Heat-stable proteases in milk: scientific, technological and physiological significance

Alan L. Kelly
School of Food and Nutritional Sciences
University College Cork, Ireland
(a.kelly@ucc.ie)

The influence of Anthony Andrews on my work

(1982a) (9)

Journal of Dairy Research (1982), **49**, 577–585 *Printed in Great Britain*

ENZYMES

The roles of native milk proteinase and its zymogen during proteolysis in normal bovine milk

BY OLIVIER DE RHAM* AND ANTHONY T. ANDREWS

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, UK

(*Received 19 February 1982 and accepted for publication 5 May 1982*)

SUMMARY. Proteolysis was measured quantitatively in normal bulk milk, either raw, pasteurized or heated (95 °C, 15 min). During incubation at 37 °C for 24 h, about 0·7 mM of peptide bonds were split in raw milk, and 1·8 mM after activation of the zymogen with urokinase. The same values were observed in pasteurized milk, and no significant activity was present in heated milk. When compared with a commercial plasmin preparation, these levels correspond to about 1·4 and 3·6 μg/ml of plasmin respectively. Most of this activity was separated in the micellar fraction, and it was suppressed by addition of soyabean trypsin inhibitor (SBTI). The remaining activity in the serum phase was not inhibited by SBTI and gave a rather non-specific breakdown with few well-defined casein fragments being produced. Upon further incubation, after the first 24 h, the activity increased, indicating that activation of the zymogen (plasminogen) occurred spontaneously. The rate of this activation was independent of the addition of more plasminogen and was higher in pasteurized than in raw milk. In pasteurized milk, all the native milk proteinase was in the form of the zymogen at the time of secretion. β-Casein was the preferred substrate for the milk proteinase (plasmin) and produced γ-caseins and proteose-peptone components 5 and 8-fast; other fragments were clearly visible on polyacrylamide gel electrophoresis, and included degradation products of α_{s1}-casein. The formation of all these fragments was enhanced by addition of urokinase alone, or of plasminogen and urokinase, or by increasing the incubation time. They were also produced by incubating the micellar fraction alone, but not the serum fraction. Additional fragments were produced when porcine plasmin was added presumably due to differences in specificity between the porcine and bovine enzymes or to contaminating enzymes. Proteolysis induced by additions of plasminogen alone, or of plasminogen plus urokinase, was closer to that observed for the native milk proteinase, and must be recommended for future work in which it is desired to enhance the level of proteinase without altering breakdown patterns, unless a very pure bovine plasmin is available.

Studies on the native milk proteinase have been reviewed recently by Humbert & Alais (1979). Its effect has been shown to be the splitting of β-casein to the γ_1-, γ_2- and γ_3-caseins and proteose-peptone components 5 and 8-fast (Andrews, 1978). The principal enzyme involved is very similar or identical to blood plasmin (Kaminogawa *et al.* 1972: Reimerdes *et al.* 1981*a*; Eigel, 1977; Eigel *et al.* 1979) although other

* Present address: Nestlé Research Department, P.O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland.

Journal of Dairy Research (1982), **49**, 587–596 *Printed in Great Britain*

Journal of Dairy Research (1975), **42**, 391–400
With 1 plate

The a

Natio

Summar
in norm
correlate
about o
micellar
by addit
serum p
Poly
degrade
identifie
changes
which w
inhibited
been pre
casein n
PAGE.
not prod

Journal of Dairy Research (1983), **50**, 57–66 *Printed in Great Britain*

Nation

Summary. S
have been s
activity of le
with Amber
single electr
activity at p
mM at pH
(K_I = 4·1 m
by F^-, Al^{3+},
was very he
(4·9), K_m fo
mM) and pyr
included F^-,
Amberlite C(
Normal bovi
mastitis sho
milk; the 2 a

Breakdown of caseins by proteinases in bovine milks with high somatic cell counts arising from mastitis or infusion with

Journal of Dairy Research (1983), **50**, 45–55 *Printed in Great Britain*

National I

(Recei

Summary. M
or had been
possessed hig
hydrolysed t
hydrolysis of
hydrolysis wa
membranes.
suggested tha
and also that
important. M
curve was a
was almost f
of proteinases
consistent w
temperature
of caseins in
was clearly
significant.

Proteinases in normal bovine milk and their action on caseins

By ANTHONY T. ANDREWS
National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, U.K.

(*Received 23 A*

Summary. Native pro
hydrolysed the casei
proteose-peptone com
of other unidentified f
proteose-peptone frac
than in raw milk, wi
α_{s1}-casein. Measureme
made and it was foun
further proteolysis wi
component 3 (PP3),
increased during stor
enzymes. Further evi
the principal protein
apparent between the
plasmin, which was
Incubations in the pre
clearly revealed that

Journal of Dairy Research (1979), **46**, 215–218

The formation and structure of some proteose-peptone components

By ANTHONY T. ANDREWS
National Institute for Research in Dairying, Shinfield, Reading, RG2 9AT

Summary. Two constituents of the proteose-peptone fraction of bovine milk have been isolated and characterized. Component 5 (PP5) has been shown to represent residues 1–105 and 1–107 of the β-casein amino acid sequence, while component 8-fast (PP8F) corresponds to residues 1–28 of β-casein. Thus, these proteose-peptones represent the N-terminal portions of the β-casein molecule, produced by proteolytic cleavages which form the γ_1-, γ_2- and γ_3-caseins from the C-terminal part. The continuing formation of the total proteose-peptone fraction, PP5, PP8F and the γ-caseins during storage of raw milk at 18 or 37 °C has also been demonstrated.

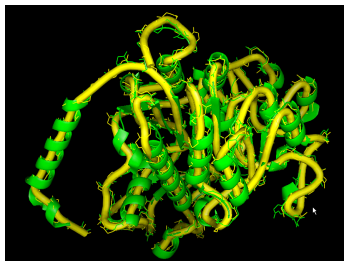
The native milk 'enzome'

- What enzymes are present?
  - first enzyme in milk reported in 1881 (lactoperoxidase)
  - around 70 identified to date, of which ~ 20 studied in detail

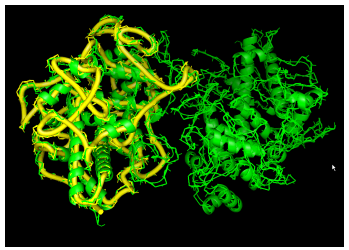
Principal Enzymes

- N-Acetylglucosaminidase
- Acid phosphatase
- Alkaline phosphatase
- Amylase
- Catalase
- Cathepsin D (and others)
- γ-Glutamyl transferase
- Lactoperoxidase
- Lipoprotein lipase
- Lysozyme
- Plasmin
- Ribonuclease
- Superoxide dismutase
- Sulphydryl oxidase
- Xanthine oxidoreductase

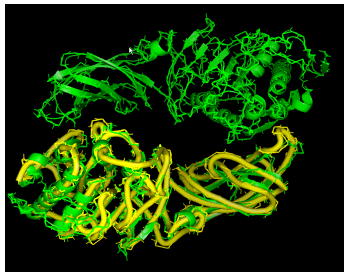
Alkaline phosphatase



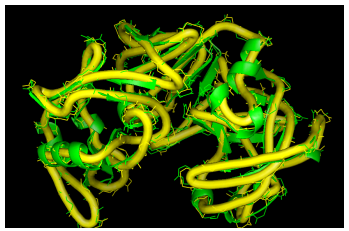
Lactoperoxidase



Lipoprotein lipase



Cathepsin D



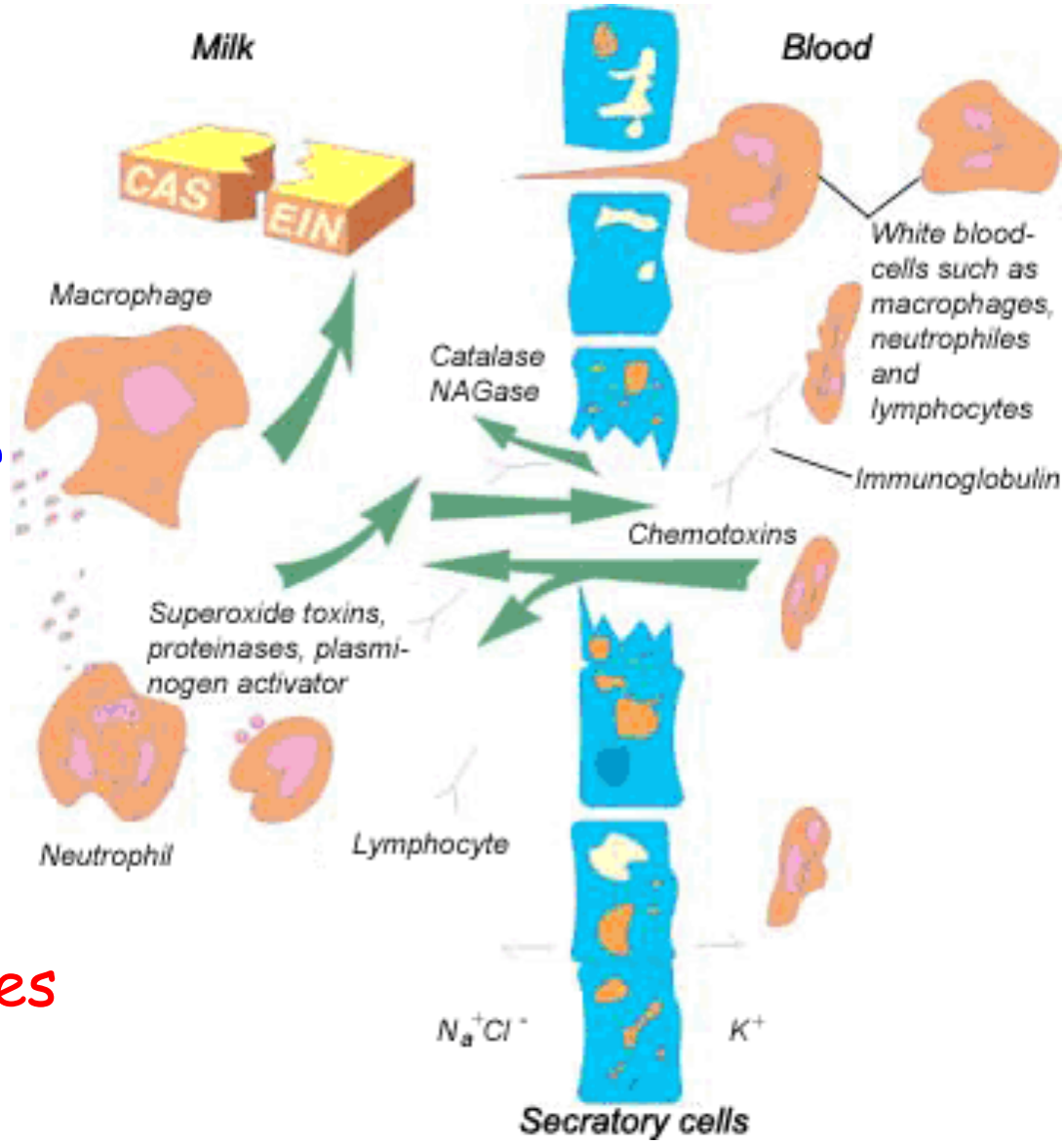
Origin of enzymes in milk

- Blood plasma
- Secretory cell cytoplasm (crescents)
- MFGM (apical cell membrane)
- Somatic cells (leucocytes)

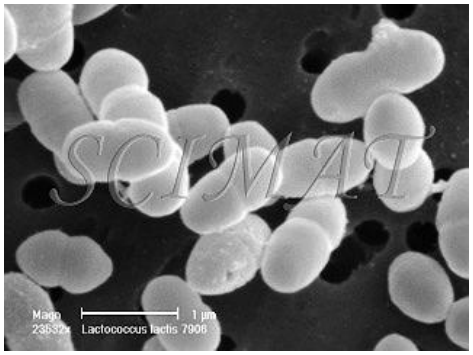
Why do enzymes occur in milk?

Leakage products from blood and/or play specific biological functions?

Origin of many milk enzymes remains ill-defined



Consider two species……



Vs.

L. lactis

Bos taurus

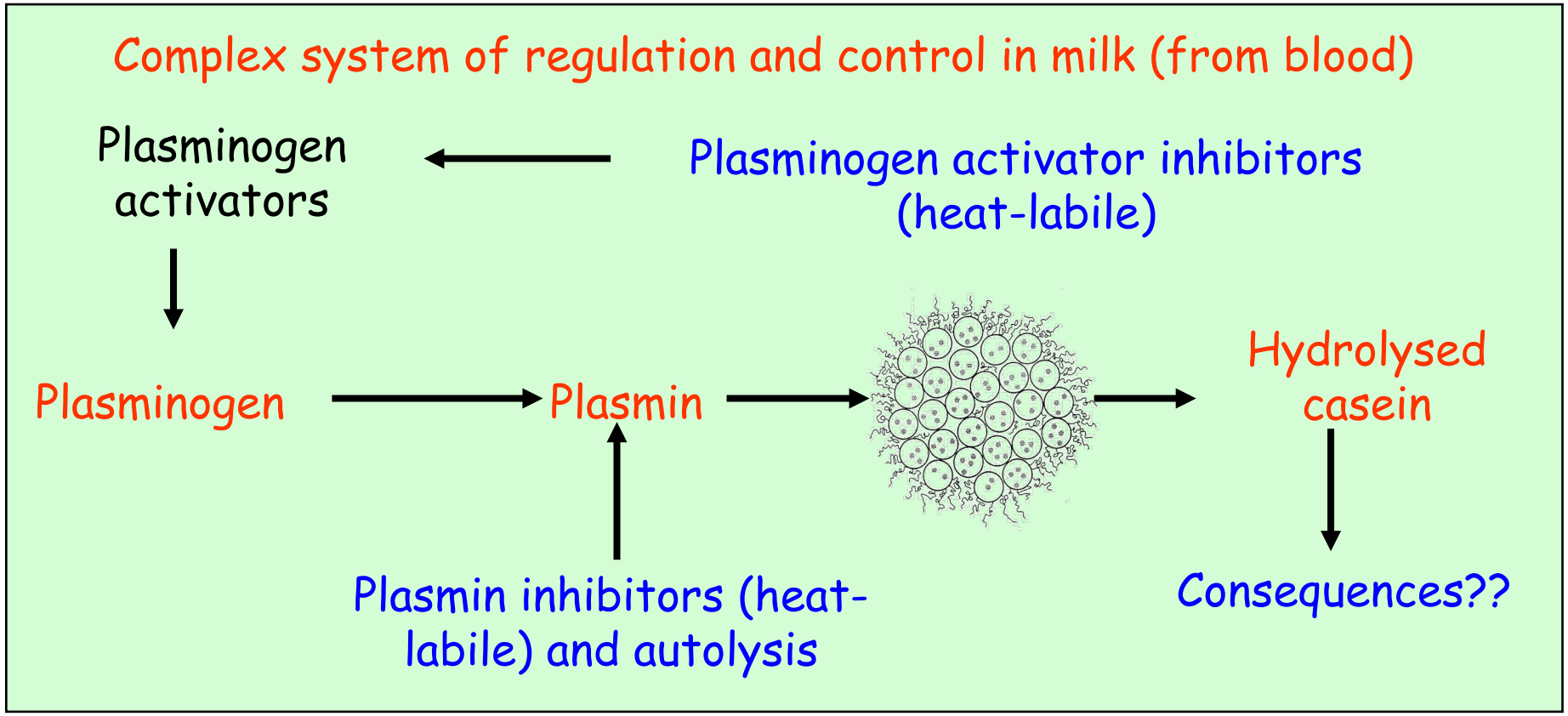
- For which do we have a better understanding of its enzyme profile?

Key questions about enzymes in milk

- What enzymes are present?
- What level of activity do these have?
- How do we measure their activity?
- Hydrolysis versus degradation?
- What physiological factors affect their level (e.g., mastitis, lactation – passive or active roles)?
- What processing factors affect their activity and how can we understand/exploit these?
- Are indigenous enzymes important for
  - dairy product manufacture
  - milk quality
  - diagnostic purposes (e.g., mastitis)
  - consumer health (positive/negative)

Plasmin: the heat-stable milk protease

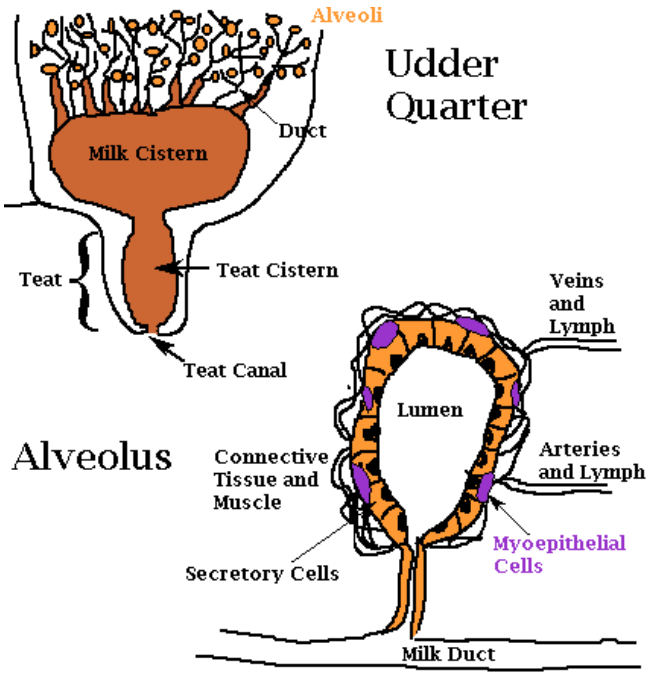
- Principal indigenous proteinase in milk
- Alkaline pH optimum
- Closely associated with substrate (casein micelles) in milk
- Principally hydrolyses β-casein to γ_1, γ_2, γ_3 -caseins, and proteose peptones; also hydrolyses α_{s2}- casein

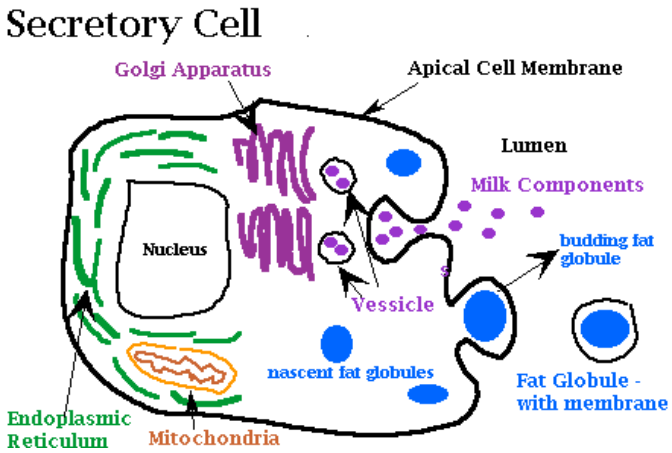


Three phases of plasmin activity that matter

1. **In ubere** (the udder)

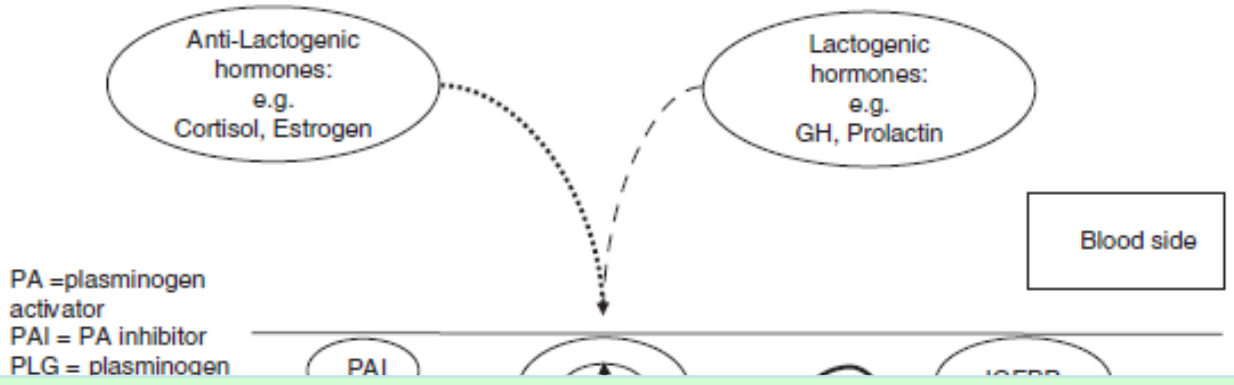
- Freshly drawn milk always shows evidence of plasmin action
- Optimum temperature
- Time to act - variable
- Physiological role (e.g., induction of involution, Silanikove **et al**.)?
- Conversion of plasminogen
- Interaction with bacteria?
- Probably determines the main consequences for dairy product quality?





Available online at www.sciencedirect.com
SCIENCE DIRECT
International Dairy Journal 16 (2006) 533–545
INTERNATIONAL DAIRY JOURNAL
www.elsevier.com/locate/idairyj

Review



All these characteristics make β-CN f (1–28) an ideal candidate for negative feedback control of milk secretion. Infusion of a solution composed of a casein digest enriched with β-CN f (1–28) into the cistern of cows, or infusion of pure β-CN f (1–28) into the cistern of goats, led to a transient reduction in milk secretion in the treated gland (Silanikove et al., 2000).

Fig. 2.
described in the text. Bold arrows indicate flow signal along the feedback loop, dotted arrows positive effects and dashed arrows suppressive effects.

2. In bulk tank (farm or factory)

- Low temperature
- Dilution of uneven milk quality?
- Time to act – can be long
- Effects of and interaction with psychrotrophic bacteria
- Slow action but can accumulate
- Self-digestion ('autolysis') of plasmin versus ctivation of plasminogen?

The progressive dilution of milk

Quarter milk

Four independent milk-producing units (quarters)
- Four milk samples per cow

Composite cow milk

Mixture of four quarters' milk
(4 x Q)

Farm bulk tank milk

Mixture of all cows' milk
(**n** x 4 x Q)

How much are quality variations 'averaged out'?

Factory bulk milk silo

Mixture of many farms' milk
(**m** x **n** x 4 x Q)

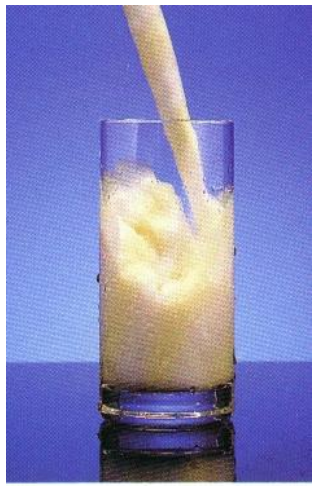
Effect of Psychrotrophic Bacteria and of an Isolated Protease from *Pseudomonas fluorescens* M3/6 on the Plasmin System of Fresh Milk[1]

C. Fajardo-Lira, M. Oria, K. D. Hayes, and S. S. Nielsen
Department of Food Science,
Purdue University, West Lafayette, IN 47907

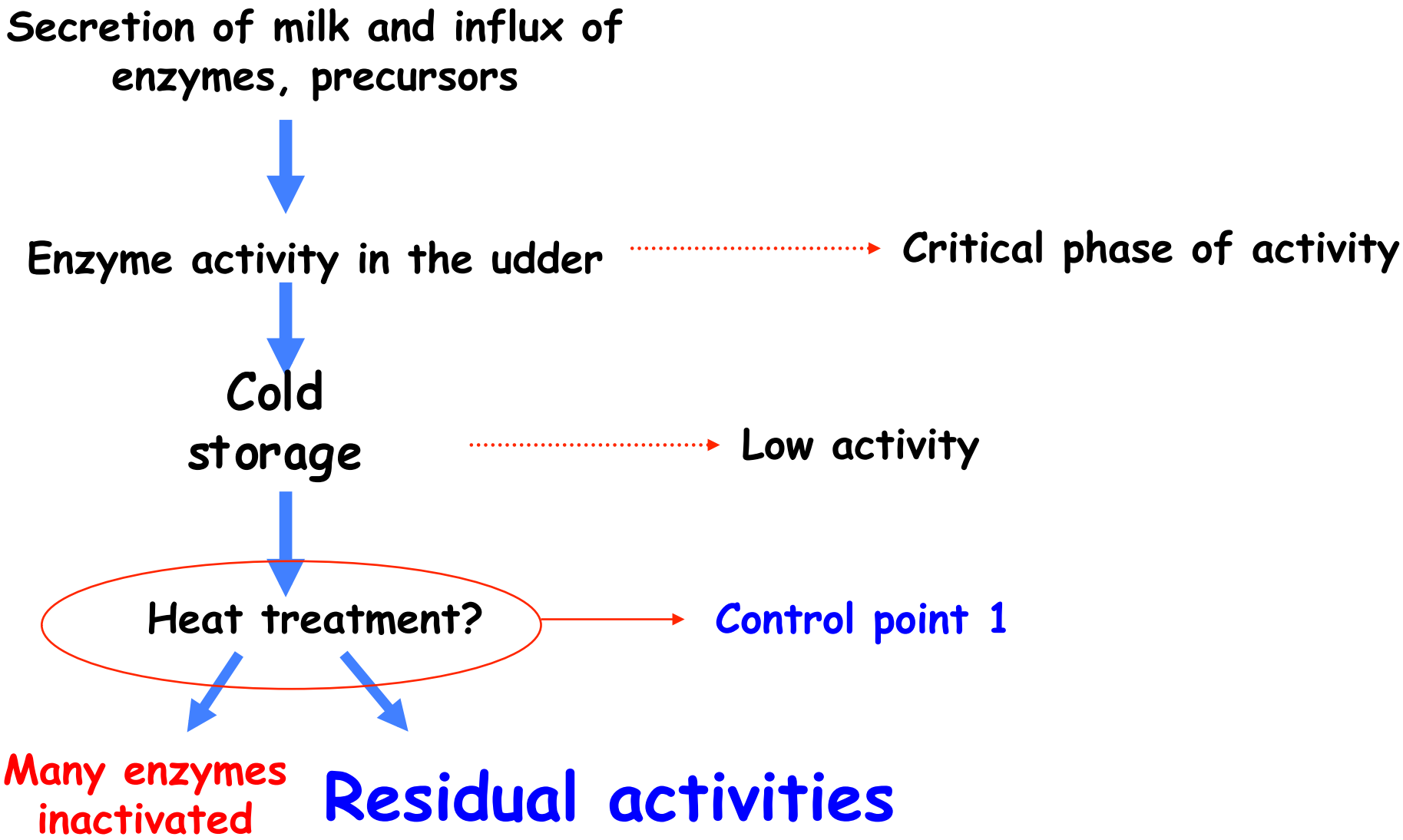
These results suggest that growth of the *Pseudomonas* strains in fresh milk, and particularly their production of extracellular proteases, may be a causative factor in the release of plasmin from the casein micelle. Such plasmin release could affect the quality of cheeses and other food products that utilize dairy ingredients.

3. In processed products

- Generally, but not always, first encounter thermal processing
- Inactivation/enhancement of action
- May be followed by short time/ low temperature or long time/ higher temperature storage
- Alternatively, milk may be separated, fractionated, converted or otherwise processed
- Enzymes, inhibitors, precursors, substrates all change or separate
- Not a natural environment for milk enzymes



Effects of milk enzymes on cheese – a complex system



Heat treatment?

Residual activity

Cheese-making → Control point 2

Loss of enzymes in whey

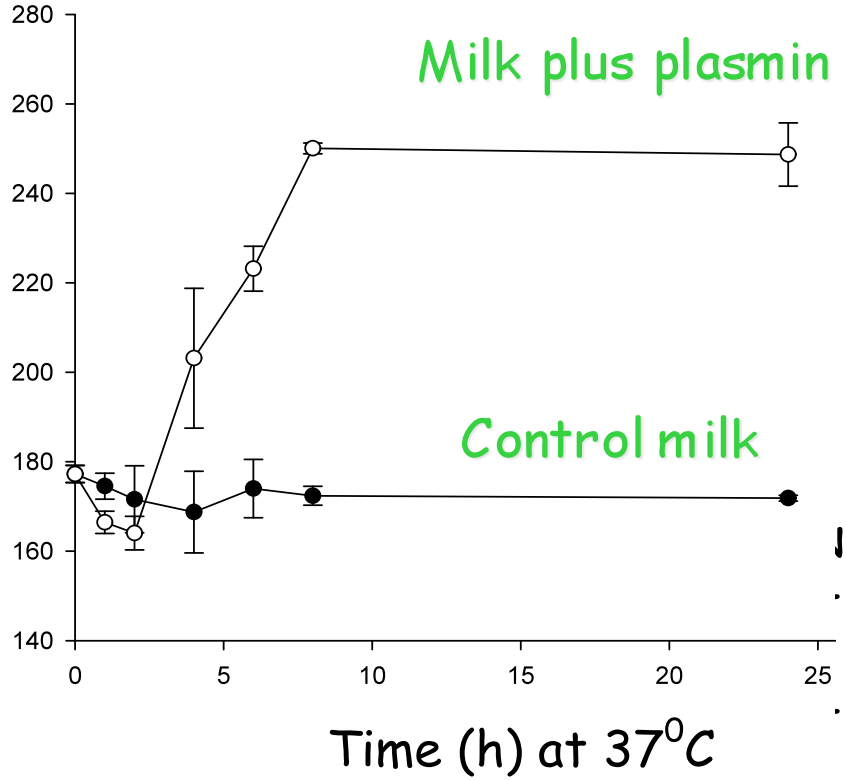
Retained activities

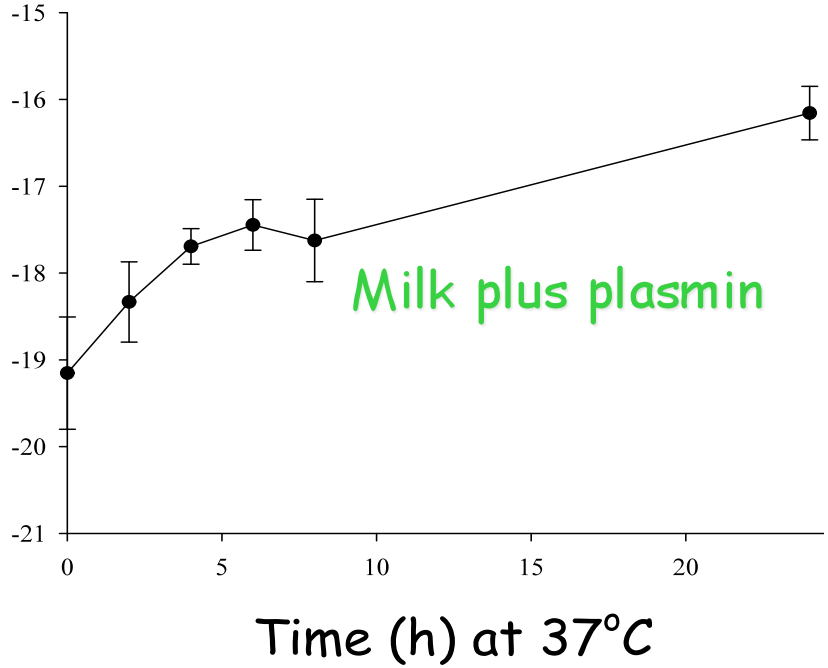
Some enzymes inactive under cheese conditions (pH, a_w etc.) → Control point 3

Certain subset of original activities influence ripening

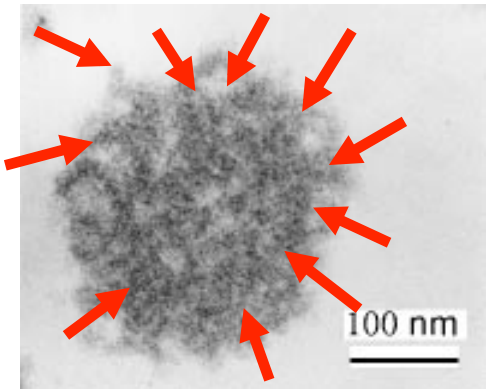
What does plasmin do to the casein micelle?

- Can plasmin cause changes in casein micelle structure that could influence processing characteristics?
- What effect does plasmin have on coagulation of milk by rennet?





Effects of plasmin on the casein micelle



Influence of plasmin hydrolysis on the curd-forming properties of milk Mara, O., Roupie, C., Duffy, A., and Kelly, A.L. **International Dairy Journal 8** 807-812 (1998)

Table 1. Milk Coagulation and Cheese-yielding Properties after Treatment with Increasing Levels of Plasmin

	Plasmin added (mg L^{-1})				Significance
	0	0.25–1.0	2.0–5.0	> 5.0	
n	4	10	6	4	
Curd yield[1]	12.0[a]	10.4[a]	9.9[a]	8.6[b]	*
Curd moisture (%)	72.1	72.8	72.0	72.8	NS
Adjusted yield[2]	11.2[a]	9.4[ab]	9.3[ab]	7.8[b]	**
Whey protein (%)	1.03[a]	1.07[a]	1.22[b]	1.43[c]	***
Rennet clotting time (RCT, min)	25.3[ab]	24.1[a]	23.5[a]	27.8[b]	**
Firming time (K20, min)	13.3[a]	13.4[a]	17.5[b]	24.8[c]	***
Curd firmness A60 (mm)	38.8[a]	38.7[a]	30.1[b]	19.6[c]	***
Cutting time (min)	38.7[a]	37.5[a]	41.0[a]	51.9[b]	***
Firming rate	0.66[a]	0.65[a]	0.58[b]	0.52[b]	***

Impact of plasmin on dairy products

- Consistent interest in impact on destablisation of UHT milk

Destabilization of commercial UHT milks: proteolysis and changes in milk particles

By Isabelle GAUCHER[1,2], Daniel MOLLE[1], Valérie GAGNAIRE[1], Joëlle LEONIL[1], Florence ROUSSEAU[1], and Frédéric GAUCHERON[1*]

[1]INRA-Agrocampus Ouest, UMR1253 Science et Technologie du Lait et de l'Œuf, 65 rue de Saint-Brieuc, 35042 Rennes Cedex, France. *E-mail : Frederic.Gaucheron@rennes.inra.fr
[2]CNIEL, 42 rue de Châteaudun, 75314 Paris Cedex 09, France

Table 2: Proteolysis indexes of the different stable and unstable UHT milks

	NCN content (g/kg)		NPN content (g/kg)		No of peptides identified in NPN filtrates, all caseins concerned	
	Stable	Unstable	Stable	Unstable	Stable	Unstable
A	3.37 ± 0.00	6.07 ± 0.01	1.04 ± 0.00	1.75 ± 0.00	31	128
B	3.47 ± 0.01	6.13 ± 0.00	1.15 ± 0.02	1.51 ± 0.02	18	77
C	3.14 ± 0.01	4.80 ± 0.00	1.05 ± 0.00	1.35 ± 0.02	32	74
D	3.14 ± 0.08	9.26 ± 0.02	1.13 ± 0.02	2.56 ± 0.02	20	144

Impact of plasmin on dairy products

- Heat-stability still area of consistent interest

Extreme high-temperature treatment of milk with respect to plasmin inactivation

A.J. van Asselt[a,*], A.P.J. Sweere[b], H.S. Rollema[a], P. de Jong[a]

[a]Department of Processing, NIZO Food Research, P.O. Box 20, 6710 BA Ede, The Netherlands
[b]Campina Innovation, P.O. Box 238, 6700 AE Wageningen, The Netherlands

Received 27 July 2007; accepted 30 November 2007

Abstract

The thermal inactivation of plasmin in combination with the denaturation of b-lactoglobulin was studied using a newly developed Innovative Steam Injection (ISI) system, a direct heating system with a very short heating time combined with very high temperatures (0.2 s, 150–188 °C). The model for inactivation of plasmin was improved by integrating the effect of the formation of (partly) denatured b-lactoglobulin during pre-heating on the kinetics of plasmin inactivation. By applying these new kinetics, the heat load of milk by currently applied UHT-treatments can be reduced while obtaining a sufficient inactivation of plasmin (i.e., o 1%). It turned out that it is possible to decrease the plasmin activity below 1% of the initial value and concomitantly to achieve 4 5.5 decimal reduction of Bacillus sporothermodurans while computer simulations showed the denaturation of b-lactoglobulin to be minimized.

Enzymes in high SCC milk

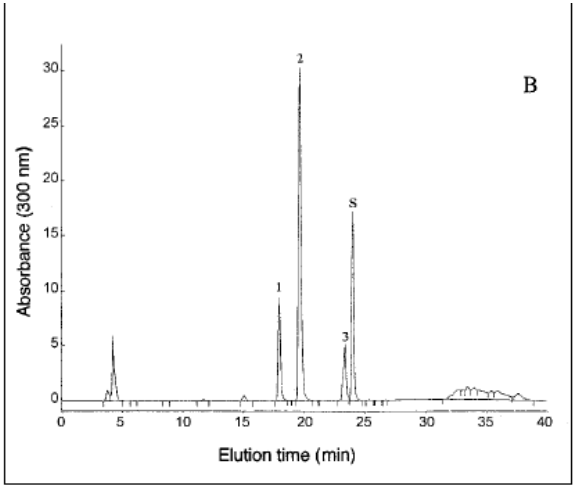


Fig. 7—RP-HPLC chromatogram of peptides produced from the heptapeptide substrate following incubation for 4 h at 37°C with (A) bovine serum extract or a (B) somatic cell extract from raw milk.

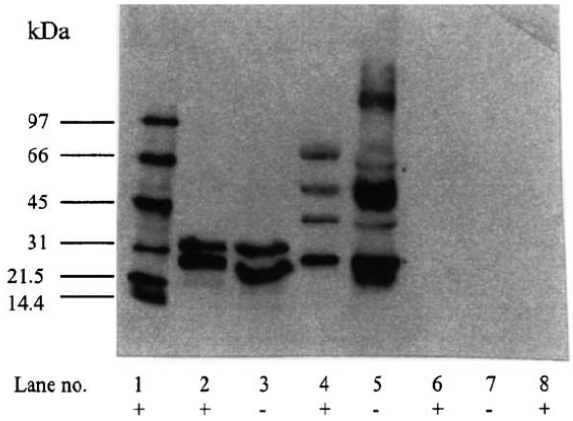


Fig. 5. Immunoblotting of partially purified fraction fIII from bovine milk using anti-bovine cathepsin B antibody against proteins separated by electrophoresis on a 4–15% acrylamide SDS-PAGE ready gel. Masses in kDa are indicated. Lane 1, low MW standard biotinylated protein mix. Lane 2, reduced Sigma bovine cathepsin B. Lane 3, unreduced Sigma bovine cathepsin B. Lane 4, reduced fIII. Lane 5, unreduced fIII. Lanes 6–8, as lanes 4, 5 and 2, respectively, except that anti-bovine cathepsin B antibody was incubated with 51 × molar excess of cathepsin B before addition to the blot.

- Rich source of enzymes amplified relative to 'normal' milk
- Plasmin no longer the sole/main player
- Several enzymes identified in high SCC milk (e.g., lysosomal cathepsin B)
- New assays developed for enzyme detection
- Extensive hydrolysis allows identification of enzymes responsible
- Correlate SCC with product quality through enzyme activities
- Different pathways of enzyme (and immune response) depending on responsible agent
- Use of antigens allows direct study of milk enzymes response

J. Dairy Sci. 95:1655–1666
http://dx.doi.org/10.3168/jds.2011-4813

Proteolytic and proteomic changes in milk at quarter level following infusion with *Escherichia coli* lipopolysaccharide

K. Hinz,* L. B. Larsen,† O. Wellnitz,‡ R. M. Bruckmaier,‡ and A. L. Kelly*[1]
*School of Food and Nutritional Sciences, University College Cork, Co. Cork, Ireland
†Department of Food Science, Aarhus University, DK-8830 Tjele, Denmark
‡Veterinary Physiology, Vetsuisse Faculty, University of Bern, CH-3001 Switzerland

Table 4. Suggested enzymes potentially responsible for the generation of the peptides S1–9, based on earlier reports[1]

Peptide	N-terminal cleavage site	Enzyme suggestion	C-terminal cleavage site	Enzyme suggestion	Reference
S1	α_{S1}-CN Phe_{24}–Val_{25}	Cathepsin B or cathepsin D or elastase	Val_{37}–Asn_{38}	Elastase or cathepsin B/D + AP[2]	Reimerdes et al., 1979; Considine et al., 2000; Hurley et al., 2000; Considine et al., 2004
S2	α_{S1}-CN Arg_{1}–Pro_{2}	AP	Arg_{22}–Phe_{23}	Cathepsin D + CP[2]	Hurley et al., 2000; Reimerdes et al., 1979
S3	α_{S1}-CN Lys_{7}–His_{8}	Cathepsin B or plasmin	α_{S1}-CN Leu_{21}–Arg_{22}	Not known[3]	Considine et al., 2004
S4	β-CN Leu_{198}–Gly_{199}	Not known[3]	β-CN Val_{209}	No cleavage[4]	
S5	β-CN Leu_{191}–Leu_{192}	Cathepsin D	β-CN Val_{209}	No cleavage[4]	Hurley et al., 2000
S6	β-CN Leu_{192}–Tyr_{193}	Cathepsin D	β-CN Val_{209}	No cleavage[4]	Hurley et al., 2000
S7	α_{S1}-CN His_{80}–Ile_{81}	Not known[3]	α_{S1}-CN Arg_{90}–Tyr_{91}	Plasmin	Le Bars and Gripon, 1993
S8	α_{S1}-CN Arg_{90}–Tyr_{91}	Plasmin	α_{S1}-CN Leu_{99}–Arg_{100}	Plasmin + CP[2]	Le Bars and Gripon, 1993; Reimerdes et al., 1979
S9	α_{S1}-CN Arg_{90}–Tyr_{91}	Plasmin	α_{S1}-CN Arg_{100}–Leu_{101}	Plasmin	Le Bars and Gripon, 1993

[1]AP = aminopeptidase; CP = carboxypeptidase. More information on peptides S1–9 is available in Table 3.

[2]An AP or a CP, eventually in combination with other protease(s), may be responsible for the cleavage.

[3]Not known indicates that a possible responsible protease for generation of this cleavage site could not be suggested.

[4]No cleavage indicates that the residue is located at the C-terminal position of the protein from which it was derived and therefore not the result of a proteolytic cleavage at that position.

An unusual subject for a dairy scientist

International Dairy Journal 20 (2010) 715–723

Contents lists available at ScienceDirect

International Dairy Journal

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

Proteins and proteolysis in pre-term and term human milk and possible implications for infant formulae

Emanuele Armaforte [a,c], Erika Curran [a], Thom Huppertz [a,d], C. Anthony Ryan [b], Maria F. Caboni [c], Paula M. O'Connor [e], R. Paul Ross [e], Christophe Hirtz [f], Nicolas Sommerer [f], François Chevalier [a,g], Alan L. Kelly [a,*]

[a] *Department of Food and Nutritional Sciences, University College, Cork, Ireland*
[b] *Cork University Maternity Hospital, Department of Paediatrics and Child Health, University College, Cork, Ireland*
[c] *Department of Food Science, University of Bologna, Cesena, Italy*
[d] *NIZO food research, Ede, The Netherlands*
[e] *Moorepark Food Research Centre, Fermoy, Ireland*
[f] *Proteomic Platform, UR1199, INRA, Montpellier, France*
[g] *Proteomic Platform, CEA-FAR/DSV-IRCM, Fontenay aux Roses, France*

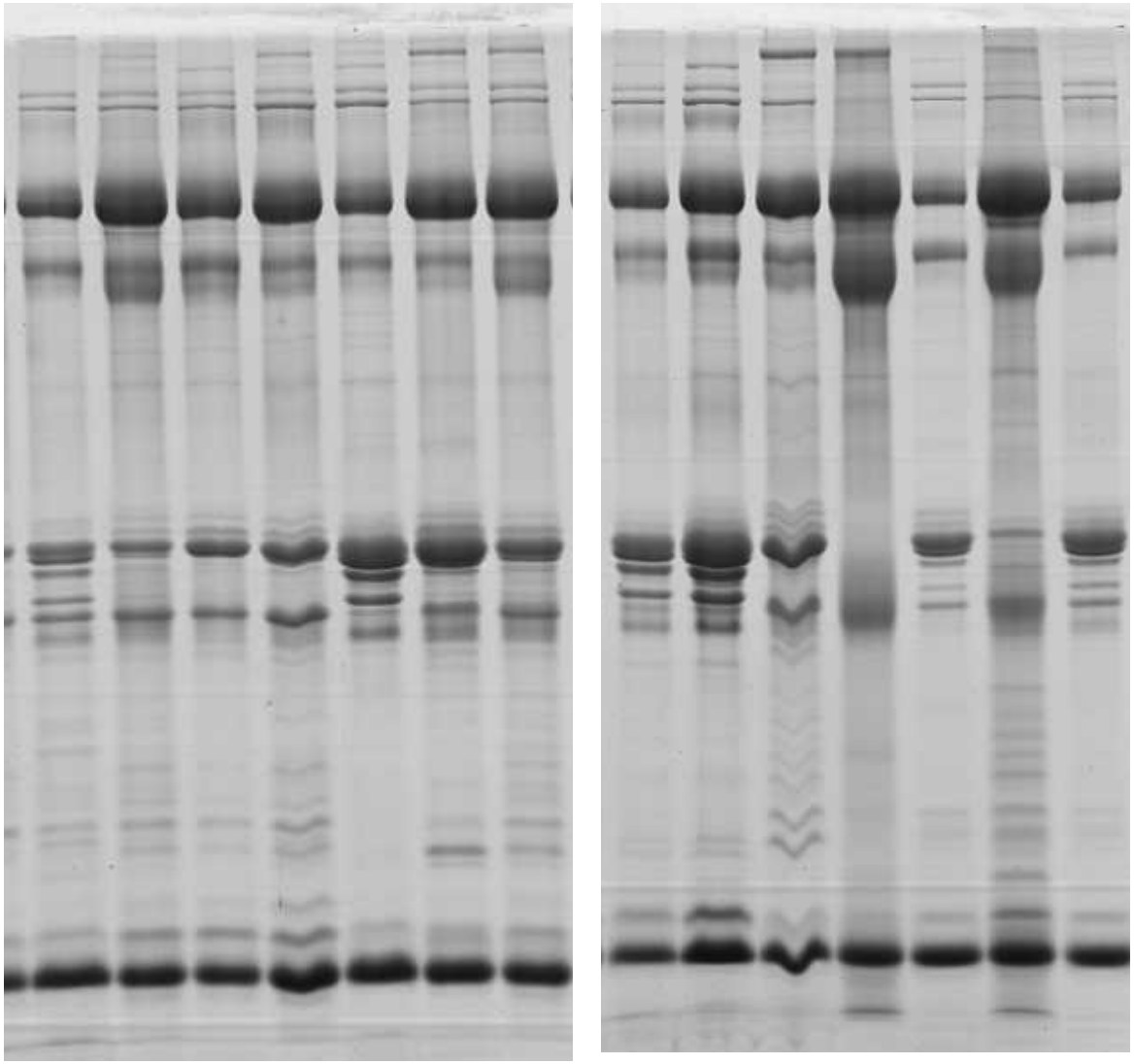
Human milk and bovine milk – a comparison

Bovine milk	Human milk
3–3.5 g protein/L	~ 1 g protein/L
Mix of four caseins (α_{s1}-, α_{s2}-, β–, κ–) is dominant protein family	Mix of three caseins (α_{s1}-, β–, κ–) is minor protein family
Two major whey proteins (α-lactalbumin, β-lactoglobulin)	Major whey protein is α-lactalbumin, high levels of lysozyme, lactoferrin
Caseins found in micelles	Caseins found in micelles
Plasmin is predominant native protease	Plasmin is predominant native protease

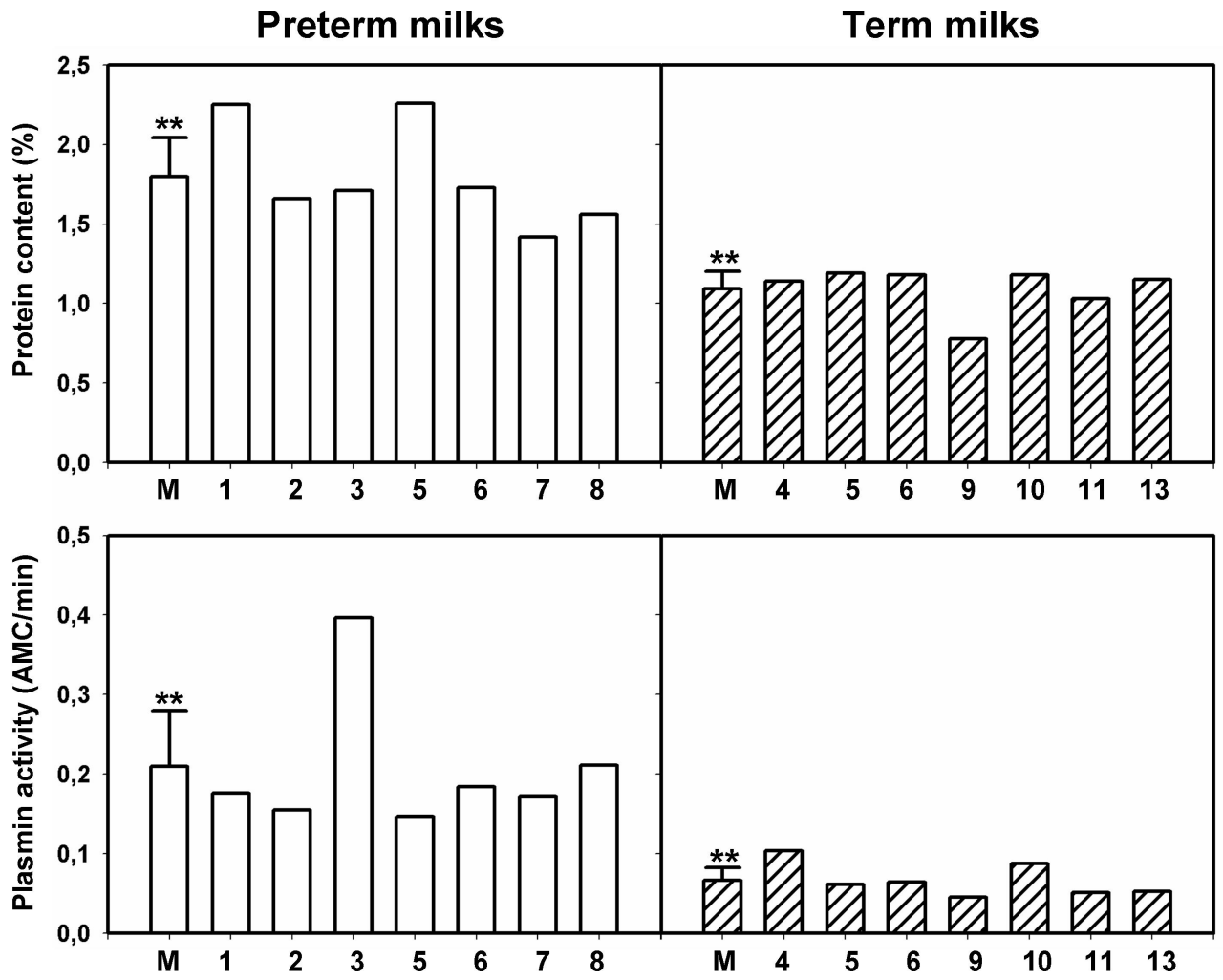
Specific interest in term and pre-term mothers' milk

Pre-term samples

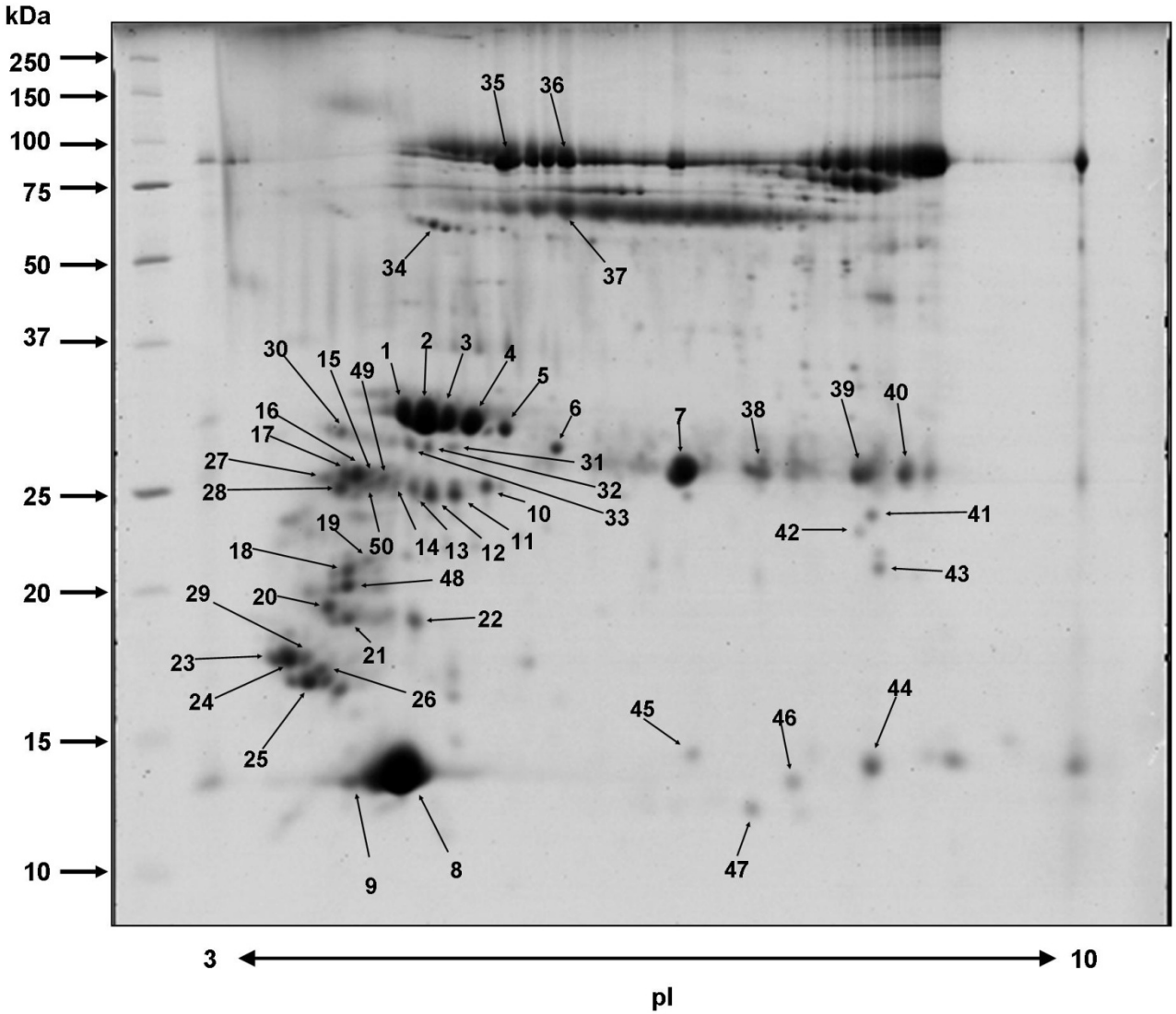
Term samples



Comparison of milk from term and pre-term mothers



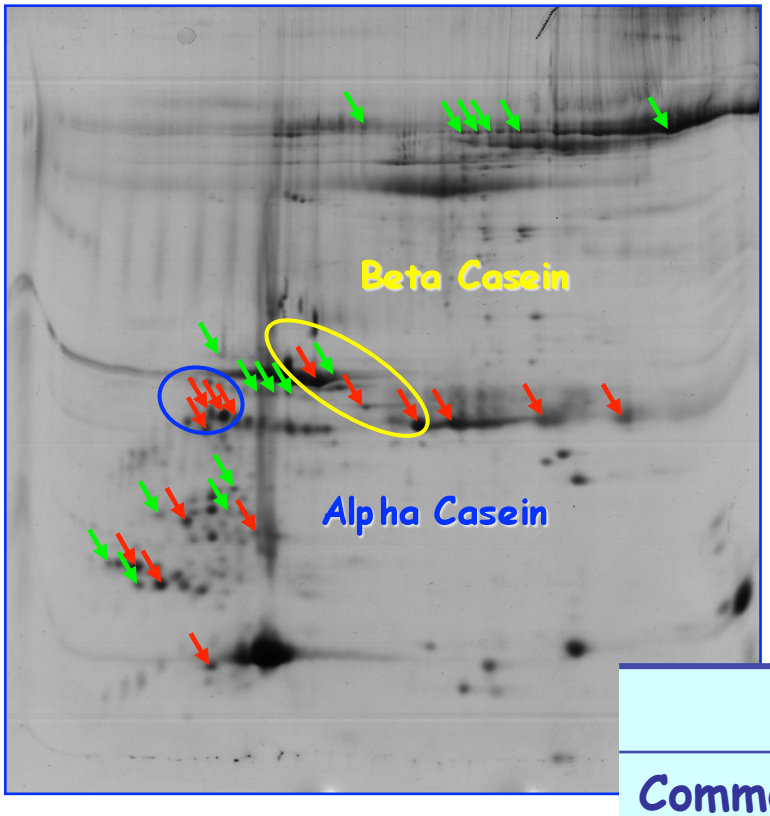
The reference human milk proteome



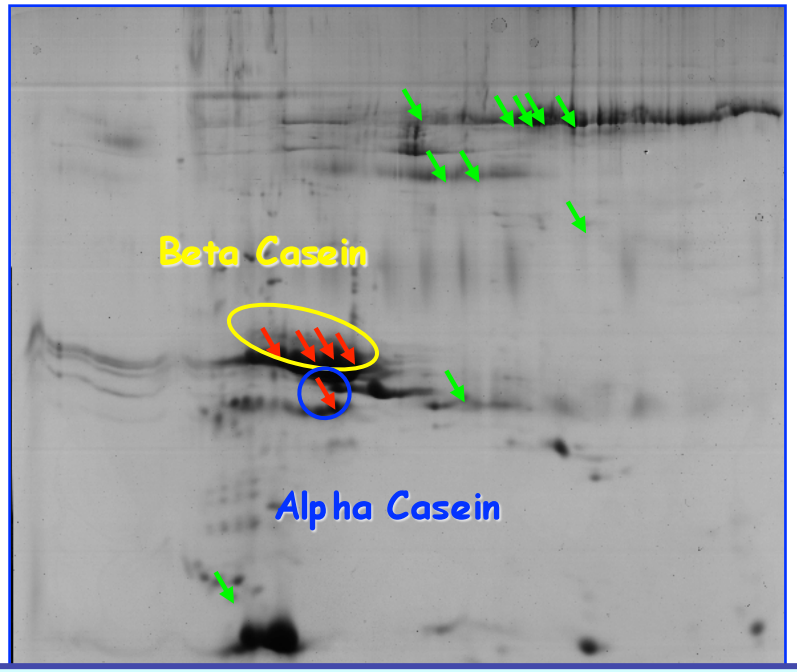
1-5, 7: β-CN
6:β-casein/immunoglobulin
8-9: α-lactalbumin
10-17: α-casein
18-19: αs1-casein/β-casein
20-22, 24-26, 30:αs1-casein
23: serine protease
27-28: immunoglobulin J 29-31: αs1-casein / β-casein / anti-pneumococcal antibody
32: αs1-casein / β-casein
33: αs1-casein / β-casein / α-lactalbumin
34: α1-antitrypsin / κ-casein
35-36: lactoferrin
37: lacto-transferrin / immunoglobulin
38-40: immunoglobulins
41-44: β-casein
45: fatty acid binding protein
46: β-casein / α-lactalbumin
47: β2-microglobulin
48: lactoferrin / αs1-casein / β-casein
49-50: αs1-casein.

Differential expression of spots between milk samples

Preterm milk



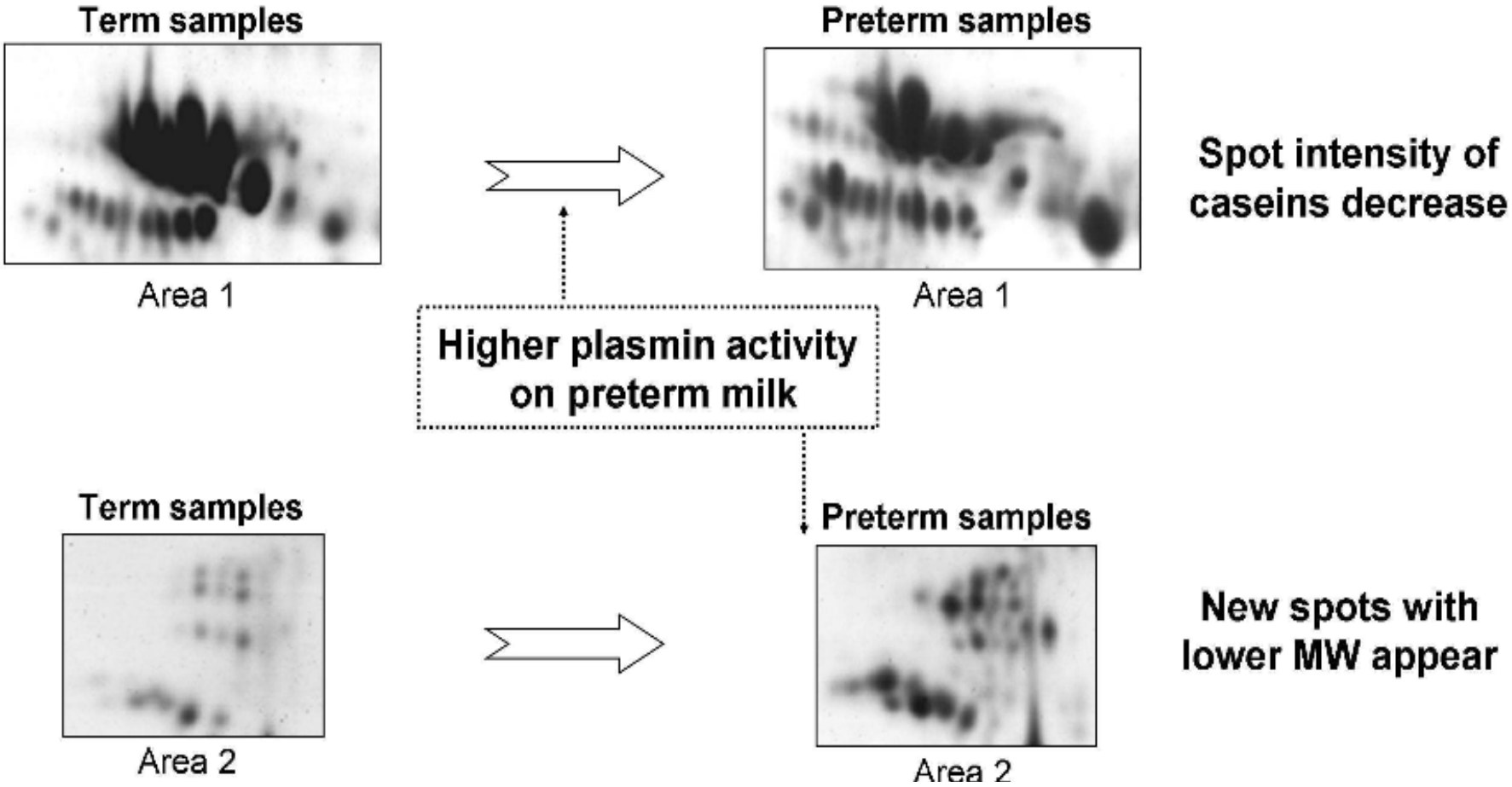
Term milk



Green = Specific
Red = over-expressed

	Preterm	Term
Common spots	84	
Specific spots	69	10
Over-expressed spots	48	7
Under-expressed spots	7	48
No differences	29	29
Total spots	153	94

A putative mechanism



Modulation of proteins and enzymes to fit needs of neonate or physiological consequence of maternal stress?

Conclusions…

- Milk enzymes a niche but rich field of research in dairy science
- Plenty of questions remain about role and significance of indigenous milk enzymes such as plasmin
- Studies of bovine and human milk have revealed new physiological aspects of enzyme roles

Thank you for the invitation to be here and for your attention!