Effect of exopolysaccharides on the proteolytic and angiotensin-I converting enzyme-inhibitory activities and textural and rheological properties of low-fat yogurt during refrigerated storage

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ABSTRACT

The aim of this study was to examine the influence of using exopolysaccharide (EPS) producing strain of *Streptococcus thermophilus* on the viability of yogurt starters, their proteolytic and angiotensin-I converting enzyme-inhibitory activities, and on the textural and rheological properties of the low-fat yogurt during storage at 4°C for 28 d. The use of an EPS-producing strain of *S. thermophilus* did not have influence on pH, lactic acid content, or the angiotensin-I converting enzyme-inhibition activity of low-fat yogurt. However, EPS showed a protective effect on the survival of *Lactobacillus delbrueckii* ssp. *bulgaricus*. Presence of EPS reduced the firmness, spontaneous whey separation, yield stress, and hysteresis loop area but not the consistency and flow behavior index of low-fat yogurt.

Key words: exopolysaccharide, proteolysis, angiotensin-I-converting-enzyme, rheology

INTRODUCTION

Exopolysaccharides (EPS) are long-chain polysaccharides consisting of branched, repeating units of sugars or sugar derivatives. Lactic acid bacteria (LAB) are able to produce several types of polysaccharides that are classified according to their location relative to the cell. Those that are excreted outside the cell wall are called exocellular polysaccharides or EPS whereas those that form adherent cohesive layers are called capsular polysaccharides. The EPS can either be loosely attached or completely excreted into the medium as a slime (Ruas-Madiedo et al., 2002). Exopolysaccharides from LAB have proved to be invaluable in their application to the improvement of rheology, texture, and mouthfeel of fermented milk products such as yogurt, particularly in low-fat yogurts. Exopolysaccharides

produced by LAB are reported to improve the texture and firmness, reduce thermal and physical shock and syneresis, and increase the viscosity of yogurt (DeVuyst and Degeest, 1999; Hugenholtz and Smid, 2002; Jolly et al., 2002; Ruas-Madiedo et al., 2002; Broadbent et al., 2003; Welman and Maddox, 2003; Sodini et al., 2004). The EPS produced by LAB are also reported to provide physiological benefits such as lowering cholesterol, immunomodulation, and antitumor activity (Hugenholtz and Smid, 2002; Welman and Maddox, 2003).

Certain strains of both of the yogurt starter cultures, namely *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, are known to produce heteropolysaccharides. The quantity of EPS ranges from 50 to 350 mg/L of fermented milk for *S. thermophilus* and 60 to 150 mg/L for *Lb. delbrueckii* ssp. *bulgaricus* (Ruas-Madiedo et al., 2002). Although these organisms produce EPS at low levels, they still contribute to the texture, mouthfeel, taste perception, and stability of the final products (Doleys et al., 2005). Growth conditions, type of nutrients available, and the strain of the organism are among the factors that influence the yield and type of EPS produced. In most industrial fermentations, EPS-producing strains of *S. thermophilus* are the rational choice for products like yogurt mainly due to their minor role in proteolysis during milk fermentation (De Vuyst et al., 2003).

Yogurt already has an established image as a healthy food. The consumption of yogurt to maintain good health is a long tradition in many countries. It is now established that several peptide sequences are encrypted in milk proteins that have the potential to provide several physiological and health benefits to the consumer. These peptides are referred to as bioactive peptides (Clare and Swaisgood, 2000; Shah, 2000). Milk fermentations using LAB release these peptides (Meisel and Bockelmann, 1999; Gobbetti et al., 2002) which are reported to provide several health benefits. Peptides that provide antihypertensive effects have caught the interest of scientists, manufacturers, and consumers. These peptides are understood to have the potential to inhibit the activity of angiotensin-I converting enzyme

Received October 8, 2008.

Accepted November 4, 2008.

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(ACE) and thereby regulate blood pressure. Several ACE-inhibitory peptides have been isolated from fermented milk and commercial dairy products (Shah, 2000; Lopez-Fandino et al., 2006; Hayes et al., 2007).

In the last few years, consumer interest has turned toward low-fat yogurts. This changing trend has generated interest in solving the major textural problem of low-fat yogurts, namely, whey separation. Spontaneous syneresis refers to the loss in the ability of the yogurt gel to entrap all of the serum phase due to the weakening of the gel network (Lucey, 2002). Moreover, yogurt texture is extremely fragile. As a result, mechanical handling of the product is difficult. Some of the common methods adopted by manufacturers to address this problem have been to increase the level of nonfat milk solids or to add sugar, proteins, natural or synthetic gums, and stabilizers. Another suggested method for improving the texture of low-fat yogurt is enzymatic stimulation of protein interactions in milk (Faergemand et al., 1999; Lorenzen, 2002; Shah, 2003; Welman and Maddox, 2003; Ozer et al., 2007; Xu et al., 2008). However, these methods are limited when faced with the increasing consumer demand of low-fat/sugar products with no additives and stabilizers (Jolly et al., 2002). A viable alternative to this is the use of EPS-producing cultures (De Vuyst et al., 2003). Hence, the in situ use of generally recognized as safe, food-grade, EPS-producing strains of LAB as functional starters in fermented food products appears to be a feasible solution.

Much of the work related to EPS-producing starters have concentrated on their influence on the viscosity, rheology, and texture of yogurt (Hassan et al., 2003a; Zisu and Shah, 2003; Guzel-Seydim et al., 2005; Amatayakul et al., 2006; Girard and Schaffer-Lequart, 2007; Purwandari et al., 2007). So far, no work has been carried out to study the influence of EPS-producing starters on the stability of the ACE-inhibitory activity of fermented milks such as yogurt. Also, very little work has been carried out to monitor the changes in the textural and rheological properties of yogurt made from EPS-producing strains of starters during storage. This study was carried out with the objective of comprehensively studying the influence of an EPS-producing strain of *S. thermophilus* on the viability of *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*, changes in pH and lactic acid content, proteolysis, and ACE-inhibitory activity, as well as on the firmness, spontaneous whey separation, and rheological parameters during storage of the low-fat yogurt at 4°C. The yield of EPS was also monitored during the period of storage.

MATERIALS AND METHODS

Propagation of Yogurt Starters

Two strains of *S. thermophilus*, one EPS producer (1275) and one non-EPS producer (1342), were obtained from the Starter Culture Collection of Victoria University (Werribee, Victoria, Australia). It has been established that *S. thermophilus* 1275 produces capsular as well as ropy EPS (Zisu and Shah, 2003). *Lactobacillus delbrueckii* ssp. *bulgaricus* 1368 was obtained from Australian Starter Culture Research Centre Ltd. (Werribee, Victoria, Australia). All 3 organisms were stored as frozen cultures in 40% (wt/vol) glycerol at -80°C. The cultures were activated by transferring first in M17 medium for *S. thermophilus* and in de Man, Rogosa, and Sharpe medium for *Lb. delbrueckii* ssp. *bulgaricus*. Thereafter, the cultures were transferred once each to sterile reconstituted skim milk (RSM; 12% wt/vol) containing 1% yeast extract and 2% glucose (wt/vol) and sterile RSM (sterilized by autoclaving at 121°C for 15 min) before preparing the bulk cultures. For each transfer, the rate of inoculation was 1% (vol/vol) and the temperature of incubation was 37°C for *S. thermophilus* and 42°C for *Lb. delbrueckii* ssp. *bulgaricus* for a period of 20 h each.

Yogurt Making

Low-fat yogurt was prepared using skim milk (Skinny Milk, Parmalat Australia Ltd., Brisbane, Queensland, Australia) that was standardized to 12% total solids with skim milk powder. The milk was preheated to 60°C, at which stage the skim milk powder was added. The heating was then continued to a temperature of 85°C and the heated yogurt mix was held at this temperature for 30 min, followed by cooling to 45°C in a water bath maintained at about 4°C. The heating and cooling processes were carried out in a closed container to minimize losses due to evaporation. This was followed by inoculation with *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*, each at the rate of 1% (wt/vol). The inoculated mix was then mixed thoroughly, dispensed in 50-mL polystyrene cups with lids, and incubated at 42°C until the pH decreased to 4.5 ± 0.1 . The fermentation was stopped by transferring the cups immediately to a walk-in refrigerator maintained at $4 \pm 1^\circ\text{C}$. Two batches of yogurt were prepared; one using the non-EPS strain of *S. thermophilus* 1342 (NEY; control batch) and the other using the EPS-producing strain of *S. thermophilus* 1275 (EY; experimental batch).

Sampling of Yogurt Mixes and Yogurt for Analyses

The samples of inoculated yogurt mixes (0 h) were removed before incubation for determining the viable counts, pH, and lactic acid content, and for measuring proteolysis and ACE-inhibition activity. Samples of both types of low-fat yogurt were removed from the refrigerated storage at 18 h postmanufacture. This was referred to as the d 1 sample. Samples were also removed at d 7, 14, 21, and 28 of storage at 4°C and analyzed for changes in pH, lactic acid content, viability of starter cultures, yield of EPS, proteolysis by *o*-phthaldialdehyde (OPA) method, and ACE-inhibitory activity, as well as for firmness, spontaneous whey separation, and rheological parameters.

Preparation of Filtrates for Analysis of Proteolysis by OPA Method and ACE-Inhibitory Activity

The filtrates of inoculated samples (0 h) were prepared by lowering their pH to 4.5 with glacial acetic acid followed by centrifugation at $4,000 \times g$ for 30 min at 4°C. Filtrates of low-fat yogurt samples stored at 4°C were prepared by centrifuging (Sorvall R2 7, Thermo Scientific, Waltham, MA) at $4,000 \times g$ for 30 min at 4°C. All the supernatants thus obtained were filtered through a 0.45- μ m membrane filter and stored at -20°C until assayed.

Measurement of pH

The changes in pH in the yogurts during preparation and storage were measured using a pH meter (model 8417, Hanna Instruments, Singapore).

Determination of Lactic Acid

The concentration of lactic acid (mg/100 g of yogurt) in all samples of low-fat yogurt stored at 4°C as well as the 0 h samples of mixes were determined by HPLC as described by Ramchandran and Shah (2008). Briefly, 40 μ L of 15.5 M nitric acid and 500 μ L of 0.005 M sulfuric acid were added to 1 g of the sample, mixed, and centrifuged for 30 min at $14,000 \times g$ in an Eppendorf 5415C centrifuge (Crown Scientific, Melbourne, Victoria, Australia). The supernatant thus obtained was passed through a 0.45- μ m membrane filter into HPLC vials. The lactic acid was separated in an Aminex HPX-87H, 300 $\times$ 7.8 mm ion exchange column (BioRad Life Science Group, Hercules, CA) fitted with a guard column maintained at 65°C. The column was attached to a Varian HPLC (Varian Analytical Instruments, Walnut Creek, CA) fitted with a UV/Vis detector. The sample injection volume was 20 μ L, which was eluted using

0.005 M sulfuric acid as mobile phase at a flow rate of 0.6 mL/min and the separated lactic acid was detected at 220 nm. The standard working solutions of L(+) lactic acid (prepared from a stock solution of 5.1990 g/50 mL) was used to compare those of the samples.

Viability of *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*

The enumeration of *S. thermophilus* 1275 and 1342 and *Lb. delbrueckii* ssp. *bulgaricus* 1368, in freshly inoculated mixes (0 h) and in the low-fat yogurts stored at 4°C, was carried out at weekly intervals by pour plate technique using M17 agar and reinforced clostridial agar, respectively (Dave and Shah, 1996). The counts were reported as log₁₀ cfu per gram of yogurt sample. In the results presented, the 0 h counts were subtracted from each of those of the yogurt samples to show the actual changes in counts due to growth of the yogurt starters during storage at 4°C for 28 d.

Determination of Crude EPS Content

The quantity of crude EPS in the EY samples was measured during the storage period of 28 d at 4°C by the method of vanGeel-Schutten et al. (1998) as described by Purwandari et al. (2007) with some modifications. The method involved centrifugation of 50 g of yogurt at $11,000 \times g$ for 10 min at 4°C. The supernatant containing the EPS was collected and mixed with 2 volumes of cold ethanol and left at 4°C for about 18 to 20 h to precipitate the EPS. This was followed by centrifugation at $11,000 \times g$ for 15 min at 4°C. The precipitates thus obtained were dissolved in 20 mL of Milli-Q water (Millipore Corp., Billerica, MA), mixed with 500 μ L of 80% TCA, and left at 4°C for another 18 to 20 h to precipitate the proteins. This was followed by centrifugation at $2,000 \times g$ for 15 min at 4°C. The protein-free supernatant was collected and mixed with 2 volumes of cold ethanol and left at 4°C for 18 to 20 h to re-precipitate the EPS. The EPS precipitates were collected by centrifuging at $2,000 \times g$ for 15 min at 4°C. The steps of protein precipitation and EPS re-precipitation were repeated once more and the EPS finally collected by centrifuging at $2,000 \times g$ at 4°C for 15 min. The collected crude EPS was dried at 40°C until 2 consecutive weights did not show a difference of more than 0.001 g. The results were expressed as milligrams of crude EPS per 100 g of yogurt.

Extent of Proteolysis by OPA Method

The extent of proteolysis was determined by measuring the free amino acid content in filtrates (prepared

as mentioned previously) of yogurt mixes (0 h) as well as of low-fat yogurt samples by the method of Church et al. (1983) as described by Ramchandran and Shah (2008). To 150 μ L of the filtrate, 3 mL of *o*-phthalaldehyde (OPA) reagent was added, vortexed for 5 s, followed by measuring absorbance at 340 nm within 2 min using NovaSpec-II spectrophotometer (Pharmacia, Biotech, Uppsala, Sweden). The readings of the 0 h samples as well as the reagent blank were deducted from the corresponding readings of yogurt samples to obtain the amount of free amino acids released because of the proteolytic activity of the starter cultures during fermentation and storage.

Determination of ACE-Inhibitory Activity

The ACE-inhibitory activity was determined in the filtrates of yogurt mixes as well as of the low-fat yogurts by the method of Cushman and Cheung (1971) as described by Ramchandran and Shah (2008). Briefly, 200 μ L of hippuryl-histidyl-leucine (5 mM in 0.1 M borate buffer) was mixed with 60 μ L of borate buffer (0.1 M solution containing 0.3 M NaCl, pH 8.3) and 30 μ L of the filtrate and incubated at 37°C for 10 min. Thereafter, 20 μ L of ACE enzyme solution (0.1 unit/mL) was added and the tubes incubated at 37°C for 30 min. The enzyme activity was terminated with 250 μ L of 1 M HCl. The hippuric acid formed was extracted by mixing in 1.7 mL of ethyl acetate. After quiescent standing for 10 min, 1.2 mL of the separated solvent layer was siphoned out and dried on a boiling water bath. The dried hippuric acid was dissolved in 1 mL of deionized water and absorbance measured at 228 nm using UV/Vis Pharmacia, LKB-UltrospecIII spectrophotometer (Pharmacia, Uppsala, Sweden). The percentage inhibition was calculated using the following formula:

$$\text{ACE inhibition (\%)} = \left[1 - \frac{C - D}{A - B} \right] \times 100,$$

where A is the absorbance in the presence of ACE and without the sample, B is the absorbance without both ACE and the sample, C is the absorbance with ACE and the sample, and D is the absorbance with the sample but without ACE. The ACE inhibition was also expressed in terms of IC₅₀, defined as the protein concentration in the sample (mg/mL) required to inhibit 50% of the ACE activity. The protein content of the filtrates was determined by the method of Lowry et al. (1951) using BSA as a standard.

Firmness of Yogurt

The firmness of the low-fat yogurts, measured as the force required to break the gel, was determined using

a texture analyzer TA-XT.2 (Stable Micro Systems, Godalming, UK) with a P20 probe (diameter 20 mm) and 25-kg load cell. The speed of penetration was set at 1 mm/s and the depth of penetration was set at 10 mm. The ratio of cup diameter to probe diameter was 3.5:1 (Amatayakul et al., 2006). The measurements were performed as soon as the samples were removed from the refrigerator. The firmness of the yogurt samples was expressed in *g*.

Spontaneous Whey Separation

Spontaneous whey separation in the stored low-fat yogurt, indicative of the whey expelled from the gel without the application of external pressure, was determined by the siphon method described by Amatayakul et al. (2006). A cup of yogurt was weighed immediately after removing from the refrigerator and tilted at an angle of 45° to collect the surface whey. The collected whey was siphoned out with a syringe to which a needle was attached. The siphoning was performed within 10 s to avoid forced leakage of whey from the curd. Thereafter, the cups were weighed and whey separation calculated by dividing the weight of whey siphoned with the initial weight of yogurt and the results were expressed as percentage spontaneous whey separation.

Rheological Measurements

The low-fat yogurt stored at 4°C was gently stirred 5 times before rheological analysis. The viscoelastic properties were determined by small amplitude oscillatory measurement using a controlled stress/controlled rate rheometer (Physica MCR 301, Anton Paar GmbH, Ostfildern, Germany). The rheometer was equipped with a temperature and moisture regulating hood and cone-plate geometry (CP50-1, 50 mm diameter, 1° angle, and 0.02 mm gap, Anton Paar). The temperature of the system was regulated by a viscotherm VT2 circulating bath and controlled at 5 ± 1°C with a Peltier system (Anton Paar). The data of the rheological measurements were analyzed with the supporting software Rheoplus/32 V2.81 (Anton Paar). A portion of the stirred samples was loaded on the inset plate and presheared at a shear rate of 500 per s for 30 s and then equilibrated for 150 s to allow structure rebuilding before small amplitude oscillatory measurement was performed. The samples were first subjected to a frequency sweep test using a frequency ramp from 0.1 to 10 Hz at a constant strain of 0.5% (determined from an amplitude sweep at 1 Hz) to ascertain the viscoelastic properties. The shear rate, storage modulus, loss modulus, and damping factors were recorded for all the samples. This was followed by a shear rate sweep to generate the flow curves. The

shear stress was measured as a function of shear rates from 0.1 to 100 per s (upward and downward sweeps). The flow behavior of the samples was determined by using the Herschel-Bulkley model which is as follows:

$$\sigma = \sigma_0 + k \cdot \dot{\gamma}^n$$

where σ_0 is the yield stress, k is the consistency index, $\dot{\gamma}$ is the shear rate and n is a dimensionless number that indicates the closeness to Newtonian flow ($n < 1$ indicates pseudoplastic liquid). The larger the value of k , the thicker the product and therefore, the more viscous the fluid (Bourne, 2002). The hysteresis loop area between the upward and downward curves was also calculated using the RheoWin Pro software (Anton Paar).

Experimental Design and Statistical Analysis

The yogurt making experiment was designed with culture (strains of *S. thermophilus*) and replications as the main plot and time as the subplot. This block was replicated 3 times with 2 subsamplings. The results of the various determinations were analyzed as split plot in time using the GLM procedure of SAS (SAS Institute, 1996). The data of EPS concentration was analyzed by one-way ANOVA and Tukey's test for multicomparison of the means. Correlational analysis was employed, where appropriate, using Microsoft Excel Statpro software. The level of significance was set at $P = 0.05$.

RESULTS AND DISCUSSION

Changes in pH

On average, EY took more time (287 min) than NEY (277 min) to reach the pH of 4.5 ± 0.1 . An early gelation in the presence of EPS-producing cultures has been reported to inhibit the mobility of the growing organisms (Hassan et al., 2002). This could explain, in part, the possible reason for the increased fermentation time observed in EY. On the contrary, Doleyres et al. (2005) have reported that yogurt prepared using EPS-producing cultures fermented faster than those prepared using non-EPS cultures.

During storage at 4°C, both the types of yogurt showed a sharp decrease ($P < 0.05$) in pH at d 7 followed by a sharp increase ($P < 0.05$) at d 21 of storage (Table 1). This was concomitant with the significant increase in lactic acid content of the yogurts (Table 1). There were no significant variations in pH of yogurts during the other periods of storage. Purwandari et al. (2007) also

observed that *S. thermophilus* 1275 stopped producing acid in yogurt after 7 d of storage at 4°C. However, the pH at the end of storage was almost the same as that at the start (d 1) for both NEY and EY. The variation in the strain of *S. thermophilus* (NEY strain vs. EY strain) did not have any influence on the changes in pH throughout the storage period. The increase in pH toward the end of storage period could be attributed to the ability of *S. thermophilus* to produce certain basic metabolites (Tinson et al., 1982). This is in contrast to the observations of Ozer et al. (2007), Purwandari et al. (2007), and Al-Kadamany et al. (2003) who observed a continuous decrease in pH of yogurts during storage whereas Salvador and Fiszman (2004) did not observe any change in pH of yogurt during storage. These differences could be due to the different types and combination of starters used for yogurt making by these researchers.

Changes in Lactic Acid

The concentration of lactic acid (mg/100 g of yogurt) in the control and experimental yogurts stored at 4°C for 28 d is shown in Table 1. The amount of lactic acid produced was not influenced by the strain of *S. thermophilus* used for making yogurt. All the yogurts showed an increase in lactic acid content ($P < 0.05$) by 0.37 to 0.39 mg/100 g of yogurt during the first week (d 7) of storage, after which there were no significant changes in the concentration of lactic acid. Amatayakul et al. (2006) did not observe any influence of the type of starter on the amount of lactic acid produced, although they reported slight increases in the lactic acid content during the 28-d storage period. On the contrary, Guzel-Seydim et al. (2005) reported significantly higher concentration of lactic acid in yogurts produced with a ropy cultures and a decrease in the content after 14 d storage at 4°C. These variations could be due to the differences in the strains of organisms used for yogurt making.

Viability of *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*

The change in the number of EY strain of *S. thermophilus*, NEY strain of *S. thermophilus*, and *Lb. delbrueckii* ssp. *bulgaricus* 1368 during storage is reported in Table 2. Among the strains of *S. thermophilus*, the counts of the NEY strain remained similar throughout the storage period, whereas the EY strain showed an increase ($P < 0.05$) at d 14 and a decrease ($P < 0.05$) at d 28, although their numbers at d 28 were similar to those at the start of storage (d 1). Also, the increase in the counts of EY strain of *S. thermophilus*

Table 1. Changes in pH and lactic acid concentration during storage of control (NEY)¹ and experimental (EY) low-fat yogurts at 4°C for 28 d²

Type of yogurt	Period of storage (d)				
	1	7	14	21	28
pH					
NEY	4.53 ^{a,A}	4.40 ^{b,A}	4.34 ^{b,A}	4.58 ^{a,A}	4.53 ^{a,A}
EY	4.52 ^{a,A}	4.44 ^{b,A}	4.33 ^{bc,A}	4.54 ^{a,A}	4.48 ^{ab,A}
SEM			0.024		
Lactic acid (mg/100 g of yogurt)					
NEY	0.75 ^{a,A}	1.14 ^{b,A}	1.21 ^{b,A}	1.21 ^{b,A}	1.20 ^{b,A}
EY	0.75 ^{a,A}	1.12 ^{b,A}	1.21 ^{b,A}	1.26 ^{b,A}	1.21 ^{b,A}
SEM			0.066		

^{ab}Means in the same row with different superscript letters are significantly different for each type of yogurt.

^{AB}Means in the same column with different superscript letters are significantly different for a particular day of storage for each parameter.

¹NEY = control yogurt prepared from skim milk standardized to 12% total solids and non-EPS-producing strain of *S. thermophilus*; EY = yogurt prepared from skim milk standardized to 12% total solids and EPS-producing strain of *S. thermophilus*.

²Values are the means of 6 observations.

was significantly higher than that of NEY strain of *S. thermophilus* at d 14 and 21 of storage indicating some protective effect of EPS. Purwandari et al. (2007) however, observed an increase in counts of EY strain of *S. thermophilus* only during the first week of cold storage of yogurt prepared using only *S. thermophilus*. On the other hand, the counts of *Lb. delbrueckii* ssp. *bulgaricus* in control (NEY) were lower ($P < 0.05$) than those in experimental (EY) yogurts. They showed sharp decreases ($P < 0.05$) in NEY in the first and second week of storage whereas in EY they decreased ($P < 0.05$) during the first and third week of storage at 4°C. It was also observed that during fermentation, *Lb. delbrueckii* ssp. *bulgaricus* exhibited an increase of only 1 log cycle in NEY while in EY the increase was by 2 log cycles. This is reflected in the higher numbers ($P < 0.05$) of *Lb. delbrueckii* ssp. *bulgaricus* in EY as compared with that in NEY. This implies that *Lb. delbrueckii* ssp. *bulgaricus* was able to grow and survive better in the presence of the EPS-producing strain of *S. thermophilus*. Thus, EPS appears to have a protective effect on *Lb. delbrueckii* ssp. *bulgaricus* and to some extent on *S. thermophilus*. Amatayakul et al. (2006) have also observed the protective effect of EPS on *Lb. delbrueckii* ssp. *bulgaricus*. Salvador and Fiszman (2004) observed a significant decrease in viability of *S. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus* in skim yogurt after 15 d of storage at 10°C.

Crude EPS Content

The EPS content (mg/100 g of yogurt) during storage of EY is presented in Table 2. During fermentation the EY strain of *S. thermophilus* produced 37.43 mg of

EPS per 100 g of yogurt. The EPS content decreased ($P < 0.05$) to almost one-third the original content at d 7 of storage. There were no significant changes in the content thereafter. The decrease in EPS content could be attributed to the presence of enzymes capable of degrading EPS (Deegest et al., 2002). Purwandari et al. (2007) made a similar observation. However, Amatayakul et al. (2006) have reported an increase in the EPS content during the 28 d storage while Doleyres et al. (2005) found the content to be stable during the 4-wk storage period. Variations in the method of estimating the EPS, differences in the types of EPS as well as strain variations could be the possible reasons for the differences observed.

Extent of Proteolysis

The extent of proteolysis, as measured by the difference in the absorbance (ΔA_{340}) of the yogurt filtrates and the absorbance of the corresponding 0 h filtrates, is shown in Table 3. Both yogurts, NEY and EY, showed similar degree of proteolysis at d 1 and 7. A significant increase was observed at d 14 (0.265 units) for EY but not for NEY. Thereafter, NEY continued to show significant increases but the extent of proteolysis in EY was similar for the last 2 wk of storage. The proteolytic activity was similar for EY and NEY during the first 2 wk of storage, higher ($P < 0.05$) in EY at d 14 and 21 but toward the end of storage (d 28), NEY showed more ($P < 0.05$) proteolysis. Guzel-Seydim et al. (2005) observed significantly higher proteolysis (measured as tyrosine value) in yogurt prepared using ropy cultures than that prepared using nonropy cultures, both of which showed an increase at d 14 of storage.

Table 2. Changes in survival of yogurt starters and EPS content during storage of control (NEY)¹ and experimental (EY) low-fat yogurts at 4°C for 28 d²

Type of yogurt	Period of storage (d)				
	1	7	14	21	28
<i>Streptococcus thermophilus</i> ($\Delta \log_{10}$ cfu/g of yogurt)					
NEY	2.06 ^{a,A}	2.06 ^{a,A}	2.17 ^{a,A}	2.12 ^{a,A}	2.03 ^{a,A}
EY	2.12 ^{a,A}	2.06 ^{a,A}	2.40 ^{b,B}	2.31 ^{b,B}	1.99 ^{a,A}
SEM			0.054		
<i>Lactobacillus delbrueckii</i> ssp. <i>bulgaricus</i> ($\Delta \log_{10}$ cfu/g of yogurt)					
NEY	1.52 ^{a,A}	1.00 ^{b,A}	0.33 ^{bc,A}	0.38 ^{bc,A}	0.24 ^{bcd,A}
EY	1.93 ^{a,B}	1.80 ^{b,B}	1.93 ^{ab,B}	1.78 ^{bc,B}	1.71 ^{bc,B}
SEM			0.048		
EPS content (mg/100 g of yogurt)					
EY	37.43 $\pm$ 17.75 ^a	11.49 $\pm$ 2.68 ^b	11.21 $\pm$ 4.05 ^b	14.02 $\pm$ 1.43 ^b	16.31 $\pm$ 4.19 ^{ab}

^{a-c}Means in the same row with different superscript letters are significantly different for each type of yogurt.

^{AB}Means in the same column with different superscript letters are significantly different for a particular day of storage for each parameter.

¹NEY = control yogurt prepared from skim milk standardized to 12% total solids and non-EPS-producing strain of *S. thermophilus*; EY = yogurt prepared from skim milk standardized to 12% total solids and EPS-producing strain of *S. thermophilus*.

²Values are the means of 6 observations.

ACE Inhibition

The changes in percentage ACE-inhibitory activity and their corresponding IC₅₀ (mg/mL) values of the yogurt filtrates are depicted in Figure 1. No ACE-inhibitory activity was detected in the 0 h samples of the 2 types of yogurt mixes indicating that ACE inhibition observed in the samples was a consequence of the proteolytic activity of the yogurt starters. An increase ($P < 0.05$) in ACE inhibition was observed at d 7 for both types of yogurt (NEY and EY). Thereafter, while NEY exhibited a decrease ($P < 0.05$) at d 14 followed by an increase ($P < 0.05$) at d 28, EY showed a decrease in ACE-inhibition being significant at d 14 and 28. Although the ACE inhibition of EY was higher at d 14 than that of NEY, the IC₅₀ values were similar because the soluble protein content (data not shown) was significantly higher in EY compared with that in

NEY. The ACE inhibition was similar in NEY and EY throughout the storage period except at d 14 and 28 ($P < 0.05$). These changes could be due to the continued proteolysis to varying extents observed in the yogurts (Table 3) that resulted in hydrolysis of the existing peptides and generation of newer peptides having ACE-inhibition potential (Ramchandran and Shah, 2008). It appears that EPS does not offer any particular protection to the ACE-inhibitory potential of the yogurts. This aspect has not been studied so far.

Firmness of Yogurt

The firmness (g) of the yogurts measured during storage for 28 d at 4°C is presented in Figure 2. The firmness of yogurt prepared from EPS-producing culture (EY) was lower ($P < 0.05$) than that of the control (NEY) at d 1. Both the yogurts showed a significant

Table 3. Changes in extent of proteolysis during storage of control (NEY)¹ and experimental (EY) low-fat yogurts at 4°C for 28 d²

Type of yogurt	Period of storage (d)				
	1	7	14	21	28
ΔA_{340}					
NEY	0.536 ^{a,A}	0.536 ^{a,A}	0.502 ^{a,A}	0.685 ^{ab,A}	0.879 ^{bc,A}
EY	0.521 ^{a,A}	0.556 ^{a,A}	0.821 ^{bc,B}	0.794 ^{bc,B}	0.722 ^{bc,B}
SEM			0.055		

^{a-c}Means in the same row with different superscript letters are significantly different for each type of yogurt.

^{AB}Means in the same column with different superscript letters are significantly different for a particular day of storage for each parameter.

¹NEY = control yogurt prepared from skim milk standardized to 12% total solids and non-EPS-producing strain of *S. thermophilus*; EY = yogurt prepared from skim milk standardized to 12% total solids and EPS-producing strain of *S. thermophilus*.

²Values are the means of 6 observations.

Figure 1. Changes in angiotensin-I converting enzyme (ACE)-inhibition (%) and IC_{50} (the protein concentration resulting in 50% inhibition; mg/mL) values of control (NEY) and experimental (EY) low-fat yogurts during storage at 4°C for 28 d. Numbers 1 to 28 following the yogurt types indicates storage period d 1 to 28. ^{a-c}Means with different lowercase letters are different within each type of yogurt; ^{A,B} means with different uppercase letters are different between each type of yogurt for a particular day of storage.

increase at d 7 after which there was no significant change in their firmness. Throughout the storage period, the firmness of EY was lower ($P < 0.05$) than that of NEY, except at d 14. This clearly indicates that EPS contributes to lowering the firmness of the yogurt gels. Several researchers have made a similar observation using different types of EPS producers (Hess et al., 1997; Hassan et al., 2003b; Amatayakul et al., 2006; Folkenberg et al., 2006). The changes in firmness showed a negative correlation ($R = -0.89$) with the changes in EPS content of EY during storage at 4°C for 28 d. Folkenberg et al. (2006) also found an inverse correlation between the presence of EPS and gel firmness. It has been reported that in yogurts made using EPS-producing strains, there is incompatibility between EPS and proteins as well as void spaces around EPS-producing bacteria that could result in a weaker network with fewer protein-protein interactions (Ruas-Madiedo et al., 2002; Hassan et al., 2003b). Also, initiation of gelation at relatively higher pH in these yogurts could result in the formation of densely aggregated networks causing the EPS to aggregate in the continuous phase as aggregation proceeds with the progress in fermentation (Hassan et al., 2003b). It has

also been reported that EPS that are weakly charged and, having low molecular weight, reduce the firmness of gels (Girard and Schaffer-Lequart, 2007).

Spontaneous Whey Separation

Figure 3 shows the changes in spontaneous whey separation observed during the storage of the 2 types of yogurt, NEY and EY. Both types of yogurt showed a significant decrease in whey separation at d 7 after which there were no changes, except in the case of NEY, which showed an increase ($P < 0.05$) to a value similar to that at d 1. However, the extent of whey separation was significantly reduced in EY, clearly indicating the influence of EPS. Similar effect of EPS has been reported by Amatayakul et al. (2006), Guzel-Seydim et al. (2005), and Hess et al. (1997). The changes in spontaneous whey separation showed a negative correlation with firmness, the correlation being better in EY ($r = -0.65$) than in NEY ($r = -0.31$). The better water-holding capacity of EPS and the modification in yogurt structure due to the presence of EPS are plausible explanations for the reduced whey separation. The decrease in whey separation observed during storage

Figure 2. Changes in firmness (g) of control (NEY) and experimental (EY) low-fat yogurts during storage at 4°C for 28 d. ^{a,b}Means with different lowercase letters are different within each type of yogurt; ^{A,B}means with different uppercase letters are different between each type of yogurt for a particular day of storage.

could in part be due to protein rearrangement (Ozer et al., 1998) and partly due to reduction in EPS content resulting in a more thermodynamically stable system with better water-holding capacity (Hassan et al., 2003b). Also, because EY took a longer time to reach pH 4.5 ± 0.1 than did NEY, it could have resulted in a more compact structure and hence lower syneresis (Hassan et al., 2003b). Castillo et al. (2006) suggested that faster acidification and coagulation reactions enhanced syneresis in cottage cheese gels. Doleyres et al. (2005) also found that yogurts prepared using EPS-producing cultures had better water-holding capacity and thereby lower syneresis, and that the water-holding capacity increased during storage. Although Folkenberg et al. (2006) reported a negative correlation between syneresis and firmness in EPS containing yogurts, they observed that syneresis was more pronounced in EPS containing yogurts.

Rheological Parameters

To model the flow behavior of the 2 types of low-fat yogurts, NEY and EY, during storage, the upward flow curves (shear stress) were fitted to the Herschel-Bulkley model to obtain yield stress σ_0 , consistency index k , and flow behavior index n (Table 4). The yield stress for NEY was higher ($P < 0.05$) than those of EY throughout the storage period suggesting the influence of EPS. This is in agreement with the higher firmness exhibited by NEY than EY (Figure 2). Doleyres et al. (2005) and Hassan et al. (2003b) made similar observations. The

decreased interactions between protein aggregates due to the presence of EPS in the continuous phase surrounding the aggregate is understood to be responsible for this (Hassan et al., 2003b). However, no change in the yield stress was observed for EY throughout the storage period, while for NEY the increase was significant only at d 21. The consistency index of EY was, surprisingly, similar to that of NEY until d 14 after which it was lower ($P < 0.05$) than that of NEY. There was no effect of storage time on the consistency index of either NEY or EY. There are conflicting reports for this in literature. Hess et al. (1997) reported a lower consistency index for EPS⁺ yogurts, while Doleyres et al. (2005) and Hassan et al. (2003b) have reported a

Figure 3. Changes in spontaneous whey separation (%) during storage of control (NEY) and experimental (EY) low-fat yogurts at 4°C for 28 d. ^{a,b}Means with different lowercase letters are different within each type of yogurt; ^{A,B}means with different uppercase letters are different between each type of yogurt for a particular day of storage.

Table 4. Flow behavior (predicted by the Herschel-Bulkley model) and hysteresis loop area of control (NEY)¹ and experimental (EY) low-fat yogurts during storage at 4°C for 28 d²

Storage period (d)	Rheological parameter									
	σ_0 (Pa) ³		k (Pa·s)		n		R^2		Hysteresis (Pa/s)	
	NEY	EY	NEY	EY	NEY	EY	NEY	EY	NEY	EY
1	8.31 ^{aA}	3.80 ^{aA}	2.05 ^{aA}	1.17 ^{aA}	0.65 ^{aA}	0.70 ^{aA}	0.99	0.98	306.28 ^{aA}	135.85 ^{aA}
7	9.61 ^{aA}	5.56 ^{aA}	1.37 ^{aA}	1.31 ^{aA}	0.73 ^{aA}	0.70 ^{aA}	0.99	0.98	249.86 ^{aA}	168.67 ^{aA}
14	12.75 ^{aA}	5.17 ^{aB}	2.35 ^{aA}	1.38 ^{aA}	0.63 ^{aA}	0.70 ^{aA}	0.98	0.99	385.01 ^{aA}	165.27 ^{aB}
21	21.95 ^{ba}	7.69 ^{aB}	2.32 ^{aA}	1.12 ^{aB}	0.74 ^{aA}	0.77 ^{aA}	0.99	0.99	605.03 ^{ba}	213.70 ^{aB}
28	23.99 ^{ba}	11.23 ^{baB}	2.92 ^{aA}	1.26 ^{aB}	0.73 ^{aA}	0.81 ^{aA}	0.98	0.98	673.41 ^{ba}	325.10 ^{baB}
SEM	2.02		0.43		0.05				63.46	

^{ab}Means in the same row with different superscript letters are significantly different for each type of yogurt.
^{AB}Means in the same column with different superscript letters are significantly different for a particular day of storage for each parameter.
¹NEY = control yogurt prepared from skim milk standardized to 12% total solids and non-EPS-producing strain of *S. thermophilus*; EY = yogurt prepared from skim milk standardized to 12% total solids and EPS-producing strain of *S. thermophilus*.
²Values are the means of 6 observations.
³ σ_0 = yield stress; k = consistency index; n is a dimensionless number that indicates the closeness to Newtonian flow ($n < 1$ indicates pseudo-plastic liquid).

higher consistency index and thus a higher viscosity in EPS-containing yogurt than non-EPS-containing ones. However, variations in the viscosifying effect due to variations in type of cultures producing different types of EPS are known (Sebastiani and Zelger, 1998). No variations in the flow behavior index (Table 4) were observed between NEY and EY, nor was there any influence of the time of storage. Doleyres et al. (2005) have also reported that the flow behavior index of yogurts produced with or without EPS-producing starter culture did not differ significantly. However, the low values (<1) of flow behavior index confirm the deviation in flow behavior of yogurts from Newtonian fluids, although there does not appear to be any influence of the presence of EPS.

The thixotropic behavior of NEY and EY as determined by the hysteresis loop area (Pa/s) between the upward and downward curves (shear rate 0.1 to 100 per s) is presented in Table 4. The higher values for NEY indicate slower structural recovery in these samples than in EY. Girard and Schaffer-Lequart (2007) have reported that weakly charged EPS and low-molecular-weight EPS allowed best recovery of the texture of milk gels after shearing. A weak gel made up of loosely bound aggregates may rebuild more easily than brittle ones. The values for NEY were higher than those for EY ($P < 0.05$) only at d 14, 21, and 28. This is in concurrence with the higher consistency coefficients of NEY than EY. The correlation of firmness to hysteresis loop area was also better for NEY ($r = 0.75$) than for EY ($r =$

Figure 4. Storage modulus of control low-fat yogurt (NEY) during storage at 4°C for 28 d, as a function of oscillatory frequency, carried out at 5°C. Reported data are means of 6 observations.

Figure 5. Storage modulus of experimental (EY) low-fat yogurt during storage at 4°C for 28 d, as a function of oscillatory frequency, carried out at 5°C. Reported data are means of 6 observations.

0.60). There was no change in hysteresis area of EY throughout the storage period while an increase ($P < 0.05$) was observed in NEY at d 21 and 28. However, Amatayakul et al. (2006) found higher loop area values (shear rate 10 to 50 per s) for EPS containing yogurts that varied during storage, whereas Purwandari et al. (2007) have observed greater hysteresis loop area in EPS containing yogurts at the end of storage. Koksoy and Kilic (2004) have found that an increase in consistency coefficient was associated with increased thixotropy.

The results of frequency sweep of NEY and EY, reported as storage modulus (G') versus log frequency, gave a straight line as shown in Figure 4 and 5 respectively. The G' of NEY (482 Pa) was higher than that of EY (161 Pa), as was the loss modulus (G'' ; 131 and 46 Pa respectively). Both G' and G'' increased with storage to 1172 and 305 Pa for NEY and 483 and 128 Pa for EY, respectively. This indicates that NEY had more solid-like (elastic) properties than EY, thereby implying that EPS conferred more viscous properties to the yogurt. This is confirmed by the higher ($P < 0.05$) firmness (Figure 2) and yield stress (Table 4) values of NEY as compared with EY. Extensive particle rearrangement during structure formation resulting in dense clusters of aggregates along with lesser protein-protein interactions could have caused lower G' values in EY. Purwandari et al. (2007), Doleyres et al. (2005), and Hassan et al. (2003b) have also made similar observations.

CONCLUSIONS

During storage of the low-fat yogurts, the presence of EPS did not have any influence on changes in pH and lactic acid content although there was a protective ef-

fect on *L. delbrueckii* ssp. *bulgaricus* and to some extent on *S. thermophilus*. The EPS content of EY dropped significantly during the first week of storage and remained stable thereafter. There was a significant increase in the extent of proteolysis in EY at d 14 and in NEY at d 14 and 21 of storage. Exopolysaccharide did not appear to have any influence on the ACE-inhibition activity. However, there was a definite influence of EPS in reducing the firmness, spontaneous whey separation, and yield stress of low-fat yogurt, whereas there was no significant influence on the consistency index and flow behavior index of the yogurts. Yogurts made from EPS-producing starter showed a faster structural recovery after shear and exhibited better viscous properties. Thus, use of EPS producers in low-fat yogurt improved the textural properties of the yogurts without influencing their ACE-inhibition potential.

ACKNOWLEDGMENT

The authors acknowledge the help provided by Todor Vasiljevic in rheological analysis of yogurt and statistical analysis of the data.

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