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Sieving Effect of Heat-Denatured Milk Proteins During Ultrafiltration of Skim Milk. I. The Preliminary Approach

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ABSTRACT

The effect of heat treatment of skim milk on the ultrafiltration process was examined. The change in permeate collection rate was explained as a function of heat-induced modifications of the milk protein system. It is suggested that there was a sieving effect which contributed to the acceleration of permeate flow-down during membrane filtration. It is thought that this resulted from formation of complex structures between heat-denatured whey proteins and casein micelles.
(**Key words:** milk, protein, heat-denaturation, ultrafiltration)

Abbreviation key: ANS = 1,8-anilinonaphthalene sulfonic acid.

INTRODUCTION

Heat treatment of milk is widely used to modify the properties of milk and milk products. The main change that occurs during heat treatment is denaturation of the whey proteins. β-Lactoglobulin and α-LA are the whey proteins that play an important role in the functional properties of heated dairy products (Singh and Newstead, 1992). The denaturation of β-LG and α-LA in milk and various model systems has been widely studied (Dannenberg and Kessler, 1988; Parris et al., 1991; Corredig and Dalgleish, 1999). Changes in milk composition influenced heat-induced interactions of proteins (Smits and van Brouwershaven, 1980). Many of these modifications cause changes in surface properties of the milk protein system. Protein surface hydrophobicity is also known to play an important role in determining several technologically relevant properties of milk. The hydrophobicity of proteins has long been interesting for its contribution to the stabilization of native protein structure and their modifications (Tanford, 1973; Bonomi et al., 1988). Many hydrophobic sites play an important role in molecular interactions (Bonomi et al., 1988; Erdem, 2000; Yuksel and Erdem, 2005). Changes in the surface hydrophobicity of milk proteins can be quantified by following the binding of a fluorescent probe, 1,8-anilinonaphthalene sulfonic acid (**ANS**), to accessible hydrophobic sites (Bonomi et al., 1988; Bonomi and Iametti, 1991; Erdem, 2000). This paper proposes a model for explaining the acceleration of permeate flux during the membrane processing of milk after heat treatment.

MATERIALS AND METHODS

Raw milk samples were obtained from the institution dairy. The samples were skimmed using a laboratory scale centrifuge, preserved by addition of sodium azide (1: 10,000), and stored at 4°C. 1,8-Anilinonaphthalene sulfonic acid was used as the fluorescent probe. All chemicals were of analytical grade.

Heat treatment of milk samples was carried out using a controlled water bath and thin wall Pyrex beakers with forced stirring. Each sample was separated into 2 aliquots. The first was ultrafiltered immediately after heat treatment as follows; 63°C for 30 min, 70°C for 1 or 5 min, 75°C for 1 or 5 min, 80°C for 5 min. The second aliquot was heat treated and stored at 4°C for 2 d and then ultrafiltered.

Ultrafiltration of heat-treated samples and unheated sample (raw) was carried out using an Amicon stirred cell (model 8200, Amicon GmbH, Witten, Germany) and Diaflo YM10 membrane (nominal molecular weight cut-off, 10 kDa) at 3 ± 0.5 kg/cm^2 pressure. All samples were concentrated 2-fold, as determined by the volume of permeate obtained. The cell was filled with 100 mL of sample, and run until 50 mL of permeate was collected into a measuring flask. The volume of permeate obtained was recorded during the run.

Undenatured whey protein index was determined according to the method of A/S Niro (1978). Surface hydrophobicity of protein in milk samples was determined by measuring the relative intensity of fluorescence with ANS as fluorescent probe using a Perkin Elmer model LS50B spectrofluorimeter (Perkin Elmer, London, UK) according to the method of Erdem (2000). Protein con-

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Table 1. Nondenatured whey protein nitrogen index (WPNI) and fluorimetric parameters of experimental milk samples.[1]

Sample	WPNI	F_{max}	K_d	$1/K_d$	F_{max}/K_d	F_{max}/P	PSH
Raw	4.91	292.57	13.39	0.075	21.85	12.25	0.915
63°C/30 min	4.56	314.71	11.93	0.084	26.38	11.24	0.942
70°C/5 min	4.24	311.84	11.12	0.090	28.04	10.95	0.985
80°C/5 min	2.18	318.90	13.66	0.073	23.35	10.89	0.797

[1]F_{max} = Maximum fluorescence at the saturated ANS concentration; K_d = ANS concentration at $F_{max}/2$; P = protein concentration in g/L; and PSH = protein surface hydrophobicity.

tent of the samples was determined by the method of Bradford (1976).

SDS-PAGE

The electrophoretic pattern of the samples was determined using a mini vertical slab gel system (mini-Protean-iii, BioRad, London, UK) for SDS-PAGE according to Laemmli (1970). All data presented were the average of 3 determinations.

RESULTS AND DISCUSSION

The spectrofluorimetric and whey protein denaturation data are given in Table 1. It is suggested that heat treatment affects surface hydrophobic sites of casein micelles. The titration of protein solutions with increasing concentration of the fluorescent probe (ANS) provides information on the number and affinity of binding sites. It was apparent that the number of surface hydrophobic sites of the milk protein system (F_{max}; the maximum fluorescence at the saturated ANS concentration, asymptotic value of the titration curve) increased with heat treatment. However, it was found that the average tightness of binding (F_{max}/K_d) between ANS and the casein complex was increased, whereas the affinity of ANS to the protein ($1/K_d$) decreased with heat treatment. The K_d is the ANS concentration at $F_{max}/2$ due to Michaelis-Menten kinetics, which denotes the dissociation constant of ANS-protein complex. The number of surface hydrophobic sites per protein (F_{max}/P) was decreased by increasing temperature or time of heat treatment, or both.

If the average tightness per protein is considered, the patterns are more clear. This parameter is called the protein surface hydrophobicity index and is calculated as follows:

$$PSH = F_{max}/K_d \cdot [P]$$

where [P] is protein concentration in g/L.

The data suggest that heat-denatured whey proteins form complexes with casein micelles because of increasing hydrophobicity or hydrophobic interactions. These increased interactions improve the size of the complex of casein-denatured whey proteins. Increasing the heat exposure during the heat treatment caused the formation of a compact structure with increased size of casein-whey protein complex. This suggestion is evaluated from data summarized in Figure 1a and 1b). In agreement with Bonomi and Iametti (1991), it is proposed that heat-induced changes in milk proteins proceeded through different steps. These steps involve an initial relaxation of the protein structure, which can then unfold completely or shrink to a compact structure. Polymerization or formation of more or less strong bonds (possibly hydrophobic) with other macromolecules (caseins) can also occur during heating.

As shown in Figure 1a, the samples treated at 63°C for 30 min (63/30) showed higher permeate flux than raw milk if ultrafiltered immediately after heat treatment. When the heat-treated sample was stored at 5°C, the membrane performance was similar to raw milk. This may be due to renaturation of α-LA and release of β-CN from micelles during cold storage. It has been reported that the degree of denaturation of α-LA, which on its own denatures semi-reversibly (de Witt and Klarenbeek, 1984), increases when β-LG is present (Elfgam and Wheelock, 1977).

If the temperature of the heat treatment was increased, the difference in permeate flux between raw and heat-treated samples became more apparent. After heating to 70°C for 1 min (Figures 1b and 1c), the difference between the fluxes was significant ($P < 0.01$) for stored and unstored samples. Following heat treatment at 75°C (Figures 1d and 1e), the difference was not significant ($P > 0.05$). It is possible that the modification in the milk protein system due to heat treatment was drastic when the temperature was increased above 70°C. It is found that renaturation of α-LA during cold storage is less significant after heating to higher temperatures. It may mean that, at higher temperatures, the complex formation between denatured whey proteins and caseins is stronger than at lower temperatures or heating times. The most drastic results were obtained for the treatment at 80°C for 5 min (Figure 1f).

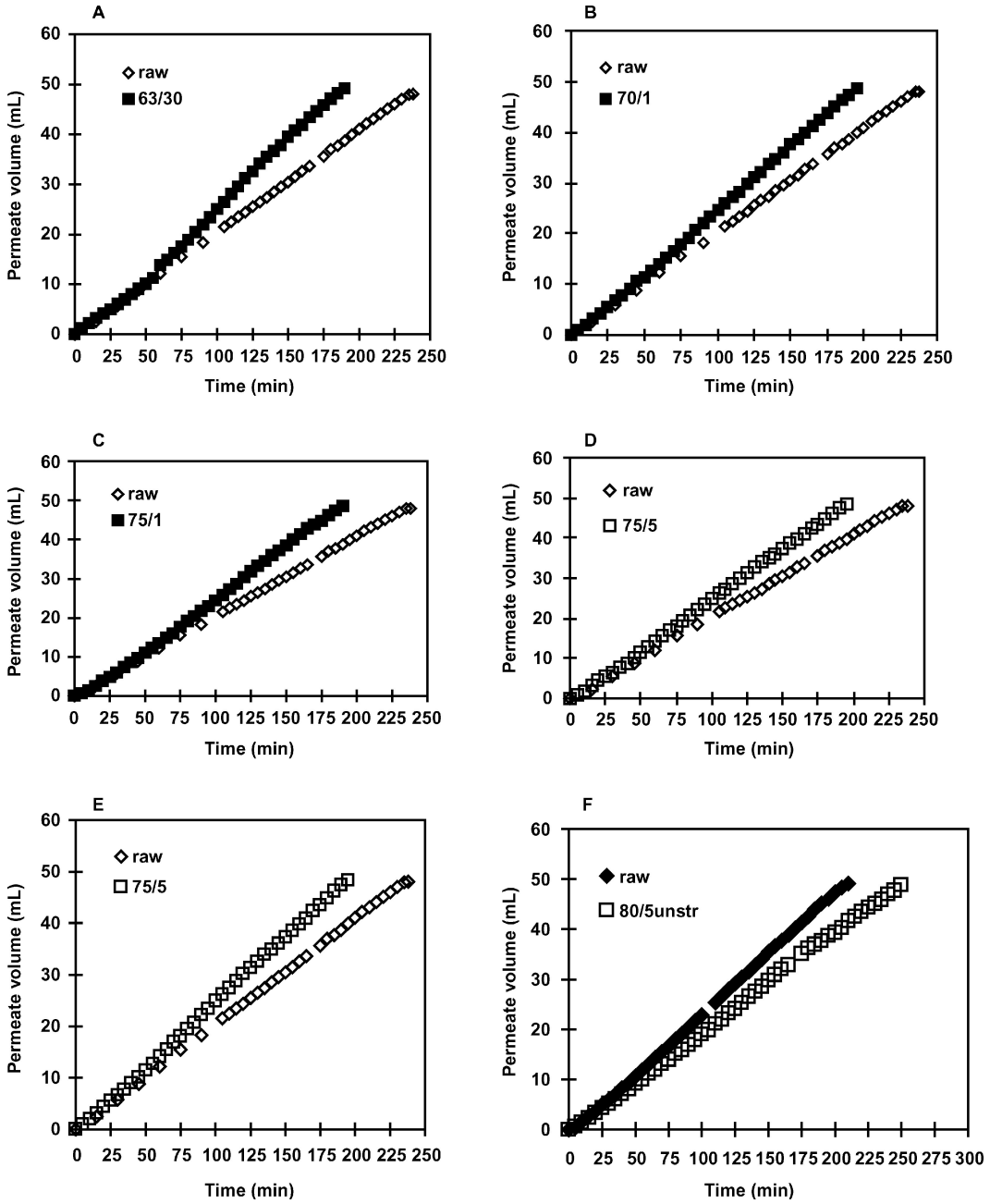

Figure 1. Permeate flow rate of heat-treated milk at 63°C/30 min (A), 70°C/1 min (B), 70°C/5 min (C), 75°C/1 min (E), 75°C/5 min (F), and 80°C/5 min (G).

According to our results, a model for the effect of heat treatment on the performance of the ultrafiltration process was proposed, as shown in Figure 2. Of the main whey proteins, β-lactoglobulin is known to be the most heat-sensitive. Heat denaturation occurs at temperatures >60°C. During heat treatment, small aggregates of β-LG are formed first. At increasing temperature or heating time, the aggregates enlarge, and larger denatured β-LG aggregates formed (Hill, 1989; Jang and Swaisgood, 1990). At increasing time or temperature of heat treatment, denaturation of α-LA begins, the denatured α-LA forms complexes with large denatured β-LG aggregates, and both proteins bind to the surface of the casein micelle (Fox, 1992; Law et al., 1994), possibly via protein-protein interactions or hydrophobic interactions. Such large denatured whey protein-casein complexes accumulate on the membrane surface (this is different from the fouling process) dur-

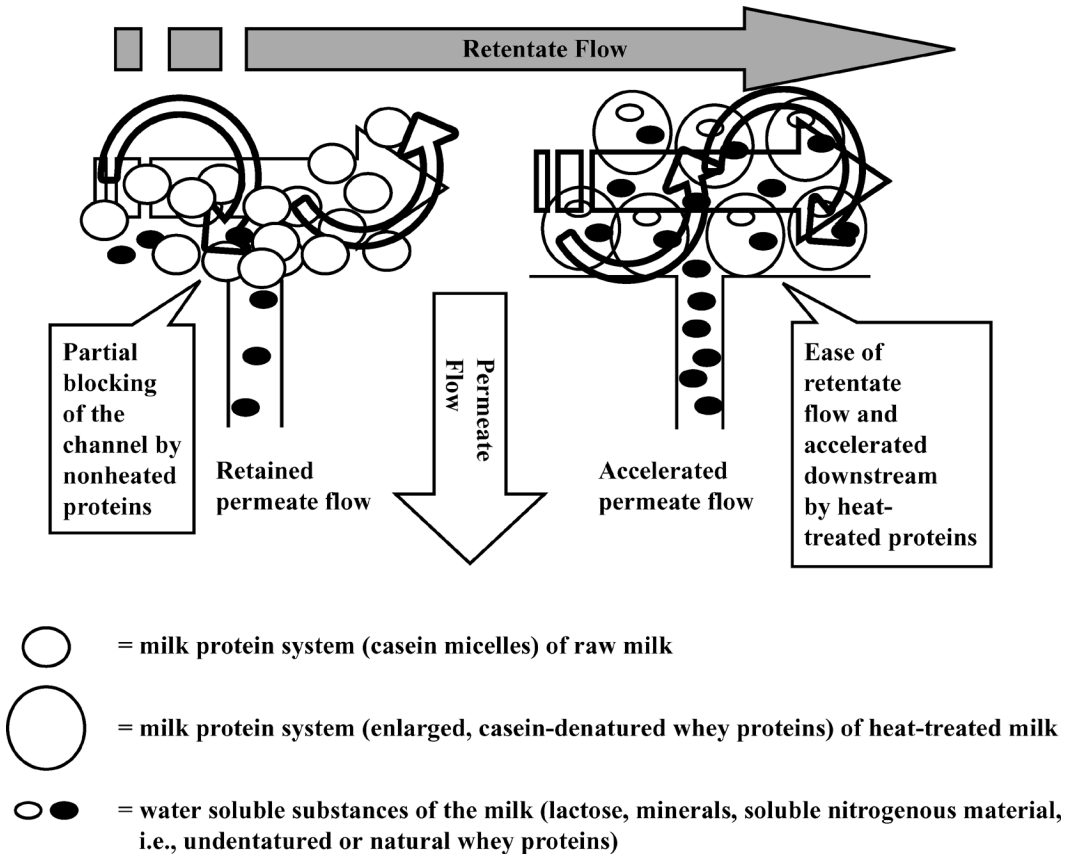

Figure 2. Preliminary model for the sieving effect of heat-modified milk proteins during ultrafiltration.

ing membrane processing. These large and almost spherical (possibly fusiform) aggregates play a sieving role during ultrafiltration. This accumulation of sieving matter (large and porous protein complex) on the membrane surface helps the permeate (small and soluble components of the milk) pass through the pores easily. Moreover, this acceleration helps to overcome membrane or pore fouling. Because of this, the permeate flux increases.

Figure 2 summarizes the sieving effect of heat-denatured whey proteins during the ultrafiltration process. The denatured whey proteins form complexes with casein micelles via attachment to their active or activated surface sites, and cause enlargement of casein micelles. During membrane processing, the macroparticles are retained on the membrane surface and protect the membrane pores from fouling and congestion. The large particles accumulated on the pore (channel) play a sieving role during filtration and, because of this, the flow of permeate accelerates. The modification of milk protein system, i.e., of whey proteins is shown in Figures 3 and 4. In these figures, the SDS-PAGE electrophoretograms are presented. It is apparent that the band intensities of α-LA and β-LG decreased at temperatures above 70°C, whereas the intensity of casein bands was increased. This helps to explain the sieving theory applied to the ultrafiltration process. The fluorimetric data also contribute to this suggestion (see Table 1). The number of surface hydrophobic sites per protein (F_{max}/P) decreased with increasing temperature and exposure of heat treatment. It means that the micelle system is becoming compacted or enlarged via hydrophobic protein-protein bindings (e.g., between casein micelles and denatured whey proteins) with increasing temperature of heat treatment.

CONCLUSIONS

The model described above explains the extent and time course of some structural rearrangements resulting from exposure of milk or milk proteins to increasing temperature during heat treatment and the effect on permeate flow during ultrafiltration. The extent of heat treatment affects the level of denaturation of whey proteins. Cold storage of heat-treated milk may cause some rearrangements in the milk protein system.

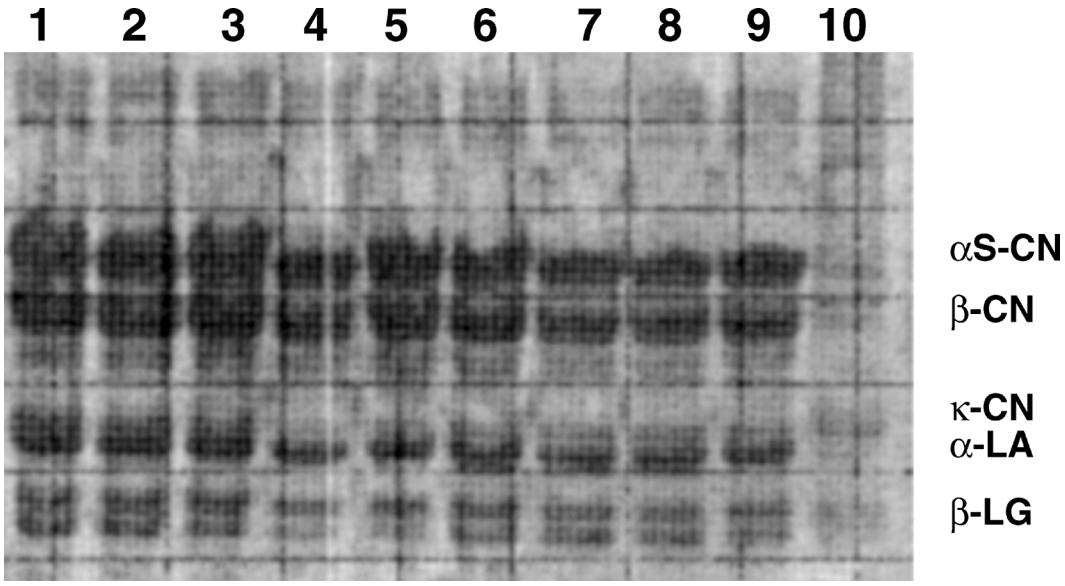

Figure 3. Sodium dodecyl sulfate-PAGE electrophoretogram of the samples. Lane 1 = 70°C/1 min heated-unstored milk's retentate; lane 2 = 63°C/30 min heated-unstored milk's retentate; lane 3 = raw milk's retentate; lane 4 = 75°C/5 min heated unconcentrated milk; lane 5 = 75°C/1 min heated unconcentrated milk; lane 6 = 70°C/5 min heated unconcentrated milk; lane 7 = 70°C/1 min heated unconcentrated milk; lane 8 = 63°C/30 min heated unconcentrated milk; lane 9 = raw, unconcentrated milk; and lane 10 = molecular weight marker.

Both of these situations affect the performance of membrane processing of milk. The denatured whey proteins form large complexes with casein micelles, and these aggregates play a sieving role on the membrane surface, which accelerates the permeation. This preliminary approach should be achieved by extensive future work with different molecular weight cut-off membranes and heat treatments.

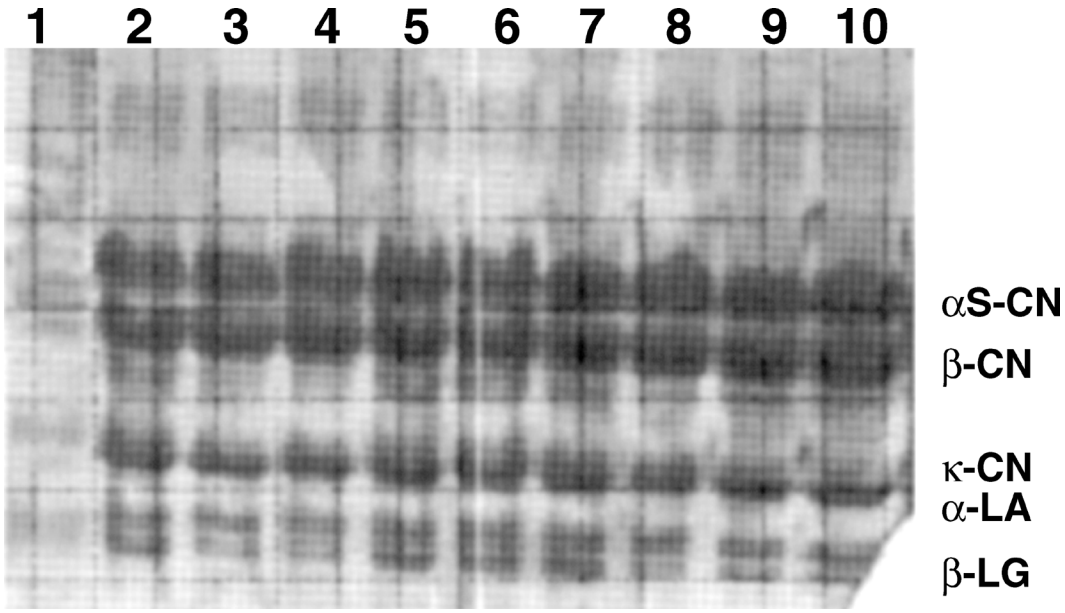

Figure 4. Sodium dodecyl sulfate-PAGE electrophoretogram of the samples. Lane 1 = molecular weight marker; lane 2 = 70°C/5 min heated-unstored milk's retentate; lane 3 = 75°C/1 min; lane 4 = 75°C/5 min heated-unstored milk's retentate; lane 5 = 63°C/30 min heated-stored milk's retentate; lane 6 = 70°C/1 min heated-stored milk's retentate; lane 7 = 70°C/5 min heated-stored milk's retentate; lane 8 = 75°C/1 min heated-stored milk's retentate; lane 9 = 75°C/5 min heated-stored milk's retentate; and lane 10 = 75°C/5 min heated-stored milk's retentate.

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