



Low molecular mass peptides generated by hydrolysis of casein impair rennet coagulation of milk

Gideon Fleminger^a, Heftsi Ragonés^a, Uzi Merin^b, Nissim Silanikove^c, Gabriel Leitner^{d,*}

^aDepartment of Molecular Microbiology and Biotechnology, The George Wise Faculty of Life Sciences, Tel-Aviv University, Tel Aviv 69978, Israel

^bDepartment of Food Science, Institute of Technology and Storage of Agricultural Products, A.R.O., The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

^cBiology of Lactation Laboratory, Institute of Animal Science, A.R.O., The Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

^dNational Mastitis Reference Center, Kimron Veterinary Institute, P.O. Box 12, Bet Dagan 50250, Israel

ARTICLE INFO

Article history:

Received 29 September 2012

Received in revised form

19 November 2012

Accepted 23 November 2012

ABSTRACT

Previously it was shown that a low molecular mass fraction isolated from the protease–peptone preparation of milk, fraction E, inhibited milk coagulation. Here, the composition and molecular mass of fraction E, and its effect on milk clotting parameters, was investigated to better understand its mechanism of action. Fraction E comprised casein-derived peptides of 1–3 kDa rich in phosphorus residues. Fraction E content increased substantially in milk from glands infected with *Escherichia coli* and *Streptococcus dysgalactiae*, and during storage of the milk. However, the specific activity of fraction E on milk clotting parameters was the same whether sampled from healthy, infected or stored milk. The inhibitory effect was reversible on adding 0.75 mM CaCl₂, suggesting that chelation of Ca by fraction E was involved in the inhibitory mechanism. However, only partial recovery was achieved and an excess of Ca was required, suggesting the involvement of additional pathways in the process.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Coagulation of milk is an important factor for a consistent production of high-quality cheese. Total protein level, the content of α _S-, β - and κ -caseins (CNs), fat level, and protein to fat ratio are recognised as the main factors influencing the quantity and quality of cheese (Guinee, Mulholland, Kelly, & Callaghan, 2007). Hydrolysis of κ -CN by rennet exposes phosphoserine residues on α _{S1}- and β -CN bound strongly by polyvalent cations, mostly Ca⁺² ions, which lead to charge neutralisation, aggregation, and eventually protein precipitation. Moreover, many of the technologically important properties of milk, such as heat stability, rennet coagulability, and the strength and rennet-induced aggregation properties of rennet gels are strongly influenced by the calcium content of milk (Dagleish & Corredig, 2012; Horne, 1998; Law & Tamime, 2010).

The major protease in milk is plasmin, an important enzyme in cheese manufacturing causing hydrolysis of casein and thus loss of curd yield. In addition, the release of casein breakdown products can yield undesirable effects, such as bitterness, off-flavours, or changes in the texture during ripening (Fox & McSweeney, 1998, chap. 10). Plasmin can hydrolyse all CNs in milk, but has a preference for β -CN

and α _{S1}-CN. It preferentially hydrolyses β -CN at positions Lys₂₈-Lys₂₉, Lys₁₀₅-His₁₀₆ and Lys₁₀₇-Glu₁₀₈, to yield three β -CN fragments γ 1-, γ 2-, γ 3-CN, and proteose peptone (p-p) (Andrews, 1983; Fox & Kelly, 2006a,2006b).

According to the fourth revision of the nomenclature of the proteins of cows' milk, the p-p fraction is characterised as a mixture of heat-stable acid-soluble (at pH 4.6) phosphoglycoproteins insoluble in 12% trichloroacetic acid (TCA) (Innocente, Corradini, Blecker, & Paquot, 1998). In earlier works, the p-p fraction appeared to consist of at least three electrophoretically distinct components, components 3, 5 and 8. Component 3 (PP3), the largest component, is a glycoprotein isolated exclusively from the whey fraction. It is characterised by high carbohydrate content (17.3%), 50% phosphorus, and a low content of aromatic amino acids. Component 5 (PP5) is distributed between the CN micelles and the serum in skim milk and it resembles CN in its phosphorus (96%) and high proline (~10.5%) contents. Component 8 (PP8) has been separated into two fractions, designated as 8-fast and 8-slow according to their electrophoretic mobility on polyacrylamide gel. Both components are high in phosphorus, at ~2.8% and 2.1%, respectively (Andrews, 1978; Brunner, 1981). More recent studies have shown that p-p is a mixture of heterogeneous proteins and peptides containing at least 38 components, the functional properties of which are not yet fully understood (Innocente et al., 1998).

* Corresponding author. Tel.: +972 3 9681745.

E-mail address: leitnerg@moag.gov.il (G. Leitner).

It is now acceptable to divide the p–p into two groups. The first is a complex mixture of peptides, mainly component 5 (now termed β -CN-5P), containing the β -CN fragments 1–105 and 1–107, and components 8-fast (β -CN fragment 1–28, now termed β -CN-4P) and 8-slow (β -CN fragments 29–105 and 29–107, now termed β -CN-1P) (Moussaoui et al., 2003). Both components β -CN-5P and β -CN-4P are multiply phosphoserilylated peptides containing the sequence SerP–SerP–SerP–Glu–Glu (Isgrove, Williams, Niven, & Andrews, 1998). The second group includes component 3, also known as lactophorin, a hydrophobic phosphorylated glycoprotein of 135 residues and a fragment (54–135), resulting from hydrolysis of PP3 (Moussaoui et al., 2003). All the casein fragments mentioned above are results of proteolysis by plasmin. In fresh good quality milk, the p–p fraction is typically present at a level of 1–2 mg mL⁻¹, or about 10–20% of the whey protein (Andrews et al., 2006).

Clinical and subclinical intramammary infections are the main diseases in modern dairy herds, affecting about 15–40% of cows in any given herd (Janosi & Baltay, 2004; Pitkala, Haveri, Pyoral, Myllys, & Honkanen-Buzalski, 2004; Wilson, Gonzalez, & Das, 1997). Intramammary infection may impair milk quality in different ways, i.e., by (i) activation of the plasmin system (Leitner, Krifucks, Merin, Lavi, & Silanikove, 2006; Leitner, Merin, & Silanikove, 2011); (ii) activation of the innate immune system, which increases the release of deteriorative enzymes (e.g., lysozyme, lactoperoxidase, and NAGase) into the milk (Barbano, Ma, & Santos, 2006; Grönlund, Hultén, Eckersall, Hogarth, & Waller, 2003; Leitner et al., 2006; Somers, O'Brien, Meaney, & Kelly, 2003; Urech, Puhán, & Schalibaum, 1999); and (iii) activation of the exogenous enzymes released by the invading bacteria (Flemingier, Ragonés, Merin, Silanikove, & Leitner, 2011; Leitner et al., 2006). Thus, milk from mastitic glands exhibits substantially increased proteolytic activity (Auldish, Coats, Sutherland, Mayes, & McDowell, 1996; Le Roux, Colin, & Laurent, 1995).

A previous study demonstrated that subclinical infection results in increased amounts of low molecular mass p–p components (Flemingier et al., 2011; Merin et al., 2008). Among these, a very low molecular mass fraction defined as Fraction E (FE), consisted of casein-degradation products, which was eluted at or just before the salt volume of a Superdex 75 gel filtration column. This fraction was dramatically increased in *Escherichia coli*- and *Streptococcus dysgalactiae*-infected glands and it impaired milk coagulation properties by increasing rennet clotting time (RCT) and decreasing curd firmness (CF) when added to fresh milk from healthy glands (Merin et al., 2008).

In the present study: fraction E isolated from healthy glands and glands infected by various bacteria, as well as fraction E peptides isolated from healthy glands during in vitro storage, were analysed for their composition and activity. A putative mechanism by which milk-clotting parameters are affected by such fractions is suggested.

2. Materials and methods

2.1. Cows and milk sampling

The cows used in the current study and milking procedures were described previously (Flemingier et al., 2011). Briefly, milk was collected from Israeli-Holstein dairy cows, in which one gland was chronically subclinically infected by *Str. dysgalactiae* (5 cows) or *Staphylococcus chromogenes* (4 cows) and from 7 cows with no bacterial finding in the glands, which were taken 30–60 days after a clinical infection with *E. coli*. In addition, 10 milk samples were collected from healthy glands. The data for somatic cell count, gross milk composition, p–p content and coagulation properties (RCT and CF) are presented in Flemingier et al. (2011).

2.2. Preparation of FE and its peptide analysis

Protease peptone fractions were prepared from milk according to the method of Andrews (1983). The acetone-washed trichloroacetic acid (TCA) precipitates obtained from 50-mL milk samples were each dissolved in 1.0 mL of 1 M NaOH and then 1.0 mL of 0.1 M ammonium carbonate buffer, pH 8.0, was added. The FE preparations were obtained by gel filtration separation, as previously described (Flemingier et al., 2011); 0.4 mL of each fraction (containing 1.5–3.5 mg p–p) was loaded on a Superdex G75 column (10 × 300 mm, GE Healthcare, Uppsala, Sweden) which was eluted at flow rate of 0.5 mL min⁻¹ with 0.1 M ammonium carbonate buffer, pH 8.0, using an Acta Purifier FPLC system (GE Healthcare, Uppsala, Sweden). The elution was monitored by UV absorbance at 280 nm. The column was pre-calibrated with bovine serum albumin, ovalbumin, myoglobin, RNase A and aprotinin (molecular masses of 67, 45, 17, 13.6, and 6.5 kDa, respectively). The chromatogram peaks were divided into fractions A–E according to their elution times, and the low molecular mass fraction eluting close to the salt volume was defined as FE (Merin et al., 2008). Quantification of eluted fractions was performed by the colorimetric protein assay of Bradford (1976) carried out in 96 well Enzyme-linked immunosorbent assay (ELISA) plates. Comparative peptide content estimation was carried out by their relative absorbance at 280 nm. Compositional analysis of peptides was conducted by liquid chromatography tandem mass spectrometry (LC-MS/MS) using the matrix-assisted laser desorption/ionisation (MALDI-TOF) mass spectrometer at the Biological Services Department of the Weizmann Institute of Science (Rehovot, Israel) as previously described (Flemingier et al., 2011).

2.3. In-vitro storage of milk

Milk of healthy glands (200 mL) was stored at 4 °C with agitation for 7 days. Samples (15 mL) were withdrawn daily and used for isolation of p–p. Gel filtration analysis was performed on a Superdex 75 column as described above.

2.4. Milk coagulation properties

Coagulation properties of milk, RCT and CF were determined with an Optigraph (Ysebaert, Frepillon, France) as previously described (Merin et al., 2008). FE samples obtained by gel filtration were dried using a SpeedVac apparatus (Thermo electron corporation, Waltham, MA, USA). The use of the volatile ammonium carbonate buffer ensured that the dry material obtained was devoid of buffer salts. The effect of the addition of FE on milk coagulation properties was analysed after the addition of the tested material, at concentrations ranging from 0.14 to 0.5 mg mL⁻¹, into milk from healthy glands before starting the Optigraph test. In some experiments, the effect of FE on milk coagulation was tested in the presence of Ca²⁺ ions. In such cases, CaCl₂ was added to the FE-containing milk tested as well as to blank samples not containing FE, at a final Ca²⁺ concentration ranging from 0.1 to 0.75 mM.

Before each Optigraph analysis, the instrument was checked three times with milk from three different healthy cows. Each Optigraph analysis of FE samples was carried out at least twice in triplicates.

3. Results

3.1. Casein hydrolysis products in healthy glands and glands infected with different bacteria

Fraction E, the smallest peptide fraction isolated from the p–p fraction, was detected in milk from healthy and infected glands. However, the amounts of FE were two to three-fold higher in the

milk obtained from *E. coli*- or *Str. dysgalactiae*-infected glands than those obtained from healthy or *S. chromogenes*-infected glands (Table 1). Bio-gel P-4 chromatography analysis showed that this fraction contains peptides of mass 1–3 kDa (data not shown).

The FE samples originated from the four different bacteria-infected and healthy glands were subjected to MS/MS analysis. As shown in Table 2, the major constituents of all four samples were small breakdown products of α_{S1} -, α_{S2} - and β -CN, β -lactoglobulin and lactophorin pp3. Most of the peptide fractions are common to the healthy milk and the different bacteria, with no significant difference between their compositions, except for β -lactoglobulin fractions, which appeared only in *Str. dysgalactiae*-infected milk, while κ -CN fractions were noted only in healthy milk. Among the various peptides detected, phosphorylated peptides α_{S1} -CN 129–141 and β -CN 1–26 or 1–27 were found in all the milk samples. However, MS/MS analysis does not provide quantitative information and therefore no information about their relative concentration was obtained.

When milk from healthy glands was stored at 4 °C for up to a week, no increase in p–p concentration was observed. However, the FE fraction of total p–p gradually increased with storage time from 3% to 13% (Table 3).

3.2. Effect of fraction E on milk clotting parameters

As previously shown, of the various fractions of the p–p (FA, FB and FE), obtained by gel filtration, FE exhibited the strongest effect of inhibiting milk clotting parameters, i.e., it increased RCT and decreased CF (Fleminger et al., 2011). Moreover, no significant differences were found among the glands infected with the different bacteria and the healthy ones when expressed in terms of mass (Merin et al., 2008). In the present study, the inhibition by FE was found to be dose-dependent, affecting both RCT and CF. Fig. 1 illustrates the effect of the addition of FE samples from healthy and from *Str. dysgalactiae*-infected glands added to uninfected milk on milk clotting parameters.

Fraction E contains phosphopeptides (Table 2), therefore, the inhibiting effect produced by the FE samples, at least in part, may be attributed to depletion of Ca^{2+} from milk due to the chelating effect of these peptides. Addition of Ca^{2+} ions (as CaCl_2) to the milk before the Optigraph analysis indeed partially reversed clotting inhibition in both FE from milk obtained from healthy and *Str. dysgalactiae*-infected glands (Fig. 2).

4. Discussion

All the infected glands demonstrated an increase in whey protein concentration and in indices of proteolysis of CN (i.e., ratios of whey protein/CN and p–p/CN), indicating that CN undergoes extensive hydrolysis in milk originating from infected glands (Le Roux, Laurent, & Moussaoui, 2003; Merin et al., 2008). Enzymatic hydrolysis of CN liberates peptides that serve as local regulators of mammary gland function (Shamay, Leitner, Shapiro, & Silanikove,

Table 2

Mass spectrometric analysis of peptide fragments in FE obtained from healthy and infected glands.^a

Fraction V	Healthy	<i>S. chromogenes</i>	<i>E. coli</i>	<i>Str. dysgalactiae</i>
α_{S1} -CN	1–21	15–22	1–27	14–34
	17–24	17–24	8–24	
	22–38	22–32	22–37	22–37
	80–921 ^P		80–93	91–104 ^P
		112–132	104–110	
α_{S2} -CN	129–141 ^{P*}	129–141 ^{P*}	129–141 ^P	129–141 ^{P*}
	87–99			89–96
	97–115			98–113
	108–113	115–126	115–126*	114–122
	108–124	160–172		160–172
β -CN	166–179	173–187		170–187
	179–207	198–207	198–207*	189–207
	1–26 ^P	1–27 ^P	1–27 ^P	1–26 ^P
	42–55	42–51	36–55	42–55
	52–71	56–70	55–80	56–71
κ -CN	82–91	80–91	71–92	83–91*
	93–104		91–103	91–103*
	125–140		119–140	123–140*
	163–183		160–185	
	175–192			
β -Lactoglobulin	191–209	190–209	190–209	190–209
	10–24			1–13*
	22–32			1–20*
Lactophorin pp3	33–56			6–20*
	1–10	1–12	1–14	
	1–21	1–25	13–24	
	128–135	13–25	1–25	
		54–67	54–68	
	63–70	54–72		
		126–135		
		130–135		

^a Each analysis was performed twice and the data represents a combined list of peptides. A superscript P indicates phosphorylated peptides; an asterisk indicates peptides that appeared in one analysis only.

2003; Shamay, Mabeesh, & Silanikove, 2002; Silanikove, Shamay, Shinder, & Moran, 2000; Silanikove, Shapiro, & Shinder, 2009). Moreover, during milk storage proteolysis of CN and/or release of breakdown products from micelles continue, resulting in lower quality milk delivered to the dairy industry (Barbano et al., 2006; Roupas, 2001). As shown in the present study, breakdown occurred even for the highest quality milk obtained from healthy glands. One of the peptides generated during proteolysis and found in FE is the peptide derived by hydrolysis of β -CN by plasmin (β -CN f1-28), which is known to down-regulate milk secretion in cows and goats; its activity has been shown to correlate with its ability to block potassium channels in the apical membranes of mammary epithelia (Silanikove et al., 2000, 2009; Silanikove, Merin, & Leitner, 2006). This peptide reduces the influx of lactose and other osmotic components from the alveoli into the gland lumen (Leitner et al., 2011). Thus, it is expected that, in bulk milk, the rate of hydrolysis of CN will increase with the addition of milk from infected glands, which contains higher amounts of products of hydrolysis of β -CN such as β -CN f1-28, thus leading to higher amount of

Table 1
Amounts of p–p and FE obtained from milk samples.^a

Preparation	Healthy (10)	<i>S. chromogenes</i> (4)	<i>E. coli</i> (7)	<i>Str. dysgalactiae</i> (5)
p–p ^a	160 ± 50	180 ± 70	360 ± 40	320 ± 60
FE ^b	7.2 ± 1.2	8.4 ± 1.4	23 ± 2.0	27.3 ± 2.2
% FE of p–p	4.5	4.4	6.3	8.4

^a Numbers in parenthesis indicate the number of glands tested. Proteose peptone determined using the Bradford (1976) method, FE estimated as the area under the OD₂₈₀ values of the chromatogram; values are given as mean and SD in mg L⁻¹.

Table 3

Amounts of p–p and FE obtained from uninfected milk during in-vitro storage.^a

Day of storage	p–p (mg L ⁻¹ milk)	FE (mg L ⁻¹ milk)	%FE in p–p
1	122	3.65	3.0
2	114	9.46	8.3
3	121	17.3	11.3
6	122	15.9	13.0

^a Proteose peptone was determined using the Bradford (1976) method; FE was estimated as the area under the OD₂₈₀ values of the chromatogram.

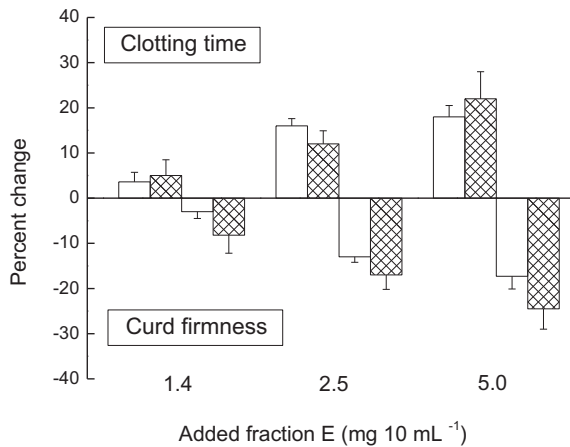


Fig. 1. Dose–response effect of the addition of fraction E (FE) from milk obtained from healthy glands (open bars) and from *Str. dysgalactiae*-infected glands (hatched bars) to milk from healthy glands on rennet clotting time and curd firmness, as assessed using the Optigraph.

breakdown products. Fraction E is the smallest group of the p–p fractions separated by gel filtration and comprises short-chain peptides (~1–3 kDa). It appears in milk originating from both infected and healthy glands, but at different concentrations. Addition of FE to uninfected milk caused inhibition of curd formation in a dose-dependent manner, regardless of the origin of FE. The fact that the same amount of FE from healthy glands caused limited inhibition of curd formation compared with FE from *Str. dysgalactiae*-infected glands suggested that the FE samples contained different peptides or ratios thereof, and that inhibition is not only a result of quantitative differences.

The increase in p–p concentration in the infected glands suggests either that CN is modified by the release of certain peptides or that a change in the CN micelle structure occurs due to changes in calcium activity (Leitner, Chaffer, et al., 2004; Leitner, Merin, & Silanikove, 2004). Both processes may hamper coagulum formation. The poor quality of the curd strongly suggests that the damage caused to the CN micelle as a consequence of the bacterial infection was irreversible. In the current study, it was shown that addition of FE-containing peptides liberated during proteolysis of CN resulted in increased RCT and decreased CF. Furthermore, these peptides

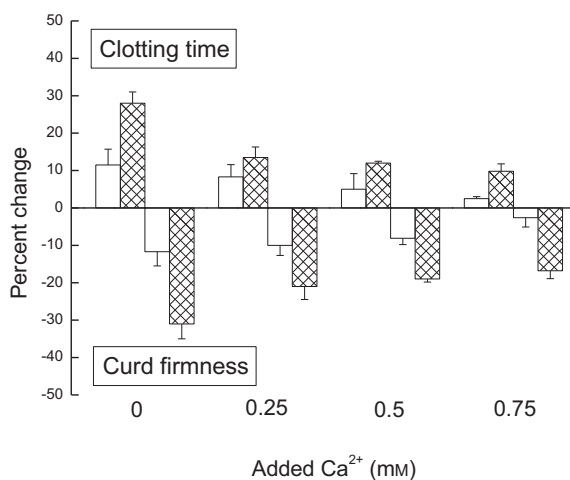


Fig. 2. The effect of addition of up to 5 mg fraction E (FE) to 10 mL of milk obtained from healthy or *Str. dysgalactiae*-infected glands, in the presence of calcium ions, on rennet clotting time (RCT) and curd firmness (CF), as assessed using the Optigraph.

and/or morphological changes in the remaining CN may affect cheese ripening, as reflected in the present study by the excessive CN breakdown in curd made from glands infected with *Str. dysgalactiae*, similarly as reported by Merin et al. (2008). Calcium activity decreases dramatically in milk of infected glands in comparison to healthy ones: thus calcium ions are most likely less available to induce their pro-coagulating effect on para- κ -CN at the initial stages of the coagulation process (Dalglish & Corredig, 2012). Raw milk behaves as if it contains Ca-chelators (Silanikove, Shapiro, & Shamay, 2003). Casein-derived phosphopeptides are Ca-chelators that can bind 4 molecules of Ca per each molecule of phosphopeptide (Mekmene & Gaucheron, 2011).

To elucidate the mechanism of the inhibiting effect of FE on clotting parameters, FE from milk of both healthy and *Str. dysgalactiae*-infected glands was added to uninfected milk in the presence of increasing concentrations of Ca²⁺ ions. Only the addition of 0.75 mM Ca²⁺ ions almost completely succeeded in preventing the inhibiting effect of FE from healthy glands, while only partial inhibition was noted with FE obtained from milk of *Str. dysgalactiae*-infected glands. This concentration is consistent with the reduction in Ca ion activity among subclinical infection in sheep and goats (Leitner, Chaffer, et al., 2004; Leitner, Merin, et al., 2004).

5. Conclusions

Fraction FE, composed of low molecular mass peptides, which are derived from the p–p fraction of milk, impaired milk coagulation properties. Most of the peptide fractions are common to the healthy milk and that from glands infected by different bacteria; however, the MS/MS does not provide quantitative information. The negative impact of fraction FE on clotting parameters, rennet clotting time and curd firmness were almost completely or partially reversed by addition of 0.75 mM Ca²⁺ to the assay mixture of uninfected milk. These phenomena suggest that these phospho-rich peptides partially induce this inhibitory effect due to their Ca²⁺-chelating properties. Further research to precisely quantify the concentration of peptides with Ca²⁺-chelating properties in milk would be helpful in understanding this phenomenon.

Acknowledgements

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

References

- Andrews, A. T. (1978). The composition, structure and origin of proteose peptone component 5 of bovine milk. *European Journal of Biochemistry*, 90, 59–65.
- Andrews, A. T. (1983). Proteinases in normal bovine milk and their action on the caseins. *Journal of Dairy Research*, 50, 45–55.
- Andrews, A. T., Williams, R. J. H., Brownsell, V. L., Isgrove, F. H., Jenkins, K., & Kanekanian, A. D. (2006). β -CN-5P and β -CN-4P components of bovine milk proteose–peptone: large scale preparation and influence on the growth of cariogenic microorganisms. *Food Chemistry*, 96, 234–241.
- Auldust, M. J., Coats, S., Sutherland, B. J., Mayes, J. J., & McDowell, G. H. (1996). Effects of somatic cell count and stage of lactation on raw milk composition and the yield and quality of cheddar cheese. *Journal of Dairy Research*, 63, 269–280.
- Barbano, D. M., Ma, Y., & Santos, M. V. (2006). Influence of raw milk quality on fluid milk shelf life. *Journal of Dairy Science*, 89, 15–19.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of protein utilization principle of protein–dye binding. *Analytical Biochemistry*, 72, 248–254.
- Brunner, J. R. (1981). Cow milk proteins: twenty-five years of progress. *Journal of Dairy Science*, 64, 1038–1054.
- Dalglish, D. G., & Corredig, M. (2012). The structure of the casein micelle of milk and its changes during processing. *Annual Reviews of Food Science and Technology*, 3, 449–467.
- Flemingier, G., Ragonés, H., Merin, U., Silanikove, N., & Leitner, G. (2011). Chemical and structural characterization of bacterially-derived casein peptides that impair milk clotting. *International Dairy Journal*, 21, 914–920.

- Fox, P. F., & Kelly, A. L. (2006a). Indigenous enzymes in milk: overview and historical aspects – part 1. *International Dairy Journal*, 16, 500–516.
- Fox, P. F., & Kelly, A. L. (2006b). Indigenous enzymes in milk: overview and historical aspects – part 2. *International Dairy Journal*, 16, 517–532.
- Fox, P. F., & McSweeney, P. L. H. (1998). Chemistry and biochemistry of cheese and fermented milks. In P. F. Fox, & P. L. H. McSweeney (Eds.), *Dairy chemistry and biochemistry*. London, UK: Blackie Academic & Professional.
- Grönlund, U., Hultén, C., Eckersall, P. D., Hogarth, C., & Waller, K. P. (2003). Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis. *Journal of Dairy Research*, 70, 379–386.
- Guinee, T. P., Mulholland, E. O., Kelly, J., & Callaghan, D. J. O. (2007). Effect of protein-to-fat ratio of milk on the composition, manufacturing efficiency, and yield of cheddar cheese. *Journal of Dairy Science*, 90, 110–123.
- Horne, D. S. (1998). Casein interactions: casting light on the black boxes, the structure in dairy products. *International Dairy Journal*, 8, 171–177.
- Innocente, N., Corradini, C., Blecker, C., & Paquot, M. (1998). Dynamic surface properties of the proteose-peptone fraction of bovine milk. *Journal of Dairy Science*, 81, 1833–1839.
- Isgrove, F. H., Williams, R. J. H., Niven, G. W., & Andrews, A. T. (1998). Aqueous two-phase systems: a novel approach for the separation of proteose peptones. *Journal of Chromatography B*, 711, 91–96.
- Janosi, S., & Baltay, Z. (2004). Correlations among the somatic cell count of individual bulk milk, result of the California Mastitis Test and bacteriological status of the udder in dairy cows. *Acta Veterinaria Hungarica*, 52, 173–183.
- Law, B. A., & Tamime, A. Y. (2010). *Technology of cheese making* (2nd ed.). London, UK: John Wiley & Sons, Ltd.
- Le Roux, Y., Colin, O., & Laurent, F. (1995). Proteolysis in samples of quarter milk with varying somatic cell counts: 1. Comparison of some indicators of endogenous proteolysis in milk. *Journal of Dairy Science*, 78, 1289–1297.
- Le Roux, Y., Laurent, F., & Moussaoui, F. (2003). Polymorphonuclear proteolytic activity and milk composition change. *Veterinary Research*, 34, 629–645.
- Leitner, G., Chaffer, M., Shamay, A., Shapiro, F., Merin, U., Ezra, E., et al. (2004). Changes in milk composition as affected by subclinical mastitis in sheep. *Journal of Dairy Science*, 87, 46–52.
- Leitner, G., Krifucks, O., Merin, U., Lavi, Y., & Silanikove, N. (2006). Interactions between bacteria type, proteolysis of casein and physico-chemical properties of bovine milk. *International Dairy Journal*, 16, 648–654.
- Leitner, G., Merin, U., & Silanikove, N. (2004). Changes in milk composition as affected by subclinical mastitis in goats. *Journal of Dairy Science*, 87, 1719–1726.
- Leitner, G., Merin, U., & Silanikove, N. (2011). Effects of glandular bacterial infection and stage of lactation on milk quality: comparison among cows, goats and sheep. *International Dairy Journal*, 21, 279–285.
- Mekmene, O., & Gaucheron, F. (2011). Determination of calcium-binding constants of caseins, phosphoserine, citrate and pyrophosphate: a modelling approach using free calcium measurement. *Food Chemistry*, 127, 676–682.
- Merin, U., Fleminger, G., Komanovski, J., Silanikove, N., Bernstein, S., & Leitner, G. (2008). Subclinical udder infection with *Streptococcus dysgalactiae* impairs milk coagulation properties: the emerging role of proteose peptones. *Dairy Science and Technology*, 88, 407–419.
- Moussaoui, F., Laurent, F., Girardet, J. M., Humbert, G., Gaillard, J. L., & Le Roux, Y. (2003). Characterization and proteolytic origins of specific peptides appearing during lipopolysaccharide experimental mastitis. *Journal of Dairy Science*, 86, 1163–1170.
- Pitkala, A., Haveri, M., Pyörala, S., Myllys, V., & Honkanen-Buzalski, T. (2004). Bovine mastitis in Finland 2001-Prevalence, distribution of bacteria, and antimicrobial resistance. *Journal of Dairy Science*, 87, 2433–2441.
- Roupas, P. (2001). On-farm practices and post farmgate processing parameters affecting composition of milk for cheesemaking. *Australian Journal of Dairy Technology*, 56, 219–232.
- Shamay, A., Leitner, G., Shapiro, F., & Silanikove, N. (2003). Casein hydrolyzates disrupt tight junction integrity, and induce involution in cows. *Journal of Dairy Science*, 86, 1250–1258.
- Shamay, A., Mabjeesh, S. J., & Silanikove, N. (2002). Casein-derived phosphopeptides disrupt tight junction integrity, and precipitously dry up milk secretion in goats. *Life Sciences*, 70, 2707–2719.
- Silanikove, N., Merin, U., & Leitner, G. (2006). Physiological role of indigenous milk enzymes: an overview of an evolving picture. *International Dairy Journal*, 16, 535–545.
- Silanikove, N., Shamay, A., Shinder, D., & Moran, A. (2000). Stress down regulates milk yield in cows by plasmin induced β -casein product that blocks K⁺ channels on the apical membranes. *Life Sciences*, 67, 2201–2212.
- Silanikove, N., Shapiro, F., & Shamay, A. (2003). Use of an ion-selective electrode to determine free Ca ion concentration in the milk of various mammals. *Journal of Dairy Research*, 70, 241–243.
- Silanikove, N., Shapiro, F., & Shinder, D. (2009). Acute heat stress brings down milk secretion in dairy cows by up-regulating the activity of the milk-borne negative feedback regulatory system. *BMC Physiology*, 9, 13.
- Somers, J. M., O'Brien, B., Meaney, W. J., & Kelly, A. L. (2003). Heterogeneity of proteolytic enzyme activities in milk samples of different somatic cell count. *Journal of Dairy Research*, 70, 45–50.
- Urech, E., Puhán, Z., & Schalibaum, M. (1999). Changes in milk protein fraction as affected by subclinical mastitis. *Journal of Dairy Science*, 82, 2404–2411.
- Wilson, D. J., Gonzalez, R. N., & Das, H. H. (1997). Bovine mastitis pathogens in New York and Pennsylvania: prevalence and effects on somatic cell count and milk production. *Journal of Dairy Science*, 80, 2592–2598.