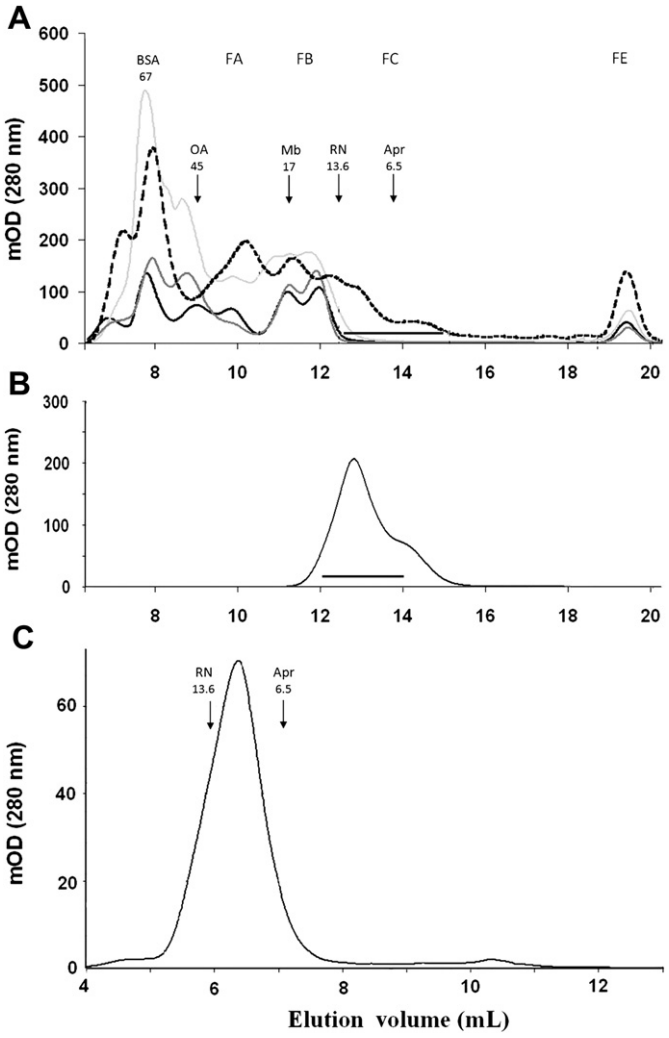

Fig. 1. (A) Size fractionation of the p–p fractions of milk from quarters infected with *Streptococcus dysgalactiae* (▬ ▬ ▬) *Escherichia coli* (▬) or *Staphylococcus chromogenes* (▬) and uninfected glands (▬) on a Superdex FPLC column. FA, FB, FC, FE denote fractions A, B, C and E, respectively. Six preparations of FC (from the region marked by a bar at A) were pooled, concentrated by Speed Vac and rechromatographed on Superdex 75. The pooled fractions (shown in B) were applied to a Superdex Peptide column (shown in C). Molecular mass markers: BSA, bovine serum albumin; OA, ovalbumin; Mb, myoglobin; RN, Rnase; Apr, aprotonin.

fragments were obtained only after chymotrypsin cleavage, but not with trypsin.

When a search for phosphoserine residues was conducted on the raw data from the MS/MS, phosphoserine residues were found only in fragments 106–119 and 153–165 of α_{S1}-CN and α_{S2}-CN, respectively. None of the phosphoserine-rich N-terminal regions at the α- or β-CN were found, indicating that the N-terminal regions of both proteins were missing from these bands. Concatenation of the overlapping peptides obtained from bands 1 and 2 revealed fragments which contained at least fragments 114–203 of α_{S2}-CN and the 126–209 C-terminal regions of β-CN.

MS-MALDI-TOF analysis revealed a main peak at 14,257 Da (Fig. 4); allowing for a <0.5% error margin for proteins at this mass range, this peak may represent the C-terminal fragment Val^{83}-Val^{209} (mass 14,288 Da) of β-CN.

In order to define the N-terminal starting point of the CN fragment in FC, bands 1 and 2 from the in vivo experiments, as well as the band from the ex vivo experiment were transferred onto PVDF membranes and subjected to N-terminal Edman sequence analysis.

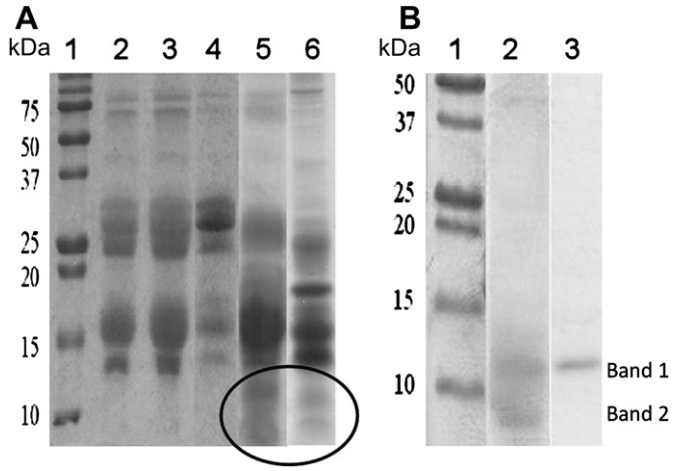

Fig. 2. (A) SDS-PAGE (15%) analysis of proteose–peptone samples from: lane 2, uninfected glands and glands infected with: lane 3, *Staphylococcus chromogenes*; lane 4, *Escherichia coli*; lane 5, *Streptococcus dysgalactiae*. Lane 6, milk inoculated ex vivo with *Str. dysgalactiae* for 24 h (B) SDS-PAGE (16.5%) analysis of p–p samples of purified FCs from (2) milk from glands infected with *Str. dysgalactiae* and (3) milk inoculated ex vivo with *Str. dysgalactiae*. Molecular mass standards are shown in lane 1. Encircled in A are lower molecular mass bands corresponding to FC.

Band 2 failed to yield any significant sequence, whereas both the in vivo Band 1 and the ex vivo band yielded similar results. As shown in Table 3, the main N-termini was observed at Val^{83}, in accordance with the MS results described above. A secondary cleavage site which was observed at Val^{82} may represent carry-over of amino acid residues from the first peptide. Additional sequences (minor peaks) starting at Val^{84}, Val^{98} and Val^{197} were also observed. None of the other proteins indicated by the MS/MS analysis shown in Table 2, α_{S1}- and α_{S2}-CN in particular, were observed in the Edman degradation analysis, except for a short fragment, Val^{200}-Leu^{207}, of α_{S2}-CN.

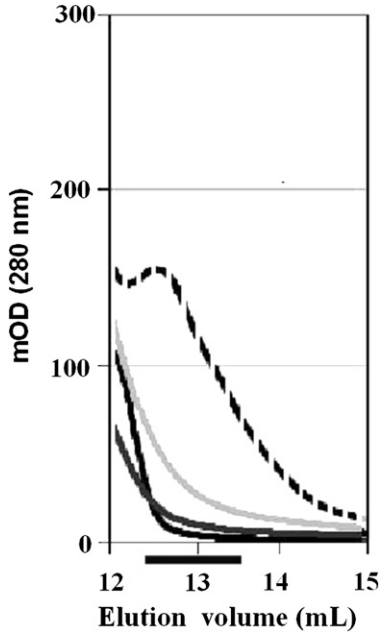

Fig. 3. Size-exclusion chromatography fractionation (partial view) of proteose–peptone fractions from milk samples inoculated with *Streptococcus dysgalactiae* (– – –) *Escherichia coli* (——) or *Staphylococcus chromogenes* (——) and uninfected milk (——); the bar marks the pooled fractions.

Table 2
Peptides in fraction C identified by liquid chromatography-tandem mass spectrometry.

Component	α_{S1}-CN	α_{S2}-CN	β-CN	β-Lactoglobulin
In vivo band 1	23–34[a]	114–125[a]	126–139[b]	92–99[a]
	91–100[a]	137–150[a]	134–143[b]	108–117[a]
	106–119[a,c]	153–165[a,c]	144–163[b]	
		159–166[a]	194–209[b]	
		164–174[b]	199–209[b]	
		174–181[a]		
		175–184[b]		
		189–197[a]		
		194–203[b]		
In vivo band 2	23–34[a]	115–125[a]	126–139[b]	
	91–100[a]	138–149[a]	194–209[b]	
		153–165[a]		
		159–166[a]		
		169–181[a]		
		175–184[b]		
		189–197[a]		
		194–203[a]		
Ex vivo band		115–113[b]	126–139[b]	99–109[b]
		115–124[b]	134–143[b]	110–118[b]
		163–178[b]	194–209[b]	

[a] Fragments obtained by trypsin digestion.
[b] Fragments obtained by chymotrypsin digestion.
[c] Fragments containing phosphoserine.

4. Discussion

Among the bacteria studied, infections with *E coli* and *Str. dysgalactiae* were found to possess a marked effect on milk quality. Both bacteria induce oxidative stress in milk (Silanikove, Shapiro, & Leitner, 2007) and impair milk coagulation (Leitner et al., 2006; Merin et al., 2008). Recently, we have shown for the first time (Merin et al., 2008) that the p–p is not merely an accumulation of CN fragments, lost with the reduction in curd yield, but that its components are actually actively involved in impairment of the coagulation process. This impairment was found for fractions C and E (the inhibitory effect of fraction E will be discussed elsewhere).

FC is a mid-sized sub-fraction of the p–p. Among the three bacterial strains tested, this appeared only in p–p of milk from glands infected with *Str. dysgalactiae*. Its presence in the milk is of major importance, because of its significant effect on milk-clotting

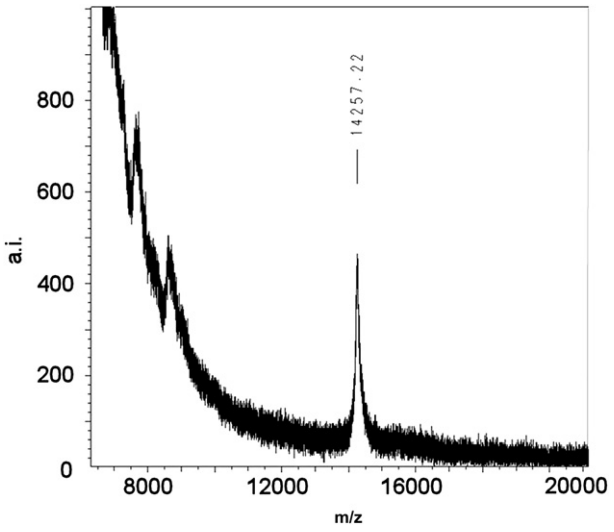

Fig. 4. Mass spectrometry (MALDI-TOF) analysis of Fraction C.

Table 3
Identification by Edman degradation of peptides in fraction C, and suggestions for enzymes that may be involved in their cleavage.

Peptide sequence[a]	Sequence origin	Possible enzyme responsible
VVVPPFLQPE[b]	β-CN (82–?)	Thermolysin[c]
VVPPFLQPEV	β-CN (83–?)	Proteinase K[c]
VPPFLQPEVM	β-CN (84–?)	HtrA[d]
VKEAMAPKHK	β-CN (98–?)	Thermolysin[c]
		Lysyl endopeptidase[c]
VLGPVRGPFP	β-CN (197–?)	Thermolysin[c]
VIPYVRYL	α_{S2}-CN (200–207)	Thermolysin[c]
		Lysyl endopeptidase[c]

[a] Residues actually found in the analysis are underlined. The most abundant peptide deduced by the analysis is in bold.
[b] This sequence may represent an artefact caused by a carry-over of amino acid residues of the second peptide.
[c] Information obtained from ExPasy Peptide Cutter. (http://expasy.org/cgi-bin/peptidecutter/peptidecutter.pl).
[d] Information obtained from Brenda Enzyme Database. (http://www.brenda-enzymes.org/php/result_flat.php4?ecno=3.4.21.107).

properties. The appearance of this fraction in uninfected milk inoculated ex vivo with *Str. dysgalactiae* indicates that this component is generated upon hydrolysis of CN by bacterial enzyme(s) and is not a product of polymorphonuclear cells (Le Roux, Laurent, & Moussaoui, 2003; Napoli, Aiello, Di Donna, Prendushi, & Sindona, 2007) and/or epithelial cells (Silanikove, Merin, & Leitner, 2006), and demonstrates a direct effect of these enzymes on milk quality. These results are consistent with those of (Haddadi, Moussaoui, Hebia, Laurent, & Le Roux, 2005) who found significant hydrolysis of CN upon incubating milk with *E. coli* lysate for 24 h.

The absence of trypsin-generated peptides among the β-CN-derived fragments in the MS suggested that either this fragment was not susceptible to trypsin or was too large to be detected by MS (>4 kDa, i.e., β-CN 105–165). This is consistent with the findings of Lapointe, Mollé, Gauthier, and Pouliot (2004) who showed that tryptic cleavage of β-CN, followed by hydrolysis with thermolysin, yielded only N-terminal peptides. In contrast, chymotrypsin yielded peptides belonging to the C-terminal part of β-CN only. It is pertinent to note that this, per se, would not indicate localization of FC within the C-terminal region of β-CN, as the N-terminal region of the protein is not susceptible to chymotrypsin (Bruins, Creusot, Gruppen, Janssen, & Boom, 2009). However, in combination with the aforementioned lack of trypsin-generated peptides of β-CN, such a conclusion appears to be reasonable. In addition, Bruins et al. (2009) identified preferential cleavage of β-CN after Leu residues by chymotrypsin, which yielded a proteolytic pattern very similar to our findings.

Both MALDI-TOF MS and Edman degradation and analysis of N-termini suggested that FC represents a C-terminal fragment of β-CN, generated by its cleavage in the Val^{82}-Val^{83}-Val^{84} region. According to the ExPasy Peptide Cutter, this sequence is cleavable by thermolysin-like enzyme(s) and proteinase K. The notion that thermolysin may be responsible for the formation of FC by cleavage at the Val-Val-Val region may be supported by the aforementioned results of Lapointe et al. (2004), who found that, upon hydrolysis of trypsin-generated β-CN fragments by thermolysin, Val^{82}-Val-Val-Pro-Pro-Phe^{87} was formed. Furthermore, in a recent study, Ho et al. (2010) found cleavage points, possibly due to the action of elastase, at the Val-Val-Val region of β-CN in milk of lactating cows and in the mammary secretion of dried-off cows. It is pertinent to note that the presence of another serine protease, HtrA (EC 3.4.21.107), preferentially cleaving Val-Val peptide bonds, has been reported in *Streptococcus pyogenes* (Cole et al., 2007), *Streptococcus mutans* (Diaz-Torres & Russell, 2001) and *Streptococcus pneumoniae* (Sebert, Patel, Plotnick, & Weiser, 2005). Thus, the identification of the enzyme responsible for the formation of FC in *Str. dysgalactiae* is far from conclusive. One should bear in mind that the list of enzymes in ExPasy Peptide Cutter is incomplete. In addition, the cleavage product may be formed by the combined action of an endopeptidase and an aminopeptidase. Nevertheless, the fact that FC was found to be associated only with *Str. dysgalactiae* infection, in vivo and ex vivo, suggests that the activity of this enzyme is unique to this bacterium.

The isolated FC in the in vivo experiments yielded two bands on SDS-PAGE analysis. The two bands showed similar peptide compositions in MS/MS analysis, but a definite N-terminal sequence was obtained for Band 1 only. Although the nature of Band 2 is not conclusive, it may represent a fragment of FC generated by further proteolysis. The ex vivo experiments yielded only one band, even though, before purification, the presence of both Bands 1 and 2 was indicated (Fig. 2). This single band showed similar properties to the in vivo Band 1 in MS/MS and in N-terminal sequence analyses. An apparent discrepancy between the results of MS/MS and sequence analysis was observed, while MS/MS indicated the presence of β-, α_{S1}- and α_{S2}-CN, Edman degradation sequencing revealed mainly β-CN fragments. This discrepancy may reflect quantitative differences between the various proteins in FC; MS/MS identifies all the peptides in the sample, whereas the Edman analysis picks up only the most abundant protein. Thus, a protein which comprises 10% of the total protein content may easily be detected by LC/MS/MS, but not by the Edman degradation.

Another discrepancy was found between the molecular mass of FC obtained by hydrodynamic analyses (gel filtration and SDS-PAGE), which was about 12 kDa, and that obtained by mass spectroscopy, i.e., 14.29 kDa. Similar and even higher deviations are often reported in the literature, for example in proteomics, in which gel-determined molecular masses may deviate from MALDI results by over 20% (Jinming et al., 2006).

When FC was added to uninfected milk, it caused interference with the clotting parameters obtained by the Optigraph analysis (Merin et al., 2008). This effect was more pronounced for CF than for RCT. The data presented here suggest that FC represents a relatively large β-CN fragment lacking the phosphoserine-rich region at the N-terminal part of the protein, which is required for stabilization of the rennet coagulum by binding the calcium phosphate formations to the clot (Horne, 2002). Based on these findings and on data related to the three-dimensional nature of milk CNs (Horne, 2002; Qi, Brown & Farrell, 2001), it was possible to construct a 3D-model (Fig. 5), by removing the Arg^{1}-Val^{82} fragment of β-CN from the molecule model. This model can partially explain the defects observed in RCT (×2.5 longer), and in the texture of cheese produced from *Str. dysgalactiae*-infected milk, as previously reported (Merin et al., 2008). Curd was fragile, resulting in a noticeable amount of curd fines and extended syneresis of opaque whey for a long time, which resulted in a softer texture at maturation and lower yields. According to the model, FC, lacking the β-CN phosphoserine-rich region, may still be incorporated into the clot, but render the complex unstable. The curd network formed from such affected milk is suggested to result in a loose or porous structure, which holds more water at the initial stages of curd formation, with lower curd yields.

Pathogenic bacteria secrete exogenous enzymes to increase their invasion and colonization in the host (Silanikove et al., 2006). One of the best known examples is the activation of the host plasmin system by secretion of plasminogen activators, which converts plasminogen to plasmin (Ward et al., 2004). The increased hydrolysis of CN by plasmin in milk as a consequence of infection with *E. coli* (Moussaoui et al., 2004) and *Streptococcus uberis* (Larsen et al., 2005), which was discussed earlier, is consistent with the

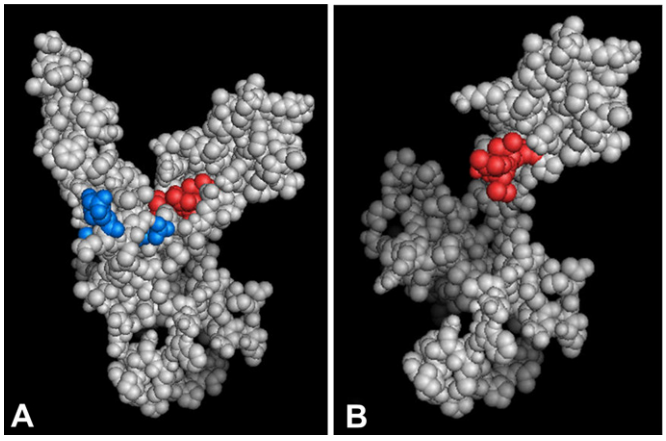

Fig. 5. (A) A 3D-model of β-CN (according to Horne, 2002). The putative cleavage region (Val^{82}-Val^{83}-Val^{84}) is marked in red, the phosphoserine-rich region is marked in blue and (B) shows the same molecule after the N-terminal region is removed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increased content of fraction E in infections with *Str. dysgalactiae* and *E. coli* (Merin et al., 2008). It is less clear why *Str. dysgalactiae* makes an effort to cleave FC from casein. It is pertinent to note that, within its sequence, FC contains peptides, in particular fractions 184–210, 193–207 and 193–209, which were reported to have antimicrobial activity (Birkemo, O'Sullivan, Ross, & Hill, 2009). Thus, it is tempting to speculate that, by the release of FC, which is relatively resistant to further degradation, *Str. dysgalactiae* protects itself by encrypting within FC antimicrobial peptides to which it is particularly sensitive.

The fact that FC was found in chronically infected animals suggests that it is relatively resistant to further degradation. Thus, its deteriorating effect may be extended to the milk tank, contributing further to impairment of milk quality during storage before pasteurization.

5. Conclusions

Bacterial invasion into the mammary gland is associated with increased content of two CN-derived fractions (C and E). *Str. dysgalactiae* secretes a unique enzyme into the milk, which is responsible mainly for cleavage of β-CN at the Val^{82}-Val^{83} sequence of the protein. The β-CN fragment generated has already been shown to be involved in impairment of curd formation, which leads to a porous curd matrix, influencing cheese quality and yield. In the present paper, its structural properties were characterized and a model, which may explain its activity, is presented.

Acknowledgements

This research was partially supported by a grant from the Israel Dairy Board. The technical assistance of S. Bernstein is highly appreciated.

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