Evaluation of the yield, molar mass of exopolysaccharides, and rheological properties of gels formed during fermentation of milk by *Streptococcus thermophilus* strains St-143 and ST-10255y

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ABSTRACT

The yield and chemical structures of exopolysaccharides (EPS) produced by many strains of *Streptococcus thermophilus* have been characterized. However, the kinetics (or production profile) for EPS during milk fermentation is not clear. In this study, we investigated whether any differences existed in the yield and molar mass of EPS when milk was fermented at the same acidification rate by 2 strains of *S. thermophilus* (St-143 and ST-10255y). The type of EPS produced by these 2 strains is different. Milk samples were analyzed for EPS concentration every 30 min during a fermentation period of 270 min (final pH 4.5) by using a modified quantification method, which was faster and validated for its recovery of added EPS. Rheological properties of milks during fermentation were also analyzed using small-strain dynamic oscillatory rheology. For the determination of molar mass, EPS extracts were isolated by ultrafiltration of whey obtained during fermentation of milk to pH values 5.2, 4.9, 4.7, and 4.5, and molar mass was analyzed using size-exclusion chromatography–multi-angle laser light scattering. During fermentation, both strains appeared to start producing significant amounts of EPS after about ~150 min, which corresponded to pH ~5.3, which was close to the point of gelation. During the remainder of the fermentation process (150–270 min), the EPS concentration from strains St-143 and ST-10255y significantly increased from 30 to 72 mg/L and from 26 to 56 mg/L, respectively. The quantity of EPS recovered by our modified method was estimated to represent ~60% of the total EPS added to milk. The molar mass of EPS produced by both strains appeared to slightly decrease during fermentation. At pH 5.2, EPS from St-143 and ST-10255y had molar masses of 2.9×10^6 and 1.4×10^6 g/mol, respectively,

which decreased to 1.6×10^6 and 0.8×10^6 g/mol, respectively, when the pH of milk was 4.5. Distinct differences were apparent in the rheological properties of gels fermented by the 2 strains. At the end of fermentation, St-143 fermented milk had weaker gels with storage modulus (G') value at pH 4.6 of 26 Pa, whereas gels made with ST-10255y were stiffer with a G' value at pH 4.6 of 82 Pa. For St-143 gels, maximum loss tangent ($LT_{\max}$) values were higher (0.50) and occurred earlier (at a higher pH value) than the $LT_{\max}$ values (0.46) for gels from ST-10255y strain. Because the fermentation conditions were identical for both strains, the observed changes in rheological properties could be due to the differences in chemical structures and molar mass of the EPS produced by these 2 *S. thermophilus* strains.

Key words: yogurt, exopolysaccharides, rheology, *Streptococcus thermophilus*

INTRODUCTION

Streptococcus thermophilus is 1 of the 2 species of lactic acid bacteria (**LAB**) that are extensively used in fermented milk products such as yogurt. Some strains of *S. thermophilus* have the ability to synthesize large polymeric carbohydrates, called exopolysaccharides (**EPS**). The presence of significant quantities of EPS in fermented milks is indicated by a sticky or stringy appearance termed “ropiness” when these gels are stirred (De Vuyst and Degeest, 1999; De Vuyst and De Vin, 2007). *Streptococcus thermophilus* strains can generally synthesize 2 types of EPS. One type is synthesized inside the cell, and when released from the cell, the EPS remains attached to the exterior of cell, forming a capsule; hence, it is called capsular EPS. Another type of EPS is liberated into the medium and provides a ropy or slimy characteristic; hence, this type is called ropy EPS (Low et al., 1998; Broadbent et al., 2003).

The ability of *S. thermophilus* strains to produce EPS has been studied since the 1980s. Giraffa and Bergère (1987) reported on 3 ropy strains of *S. thermophilus*, namely CNRZ 404, CNRZ 783, and CNRZ 784, that

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produced 21 to 39 mg of EPS per liter in reconstituted skim milk supplemented with additional nutrients. Another 3 rosy strains of *S. thermophilus* were reported to produce 29 to 185 mg of EPS per liter of reconstituted skim milk, and the EPS content was greatly reduced when isolated EPS was subjected to protein hydrolysis followed by dialysis (Cerning et al., 1988). At the present time, at least 60 strains of *S. thermophilus* that produce EPS have been studied during the fermentation of milk, and the yield of EPS obtained from these various strains varies markedly (8–290 mg/L) from strain to strain (Giraffa and Bergère, 1987; Cerning et al., 1988; De Vuyst et al., 2001; Broadbent et al., 2003; Pachekrepapol et al., 2017).

Different methods have been used for the isolation and quantification of EPS from various types of fermentation media. To isolate EPS from milk, removal of milk protein has been carried out by (optional) enzymatic hydrolysis of protein, trichloroacetic acid (TCA) precipitation, or both. To isolate EPS from the resulting supernatant, ethanol, acetone, or both have been used; ethanol is the most commonly used solvent (Ruas-Madiedo and de los Reyes-Gavilán, 2005; Leroy and De Vuyst, 2016). Different strains may produce different amounts of EPS in milk depending on the availability of nutrients and fermentation conditions; the EPS yield is also affected by the methods used for isolation and quantification of EPS (Leroy and De Vuyst, 2016). Giraffa and Bergère (1987) and Cerning et al. (1988) isolated EPS by first hydrolyzing milk protein using Pronase enzyme after the fermentation was completed, followed by cell removal via centrifugation and multi-step precipitation of EPS by ethanol. Initially, Cerning et al. (1988) determined the EPS content by the phenol sulfuric acid method. When EPS was further subjected to dialysis, the net EPS content in the extract was markedly reduced, indicating ethanol precipitation may have been insufficient to remove all the small sugars, particularly lactose, that could interfere with the estimation of the actual quantity of EPS (Cerning et al., 1988). In another study by Faber et al. (1998), the yield of EPS was determined by removing milk protein by TCA precipitation and extensive dialysis of the supernatant. The EPS content in the supernatant was determined by high-performance gel permeation chromatography, using dextran standards. De Vuyst et al. (2003) used a method consisting of protein removal by TCA precipitation, EPS isolation by acetone precipitation, and residual protein precipitation by TCA followed by re-precipitation by acetone to finally isolate EPS free of small sugars and protein. The yields of EPS reported for the *S. thermophilus* strains used by De Vuyst et al. (2003) and Vaningelgem et al. (2004) were higher in comparison to other reports.

Not many reports exist for the yield of EPS from the same strains of *S. thermophilus* quantified using different isolation methods. Rimada and Abraham (2003) compared 8 different methods for the isolation and quantification of kefiran (the type of EPS in kefir) from milk-based media and found marked differences in the kefiran yields. Yields were higher for methods that used heating as an initial step and when multiple ethanol precipitation steps were used. Trichloroacetic acid precipitation at the beginning of the isolation procedure caused an almost 50% loss in EPS yield (Rimada and Abraham, 2003). Recently, EPS from *S. thermophilus* strains St-143 and DGCC 7710 was isolated by 2 different methods. In the study by Pachekrepapol et al. (2017), milk protein was removed from the fermented milk by TCA precipitation, and EPS was isolated from the supernatant by repeated ethanol precipitation followed by removal of small sugars by dialysis. The EPS content in freeze-dried isolate produced by DGCC 7710 and St-143 strains were 77 and 66 mg of glucose equivalent (GE)/L of medium, respectively (Pachekrepapol et al., 2017). Mende et al. (2012) also isolated this EPS by enzymatic hydrolysis, TCA precipitation of protein followed by precipitation with acetone and dialysis. The EPS contents in their dried sample were 110 and 137 mg of GE/L for the strains St-143 and DCGG7710, respectively (Mende et al., 2012). Goh et al. (2005) evaluated 2 methods of EPS isolation from synthetic media. In one method, protein was hydrolyzed by Pronase and precipitated by TCA. The resultant supernatant was dialyzed for 48 h. In another method (Goh et al., 2005), cells were removed by centrifugation and EPS was directly precipitated by ethanol and dialyzed for 48 h. A comparison of these 2 methods revealed a marked difference in EPS yields.

Since Doco et al. (1990) first determined the molar mass of EPS isolated from *S. thermophilus* strains, at least 40 strains of this species have been investigated for the molar masses of their isolated EPS. The reported molar mass values of the different EPS isolated from *S. thermophilus* strains have varied greatly. More than 26 *S. thermophilus* strains have been reported to produce EPS with molar mass greater than 1×10^6 g/mol. At least 14 strains have been reported to produce EPS with molar mass less than 1×10^6 g/mol, whereas 10 strains have been reported to produce 2 distinct EPS fractions, one type having molar mass values larger than 1×10^6 g/mol and another fraction with molar mass values below 1×10^6 g/mol (Doco et al., 1990; Lemoine et al., 1997; Faber et al., 1998; Marshall et al., 2001; Vaningelgem et al., 2004). The molar mass determination techniques were mainly based on gel filtration chromatography, which compared the molar mass of EPS with standards (dextran) of known molar mass

values. In more recent studies (e.g., Pachekrepapol et al., 2017), size-exclusion chromatography coupled with multi-angle laser light scattering (**SEC-MALLS**) has been used, which is a method that can directly measure the molar mass of polymers.

Reviewing the different methods for the isolation and yield determination of EPS, 2 major limitations of the various methods are obvious. Most of the EPS isolation and purification methods are very time consuming. In addition, uncertainties are present in these methods about the ability to recover the total amount of EPS present in a milk-based medium (Leroy and De Vuyst, 2016). Moreover, all the milk studies conducted to date on the production of EPS from *S. thermophilus* were focused on the final EPS yields (i.e., at the end of fermentation). Information on quantities and structural properties of EPS produced during the fermentation period (i.e., the kinetics of EPS production) are limited.

In this study, our objective was to determine the EPS concentrations at regular intervals during the fermentation of milk by using a relatively quicker quantification method, which was validated for its recovery of added EPS. Another objective was to determine the physical properties, including molar mass, size, and the polydispersity of EPS produced during fermentation, and to understand the relationship between the physical properties of EPS and the rheological properties of fermented milk gels.

MATERIALS AND METHODS

Reconstitution of Milk

Low heat nonfat dry milk powder was obtained from Dairy America (Fresno, CA) and the whey protein nitrogen index was determined as 7.56 mg of undenatured whey protein per gram of powder, using the method suggested by Wehr and Frank (2004). Milk was reconstituted by dispersing 12 g of powder in 100 g of deionized water and stirring at room temperature for 3 h. Reconstituted milk was heated at 85°C for 30 min in a water bath, cooled to ~5°C, and stored in refrigerator for up to 24 h before use.

Preparation of EPS Cultures

Two EPS-producing *S. thermophilus* strains, St-143 and ST-10255y, obtained from Chr. Hansen (Hørsholm, Denmark) were used for this study. Both strains are known to produce EPS when grown in a milk medium. These 2 strains were selected based on the differences in their likely EPS yields as well as differences in repeating unit structures (Pachekrepapol et al., 2017).

Strain St-143 has been reported to produce EPS yields ranging from 65 to 110 mg of GE/L, whereas strain ST-10255y has been reported to produce 27 mg of GE/L of EPS when grown in reconstituted milk (Mende et al., 2012; Pachekrepapol et al., 2017). Strain St-143 has been reported to produce EPS with a hexasaccharide repeating unit, whereas the ST-10255y EPS repeating unit was reported to be tetrasaccharide (Pachekrepapol et al., 2017).

Reconstituted skim milk, prepared as above, was autoclaved for 15 min and cooled to 40°C. Frozen cultures (−80°C) were thawed, and 2 mL of these cultures was inoculated into 500 mL of the sterilized skim milk at 40°C and incubated at 40°C until the milk gelled (pH ~5.0). The fermented milk was aseptically filled into 5-mL sterile cryogenic vials (Fisher Scientific, Pittsburgh, PA) and stored frozen (−80°C) to use as a stock culture. Starter culture was prepared immediately before use by inoculating 1 mL of the thawed stock culture into 80 mL of the sterilized milk at 40°C. Inoculated milk was incubated at 40°C for ~4 h (pH ~5.0).

Fermentation of Milk and Monitoring of pH

Both of these strains had different abilities to acidify milk, mainly due to minor differences in their proteolytic activity. To obtain similar acidification rates, the milk used for fermentation by St-143 and ST-10255y strains was supplemented with 3.0% (wt/wt) and 0.2% (wt/wt) peptone, respectively, as suggested by Pachekrepapol et al. (2017). After cooling to 40°C, 500 mL of milk was inoculated with 2% (vol/vol) of the freshly prepared starter culture of each individual strain. Exactly 50 mL of inoculated milk was poured into sterile 250-mL polycarbonate centrifuge bottles (Fisher Scientific, Waltham, MA), and bottles were closed with an air-sealed cap before incubation at 40°C in a Precision Low Temperature incubator (Thermo Fisher Scientific, Marietta, OH). To monitor the change in pH during fermentation, 50 to 70 mL of milk was poured in a 100-mL glass vial and a pH probe of a computer-connected model PCM 700 Orion Sensor Link system (Orion Research Inc., Beverly, MA) was immersed into the milk. The surface of the milk in the vials was covered with vegetable oil to make a similar (anaerobic) environment as used in the measurement of rheological properties. The system recorded pH values every 5 min during fermentation.

Rheological Properties of Milk During Fermentation

To determine the rheological properties of milk gels during fermentation, a Paar Physica rheometer, MCR

301 (Anton Paar, Ashland, VA) was used. Nondestructive, small-strain dynamic oscillatory measurements were performed with a cup-and-bob geometry (CC27-SN23928) consisting of 2 coaxial cylinders with diameters of 26.7 and 28.9 mm. The temperature of the system was set at 40°C. Inoculated milk was aseptically transferred to the rheometer. The milk surface of the measuring geometry was covered with vegetable oil to avoid evaporation. An oscillatory frequency of 1 Hz, with 0.5% strain, was applied to milk during the entire fermentation period. Data for storage modulus (G') and loss tangent (LT) were recorded every 5 min until the end of fermentation (270 min, pH ~4.5). During fermentation, milk was considered to be gelled when the G' values of the gels exceeded 1 Pa (Lucey et al., 1998a).

Determination of EPS Concentration During Milk Fermentation

The EPS content of fermented milk was determined using the method for EPS quantification described by Enikeev (2012) with some modifications. Originally, this method was used to determine the EPS content in kefir. This method was modified to standardize conditions and steps and to determine its efficiency to recover added EPS from milk.

Modification of EPS Determination Method and Recovery of EPS. Exactly 50 mL of milk containing EPS was transferred into 250-mL Fisherbrand polycarbonate centrifuge bottles (Fisher Scientific, Pittsburgh, PA), and the pH value was adjusted to pH ~4.6 (if necessary) by the addition of 10% (vol/vol) lactic acid solution. Milk was heated at 98°C in a water bath for 30 min with constant stirring to inactivate the culture and precipitate proteins. The heated milk was then centrifuged at $6,000 \times g$ for 15 min at 25°C. The pellet was discarded, and the supernatant was treated with 5 mL of 12 M HCl at 70°C, cooled, and neutralized to pH 7.0 by the addition of 20% (wt/vol) NaOH. This supernatant was again centrifuged at $6,000 \times g$ for 15 min at 25°C, and the supernatant was treated with a double volume of cold (−20°C) absolute ethanol and kept at −20°C for 3 h to facilitate precipitation of EPS. After precipitation, the entire contents of each bottle was centrifuged at $16,000 \times g$ for 20 min at 4°C, and the supernatant was discarded. The pellet was dissolved in 40 mL of 55°C deionized water, and the solution was centrifuged at $6,000 \times g$ for 15 min at 25°C to remove undissolved impurities as a pellet. The supernatant was treated with equal volume of cold absolute ethanol and kept at −20°C for 3 h. The supernatant was centrifuged at $16,000 \times g$ for 20 min at 4°C, and the supernatant

was discarded. The pellet was washed twice with 50% cold ethanol to remove any remaining small sugars and dissolved in 40 mL of water at 55°C. Finally, the aliquot was centrifuged at $6,000 \times g$ for 15 min at 25°C to remove insoluble materials, and the volume of the supernatant was adjusted to 50 mL.

To test the efficiency of the extraction method, known concentrations of model EPS [i.e., dextran (Sigma-Aldrich, St. Louis, MO) with an average molar mass of 2×10^6 g/mol] was added to (unfermented) milk at various dextran concentrations ranging from 0 to 800 mg/L. The amount of dextran in these milks was determined by our modified Enikeev method.

Recovery of added EPS by the modified Enikeev method was calculated using the following equation:

$$\text{Recovery (\%)} = \frac{\text{Measured dextran by modified method (mg/L)}}{\text{Added dextran to milk (mg/L)}} \times 100.$$

To ensure that we had removed all the small sugars, the lactose, glucose, and galactose contents in the final EPS aliquot were determined by an HPLC method (Mullin and Emmons, 1997). Protein content in the final EPS aliquot was also determined by bicinchoninic acid assay using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). The amount of EPS synthesized by the *S. thermophilus* strains in milk during the fermentation period of 270 min was determined at 30-min intervals. Exactly 50 mL of milk (in 250-mL centrifuge bottles) was fermented for each interval and subjected to EPS determination.

Quantification of EPS Concentration. The phenol sulfuric method (Dubois et al., 1956), with some modifications, was used for the determination of EPS (sugar) contents in the final EPS aliquots. A standard curve for the phenolic sulfuric acid method was obtained using various known concentrations of dextran (Sigma-Aldrich); the dextran had an average molar mass of 2×10^6 g/mol, which was a similar molar mass to the EPS produced by *S. thermophilus* strains. An aliquot was also extracted from an inoculated (but not fermented) milk sample to use as the blank for phenol sulfuric acid analysis of the EPS extracts from fermented samples.

Determination of Molar Mass of Isolated EPS

In determining the molar mass of EPS, a concern existed that precipitation of EPS during extraction with ethanol, in conjunction with lyophilization, could alter its measured molar mass when rehydrated (Pachekre-

papol et al., 2017). Therefore, we instead isolated the EPS by ultrafiltration. One liter of reconstituted skim milk with added peptone was prepared as described earlier, and 30 mL of starter culture was added into this milk. Inoculated milk was incubated at 40°C until pH values of 5.2, 4.9, 4.7, and 4.5. When milk reached these selected pH values, fermentation was stopped by heating milk at 85°C for 10 min with constant stirring. The mixture was cooled and centrifuged at $6,000 \times g$ for 10 min at 25°C, and the supernatant (whey) was collected. The pellet (curd) was suspended into 500 mL of water at 50°C, cooled to 25°C, and centrifuged again. The supernatant was collected, and the pellet was discarded. The 2 supernatants were pooled and ultrafiltered through 100-kDa Prep/Scale TFF ultrafiltration membrane cartridges (Millipore, Bedford, MA). The permeate was discarded, and the retentate was diafiltered with distilled water up to 12 times to remove small sugars (as indicated by a minimal carbohydrate content of the permeate by the phenol sulfuric acid analysis method). After ultrafiltration, the retentate was collected and pH was adjusted to 4.6 by addition of 0.1 N HCl. The retentate was then heated without stirring at 80°C for 5 min and centrifuged at $10,000 \times g$ for 15 min at 25°C. For the EPS sample from strain ST-10255y, residual protein content in the supernatant was removed by 12% TCA precipitation followed by repeated ultrafiltration to remove any residual TCA. Final retentate was concentrated to one fifth of its volume by using an Amicon stirred ultrafiltration cell (Millipore) with a 10-kDa Millipore disc membrane (Millipore).

Next, SEC-MALLS was used to determine the molar mass of the isolated EPS, as described by Wang and Lucey (2003). The SEC system consisted of Waters 600 HPLC unit (Waters, Milford, MA) connected to SEC Superose 12HR 10/300 and 6HR 10/300 columns connected in series (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The system consisted of 3 detectors, namely a model 2998 photodiode array detector operating at 280 nm (Waters), a DAWN EOS MALLS photometer system (Wyatt Technology, Santa Barbara, CA) fitted with a helium neon-laser ($\lambda = 690$ nm) and a K-5 flow cell, and a model 2414 refractive index (RI) detector (Waters).

The EPS extracts were first filtered using a 0.22- μ m syringe filter (Millipore) to remove any dust or impurities. Sample (100 μ L) was injected into the SEC system, which was operated at a flow rate of 0.5 mL/min. The chromatograms were analyzed for the intensities of the RI and UV signals. The peaks with higher RI signals and correspondingly low or no UV signals at 280 nm were confirmed as EPS peaks. Molar mass, polydisper-

sity, and radius of gyration (R_g) of the EPS molecules were determined by ASTRA software version 4.73.04 (Wyatt Technology) using the Debye fitting method (Wang and Lucey, 2003). Refractive index increment value of 0.14 mL/g was used for polysaccharides (EPS) (di Cagno et al., 2014). The SEC-MALLS system was calibrated using monomeric bovine serum albumin (molar mass of 66.4×10^3 g/mol; Sigma-Aldrich).

Statistical Analysis

Experiments for the determination of EPS yield were repeated 3 times with duplicates in each experiment ($n = 6$). Experiments for dextran recovery and rheological properties of milk gels were also repeated at least 3 times. Experiments for molar mass determination were carried out twice. Means were compared by ANOVA and Tukey-Kramer HSD using JMP Pro 11.0 (SAS Institute Inc., Cary, NC) software with significance indicated at $P < 0.05$.

RESULTS

Fermentation Time and pH

The pH values during the fermentation of milk is shown in Figure 1. The initial pH of inoculated milk was close to 6.5 and reached pH 4.5 after 270 min, when fermented by both strains. Similar pH profiles of fermentation were observed for both strains. Until 90 min, milk pH did not noticeably change; after 150 min, pH decreased steeply until 210 min, when it decreased

Figure 1. Change in pH values of milk during fermentation by *Streptococcus thermophilus* St-143 (○) and ST-10255y (Δ) at 40°C. Error bars indicate standard error of means.

Table 1. Recovery of added dextran in milk by the modified isolation method, and the lactose and protein contents in the final dextran extracts¹

Dextran added to milk (mg/L)	Recovered dextran (mg/L)	Recovery of dextran (%)	Lactose content in final EPS ² extract (mg/L)	Protein content in final EPS extract (mg/L)
0	0.0	0.0	0.5 ± 0.1 ^a	1,157 ± 356 ^b
50	22 ± 3	44 ± 5 ^b	0.6 ± 0.1 ^a	1,491 ± 331 ^{ab}
100	54 ± 4	53 ± 4 ^{ab}	0.8 ± 0.1 ^a	1,584 ± 262 ^a
200	97 ± 3	48 ± 1 ^b	0.8 ± 0.0 ^a	1,336 ± 327 ^{ab}
400	242 ± 16	60 ± 4 ^a	0.6 ± 0.1 ^a	1,302 ± 222 ^{ab}
800	489 ± 35	61 ± 4 ^a	0.7 ± 0.1 ^a	1,754 ± 249 ^a

^{a,b}Different superscripts in the same column indicate significant difference ($P < 0.05$).

¹Values are means ± SEM for $n = 4$ for recovery of dextran and $n = 6$ for lactose and protein contents.

²EPS = exopolysaccharides.

relatively slowly until fermentation was stopped at pH values close to 4.5.

Evaluation of Modified Enikeev Method for Determination of EPS Concentration

Recovery of Added EPS. The recovered EPS content from milk that originally contained 50 mg/L of dextran was 22 mg/L, which corresponded to 44% recovery. Lower recoveries were obtained for samples containing relatively lower dextran concentration, whereas recovery exceeded 60% for milk containing more than 400 mg/L of added dextran (Table 1).

The HPLC analysis indicated that the concentrations of lactose, or simple sugars, were less than 1 mg/L (Table 1), and only trace levels of glucose and galactose (result not shown) were present in the final EPS extracts. This result indicated that the total carbohydrate contents determined for EPS extracts were not significantly affected by any potential contamination by residual lactose, glucose, or galactose. The isolated EPS (dextran) extracts were also analyzed for protein content. Regardless of the added dextran concentration in the extracts, the residual protein content varied from 1,157 to 1,753 mg/L (Table 1). The phenol sulfuric acid method was evaluated for any possible interference due to residual protein or ethanol, and no interferences were observed.

Yield of EPS. The yields of EPS produced by *S. thermophilus* strains St-143 and ST-10255y during fermentation of milk are shown in Figure 2b. For about the first 120 min of the fermentation period (pH ≥ 5.9), the milks fermented by either strains had no significant amount of EPS. The yield of EPS increased for both strains as the fermentation continued (pH decreased < 5.9). Final EPS yield at the end of fermentation (pH 4.5) was significantly higher for the strain St-143 compared with ST-10255y (Table 2, Figure 2b).

Rheology of Acid Milk Gels

The rheological properties of milk samples fermented by *S. thermophilus* strains St-143 and ST-10255y are shown in Figure 2a. The gelation point (i.e., $G' > 1$ Pa) was similar (~152 min) for milk fermented by

Figure 2. (A) Rheological properties of acid milk gels, and (B) exopolysaccharide (EPS) production during fermentation of milk at 40°C by *Streptococcus thermophilus* strains St-143 (○), and ST-10255y (△). (A) Dark gray symbols indicate storage modulus (G'), and white symbols indicate loss tangent (LT); (B) error bars indicate standard error of means.

Table 2. Summary of rheological properties of acid gels, and yield and properties of exopolysaccharides (EPS) isolated from milk fermented by *Streptococcus thermophilus* strains at 40°C

Parameter ¹	<i>S. thermophilus</i> strains ²	
	St-143	ST-10255y
Gelation time (min)	152 ± 2 ^a	153 ± 2 ^a
pH at gelation	5.26 ± 0.02 ^a	5.25 ± 0.03 ^a
LT _{Max}	0.498 ± 0.003 ^a	0.456 ± 0.001 ^b
pH at LT _{Max}	4.9 ± 0.03 ^a	4.7 ± 0.02 ^b
G' at pH 4.6 (Pa)	26.1 ± 1.7 ^b	82.1 ± 4.9 ^a
Time to reach pH 4.5 (min)	270 ^a	270 ^a
Max EPS yield ³ (mg/L)	71.3 ± 2.3 ^a	55.8 ± 2.8 ^b
Molar mass of EPS ³ (10 ⁶ g/mol)	1.57 ± 0.27 ^a	0.78 ± 0.00 ^b
R _g of EPS ³ (nm)	54.0 ± 0.1 ^a	42.1 ± 0.1 ^b
PD ratio of EPS ³	1.15 ± 0.03 ^a	1.01 ± 0.00 ^b

^{a,b}Different superscripts in the same row indicate significant difference ($P < 0.05$).

¹LT_{Max} = maximum loss tangent; G' = storage modulus; R_g = radius of gyration; PD = polydispersity.

²Values are means ± SEM for n = 3 for rheological properties, and n = 6 for Max EPS yield.

³EPS isolated from milk samples fermented to pH 4.5.

both strains St-143 and ST-10255y (Table 2). The pH of gelation for both strains was pH ~5.25 (Table 2). The G' profile for strain St-143 exhibited an initial increase after gelation, but the G' values then decreased around pH 4.9 before increasing again at pH values ≤ 4.8 (Figure 2a). The decrease in G' values corresponded to the pH (pH ~4.9) at which the maximum loss tangent (LT_{Max}) values (0.498) of the gel was also observed (Figure 2a). The G' profile of milk fermented by ST-10255y exhibited a shoulder (flattening of the G' profile) between pH 4.9 and 4.6, while at lower pH values (≤4.6), it increased sharply (Figure 2a). The LT_{Max} (0.456) was also observed to occur at a lower pH values (pH 4.7) for the gels made with ST-10255y (Figure 2a, Table 2). For strains St-143 and ST-10255y, the G' values of milk gels at pH 4.6 were 26 and 82 Pa (Table 2), reaching 35 and 145 Pa, respectively, when pH was 4.5.

Molar Mass of EPS During the Fermentation Period

During fermentation, EPS was isolated without precipitation by ethanol, and the molar mass of EPS was determined in ultrafiltration extracts. Peaks referring to EPS were selected by examining both RI and UV signals. Peaks with high UV signals were considered likely because of high levels of protein contamination and were ignored. The cumulative weight fraction and molar mass plots of the EPS indicated a decrease in the molar mass values of both EPS types during fermentation (Figure 3). For the EPS isolated from strain St-143, the average molar mass of the EPS obtained at pH 5.2 during fermentation was 2.9×10^6 g/mol, which then gradually decreased to 1.6×10^6 g/mol in the sample isolated at pH 4.5 (Figure 4). A significant de-

crease was also observed in the molar mass of EPS during fermentation by strain ST-10255y. The R_g values, which reflect the molecular size of the polysaccharides (Tuinier et al., 1999), was 56 to 42 nm for EPS from St-143 and ST-10255y strains, respectively (Table 2). Fermentation pH had no significant effect on these R_g

Figure 3. Cumulative molar mass profile of exopolysaccharides obtained from *Streptococcus thermophilus* St-143 (A) and ST-10255y (B) at different pH values during fermentation of milk at 40°C, obtained by size exclusion chromatography multi-angle laser light scattering (SEC-MALLS). Solid lines = pH 5.2; long dashed lines = pH 4.9; short dashed lines = pH 4.7; dotted lines = pH 4.5.

values (results not shown). The polydispersity values of the EPS, calculated as the ratio of weight-average molecular weight to the number-average molecular weight, for all the EPS samples were 1.1 to 1.2 (result not shown), indicating that these isolated molecules were fairly monodisperse.

DISCUSSION

Streptococcus thermophilus strains started producing EPS when pH values reached ~5.3 (close to gelation) (Figure 2b). Thereafter, the amount of EPS increased until the end of fermentation (pH 4.5). Most of the past studies using milk were focused on determining the final yield of EPS at the end of fermentation, so it was not clear from them when *S. thermophilus* strains produced EPS. When milk was fermented at constant pH of 5.5, EPS production started after ~4 h of fermentation with *S. thermophilus* LY03, suggesting that EPS production was faster when fermentation was carried out at pH values close to the gelation point of milk (De Vuyst et al., 1998). In case of milk fermented without pH control, Frengova et al. (2000) reported for pure or mixed yogurt cultures of *S. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* that the production of EPS began within 1 h of the start of fermentation. These reports suggest that EPS synthesis does not start immediately at the initial phase (lag phase) of fermentation. It appears that EPS production may occur toward the exponential phase of growth. After 90 to 150 min, the rate of pH decrease (related to bacterial

growth rate) was the highest until milk fermentation reached pH ~5.3. At that point, the actively growing bacterial cells were likely to sense stress due to the decrease in pH, which possibly helped to promote EPS synthesis. Biosynthesis of cell wall polymers and EPS by LAB needs nucleotide sugars as precursors (De Vuyst and Degeest, 1999; Laws et al., 2001). Because active cell growth needs more energy at an early stage of fermentation, metabolism may be directed toward glycolysis, with the available nucleotide sugars mostly used for biosynthesis of cell wall polysaccharides (De Vuyst et al., 1998). Toward the end of active growth phase (near pH 5.3 during fermentation of milk), it is possible that cell division slows down, energy requirements decline, biosynthesis of cell wall polysaccharides reduces, and more nucleotide sugars are available for EPS biosynthesis (De Vuyst et al., 1998).

The yield of EPS during fermentation of milk by *S. thermophilus* St-143 and ST-10255 increased during fermentation. The final yields of EPS for both strains were in agreement with the previously reported EPS yields for these strains. In past studies, EPS-producing *S. thermophilus* have been investigated for their final EPS yields in different types of media, including milk (Giraffa and Bergère, 1987; Cerning et al., 1988; Faber et al., 1998; De Vuyst et al., 2003; Vaningelgem et al., 2004; Mende et al., 2012; Pachekrepapol et al., 2017). Depending on the strains and fermentation media and conditions, reported yields of EPS produced by the *S. thermophilus* strains ranged from 8 mg/L (Pachekrepapol et al., 2017) to 290 mg/L (Vaningelgem et al., 2004). In most of the past studies, in which milk-based media were fermented at temperatures in the range of 37 to 42°C for extended periods, the final yield of EPS was relatively high (>100 mg/L) (De Vuyst et al., 2003; Vaningelgem et al., 2004), whereas, for relatively shorter fermentation periods, the final yield of EPS was less than 100 mg/L (Mende et al., 2012; Pachekrepapol et al., 2017). The yields of EPS from *S. thermophilus* St-143 and ST-10255y were reported to be 65 and 27 mg GE/kg, respectively, when isolated by ethanol precipitation of whey obtained from fermented milk (Pachekrepapol et al., 2017). These results indicated that the yield of EPS is higher when milk is fermented for an extended period of time. Studies have also highlighted that EPS biosynthesis is positively associated with bacterial growth (De Vuyst et al., 1998; Degeest et al., 2001). For example, EPS yield coefficients, calculated as grams of EPS produced per gram of lactose present in the medium, was maximal when cultures attained maximum specific growth rate, indicating that EPS synthesis was highly associated with bacterial growth rate (De Vuyst et al., 1998). Increased yields of

Figure 4. Molar mass of exopolysaccharides obtained from *Streptococcus thermophilus* strains St-143 (dark gray bars) and ST-10255y (light gray bars) at different pH values during fermentation of milk at 40°C. Error bars indicate standard error of means.

EPS parallel to growth rate, fermentation duration, or both have also been reported in other LAB such as *L. delbrueckii* ssp. *bulgaricus* (Grobben et al., 1995) and *Lactobacillus rhamnosus* (Pham et al., 2000). Increased yields of EPS during the fermentation period of ~5 h in our study are consistent with the trend for reported growth-dependent EPS production in *S. thermophilus* as well as other LAB.

The amount of EPS isolated from a medium depends on the complexity of the medium and the method used for isolation. Methods based on ultrafiltration (Goh et al., 2005), TCA precipitation of protein and subsequent EPS precipitation by ethanol or acetone (Cerning et al., 1994), and enzymatic digestion and TCA precipitation of protein (Kimmel et al., 1998) have all been used for isolation of EPS from milk. The presence of protein (~3.5%) in milk makes it a more complex medium for EPS isolation; however, the quantity of EPS synthesized by *S. thermophilus* in fermented milk is very low (<300 mg/L) (De Vuyst et al., 2003; Mende et al., 2012) in comparison with the higher concentration (>45,000 mg/L) of the native milk carbohydrate (lactose) (Fox, 2009). Therefore, an ideal method of EPS isolation should be efficient to prevent excessive loss of EPS during the isolation process and to effectively remove small sugars such as lactose, glucose, and galactose (Rimada and Abraham, 2003). The method for isolation of EPS used in this study had a recovery efficiency of 44 to 61%, depending on the concentration of added dextran (a model EPS) in milk (Table 1), which was higher than reported past results. Goh et al. (2005) reported 27 to 30% recovery of dextran by ethanol precipitation from milk permeate. Methods that solubilize EPS at an early stage of isolation process by heating of milk, avoid TCA precipitation of protein, and use multiple steps of ethanol precipitation resulted in increased EPS recovery (Rimada and Abraham, 2003). Heating of milk before protein removal and multiple ethanol precipitation steps might have contributed to the higher (~50%) recovery of added EPS in this study.

The molar mass of EPS significantly decreased during fermentation as pH decreased from 5.2 to 4.5 (Figure 4). No previous published reports exist (that we are aware of) on the molar mass of EPS isolated at various time points during fermentation of milk. However, the results for the overall range of molar mass obtained for EPS in this study are in general agreement with previous studies. Past studies showed that the molar mass of EPS produced by *S. thermophilus* ranged from 1×10^6 to 5×10^6 g/mol. One of the earliest report on molar mass of isolated EPS samples from *S. thermophilus* strains CNCMI 733, 734, and 735 was about 1.0×10^6 g/mol (Doco et al., 1990). The molar mass of EPS

synthesized by *S. thermophilus* strains SFi39 and SFi12 in milk medium was higher than 2×10^6 g/mol when analyzed by gel filtration chromatography (Lemoine et al., 1997). Milk fermented by 2 EPS-producing *S. thermophilus* strains (Rs and Sts) yielded EPS of molar mass that ranged from 2.6×10^6 to 3.7×10^6 g/mol when analyzed by permeation chromatography combined with static light scattering and RI (Faber et al., 1998). Considerable variation has also been reported in the molar mass of EPS isolated from the same strain. For example, Mende et al. (2012) reported that the molar mass of EPS from *S. thermophilus* strain St-143 was 4.3×10^6 g/mol when analyzed by gel filtration chromatography, whereas molar mass of EPS from the same strain was reported as 1.4×10^6 g/mol when analyzed by SEC-MALLS (Pachekrepapol et al., 2017).

Notably, in most of those studies, EPS was isolated from milk after the fermentation was ended. Information on the molar mass of EPS samples isolated from milk at intermediate fermentation periods is not available. However, some indications exist that EPS molecules may undergo some enzymatic hydrolysis if the EPS remain in the fermentation medium for an extended period of time. Cerning et al. (1988) discussed the possibility of the production of exocellular glycohydrolases during fermentation of milk by *S. thermophilus* that may be activated during fermentation, which would result in the hydrolysis of EPS. A decrease in the molar mass of EPS from *S. thermophilus* (BCRC 14085) during the pH controlled fermentation (pH 5.0) of skim milk for >12 h also indicated the possibility of enzymatic degradation of EPS (Lin and Chien, 2007). Frengova et al. (2000) also reported a reduction in the quantity of EPS from *S. thermophilus* during refrigerated storage of fermented milk, possibly due to hydrolysis of EPS by glycohydrolase enzymes. The presence of EPS-hydrolyzing glycohydrolase enzymes α -glucosidase, β -glucuronidase, β -D-glucosidase, α -D-galactosidase, β -D-galactosidase, and α -L-rhamnosidase in the cell extract of *L. rhamnosus* were also reported by Pham et al. (2000). In our study, some partial hydrolysis of EPS during fermentation might possibly have occurred causing the decrease in molar masses. Another likely possibility is that the EPS isolated at different pH values during fermentation could have varying levels of contamination by protein, which might have affected the estimated molar mass of the isolated EPS.

Milk fermented by both strains gelled when the pH of milk was ~5.25 (Table 2). The pH of gelation was slightly higher than previous results for these *S. thermophilus* strains (pH 5.1–5.2) reported by Mende et al. (2012). Small variations in the gelation pH of milk during fermentation can be caused by differences in the

extent of whey protein denaturation during preheating of milk as well as the level of starter inoculation (Lucey et al., 1997; Lee and Lucey, 2004). Severe heating of milk under conditions that cause denaturation of most whey proteins, particularly β -lactoglobulin, helps milk to gel at pH values close to the isoelectric pH (pH $\sim$ 5.3) of β -lactoglobulin (Lucey and Singh, 2003).

Milk gelled by *S. thermophilus* strain St-143 showed a significant decrease in the G' curve between pH values 5.1 and 4.9, whereas the gel fermented by *S. thermophilus* strain ST-10255y showed only a shoulder or flattening in the G' curve between pH 5.0 and 4.7 (Figure 2a). An increase in G' values after gelation and a slight shoulder in the G' curve, before increasing again at low pH values is a typical rheological pattern of fermented milk gels made from heated milk (Lucey et al., 1998b; Lucey, 2002; Peng et al., 2009). When milk pH reaches $\sim$ 5.3, casein particles bridged by denatured whey proteins initiate gelation. In the pH region from 5.0 to 5.2, gel stiffness may decrease mainly because colloidal calcium phosphate (CCP) solubilizes. The CCP nanoclusters are important internal crosslinking material within the casein particles. A decrease in casein particles' internal crosslinking can lead to a decrease in gel stiffness (Lucey, 2002; Lucey and Singh, 2003). Gel stiffness increases again at lower pH values approaching the isoelectric point of casein, leading to stronger casein-casein interactions (Lucey, 2002; Lucey and Singh, 2003). With the solubilization of CCP and the weakening of initial (weak) gel network, the LT values increase to reach a maximum near pH 5.0 (Lucey, 2002). Higher LT_{Max} values indicate greater bond mobility in the gel network that could promote more bond relaxation and greater network rearrangements (Lucey et al., 1997; Lucey and Singh, 2003). The gel formed by strain St-143 had a higher LT_{Max} value compared with gel formed by strain ST-10255y. A higher LT_{Max} value for the gel made by strain St-143 could have resulted in more extensive rearrangement of the network, contributing to a decrease in G' values in that pH region. Because the LT_{Max} occurred at a higher pH value (pH 4.9) in gels made with St-143 (Table 2), these newly formed gels were possibly more prone to extensive rearrangements that ultimately resulted in a sharper decrease in the G' curve compared with the gels made with ST-10255y, for which the LT_{Max} occurred at pH 4.7 (Figure 2a).

Because the milk composition, heat treatment, fermentation conditions, and gelation pH values were all identical in these samples, the observed differences in rheological properties during the formation of gels made by strains St-143 and ST-10255y were likely related to the properties of EPS produced by these 2 strains. Polysaccharides of higher molar mass and size are less likely to be compatible with proteins and may promote

greater depletion flocculation type effects (de Kruif and Tuinier, 2001; Corredig et al., 2011; Pachekrepapol et al., 2014). The molar mass of EPS produced by strain St-143 was almost double the molar mass of EPS produced by ST-10255y, and the R_g value was also larger (Table 2). It is unclear why the gels made with St-143 had a higher value of LT_{Max} that also occurred at a higher pH. Because the EPS concentration was also significantly higher for strain St-143 in the pH region where the LT_{Max} was observed, we speculate that the EPS produced by strain St-143 could have promoted more incompatibility with the protein network, which could also have contributed to the observed decrease in G' values (Figure 2a).

Folkenberg (2005) studied the microstructures of acid gels and reported that the EPS from strain St-143 was likely incompatible with the protein in the gel network because EPS was mostly observed in the pores (serum phase) of the network. On the other hand, they considered that the EPS from strain ST-10255y was co-located in the network. The EPS produced by strains St-143 and ST-10255y are also different in their structure. The repeating unit of EPS produced by ST-10255y had 4 sugars (2 β -galactose, 1 α -glucose, and 1 N-acetylgalactosamine), 3 in a linear chain and 1 branched. Sugars in the linear chain were connected by β (1 $\rightarrow$ 3) linkages. On the other hand, the EPS from strain St-143 had 6 sugars in its repeating units, mainly joined by β (1 $\rightarrow$ 4) linkages (Pachekrepapol et al., 2017). The role of types of glycosidic linkages on the physical properties of EPS is not well understood. However, it has previously been suggested that carbohydrate polymers with β (1 $\rightarrow$ 4) linked structures were stiffer than with β (1 $\rightarrow$ 3) linked structures (Welman and Maddox, 2003). Less flexible β (1 $\rightarrow$ 4) EPS polymers in St-143 samples may have possibly caused more localized phase separation in the weak initial protein network.

CONCLUSIONS

The modified Enikeev method for EPS determination in fermented milk that was used in this study could be completed in less than 1 d and was able to recover $\sim$ 60% of added EPS. The 2 strains of *S. thermophilus* studied produced a significant amount of EPS only after $\sim$ 150 min of fermentation, which was around the time when milk started to gel. When fermentation had progressed further, the quantities of EPS increased, but the molar mass of EPS slightly decreased. The strain St-143 produced higher concentrations of EPS that had higher molar mass, larger R_g values, and longer repeating unit structures, which resulted in weaker acid gels in comparison with the gels made by strain ST-10255y.

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