Chromatographic analysis of serum protein in bulk milk – a possibility to detect changes of the mammary tissue as well as of the manufacturing properties of milk

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Abstract

Chromatographic analysis of serum protein in bulk milk (BM) samples using SE-FPLC was applied to deduce on the average udder health status and on the manufacturing properties in the dairy industry. The manufacturing properties were adversely affected by an increase in proteinase activity due to effects of mastitis. The increased proteinase activity in BM samples was correlated to an increase in free individual casein and bovine serum albumin but to a decrease in α-lactalbumin and lactose.

1. Introduction

It is now widely accepted that bulk milk somatic cell count (BMSCC) can provide a reliable estimate of the prevalence of infection within herds and cows and that the main contribution to an elevated BMSCC is mastitis, either sub-clinical or clinical (Eberhard *et al*., 1982; Holdaway *et al.*, 1986a-c). This acceptance has also led to a clearer understanding in the relationship between mastitis, milk composition and dairy product quality.

Casein-derived products are most severely affected by an elevated SCC and intense research has addressed the effects on cheese yield and quality. Milk from mastitic udders exhibits greatly increased proteolytic activity (Grieve & Kitchen, 1985; Senyk et al., 1985; Seaman *et al*., 1988; Urech *et al*., 1999). Involved changes on the surface hydrophobicity of native casein micelles were irreversible and may be applied to conclude on the udder health at a time before effects of the cellular immune defence are detected (Lieske, 1999).

The new findings were to confirm by ongoing investigations in related effects on the composition of proteins in whey or milk serum due to *(i)* proteolysis of micellar casein, *(ii)* an increasing cellular immune defence (Coffin *et al*., 1983; Rogers *et al*., 1989; Urech *et al*. 1999), and *(iii)* the "leakage" of serum constituents into milk and *vice versa* (Poutrel *et al*. 1983; Paape *et al*., 1995; Lacy-Hulbert *et al*., 1996). It was realised by chromatographic separations of the protein present in the serum of bulk milk samples exhibiting a moderately increased proteinase activity.

2. Material and methods

2.1. Origin and preparation of milk samples

Two kinds of bulk milk (BM) were investigated: first, *(i)* BM from a local farm collected from 250 lactating cows. The mean BMSCC was estimated between 460000 and 755000 ml^{-1} and values of colony forming units (cfu) were between 14000 and 22000 ml^{-1} during the time of this study (5 weeks). Using this BM in cheese making (Mozzarella and a Tilsit cheese) resulted in a poor quality and a reduced yield. The other *(ii)* BM was collected from a dairy factory silo. Data of BMSCC and cfu were unknown due to the use of preservatives before sending off. Using this BM to produce functional milk protein concentrates resulted in product qualities which were difficult to predict or to give a reliable guarantee. In both milks,

(*i*) and (*ii*), effects of an increased proteolysis of micellar casein were detected in our laboratory.

2.2. Sample preparation

Proteolysis was taken into account for preparing the samples. These were skimmed at 3000 *g*, 15 °C for 20 min. The skimmed milk was stabilised by addition of 0.02% (w/v) NaN_3 and by aprotinin (3 mg l^{-1}) purchased from Sigma (Deisenhofen, Germany) for preventing bacterial growth and proteolysis by serin proteinases, respectively. In this way stabilised milk samples were centrifuged at 45000 *g*, 20°C for 1 h to separate the milk serum from casein.

2.3 Chromatographic analysis

The equipment used for chromatographic separation was the FPLC system of Pharmacia (Freiburg, Germany) which was connected to a variable UV-detector Uvicord VW 2251 and a Shimadzu C-R 6AJ integrator. Milk serum was separated by size exclusion (SE) in a Superose 12 column HR 10/30 (Pharmacia LKB) whereas the casein present in milk serum was collected from SE-eluate and separated by hydrophobic interaction (HIC) in a Phenyl-Superose HR 5/5 column (Pharmacia LKB). Conditions used for chromatographic separations were indicated with the profiles shown in Figs. 1, 2.

2.3. Chemical analysis

For quantifying the lactose in skimmed milk the cuprous oxide method (IDF 1967) was used.

3. Results and Discussion

A typical chromatographic SE profile of BM serum is shown in Fig. 1. The serum protein is separated in 6 individual protein fractions which were identified as immunoglobulin (Ig) M and G (fraction 1 and 2), bovine serum albumin (BSA, fraction 3), β-lactoglobulin (β-Lg, fraction 5) and α-lactalbumin (α-La, fraction 6).

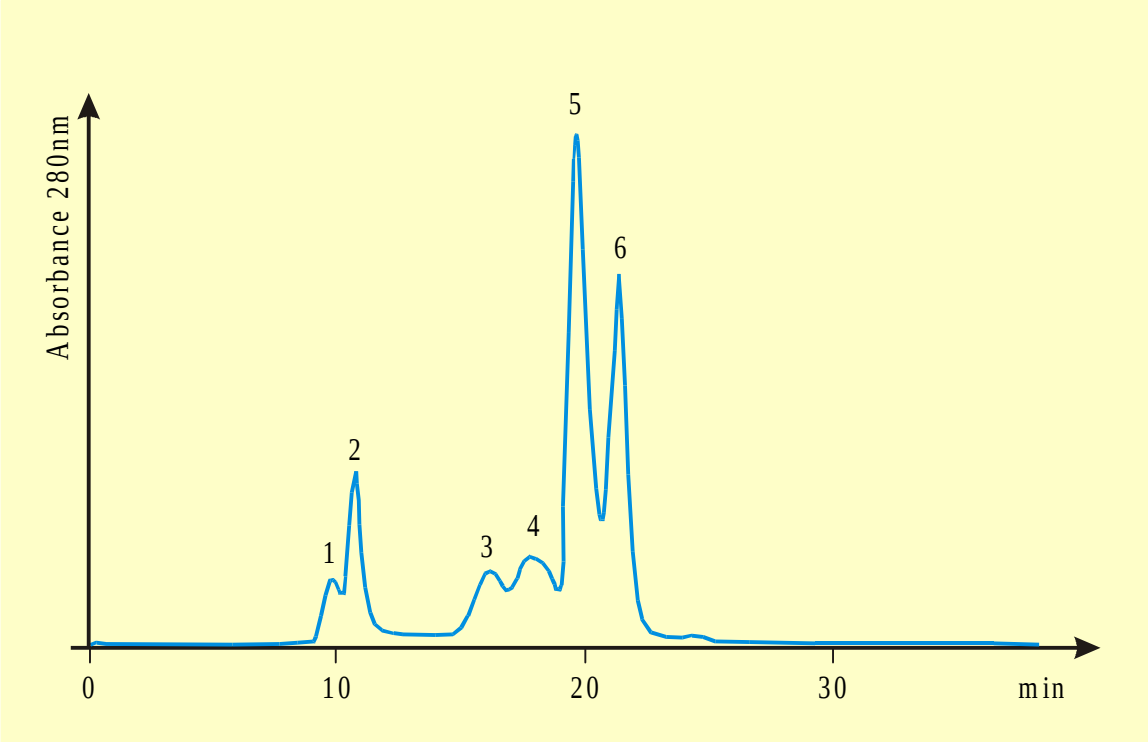

Fig. 1: Separation of serum proteins from BM samples by SE-FPLC on a Superose 12 HR 10/30 column at 20 °C and with a phosphate buffer (0.05 M NaH_2PO_4 / 0.15 M NaCl) pH 7.0. All sera were desalted on an Econo Pak 10 DG column prior to the application of 25 µl serum to the column. The flow rate was 0.7 ml min^{-1}; detection 280 nm.

The origin of the protein in fraction 4 was unknown, but in presence of chymosin or at pH 4.6 most of the protein in this fraction was precipitated what is indicating to soluble casein (Cn). To confirm this fraction 4 was collected from the eluate of the Superose 12 column. The protein in this fraction was isolated and analysed by a further separation in a HIC column as shown in Fig. 2. In the profile three protein fractions are seen belonging to κ-Cn, α_{S2}-Cn and α_{S1}-Cn. From the chromatographic separations obtained by SE-chromatography it was calculated that both BM samples released 13.31 ± 0.32% soluble Cn in the milk serum on centrifugation at 45000 *g*, 20°C for 1h. When the same conditions were applied to reconstituted milk samples from "low heat" milk powder (Nilac, NIZO, The Netherlands) then the soluble Cn in whey amounted to 3.65 ± 0.1%. With raw milk from healthy udders the free Cn may by estimated below this value.

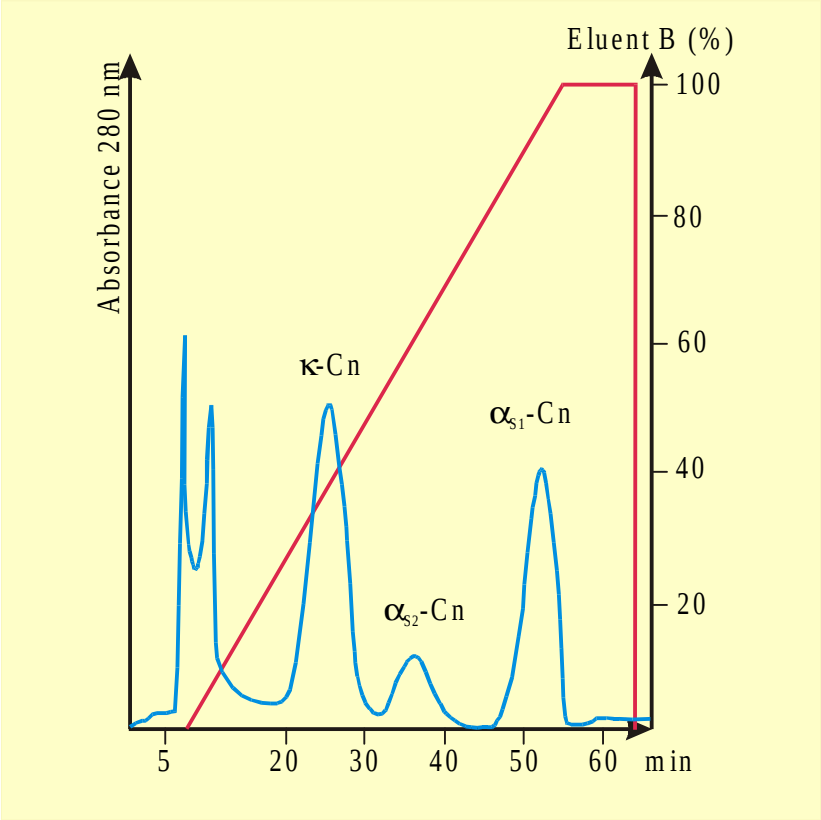

Fig2: HI-FPLC separation of individual caseins in milk serum, isolated by isoelectric precipitation; the precipitate was washed twice at pH 4.6 and dissolved in 0.48 M sodium phosphate, 2.5 M urea, 0.1 % dithiothreitol, pH 6.3; a 50µl sample was applied to the column and eluted at a flow rate of 0.3 ml min^{-1} using a 0.48 - 0.037 M sodium phosphate gradient together with a pH 6.3 - 6.5 gradient in 2.5 M urea at 20 °C

The increase in soluble Cn in the serum of BM samples is a result of an enhanced proteolytic activity affecting the colloidal stability of casein micelles and thus the processing properties of the milk. The analysed Cn is an intermediate product of proteolysis and was degraded further to peptides of different sizes as soon as proteinase inhibitors were omitted. The proteolysis lead to a differentiated decrease in the relative proportion of soluble Cn in raw milk serum as shown in Fig. 2. In the chromatographic profile of BM samples no β-Cn was analysed due to an early digestion by plasmin proteinases that occurred prior to the addition of stabilising agents. The high proportion of soluble κ-Cn may be explained by the known resistance to proteolytic degradation caused by plasmin.

In Tab. 1 the results of chromatographic analysis of whey protein are summarised comparing the quantitative proportions between the individual whey proteins in dependency on the origin of the milk samples. The calculation is based on the known coefficients of extinction at 280 nm according to Eigel *et al.* (1984). The quantity of Ig's was similar in all substrates compared in Tab. 1. It is seen that no relationship exists between BM samples with elevated

SCC or reconstituted milk and the Ig-content. For BM samples the SCC seemed to be the more reliable indicator of the cellular immune defence than the concentration of Ig's.
The concentration of BSA in BM samples increased by double the amount of that in milk from healthy udders. This increase in protein of blood serum origin is possibly due to a disruption of the integrity of the mammary epithelia allowing 'leakage' of serum constituents into milk and milk constituents into the blood (Paape *et al*., 1995). As seen in Tab. 1, the concentration of β-Lg in both BM samples remained constant. This protein is known to be relatively resistant to proteolytic attack due to structural properties in the native status (Konrad & Lieske, 1997).
The other whey protein synthesised *de novo* in the mammory gland is α-La. It showed a decrease by about 33% in both BM samples that was corresponded to a decrease in the lactose content by about 2.6 g kg^{-1} (Tab. 1).

Chromatographic fraction of	Bulk milk samples *(i)*	*(ii)*	Reconstituted low-heat powder[a]	Data from Walstra & Jenness
IgM plus IgG	12.3 ± 0.25	13.8 ± 0.30	12.8 ± 0.20	13.7
BSA	16.0 ± 0.20	15.3 ± 0.22	8.1 ± 0.30	7.2
β-Lg	57.4 ± 0.41	56.4 ± 0.42	60.7 ± 0.47[b]	57.7
α-La	14.3 ± 0.20	14.5 ± 0.20	18.3 ± 0.10[b]	21.6
Lactose (g kg^{-1})	43.3	43.5	47.0	46.0

[a] "Nilac" skimmilk powder (NIZO)
[b] partially complexed protein due to thermal effects

Tab. 1: Quantitative proportion of individual whey protein components in the serum of bulk milk samples and reconstituted milk compared with data of Walstra & Jenness (1984) (expressed as percentage)

The biological activity of α-La is the interaction with galactosyl transferase to promote the transfer of galactose from UDP galactose to glucose to form lactose (Walstra & Jenness, 1984). This interrelation may explain the stated deficiency in the concentration of lactose and it has been suggested that lactose may by used as an indicator of mastitis (Renner, 1975). However, the decrease of α-La is probably caused by the leakage of this protein out of the milk into the extracellular fluid. Support for this theory is afforded by the detection of elevated concentrations of α-La in the blood of cows with elevated SCC (Mc Fadden *etal*., 1988).

4. Conclusion

Subclinical mastitis is known to have a multitude of effects on the quantity, quality and processing properties of the raw milk. In the dairy practice the detrimental effects of processing such milk are less well known due to the relationship between BMSCC and the actual mastitis situation in a herd was found to be rather poor (Minjen *et al*., 1982). Both BM samples studied were affected by an increased proteinase activity contributed by milk collected from infected udders. It requires a greater attention to the control of mastitis in the dairies to prevent that such milk for further refinement is processed for further refinement.
The chromatographic approach proposed is a powerful method to obtain this information by one fast separation in a SE-column. The quantitative analysis of the individual proteins fractionated from milk serum allows a differentiated insight in, first, the colloidal status of casein micelles indicated by the concentration of individual free Cn and, second, in the status of udder health indicated by the concentrations of BSA and α-La in milk serum.

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