Interactions Between Milk Proteins and Exopolysaccharides Produced by *Lactococcus lactis* Observed by Scanning Electron Microscopy

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ABSTRACT

The interactions between exopolysaccharides produced by *Lactococcus lactis* ssp. *cremoris* JFR1 and dairy proteins (caseins and whey proteins) in fermented media (milk permeate and buttermilk) were observed using scanning electron microscopy. An immobilization technique by crosslinking was employed to bind the protein to the observation surface, so that a washing step could be performed to remove noninteracting material. The use of this novel technique allowed us, for the first time, to confirm that the exopolysaccharide molecules interact with dairy proteins. Exopolysaccharides appear as filament strands attached to the protein aggregates and to the bacterial cells. This new sample preparation technique proved to be very valuable for observing molecular interactions in fermented media.

Key words: scanning electron microscopy, self-assembled monolayer, exopolysaccharide

INTRODUCTION

Exopolysaccharides (EPS) produced by some strains of lactic acid bacteria have caught the attention of food scientists because of the characteristics that they impart when present in some dairy products (most commonly yogurt and cheese). The presence of EPS has been shown to modify texture and increase water retention in fermented dairy products and cheese (Broadbent et al., 2001; Ruas Madiedo et al., 2002).

Although the microstructure and rheological properties of various dairy products containing EPS have been extensively described (Hassan et al., 1995; Skriver et al., 1995; Bhaskaracharya and Shah, 2000; Hassan et al., 2002; Folkenberg et al., 2005, 2006), very little is known about the mechanisms that generate the changes in texture observed. It remains unclear if or how EPS interact with milk proteins. Indeed, it

is known that there is an effect on texture, but the details of the interactions between the proteins and polysaccharides that may generate this effect are yet not understood. In the present research, we have attempted to improve our understanding in this area by using scanning electron microscopy (SEM) with a novel sample preparation technique.

Scanning electron microscopy, a well-established technique often employed to observe the microstructure of dairy products, has been used extensively to observe milk fermented by EPS-producing cultures (Schellhaass and Morris, 1985; Tegatz and Morris, 1990; Hassan et al., 2003). This microscopy technique has some inherent disadvantages because sample preparation involves a dehydration step with consequent structure distortion. This is especially important when trying to observe highly hydrated molecules such as polysaccharides (Hassan et al., 2003), because they are not efficiently fixed for observation. With SEM, using a conventional sample preparation technique (e.g., cryo-SEM), EPS appear as filaments associated with bacterial cells and the casein network or as void spaces in a protein matrix, depending on the dairy product observed (Bhaskaracharya and Shah, 2000; Hassan et al., 2003). Previous reports have suggested that the filamentous appearance of EPS in SEM images results from the EPS structure collapsing during the sample dehydration step (Kalab, 1993). Nevertheless, because of its unique resolution and imaging characteristics, several attempts have been carried out to employ SEM techniques for the observation of the interactions between EPS and milk proteins in different dairy products such as cheese and yogurt (Tegatz and Morris, 1990; Skriver et al., 1995; Bhaskaracharya and Shah, 2000; Broadbent et al., 2001).

Bhaskaracharya and Shah (2000) used SEM to study the microstructure of Mozzarella cheese produced with EPS-producing and nonproducing cultures. Exopolysaccharides appear as filaments extending from the protein matrix; however, because everything is dried in place, it is not possible to obtain definite evidence of the presence or absence of interactions between EPS and the milk protein aggregates. Cryo-SEM has also

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been employed to observe the microstructure of 2 types of cheese (Karish and Feta) and milk fermented with different ropy and nonropy strains of lactic acid bacteria (Hassan et al., 2003). With cryo-SEM it was possible to better define the microstructure of the milk gels fermented with moderately and highly ropy strains: large domains with dense filaments are contained in the pores of the cheese protein network. The observations suggested that in both cheese and fermented milk, EPS and the casein gel are segregated and the polysaccharides form a network-like structure in the pores. However, it could not be concluded if direct interactions occur between EPS and the protein molecules.

The observation of biomolecules by electron microscopy techniques may be carried out after immobilization to solid substrates. Immobilization via covalent bonds between the linker molecule and the protein would result in a protein layer that can withstand washing (Martin et al., 2006). Molecules can be adsorbed on a solid surface in self-assembled monolayers (SAM), in which the linker molecules organize spontaneously into crystalline (or semicrystalline) structures. When the molecules in the SAM have a functional group it is possible for a particular substrate (e.g., proteins) to specifically react with the SAM (Love et al., 2005). The SAM of small molecules can be employed to crosslink proteins. Alkanethiols are often used because their chain length can be selected to modulate the spacing between the support and the immobilized molecule. Alkanethiols with a carboxylic acid headgroup can react covalently with proteins through carbodiimide chemistry. The reaction involves the formation of an ester group that reacts with the amino groups of the protein. Multiple protein-SAM linkages occur for a single protein (Ostuni et al., 1999).

It has been shown that milk proteins can be immobilized to a SAM of 11-mercaptoundecanoic acid (Uricanu et al., 2004), and the technique has been employed for the observation of the interactions of casein micelles with polysaccharides (Martin et al., 2006). Among the main advantages of this new technique, and what makes it particularly interesting for the study of dairy systems containing EPS, is that the covalent attachment of the protein particles to the SAM allows the introduction of a washing step in the sample preparation procedure. Therefore, anything that remains after the washing step can be safely assumed to be interacting with the proteins.

The objective of this research was to observe dairy proteins in the presence of EPS-producing cultures. Results from this work will help further clarify the molecular details that cause structuring of EPS in dairy products.

MATERIALS AND METHODS

Bacterial Strain

Lactococcus lactis ssp. *cremoris* (JFR1), a highly ropy strain (Hassan et al., 2003), was obtained from A. Hassan's collection at South Dakota State University (Brookings). The strain was received in milk, propagated twice in M17 medium with lactose as a carbon source, and then stored at -80°C in 10% (wt/vol) reconstituted skim milk with 25% glycerol. The stock culture was propagated 3 times using 1% (vol/vol) inoculum for 12 to 16 h at 32°C in M17 containing 0.5% (wt/vol) lactose.

Sample Preparation

Different substrates were used to determine the feasibility of this methodology for the observation of the interaction of EPS with milk proteins. The different media were pasteurized at 63°C for 30 min and inoculated with 2% (vol/vol) *L. lactis* ssp. *cremoris* JFR1.

Fresh buttermilk (by-product of butter making, containing lactose, fragments of the milk fat globule membrane, caseins, and whey proteins) was obtained from a local dairy plant (Gay Lea Foods, Guelph, Ontario, Canada) and had a composition similar to that of milk and a pH of 6.6. Fermentations of buttermilk were performed at a controlled pH of 5.8 in a BioFlo-3000 bioreactor (New Brunswick Scientific Co., Edison NJ), at a temperature of 30°C and with agitation of 100 rpm and controlled nitrogen addition. Samples were taken at 24 h, immediately cooled on ice, and kept frozen until required.

Fermentations were also carried out on ultrafiltration (UF) permeate with various amounts of whey protein added. The permeate was prepared in the pilot plant facility of Parmalat R&D Canada (London, Ontario, Canada). The original milk permeate was obtained by UF of milk and then concentrated using a reverse osmosis system, obtaining a final product of approximately 8% (wt/vol) solids. This permeate contained minimal amounts of protein (0.02% as determined by Dumas combustion method in an FP-2000 protein analyzer, Leco Corporation, St. Joseph, MI) and a lactose content of 5.45% (determined using lactose/D-galactose UV method for enzymatic analysis; Roche R-Biopharm, Darmstadt, Germany). The permeate samples were then supplemented with various concentrations (0, 2, 4, 6, and 8%) of whey protein isolate containing 95% protein (Davisco Foods International, Le Sueur, MN).

Two reference treatments containing no EPS were also prepared. The first control treatment was a sample of permeate with 6% whey protein added, inocu-

Figure 1. Scanning electron microscopy images obtained from ropy samples of buttermilk fermented at pH 5.8. Panel A scale bar = 3,000 nm; panel B scale bar = 1,200 nm. E = exopolysaccharide; P = protein; B = bacterial cells.

lated with the EPS-producing culture, *L. lactis* ssp. *cremoris* JFR1, and taken immediately after inoculation. Because this control had a high pH, a second control was prepared by fermenting concentrated permeate with 6% whey protein added, with a commercial non-EPS-producing strain of *L. lactis* (MA Choozit, Danisco Canada Inc., Scarborough, Ontario, Canada).

All media were pasteurized at 63°C for 30 min before inoculation.

Sample Immobilization on Functionalized Substrates

The procedure developed by Martin et al. (2006) for the SAM preparation and immobilization of the sam-

Figure 2. Field emission scanning electron microscopy images of buttermilk fermented at pH 5.8. Panel A scale bar = 1,500 nm; panel B scale bar = 750 nm. E = exopolysaccharide; P = protein; B = bacterial cells.

ples was employed with some modifications. Briefly, clean and dry polished carbon planchets (diameter 12.7 mm; Canemco-Marivac, Lakefield, Quebec, Canada) were sputter-coated with a layer of ~35 nm thickness of Au:Pd (ratio 60:40; Emitech K550, Ashford, Kent, UK), directly immersed in a 2 mM solution of 11-mercaptoundecanoic acid in 100% ethanol, and left for at least 18 h to form the SAM. The terminal functional group of the SAM was modified by carbodiimide chemistry using equal volumes of 0.1 M N-hydroxysuccinimide (NHS) and 0.4 M N-ethyl-N-(dimethylaminopropyl) (EDC) carbodiimide (Martin et al., 2006).

The activated carbon planchets were immersed in the different samples for at least 30 min and then rinsed with 10 mM imidazole buffer containing 1 mM CaCl_2 (adjusted to the same pH of the samples). After the rinsing step, the specimens were fixed by immersion in a 1.5% (vol/vol) glutaraldehyde solution in 10 mM imidazole buffer (adjusted to pH of the sample) for 30 min. The carbon planchets were then rinsed with ultrapure water and subjected to dehydration in increasing concentrations of ethanol (10 min in 70%, 10 min in 90%, and 3 times in 100% alcohol for 10 min) before performing critical point drying with CO_2 . Before the observations with SEM, the sample was sputter-coated with a thin layer (~15 nm) of Au:Pd (60:40).

Imaging

The samples were observed with an Hitachi S-570 SEM or an Hitachi S-4500 field emission SEM (Hitachi, Tokyo, Japan). In both cases, an acceleration voltage of 10 kV and emission current of 10 μA were used. All images were acquired digitally using Quartz PCI software (Vancouver, British Columbia, Canada).

RESULTS AND DISCUSSION

Buttermilk Samples

Buttermilk was used as a substrate for the fermentation because it contains all major proteins present in milk as well as fragments of the milk fat globule membrane. This would allow for a detailed analysis of the interactions (if existing) between different proteins. Preliminary experiments demonstrated that fermentation of buttermilk at a controlled pH of 5.8 generated highly ropy material after 24 h. This suggested the presence of EPS in the fermented media. Representative SEM images of the buttermilk samples after attachment on the SAM, washing, fixation, and dehydration are shown in Figure 1. The EPS appear as strings attached to the bacteria and to the protein complexes.

At this pH the proteins do not form a visible gel; however, heat-induced aggregates were present in the media.

The bacteria were attached to the protein network through filamentous strands of the polysaccharide emerging from them. At higher magnification (Figure 1B), it is possible to observe that these strings or filaments were well attached to the protein aggregates. The images clearly demonstrate that the EPS strings are intertwined in the protein matrix.

The EPS structures are similar to those observed in previous SEM reports (Bhaskaracharya and Shah, 2000; Hassan et al., 2003) and the filamentous appearance has been attributed to the dehydration during sample preparation. The EPS molecules in their hydrated state may occupy much more volume than is observed in these images. The background shows partial coverage by milk proteins attached to the SAM, in agreement with previous observations (Martin et al., 2006). Isolated clumps of aggregated protein are detectable in conjunction with the bacteria, but no continuous network was formed, because at this pH, the system was not gelled. The formation of these protein aggregates is probably caused by the heat treatment received by the cream before butter making and during buttermilk pasteurization (63°C for 30 min) coupled with the further acidification at pH 5.8. Unlike the observations on κ -carrageenan systems in milk (Martin et al., 2006), where κ -carrageenan seems to form a network between individual caseins or small aggregates, in this case, EPS strands are embedded in the casein aggregates.

It is important to emphasize that the protein aggregates are covalently linked to the SAM and only material interacting with the protein can be observed, because the rest is washed off by rinsing with imidazole and calcium buffer. These images are the first direct evidence of the interactions of EPS with milk proteins. In previous work, Martin et al. (2006) used this technique to observe samples of casein micelles with noninteracting (guar gum) and interacting (carrageenan) polysaccharides, and only the interacting carrageenans were detected by microscopy, whereas guar gum was not observed after the washing step.

Further detail of the protein complexes could be observed using a greater resolution field-emission SEM. As shown in Figure 2, the strands of the EPS emerging from the bacteria are embedded in the protein network.

These images further demonstrate the entanglement of the EPS within the protein network. Although SEM allows only for clear observation of the surface, bacteria and EPS are most possibly contained inside the protein complexes, as can be observed in Figure 2A.

Milk Permeate

To establish if EPS interacted with whey protein, samples of permeate with different levels of whey proteins (0, 2, 4, 6, and 8%) were fermented with *L. lactis* ssp. *cremoris* JFR1. In these experiments, the pH was not kept constant, because 12 h of fermentation to pH 4.5 caused significant ropiness of the fermented substrate. High ropiness was obtained from fermentations performed in milk permeate with 6 and 8% added protein. The milk UF permeate without whey protein was not ropy and did not support bacterial growth, as evidenced by the lack of acidification observed after 12 h of incubation. Although the 8% protein sample was visibly more ropy than the fermented media with 6% protein added, it was not suitable for microscopic observations because the protein complexes were too concentrated, which prevented observation of important structural features that could be appreciated in the 6% protein samples (Figure 3B and 3C).

All samples showed large aggregate structures of whey proteins, which formed during heating and subsequent acidification of the fermentation media. These particulate, granular whey protein aggregates have been previously visualized using atomic force microscopy (Ikeda and Morris, 2002). Samples of permeate containing 2 and 4% whey protein showed less EPS (Figure 3A) than those of permeate containing greater amounts of whey proteins (Figure 3B and 3C). This may suggest that the amount of protein in the medium influences the amount of EPS observed in the images. As can be seen in Figure 3, the background in these images showed no attached proteins, because no caseins were present and nonaggregated whey proteins (if any) were too small to be observed at this resolution.

Observation using the field emission SEM provided more detail on the EPS and the protein network. Figure 4 shows representative micrographs for permeate fermented with 6% whey protein. These images clearly indicate that EPS interacted with whey protein aggregates at this pH. The EPS appear as long strings intertwined with the aggregated protein and attached to bacterial cells. From the microscopic observations, it could be seen that washing did not remove EPS, which were either attached to the bacterial cells or interacting with the protein network. Bacterial cells and proteins were also found attached to the SAM, as expected considering their chemical composition.

The novel aspects of these observations rely on the fact that although it is not possible to observe the interactions of the protein with the polysaccharide in great detail, we can conclude that EPS are interacting and not simply entrapped or located in the surface or in void spaces of the protein complexes.

Figure 3. Conventional scanning electron microscopy images of concentrated milk permeate with A) 2%; B) 6%; and C) 8% protein added. Scale bars: A = 1,500 nm, B = 1,200 nm, C = 1,000 nm. E = exopolysaccharide.

Figure 4. Field emission scanning electron microscopy images of fermented concentrated milk permeate with 6% protein added. Panel B (scale bar = 600 nm) is a magnification of panel A (scale bar = 1,200 nm).

Reference Samples

The 2 reference samples (*L. lactis* ssp. *cremoris* JFR1 at the beginning of fermentation and the non-EPS-producing *L. lactis*) successfully demonstrated that the observed filaments were, in fact, EPS interacting with the protein and not artifacts generated during sample preparation. They also confirmed the ability of protein and bacterial cells to attach to the SAM. Figure 5 shows field emission images of the milk permeate with 6% whey protein inoculated with *L. lactis* ssp. *cremoris* JFR1 at the beginning of the fermentation (immediately after inoculation). The pH of this sample was around 6.3.

As can be appreciated, no EPS strings were visible in the protein complex or emerging from the bacterial cells, even when some bacteria appeared attached to the protein. Figure 6 shows concentrated milk permeate with 6% (wt/vol) protein addition inoculated with a commercial strain of non-EPS-producing *L. lactis* and fermented for 12 h at 30°C without agitation, under the same conditions as the samples fermented with EPS-producing cultures. The concentration of 6% protein was chosen because it proved to be the most suitable for observation in the SEM as described above. As can be clearly observed, the bacterial cells did not show the characteristic EPS filaments protruding, nor could they be seen in the protein network formed.

Figure 5. Field emission scanning electron microscopy images of milk permeate with 6% protein inoculated with *Lactococcus lactis* ssp. *cremoris* JFR1; samples were taken at the beginning (0 h) of fermentation. Panel A and B scale bar = 1,200 nm.

Figure 6. Conventional (A) and field emission (B) scanning electron microscopy images of concentrated milk permeate with 6% (wt/vol) protein added, fermented for 12 h with nonropy strain of *Lactococcus lactis*. Panel A scale bar = 2,000 nm; panel B scale bar = 860 nm.

CONCLUSIONS

Scanning electron microscopy of samples immobilized on self-assembled monolayers proved to be a valuable technique for the observation of EPS and their interactions with proteins. The EPS molecules clearly interact not only with caseins (see buttermilk images), but also with whey proteins (see permeate experiments) and play an active role in the formation of the aggregates. This may explain the viscosifying effect that presence of EPS has in fermented dairy products. It was observed that caseins, whey proteins, and bacterial cells could be successfully linked to the SAM and that EPS were firmly attached to them after the washing procedure. More EPS was observed in samples containing a greater concentration of whey protein. As previously discussed (Martin et al., 2006), the cross-linking of the proteins to SAM and the subsequent washing of the samples results in the elimination of noninteracting components, bringing direct evidence of the interactions of EPS with dairy proteins. This novel approach to sample preparation of SEM complements previous microscopy techniques employed to observe the effect of EPS on the microstructure of dairy products (Hassan et al., 2003; Folkenberg et al., 2005).

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