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Dragoljub D.J. Gavaric

Marijana Caric

Miloslav Kalab

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## EFFECTS OF PROTEIN CONCENTRATION IN ULTRAFILTRATION MILK RETENTATES AND THE TYPE OF PROTEASE USED FOR COAGULATION ON THE MICROSTRUCTURE OF RESULTING GELS

Dragoljub Dj. Gavarić, Marijana Carić, and Miloslav Kaláb\*

Faculty of Technology, University of Novi Sad, 21000 Novi Sad, Yugoslavia, and  
\*Food Research Centre, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6

### Abstract

Milk retentates (35% total solids, 13% protein) obtained by ultrafiltration were diluted with the permeate to 3.2, 5.0, 6.5, 10.0, and 13.0% protein and coagulated using commercial proteases. Rennet or one of microbial proteases isolated from *Bacillus polymyxa*, *Endothia parasitica*, *Mucor miehei*, or *Mucor pusillus* were used. Coagulation times were decreased with the *Mucor* proteases as the protein concentrations in the retentates were increased but the coagulation times were increased with *B. polymyxa* and *E. parasitica* proteases under similar conditions. Firmness was higher in gels made from homogenized retentates than from nonhomogenized retentates of the same protein concentrations. Scanning as well as transmission electron microscopy showed increasing densities of the protein matrices in the gels as the protein concentrations were increased. Large fat globules and their clusters were noticeable in gels made from nonhomogenized retentates. Gelation of homogenized retentates resulted in uniform matrices with the dimensions of the disintegrated fat particles similar to those of casein micelles. These minute fat particles were closely associated with the protein matrix. Firm gels made by coagulating the retentates with rennet consisted of extensively branching casein particle chains whereas softer gels made with *B. polymyxa* protease consisted of small casein particle clusters.

### Introduction

Ultrafiltration (UF) of milk is rapidly becoming one of the most important processes in the dairy industry, particularly because it reduces the volume of the milk by removing a substantial amount of water from it. A part of lactose and mineral salts present in the milk is also removed. Thus, the retentate has a higher protein content than the original milk whereas the lactose and mineral contents remain at about the same levels as in the milk.

The use of milk retentates in cheese manufacture has, therefore, numerous advantages which are of both technological and economical nature. The most important advantages are higher yields (5-15%), continuity of processing following the introduction of automation, decrease of up to 50% in the requirement for starter and rennet, increased nutritive value because whey proteins are retained in the product, a lower biological oxygen demand for the breakdown of the waste materials, and a closed sanitary system. However, complications may arise from differences in the chemical composition and physical properties of the retentates and the milk, e.g., the changed ratios of the individual components resulting in an increased buffering capacity of the retentate, a higher calcium concentration in it, and a higher viscosity of the retentate. The effects of proteolytic enzymes other than calf rennet (which is in short supply on the global scale) on UF retentates may also differ from the effects of this enzyme on milk. Only proteases isolated from a few species of fungi, such as *Mucor miehei*, *M. pusillus*, and *Endothia parasitica*, and bacteria, such as *Bacillus subtilis*, *B. cereus*, and *B. polymyxa* meet the criteria for good quality cheese production.

The objective of the study was to examine the effects of commercial proteolytic enzymes of various origins on the coagulation of UF retentates having varying protein concentrations, to test the resulting coagula for firmness, and to correlate firmness and the microstructure of the protein matrices as seen under the electron microscope.

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Direct inquiries to D. D. Gavarić  
Telephone number: 38-21-59549

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Table 1. Effect of ultrafiltration on chemical composition of milk retentates\*

Component	Milk	Retentates	
		Nonhomogenized	Homogenized
Total solids (%)	12.13 ± 0.21	34.77 ± 0.85	35.13 ± 0.58
Total protein (%)	3.24 ± 0.13	13.14 ± 0.13	13.07 ± 0.03
Fat (%)	3.87 ± 0.10	16.44 ± 0.59	16.57 ± 0.41
Lactose (%)	4.30 ± 0.18	3.65 ± 0.60	4.00 ± 0.24
Casein (%)	2.74 ± 0.12	11.75 ± 0.15	11.73 ± 0.13
Whey proteins (%)	0.37 ± 0.04	1.28 ± 0.19	1.23 ± 0.08
Total nitrogen (%)	0.51 ± 0.02	2.06 ± 0.21	2.05 ± 0.20
Nonprotein nitrogen (%)	0.021 ± 0.0041	0.017 ± 0.0021	0.017 ± 0.002
Ash (%)	0.71 ± 0.03	1.46 ± 0.07	1.42 ± 0.02
Calcium (mM/L)	27.88 ± 0.66	93.80 ± 5.02	96.19 ± 3.60
Phosphorus (mM/L)	30.47 ± 1.58	84.46 ± 4.68	84.15 ± 1.25
Acidity (°SH)	6.61 ± 0.03	16.79 ± 1.04	16.75 ± 1.32
Acidity (pH)	6.67 ± 0.02	6.65 ± 0.14	6.65 ± 0.10

\* A total of 15 milk batches and 15 retentate batches were analyzed. Relatively large standard deviations are the result of milk batches obtained from various sources over a period of time.

### Materials and Methods

#### Preparation of milk retentates and their analysis

High-temperature short-time (HTST) pasteurized commercial milk was standardized to 3.9% milk fat. The milk was concentrated at 55°C by UF to the final concentration of 36% total solids using an industrial line Pasilac DDS Model 35 equipment (Nakskov, Denmark) furnished with GR 61PP membranes. Pressures at the milk inlet and the retentate outlet were 3.0 bar (300 kPa) and 1.5 bar (150 kPa), respectively. Each retentate was divided into two aliquots, one of which was repasteurized at 81°C for 10 s and homogenized at 70 bar (7 MPa). The nonhomogenized and homogenized retentates were diluted with the permeate to obtain solutions containing 5.0, 6.5, 10.0, and 13.0% protein. Their mean fat contents were 6.0, 7.9, 12.3, and 16.2%, respectively. The original milk which contained 3.2% protein (3.9% fat) was used for control. A total of 15 retentates were prepared.

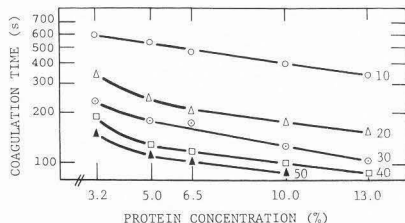


Fig. 1. Effect of protein concentrations in UF nonhomogenized retentates on their coagulation times using rennet (10, 20, 30, 40, and 50 mg/50 mL) as the coagulant.

The following parameters were determined in the retentates and retentate gels:

Dry matter was determined by drying at 102 ± 2°C according to the AOAC methods [4]. Milk fat content was determined by the method of Gerber [4]. Total and nonprotein nitrogen was measured according to the AOAC methods [4]. Lactose was determined according to the method recommended by the International Dairy Federation [22]. Ash was determined by mineralization at 550°C [4]. Calcium was determined using a complexometry technique [26] while phosphorus was determined by spectrophotometry [4].

#### Preparation of retentate gels

Protease solutions (a) to (e), 100 mL each, were prepared by dissolving the following: (a) Rennet (Astra, Nova Pazova, Yugoslavia), 1.00 g, (b) *Mucor miehei* protease (Novo Industry, Copenhagen, Denmark), 0.25 g, (c) *Mucor pusillus* protease (Meito Sanyo Co., Tokyo, Japan), 0.15 g, (d) *Endothia parasitica* protease (Pfizer Chemie, Wiesbaden, Federal Republic of Germany), 1.00 mL,

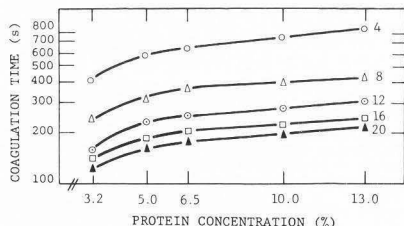


Fig. 2. Effect of protein concentrations in UF nonhomogenized retentates on their coagulation times using *Bacillus polymyxa* protease (4, 8, 12, 16, and 20 mg/50 mL) as the coagulant.

## Ultrafiltration Milk Retentate Gels

Table 2. Effect of protein concentration in homogenized (H) and nonhomogenized (NH) UF retentates on gel firmness

Protease used	Test time (min)	Depth of penetration (mm)*									
		3.2%		5.0%		6.5%		10.0%		13.0%	
		NH	H	NH	H	NH	H	NH	H	NH	H
Rennet	30	1.19 <sup>a</sup>	0.69 <sup>a</sup>	0.73 <sup>a</sup>	4.07 <sup>a</sup>	2.82 <sup>a</sup>	2.21 <sup>a</sup>	0.99 <sup>a</sup>	0.72 <sup>a</sup>	0.18 <sup>a</sup>	
	45	1.03 <sup>b</sup>	0.60 <sup>b</sup>	0.59 <sup>b</sup>	2.11 <sup>b</sup>	1.78 <sup>b</sup>	1.68 <sup>b</sup>	0.90 <sup>b</sup>	0.66 <sup>a</sup>	0.16 <sup>a</sup>	
<i>M. miehei</i> protease	30	1.46 <sup>c</sup>	0.79 <sup>c</sup>	0.72 <sup>a</sup>	4.44 <sup>c</sup>	3.54 <sup>c</sup>	2.46 <sup>c</sup>	0.89 <sup>b</sup>	0.76 <sup>a</sup>	0.55 <sup>b</sup>	
	45	0.96 <sup>d</sup>	0.62 <sup>b</sup>	0.60 <sup>b</sup>	2.25 <sup>b</sup>	2.32 <sup>d</sup>	1.62 <sup>b</sup>	0.60 <sup>c</sup>	0.59 <sup>b</sup>	0.18 <sup>a</sup>	
<i>M. pusillus</i> protease	30	1.51 <sup>c</sup>	0.72 <sup>a</sup>	0.70 <sup>a</sup>	3.75 <sup>d</sup>	3.79 <sup>c</sup>	1.83 <sup>d</sup>	1.07 <sup>a</sup>	1.18 <sup>c</sup>	0.55 <sup>b</sup>	
	45	0.96 <sup>d</sup>	0.60 <sup>b</sup>	0.64 <sup>b</sup>	3.29 <sup>e</sup>	2.82 <sup>a</sup>	1.41 <sup>e</sup>	0.94 <sup>b</sup>	0.52 <sup>b</sup>	0.23 <sup>a</sup>	
<i>E. parasitica</i> protease	30	1.34 <sup>e</sup>	0.74 <sup>a</sup>	0.71 <sup>a</sup>	4.71 <sup>c</sup>	2.36 <sup>d</sup>	2.04 <sup>a</sup>	0.99 <sup>a</sup>	1.01 <sup>d</sup>	0.80 <sup>c</sup>	
	45	1.14 <sup>a</sup>	0.64 <sup>b</sup>	0.61 <sup>b</sup>	2.27 <sup>b</sup>	2.12 <sup>b</sup>	1.85 <sup>d</sup>	0.96 <sup>a</sup>	0.69 <sup>a</sup>	0.72 <sup>d</sup>	
<i>B. polymixa</i> protease	30	1.68 <sup>f</sup>	0.82 <sup>c</sup>	0.81 <sup>c</sup>	5.90 <sup>f</sup>	5.06 <sup>e</sup>	2.45 <sup>c</sup>	1.77 <sup>d</sup>	1.88 <sup>e</sup>	0.89 <sup>e</sup>	
	45	1.50 <sup>c</sup>	0.69 <sup>a</sup>	0.68 <sup>a</sup>	4.65 <sup>c</sup>	4.93 <sup>e</sup>	2.12 <sup>a</sup>	1.47 <sup>e</sup>	1.15 <sup>c</sup>	0.84 <sup>c</sup>	

\* Gels containing 3.2% and 5.0% protein were measured using a probe with a force of 0.71 g/cm<sup>2</sup> and gels having higher protein concentrations were measured using a probe at 3.51 g/cm<sup>2</sup>.

Firmness values which are marked in each column with identical superscript (a to f) do not significantly ( $P < 0.05$ ) differ from each other.

(e) *Bacillus polymixa* protease (Godo Shusei Co., Tokyo, Japan), 0.45 g.

Coagulating activities of the individual enzymatic preparations were not determined but the enzyme concentrations were adjusted in such a way that similar coagulation times were obtained. The need to use working enzyme solutions which varied in concentration indicates that specific activities of the enzyme preparations differed from each other. Standardization of the coagulation time was introduced in order to compare other characteristic properties of the retentates [14].

Retentate aliquots (50 mL) were coagulated in glass beakers using the diluted protease solutions (1 mL). The temperatures of coagulation were 29.0, 32.0, and 35.0°C for gels made at pH 6.65; the temperature of 32°C was also used for gels made at pH 6.3 and 6.5. The retentates were adjusted with 2 M NaOH or 2 M HCl to obtain the predetermined pH values.

#### Determination of coagulation time

The onset of the primary and secondary coagulation phases, induced by fungal proteases [3], were established using the method by Foltman [11] developed for rennet. Coagulation curves were obtained by plotting reciprocal values of the enzyme concentrations (1/c) against coagulation time (t). The slope of the resulting line indicated the duration of the primary phase, and the intercept for  $t = 0$ , indicated the duration of the secondary phase.

#### Firmness measurement

Firmness was measured in gels made from homogenized as well as nonhomogenized retentates containing 5.0, 6.5, 10.0, and 13.0% protein. The measurements were carried out using a Höppler consistometer (VEB Prüfgeräte-Werk, Medingen, Dresden, German Democratic Republic). Gels

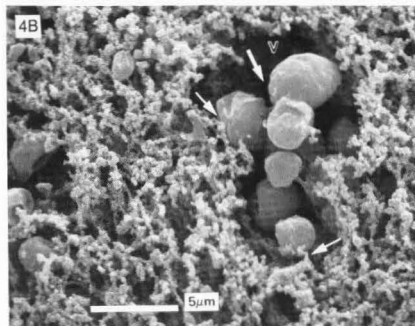
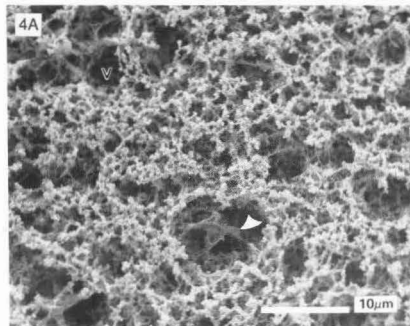
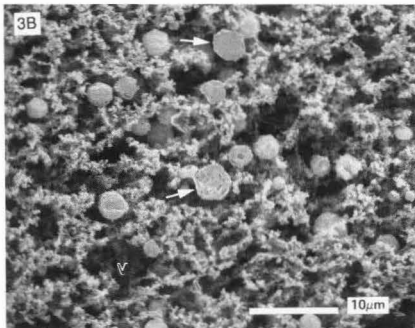
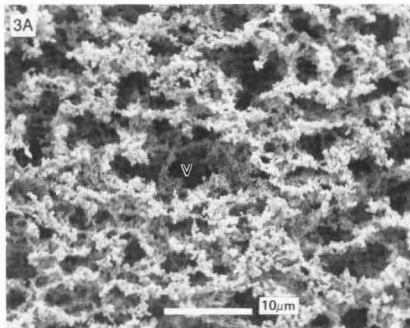
containing 3.2 and 5.0% protein were tested with a spherical weight of 40.2 g (diameter of 85 mm, 0.71 g/cm<sup>2</sup>) and gels containing 6.5, 10.0, and 13.0% were tested with a weight of 99.0 g (diameter of 60 mm, 3.51 g/cm<sup>2</sup>). Firmness was expressed in the depth (mm) to which the weights penetrated the gels 30 or 45 min after gelation.

#### Electron microscopy

The gels were sampled for electron microscopy according to a formula: Sampling Time = 3 x Coagulation Time. Gel particles, approximately 10 mm in diameter, were fixed in a 2.8% glutaraldehyde solution at 20°C for 24 h and mailed to Ottawa for electron microscopy [1].

For scanning electron microscopy (SEM), the particles were trimmed to form prisms, approximately 10 mm long, 1 mm<sup>2</sup> in cross section. In order to visualize fat globules, selected samples were postfixed in an imidazole-buffered 2% osmium tetroxide solution [2]. All samples were then dehydrated in a graded ethanol series. Fat was removed by extraction with chloroform from samples which had not been postfixed with osmium tetroxide. All samples were impregnated with absolute ethanol, frozen in Freon 12 at -150°C, and freeze-fractured under liquid nitrogen. The fragments were thawed in ethanol, critical-point dried from carbon dioxide, mounted on SEM stubs, sputter-coated with gold, and examined at 20 kV in an ISI DS-130 scanning electron microscope equipped with an external oscilloscope [5]. Micrographs were taken on 35 mm film.

For TEM, the samples fixed in glutaraldehyde were cut into particles approximately 1 mm in diameter and postfixed in a 2% osmium tetroxide solution in a 0.05 M veronal-acetate buffer, pH 6.75. The postfixed samples were embedded in a Spurr's low-viscosity medium (J. B. EM Service,



Figs. 3 and 4. Gel made from nonhomogenized milk (Fig. 3: 3.2% protein, 3.9% fat) and from diluted nonhomogenized UF retentate (Fig. 4: 5% protein, 6% fat) using rennet. Void spaces (V) were present in gels prepared for SEM by extracting the fat (Figs. 3A and 4A). An arrowhead points to fat globule membrane fragments (Fig.

4A). Fat globules were retained (Figs. 3B and 4B) by postfixing the gels with osmium tetroxide [2]. Some of the fat globules are fractured (arrows). Fig. 4B shows a detail of a fat globule aggregate (large arrow) surrounded with a void space (V) except for a few points of contact (small arrows) with the protein matrix.

Pointe Claire, Dorval, Quebec, Canada). Thin sections were stained with uranyl acetate and lead citrate solutions [29] and examined in a Philips EM-300 electron microscope operated at 60 kV [23].

### Results and Discussion

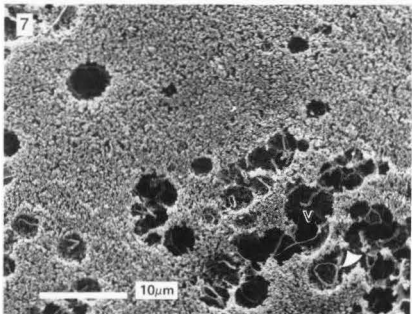
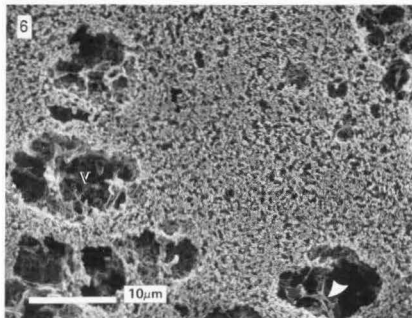
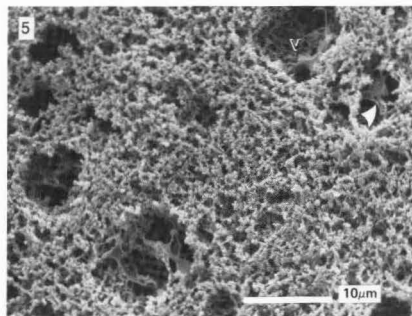
#### Chemical composition

Ultrafiltration of milk resulted in considerable changes in its chemical composition. Individual analyses of the 15 retentates prepared were reported elsewhere [14]. Mean values have been summarized in Table 1. The total solids content of 12.1% in the initial milk was increased by ultrafiltration to 35%. In a similar way, protein was increased from 3.2% to 13.1% and fat was increased from 3.9% to 16.5%. Lactose and nonprotein nitrogen contents were slightly lower in the retentates. This is to be expected as they are likely

to be at the same concentration in the aqueous phase, which is a smaller proportion of the total in the retentates. The relatively high standard deviations were the result of milk batches obtained from various suppliers. Calcium and phosphorus, which in the form of calcium phosphate are part of the casein micelles, were retained in the retentates and their concentrations were increased from 27.9 and 30.5 mM/L, respectively, in the milk to 93.8 and 84.4 mM/L, respectively, in the retentates.

#### Coagulation time

In order to study the effect of protein concentration on the gelation of the retentate, the final retentates were diluted with the permeate in varying proportions. As the protein concentrations in the retentate solutions were increased, the times needed to coagulate them with rennet, and the *M. miehei* and *M. pusillus* proteases decreased



**Figs. 5 - 7.** Gels obtained by coagulating a diluted nonhomogenized UF retentate (Fig. 5: 6.5% protein, 7.9% fat; Fig. 6: 10% protein, 12.3% fat; Fig. 7: 13% protein, 16.2% fat). Void spaces (V) indicating the presence of fat in the original gels before extraction for SEM may contain fat globule membrane residues (arrowheads).

but increased when the *E. parasitica* and the *B. polymixa* proteases were used. Results on this subject have been published in detail elsewhere [14]. *Mucor* proteases and proteases obtained from *E. parasitica* and *B. polymixa* have shown, in general, a higher proteolytic activity than rennet. *E. parasitica* protease, e.g., hydrolyzed 29% of the peptide bonds in casein compared to only 10.2% of the peptide bonds hydrolyzed by rennin [32]. Because the *E. parasitica* protease hydrolyzes whey proteins in addition to the caseins ( $\kappa$ -casein as well as  $\alpha$ <sub>S</sub>- and  $\beta$ -casein), this action has to be taken into consideration when selecting a protease for the manufacture of a particular cheese variety [15].

Characteristic examples obtained with non-homogenized retentates are shown in Figs. 1 and 2 using rennet and *B. polymixa* protease, respectively, to coagulate the milk proteins. Coagulation times with homogenized retentates were shorter than the coagulation times with nonhomogenized retentates.

The reduction in the coagulation times, as the protein concentrations of the retentates coagulated with rennet were increased, may be attributed to a number of factors such as an increased number of effective collisions between the enzyme and the casein molecules [21], a lower number of indispensable hydrolyzed casein micelles to start coagulation [13], and a high concentration of calcium although most of it was in colloidal form.

The properties of the *M. miehei* and *M. pusillus* proteases were anticipated to be similar because both enzymes belong to so-called aspartic proteases [12]. In contrast, the *B. polymixa* protease belongs to metalloproteases, i.e., a group of enzymes considerably different from the previous group as far as their structure and mechanism of splitting protein molecules are concerned. These differences may be responsible for the rate of the proteolysis of  $\kappa$ -casein and the final value of coagulation time.

The behaviour of the *E. parasitica* protease in the retentates having higher protein concentrations differed from the anticipated behaviour. This enzyme possesses an aspartate group in the active centre and, thus, should follow the proteolytic pattern of the other proteases of fungal origin. However, the absence of the carbohydrate moiety in the molecule of this enzyme, which is in contrast to the *Mucor* proteases [10], a different structure of this enzyme and a different pH of optimal activity [28] considerably affected the behaviour of this enzyme in the presence of high-protein substrates.

However, M. L. Green (personal communication) has suggested that the differences in the behaviour of the proteases relate to the differences in specificity rather than to differences in structure. The Michaelis-Menten equation predicts that the subsidiary substrates will compete better with the main substrate as the total substrate concentration increases. This will have a considerably greater effect on the relatively nonspecific enzymes. Data in Table 2 also suggest that nonspecific proteolysis is favoured in the more concentrated milk retentates.

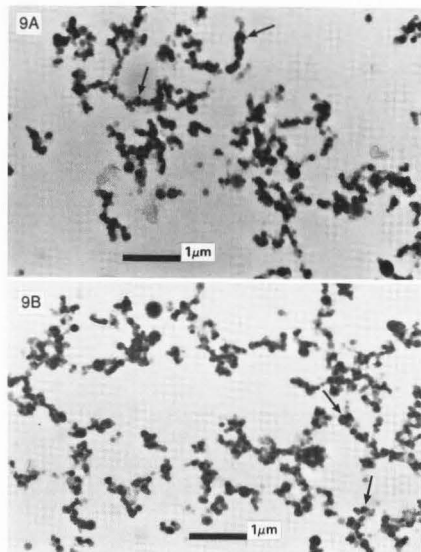
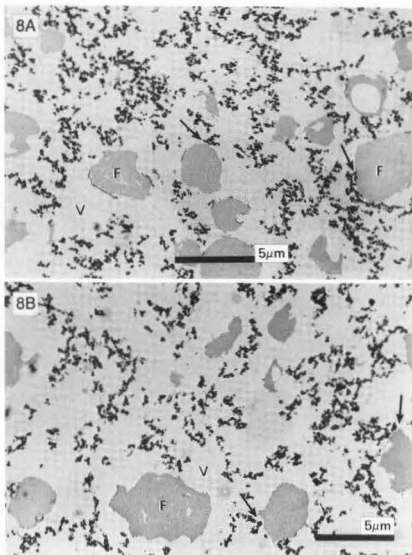
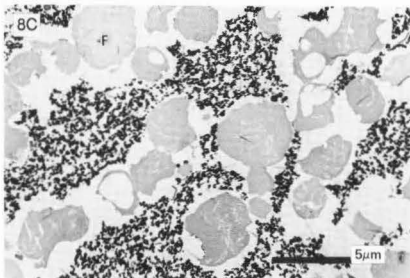
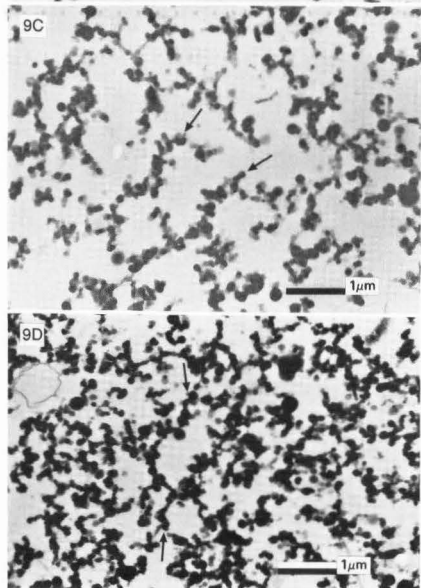


Fig. 8. TEM of gels made from nonhomogenized milk (Fig. 8A: 3.2% protein) and from a nonhomogenized diluted UF retentate (Fig. 8B: 6.5% protein; Fig. 8C: 13% protein) using rennet. Fat globules (F) are relatively evenly distributed in the low-protein gels (Figs. 8A and 8B) but appear to be in the form of clusters in the 13% protein gel (Fig. 8C). There are void spaces (V) around most fat globules with only a few points of contact (arrows) between the fat globules and the protein matrix. Fig. 9. Details of the protein matrices in gels made by rennet from a nonhomogenized UF retentate at varying protein concentrations (Figs. 9A: 5%; Fig. 9B: 6.5%; Fig. 9C: 10%, and Fig. 9D: 13%). Branching of the casein micelle chains (arrows) is increased with the increased casein micelle cluster formation.



### Gel firmness

Gel firmness was another property of the gels where correlations with the protein concentration and the type of enzyme used have been clearly established. These characteristics have been presented and discussed in detail elsewhere [14] in an attempt to analyze the relationships between the nature of the proteolytic enzymes of various origins used and the rheological properties of the retentate gels formed.

It is evident from Table 2 that gel firmness was affected by the time when it was measured. The firmness was found to be higher, *i.e.*, the depth of the penetrating probe was decreased, when the gels were measured 45 min after gelation as compared to measurements carried out 30 min after gelation. This difference may be explained by proteolysis extended for another 15 min leading to a more advanced aggregation of casein micelles and formation of a higher number of linkages between them. In comparison to milk, interactions of casein micelles were facilitated in high-protein retentates. According to Storry and Ford [31], gel formation in milk starts about 2.5 min after coagulation time but Mocquot [25] reported that in retentates, gelation started considerably sooner. Although the observed start of gel formation depends on the measurement method [16], accelerated gelation in the retentates may be explained by shorter distances between the micelles. Whereas in milk the mean distance between 2 casein micelles is approximately 3 micelle diameters, in milk retentates the casein micelles are, on an average, only about 1 micelle diameter apart [18]. The higher curd firmness in the retentates than in the milk is related to the increased solids content in the retentates rather than to the rate of casein aggregation [17].

In the experiments summarized in Table 2, the firmest gels were obtained using rennet and the softest gels were produced by the *B. polymixa* protease. The differences were relatively smaller in gels made from nonhomogenized retentates containing 3.2% protein (1.19 mm penetration depth for rennet vs. 1.68 mm for *B. polymixa* protease) than in gels containing 13% protein (0.72 mm vs. 1.88 mm, respectively).

Effects of protein concentrations and homogenization on firmness of the resulting gels are closely related to their structures and will be discussed below.

### Gel structure

In general, there are two different microstructures of the milk retentate gels under study depending on whether the retentates had been or had not been homogenized prior to coagulation. In gels made from nonhomogenized retentates, large fat globules and their aggregates were embedded in a protein matrix which was formed by casein micelle chains and clusters. The heterogeneity of these gels is noticeable even at a low magnification. In contrast, gels made from homogenized retentates consisted of protein matrices in which minute fat globules were evenly distributed. In both kinds of gel, the densities of the protein matrices varied depending on the protein content of the gels. This is shown by both scanning electron microscopy (SEM) and transmission electron

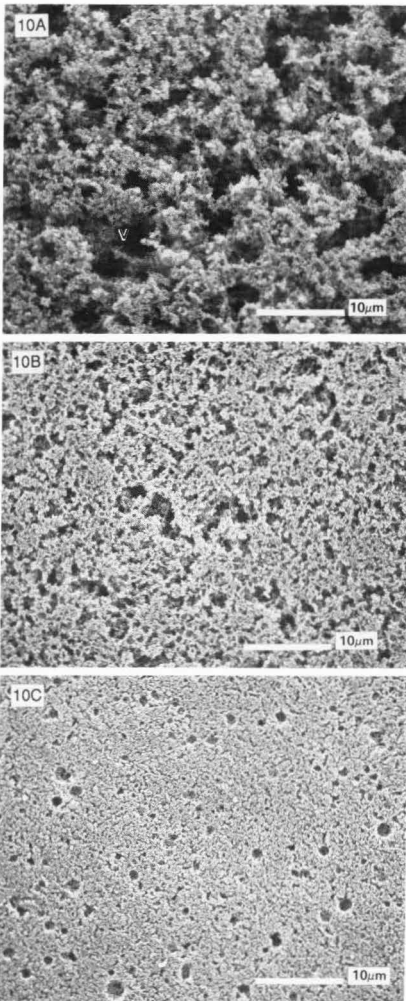


Fig. 10. Gels made by rennet from homogenized milk and UF retentates from which fat had been removed prior to the SEM examination. Fig. 10A: 3.2% protein; Fig. 10B: 5% protein; Fig. 10C: 13% protein. Void spaces (V) are considerably smaller than in the gels made from nonhomogenized milk and UF retentates.



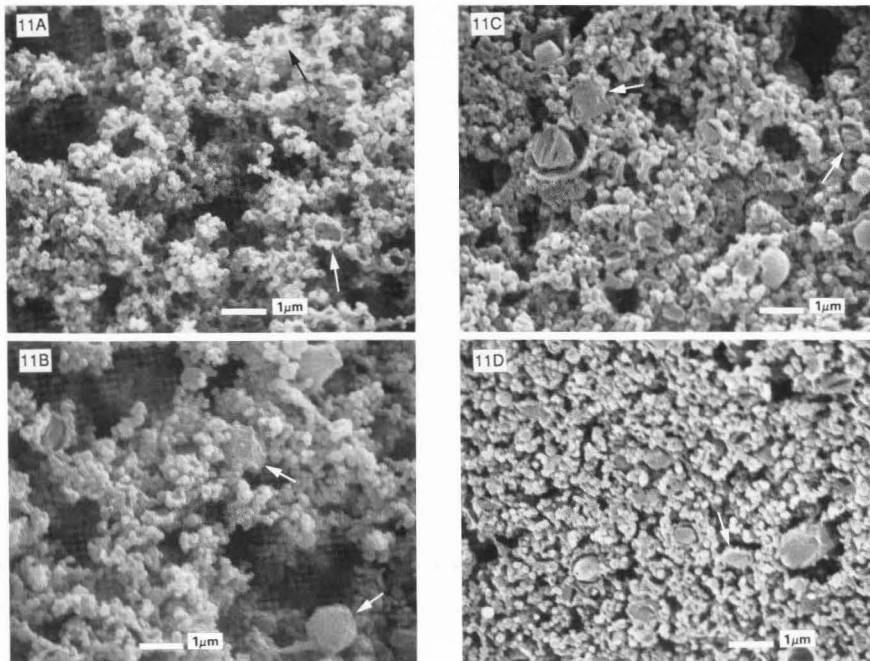


Fig. 11. Details of the structures of gels made from homogenized milk and UF retentates using rennet. Fig. 11A: 3.2% protein; fat had been removed but the protein envelopes of the fat particles are noticeable (arrows). Figs. 11B (3.2% protein), 11C (5% protein), and 11D (13%

protein) show the homogenized fat particles (arrows) integrated in the protein matrices, the densities of which are increased with increased protein concentrations.

microscopy (TEM) using gels made from nonhomogenized retentates. In the SEM micrographs, void spaces in the protein matrices were noticeable (Figs. 3 - 7). The largest void spaces were present in gels made from the initial milk (3.2% protein) but it was difficult to assess as to whether all the void spaces had initially been occupied with fat (Fig. 3A) prior to the extraction of the fat during the preparation of the samples for SEM. Fixation of the fat globules with imidazole-buffered osmium tetroxide [2] revealed (Fig. 3B) that fat had occupied only some of the void spaces whereas others had been filled with whey. The fat globules and their aggregates were not associated with protein. They were surrounded with void spaces and only a few points of contact with the protein matrix could be seen (Fig. 4B). As the protein content of the gels was increased, the protein matrices became denser (Figs. 4 - 7). In gels containing 10.0 or 13.0% protein (Figs. 6 and

7, respectively), the presence of fat globules and their clusters was clearly evident from the void spaces in the protein matrices. Most such void spaces contained residues of the fat globule membranes.

These findings were confirmed by TEM. Large fat globules were present in all gels made from nonhomogenized retentates (Fig. 8). Only a small proportion of the fat globules was associated with protein and void spaces surrounded individual fat globules in the low-protein gels (Figs. 8A and 8B). Thus, no imprints by the fat globules have been left in the protein matrix following their removal in contrast to the high-protein gels, where the casein particles were tightly packed with no free space left around the fat globules (Fig. 8C). Individual fat globules having diameters of up to 5  $\mu$ m prevailed in low-protein gels but were mostly clustered in high-protein gels.

A great proportion of the protein was in the

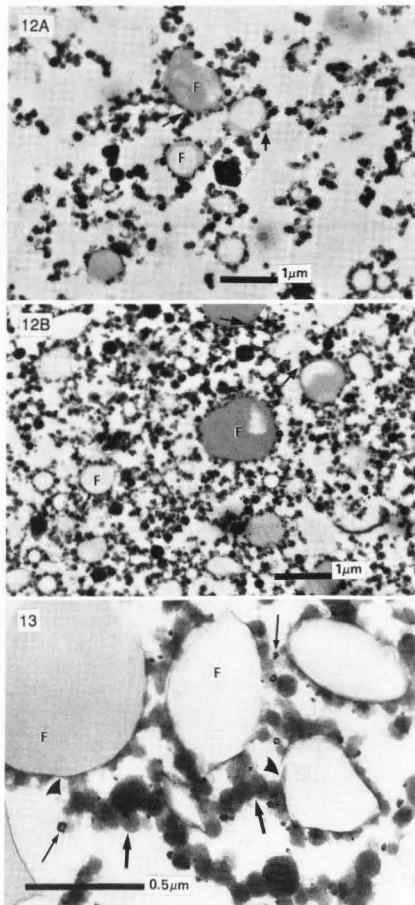


Fig. 12. TEM of a gel made from homogenized milk (3.2% protein, Fig. 12A) and a gel made from a homogenized UF retentate (13% protein, Fig. 12B). Both gels were made by rennet. Homogenized fat particles (F) are coated with protein particles (arrows) and are integrated in the protein matrices.

Fig. 13. Detail of protein (arrowheads) adhering to homogenized fat particles (F) in a gel made from a UF retentate (13% protein) using *M. miehei* protease. Casein micelle chains (large arrows) connect the fat particles with each other. Minute black particles (small arrows) are an artefact [27].

form of casein micelle chains noticeable even at a low magnification in low-protein gels (Figs. 8A and 8B). The chains became less evident as the density of the protein matrix was increased (Fig. 8C). At a higher magnification, structural details of the protein matrices were revealed more clearly (Fig. 9). As most of the fat was concentrated in the form of large fat globules, the matrices were composed almost exclusively of casein particles. Their chains were longest (up to 2  $\mu\text{m}$ ) in the low-protein gels (Figs. 9A and 9B) but chains of a similar length were found even in the high-protein gels (Figs. 9C and 9D) where shorter chains were attached at their sides.

Homogenization of the retentates considerably reduced the dimensions of the fat globules. Coagulation of homogenized retentates resulted in gels having more uniform structures (Fig. 10) than the gels made from nonhomogenized retentates. Although the differences were small in the gels made from the initial milk (3.2% protein) as follows from a comparison of Figs. 3A and 10A, they were quite noticeable as the protein concentrations were increased, as may be seen by comparing Figs. 4A and 10B (5% protein) and Figs. 7 and 10C (13% protein). Because of the increased homogeneity of the gels made from homogenized milk and UF retentates, higher magnifications than those used with gels made from nonhomogenized retentates had to be used to show the characteristic features. The disintegrated fat particles were relatively evenly distributed throughout the protein matrices (Figs. 11 and 12). SEM of a gel made from homogenized milk (3.2% protein) from which fat had been extracted (Fig. 11A) shows that protein had been attached to the fat particles. This finding was confirmed by retaining the fat particles in the gels (Figs. 11B-11D) and also by TEM. The latter technique showed the association between the small fat globules and protein particularly well (Figs. 12 and 13). The protein attached to the fat globules is part of the matrix, thus linking the neighbouring fat globules and making them part of the entire gel structure.

The micrographs shown above are in agreement with the findings that homogenization results in the disintegration of the original fat globules into smaller droplets, and that the combined surface of the fat particles, which is increased 5 to 6 fold [7], becomes rapidly covered with milk proteins [20]. This coating facilitates the integration of the minute fat particles with the protein matrix and contributes to a higher firmness in the gels made from homogenized retentates (Table 2). The participation of homogenized fat particles in the formation of the gel structure is particularly clearly noticeable in TEM micrographs (Figs. 12 and 13). The micrographs in Fig. 12 are of the same magnification as Fig. 9 to make a comparison of the structures possible. The close association of the homogenized fat particles with the protein component of the gels is particularly well evident at a higher magnification in Fig. 13, where a gel obtained using the *M. miehei* protease is shown. In contrast, the large fat globules and their clusters in gels made from nonhomogenized retentates are not in close contact with the protein (as was shown in Figs. 3B, 4B, 8A, and 8B) and probably act as weak areas in the gel matrices making the gels softer. Apparently, these findings

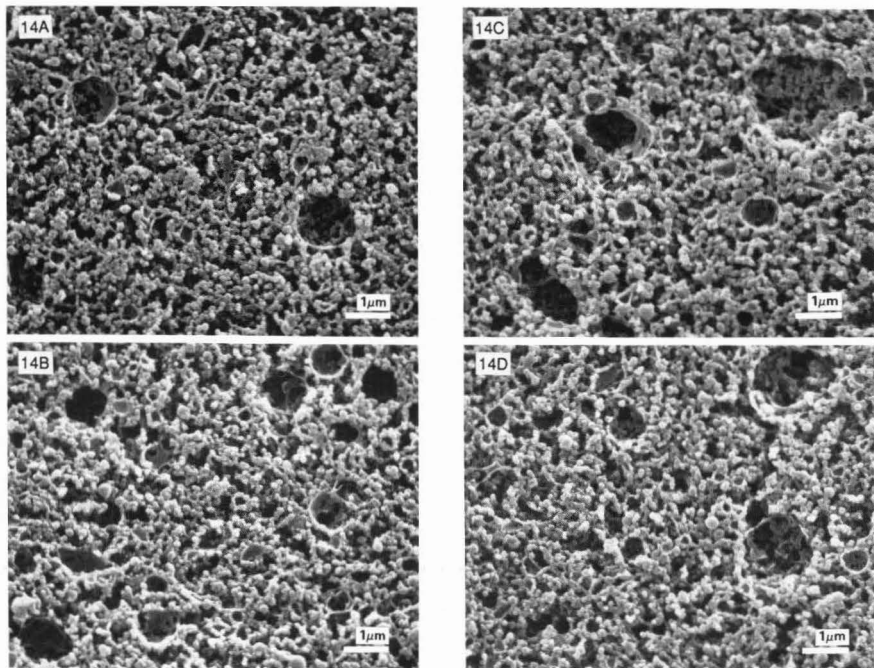


Fig. 14. Details of the protein matrices (fat removed) of gels made from a homogenized UF retentate (13% protein) using rennet (Fig. 14A), *M. pusillus* protease

(Fig. 14B), *M. miehei* protease (Fig. 14C), and *E. parasitica* protease (Fig. 14D).

may be related to studies in which glass particles and oil droplets were incorporated in protein gels [6, 24]. SEM showed that particles with a hydrophilic surface became an integral part of the gel whereas hydrophobized particles incorporated in a similar gel failed to develop any strong links with the protein matrix and easily separated from it when the gel was fractured. The presence of particles with hydrophilic surfaces in the gels provided a greater strength in compression than did particles with hydrophobic surfaces [24].

Based on the micrographs presented, the differences in gel firmness related to protein concentrations or to homogenization of the retentates may be understood from the microstructures of the gel matrices as viewed at low-magnification SEM and/or TEM. In some respects, similar results were obtained with yoghurts made at different concentrations of total solids [19]. The differences in gel firmness caused by the nature of the proteases used, however, are more difficult to correlate to the microstructure as they may be more subtle. At

low-magnification SEM, the gels made from nonhomogenized retentates (13.0% protein) using rennet and proteases of microbial origin appeared to have similar microstructures and, likewise, the microstructures of the gels made from homogenized retentates at the same protein level also resembled each other (Figs. 14A - 14D). In order to facilitate the comparison of the protein matrices, fat had been extracted from the samples shown. Extraction of fat particles about 1  $\mu\text{m}$  in diameter resulted in the development of noticeable void spaces, but the extraction of smaller fat particles made the gel structures appear to be more porous than the gel (13% protein, coagulation by rennet) with the fat retained which is shown in Fig. 11D at the same magnification. However, differences in the microstructures are apparent when high-magnification TEM micrographs of a firm gel produced by coagulating a nonhomogenized retentate (13% protein) with rennet are compared with micrographs of a soft gel produced by coagulating the same retentate using the *B. polyrika* protease

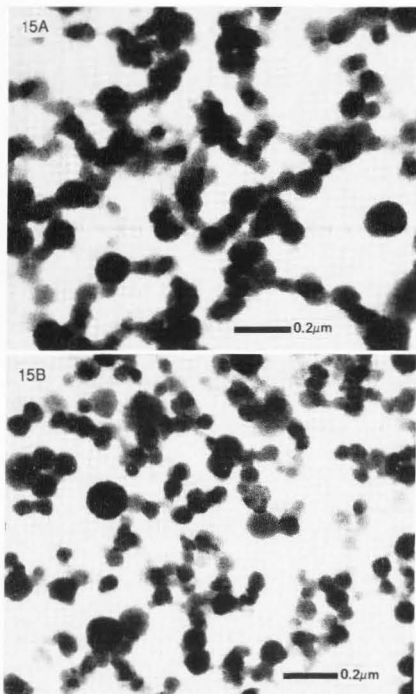


Fig. 15. Details of the protein structures in gels made from a nonhomogenized UF retentate (13% protein) using rennet (Fig. 15A) or *B. polymixa* protease (Fig. 15B). Rennet produced a protein matrix consisting mostly of long casein micelle chains linked with each other. The gel made using *B. polymixa* protease consisted mostly of short casein micelle chains and small clusters.

(Table 2). The protein matrix of the firm gel consisted of casein particle chains branching extensively in many directions (Fig. 15A) as was already shown in rennet-coagulated gels featured at lower magnifications (Figs. 8 and 9), whereas the soft gel was formed by shorter chains and small clusters with considerably fewer linkages among them (Fig. 15B).

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#### Discussion with Reviewers

Y. Kakuda: The authors have stated that the milk was purchased from various sources and was the cause for the high standard deviations in the compositional data. It would be helpful to know the following: How many dairies were involved? Over what time period was the milk collected? Could seasonal variations in the milk affect the result presented? Was bulk milk used?

Authors: The milk was obtained from one dairy plant but it was bulk milk collected from a number of various producers. The studies were carried out over a period of one and a half years which means that seasonal variations played a role.

Y. Kakuda: How were the individual protease solutions prepared: in distilled water or in a buffer? Were the enzymes purified prior to use?

Authors: The proteases were dissolved in distilled water (although some other researchers use buffer solutions for this purpose) in order to simulate conditions in commercial dairy plants. The proteases were used without purification since another objective was to assess their commercial quality.

Y. Kakuda: Please explain how the enzyme solutions were made to carry out the experiments featured in Figs. 1 and 2, where the enzyme concentrations are shown to be as high as 50 mg/50 mL. What was the pH and the temperature in these studies? Were these studies done on all 15 retentates using all enzyme preparations? If not, how many replicates were analyzed?

Authors: To obtain higher enzyme concentrations, 1 to 5 mL of the stock solution were diluted to make 50 mL of the working solution. Temperature was 32°C and pH was 6.65. Data on the effects of homogenization and protein concentration on coagulation time using all 5 enzyme preparations were obtained using aliquots from the same batch of retentate. All results reported are the means of 6 replicates. In other experiments, different batches of retentate were coagulated with the individual proteases. The results have been reported in the PhD thesis [14] (with table and figure legends and a summary in English) and will be published separately.

Y. Kakuda: Were all 15 retentates coagulated and measured for firmness? It is not clear whether the values in Table 2 represent averages or individual results.

Authors: Only one retentate was used for firmness measurements. Aliquots obtained from the same batch of retentate were coagulated with all 5 proteases, each in 6 replicates. The firmness measurements are the means of the replicates.

Y. Kakuda: The reference for AOAC [4] should be more specific and indicate the number codes for the individual assays.

Authors: The number codes are as follows: Dry matter - 16.032, milk fat - 16.064, total and nonprotein nitrogen - 2.057, 16.036, 16.047, 16.050, ash - 16.035, phosphorus - 11.032.

**Y. Kakuda:** Were the coagulation times with homogenized retentates shorter than with nonhomogenized retentates at all levels of solids and with all proteases? Can you suggest reasons why homogenization decreases the coagulation time? Was this a common effect seen with all proteases and for all protein concentrations tested?

**Authors:** A separate study has been prepared which answers these questions: in all cases, the coagulation times were shorter with homogenized retentates than with nonhomogenized retentates. The differences in the coagulation times were, however, smaller at high protein concentrations than at low protein concentrations.

The reasons for this phenomenon were studied by Robson and Dalgleish [30]. They observed reduced concentration of casein micelles in the milk serum due to their adsorption on the homogenized fat globules.

**R. Cartwright:** You explained differences in casein micelle chain structure between rennet and *B. polymixa*. What differences did you notice between rennet and the other enzymes used?

**Authors:** The differences between the effects of rennet and the *B. polymixa* protease were most clearly noticeable: the protein matrices of gels made using rennet consisted mostly of branching casein micelle chains whereas casein micelle clusters were predominant in gels made using the

*B. polymixa* protease. The ratios of chains to clusters varied in the other gels which were made using the other microbial proteases (Figs. 16 - 20) and the ratios were to some degree related to the gel firmness. In addition, firm gels had the casein particle aggregates more robust (Figs. 15A, 16, and 17) than the soft gels (Figs. 15B and 18-20).

**Y. Kakuda:** Is it possible that increased proteolysis and nonspecific proteolysis may be interfering with the coagulation process at high protein concentrations (in the case with bacterial proteases) resulting in increased coagulation times?

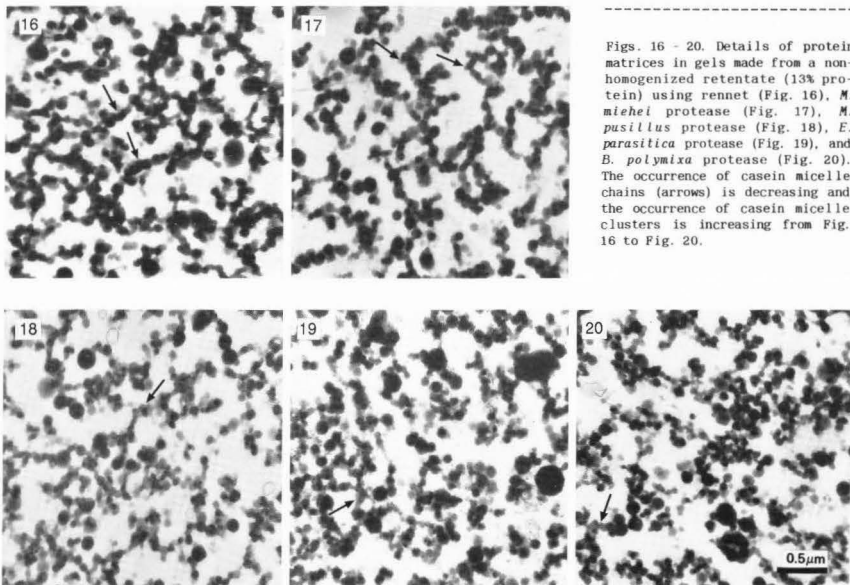
**Authors:** Yes, it is possible.

**R. Cartwright:** How would you expect homogenization of the fat to affect fat loss in the whey?

**Authors:** This aspect was not investigated in this study but according to Davis [8], homogenization leads to less fat loss in the whey.

**D. P. Dylewski:** My question concerns the degree of whey protein denaturation and its possible role in textural properties and gel strength. Was an attempt ever made, through the use of electrophoresis, to determine the degree of denaturation?

**Authors:** Polyacrylamide gel electrophoresis showed very low concentrations of serum proteins in the



Figs. 16 - 20. Details of protein matrices in gels made from a non-homogenized retentate (13% protein) using rennet (Fig. 16), *M. miehei* protease (Fig. 17), *M. pusillus* protease (Fig. 18), *E. parasitica* protease (Fig. 19), and *B. polymixa* protease (Fig. 20). The occurrence of casein micelle chains (arrows) is decreasing and the occurrence of casein micelle clusters is increasing from Fig. 16 to Fig. 20.

aqueous phase of the retentate gels but the results were not evaluated quantitatively.

R. Cartwright: What role would you expect the liberated fat globule membrane material to play in the gel formation process with regard to water retention and gel strength?

Authors: Since the total surface area of the fat globules is considerably (5 to 6-fold) increased as the result of homogenization, a part of the membrane fragments may be adsorbed on the newly formed surface. A potential exists for the fat globule membrane fragments to participate in emulsion stability through interactions with the phospholipid component of the membrane fragments.

R. Cartwright: What effect does homogenization have on the flavour and structure of the finished cheese?

Authors: Cheese made from homogenized milk has a smoother texture and an enhanced flavour compared to cheese made from nonhomogenized milk. Cheddar cheese made from homogenized low-fat milk was found by Emmons et al. [9] to be firmer than Cheddar cheese made from whole nonhomogenized milk although both cheeses had the same moisture levels

in the nonfat matter. Curd granule junctions and milled curd junctions in the cheese made from homogenized milk were less apparent than in the other cheese because the protein-dense areas constituting the junctions (which in regular cheese result from the loss of fat globules during the cutting of curd) were considerably thinner. Microstructure of cheese made from nonhomogenized whole milk (3.6% fat) and from low-fat (1.4%) milk which had not been homogenized or had been homogenized at 5,200 and 1,700 kPa or 13,800 and 3,400 kPa was shown by SEM and TEM.

D. P. Dylewski: If and when do you think microbial proteases will replace traditional rennet on a significant and commercial basis?

Authors: Microbial proteases with specificity resembling that of rennet are already being used on a commercial scale. However, they are used mostly in soft and semi-hard cheeses which have relatively shorter ripening times. A more intensive proteolysis, which takes place in hard cheeses, may produce bitter-tasting peptides and off-flavours. An objective for genetic engineering has arisen: to tailor microbial proteases for use in cheese manufacture.