Production of an Exopolysaccharide-Containing Whey Protein Concentrate by Fermentation of Whey

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ABSTRACT

Using whey as a fermentation medium presents the opportunity to create value-added products. Conditions were developed to partially hydrolyze whey proteins and then ferment partially hydrolyzed whey with *Lactobacillus delbrueckii* ssp. *bulgaricus* RR (RR; an EPS-producing bacterium). In preliminary experiments, pasteurized Cheddar cheese whey was treated with Flavourzyme to partially hydrolyze the protein (2 to 13% hydrolyzed). Fermentation (2 L, 38°C, pH 5.0) with RR resulted in EPS levels ranging from 95 to 110 mg of EPS per liter of hydrolyzed whey. There were no significant differences in the amount of EPS produced during fermentations of whey hydrolyzed to varying degrees. Since a high level of hydrolysis was not necessary for increased EPS production, a low level of hydrolysis (2 to 4%) was selected for future work. In scale up experiments, whey was separated and pasteurized, then treated with Flavourzyme to hydrolyze 2 to 4% of the protein. Following protease inactivation, 60 L of partially hydrolyzed whey was fermented at 38°C and pH 5.0. After fermentation, the broth was pasteurized, and bacterial cells were removed using a Sharples continuous centrifuge. The whey was then ultrafiltered and diafiltered to remove lactose and salts, freeze-dried, and milled to a powder. Unfermented hydrolyzed and unhydrolyzed whey controls were processed in the same manner. The EPS-WPC ingredients contained approximately 72% protein and 6% EPS, but they exhibited low protein solubility (65%, pH 7.0; 58%, pH 3.0).

(Key words: exopolysaccharide, whey protein concentrate, *Lactobacillus delbrueckii* ssp. *bulgaricus* RR)

Abbreviation key: BOD = biological oxygen demand, DH = degree of hydrolysis, EPS = exopolysaccharide, LAB = lactic acid bacteria, MWCO = molecular weight cut-off, RR = *Lactobacillus delbrueckii* ssp. *bulgaricus* RR, WPC = whey protein concentrate.

INTRODUCTION

Whey comprises 80 to 90% of the volume of milk entering the cheesemaking process and contains about 50% of the solids present in the original whole milk, including 20% of the protein and most of the lactose, minerals, and water-soluble vitamins (Marshall, 1982). Annually, 33 million metric tons of whey is produced in the United States (ADPI, 1999) and 110 million metric tons is produced worldwide through cheese manufacture (IDFA, 1999). However, only about 55% of the whey produced is further processed in the United States (ADPI, 1999). Disposal of whey is difficult and costly because of its high biological oxygen demand (BOD) (Marshall, 1982). New high value-added products and technologies are crucial for the dairy industry to decrease the expenses of waste disposal (Yang and Silva, 1995).

One approach to transform whey and lactose into value-added products is to use the whey as a fermentation feedstock. According to Yang and Silva (1995), fermentation should be used as a means of modifying the functional properties of the whey to add value. Use of whey or whey permeate as a fermentation medium reduces the lactose content, resulting in a decrease in the BOD and associated disposal costs (Kilara and Patel, 1992).

Some strains of lactic acid bacteria (LAB) possess the ability to produce exopolysaccharides (EPS). EPS refers to all forms of bacterial polysaccharide, both slime and capsule, found outside of the cell wall (Sutherland, 1972). EPS produced by lactobacilli are typically neutral (noncharged) heteropolysaccharides. Those EPS produced by dairy LAB that are food grade may lend several important functional properties to food systems. Schellhaass and Morris (1985) observed decreased gel strength and syneresis and increased viscosity at higher shear rates when skim milk gels were fermented with ropy strains (including *Lactobacillus delbrueckii* ssp. *bulgaricus* RR; RR) compared with other nonropy strains. Hess et al. (1997) demonstrated that yogurts made with EPS-producing strains of *L. delbrueckii* ssp. *bulgaricus* were less susceptible to syn-

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eresis, required less force to penetrate the gel, and were more extensible than yogurts made with nonropy strains. In a fermented food system like yogurt, EPS appears to interact with the surface of bacterial cells and is associated with the protein matrix, affecting the viscosity and stability of the milk gel (Schellhaass and Morris, 1985).

Ludbrook et al. (1997) isolated EPS produced through fermentations of skim milk supplemented with yeast extract, peptone, and glucose by several strains of LAB. The viscosity of solutions of purified EPS (0.2% w/v) in water did not exhibit thickening properties. However, when EPS (0.2% w/v) was combined in a solution with whey protein concentrate (WPC) (0.2% w/v), viscosity increases were observed, which suggests potential interactions between the two components. Tuinier et al. (2000) examined the interactions between aggregated whey proteins and an EPS produced by *Lactococcus lactis* ssp. *cremoris* B40 and described the concentration-dependent depletion-induced phase separation behavior of these polymers. It is important to note the polymer produced by B40 has a negative charge. The EPS produced by RR is not charged (Gruter, 1993).

Whey is not always an economical or feasible industrial fermentation substrate. Whey and whey permeate lack sufficient low molecular weight nitrogen, which presents a challenge to the growth of many industrial microorganisms, so they often require supplementation (Amrane and Prigent, 1993). *L. delbrueckii* ssp. *bulgaricus* is typically weakly proteolytic and grows poorly in unsupplemented media. While whey or whey permeate is often supplemented with yeast extract for fermentation in a research setting (Roy et al., 1986; Christopherson and Zottola, 1989; Ludbrook et al., 1997; Ricciardi et al., 1997), this represents an increased cost that is generally not feasible on an industrial scale (Tejayadi and Cheryan, 1988). An alternative to supplementation of the whey is partial hydrolysis of the native protein.

Leh and Charles (1989) fermented protein-hydrolyzed whey with *L. bulgaricus*. A mixture of whey hydrolyzed with a bacterial endoprotease and whey permeate (25:75) stimulated growth of the microorganism. Rates of cell mass and acid production were greater when the hydrolyzed whey-permeate mixture was fermented compared with fermenting the hydrolyzate alone (Leh and Charles, 1989).

While unsupplemented whey has not been used as a medium for *Lactobacillus delbrueckii* ssp. *bulgaricus* RR, the research of Leh and Charles (1989) with protein-hydrolyzed whey demonstrates potential for the growth and EPS production of this organism. If *Lactobacillus delbrueckii* ssp. *bulgaricus* RR can be grown in protein-hydrolyzed whey and produce EPS, then whey-derived ingredients (such as whey protein concentrates)

containing EPS could be produced. Based on the rheological characteristics this EPS exhibits in dairy-based systems, it is possible that whey-derived ingredients with modified or enhanced functional properties could be produced. This would enable utilization of a nutrient-rich waste stream while generating increased opportunities for use of whey-derived ingredients.

The objectives of the present research were to determine the level of protein hydrolysis needed to support growth and EPS production by RR in whey and to manufacture a fermented whey protein concentrate containing EPS from *Lactobacillus delbrueckii* ssp. *bulgaricus* RR.

MATERIALS AND METHODS

Bacterial Strain Maintenance and Culture Preparation

Lactobacillus delbrueckii ssp. *bulgaricus* RR, an EPS-producing strain, was originally obtained from the laboratory of H. A. Morris (University of Minnesota, St. Paul, MN) and maintained in MRS broth (Difco, Detroit, MI). Stock cultures were prepared by mixing a pure culture grown in MRS broth for 14 to 15 h at 42°C with an equal volume of 20% sterile glycerol. This mixture was dispensed into sterile cryogenic vials (Nalgene, Rochester, NY) and stored at -70°C. Working cultures were prepared by transferring the culture to fresh MRS media and incubating at 42°C (16 to 24 h). Culture was transferred three times from the frozen stock prior to inoculation. Fermentation inoculum was prepared by transferring a 0.2-ml aliquot of turbid culture into 100 ml of MRS broth and incubating at 42°C for 12 h, then centrifuging at 8000 × *g* and 4°C for 10 min to pellet the cells. Cells were washed twice with 100 ml of sterile phosphate buffer (pH 7.0) to remove residual medium components, then repelleted under the above centrifugation conditions and resuspended in 20 ml of sterile phosphate buffer. A 10-ml aliquot of the washed cell suspension was used to inoculate 2.0-L volumes of fermentation medium.

Culture preparation for scale up fermentations. For scale up fermentations, culture was prepared by transferring a 3-ml aliquot of turbid culture to 1.5 L of MRS and incubating at 42°C until an OD₆₀₀ of 0.900 (mid-late log phase) was achieved. This culture was transferred to the 80-L fermentation vessel using a peristaltic pump.

Preparation of Fermentation Media

Cheddar cheese whey obtained from the Pennsylvania State University Creamery (University Park, PA) was strained into sanitized milk cans using cheesecloth

bags to remove cheese fines and then separated using a pilot plant-scale Westfalia cream separator to remove fat. Clarified whey was pasteurized (74°C, 15 s) using an APV pilot plant-scale high-temperature short-time pasteurizer to destroy the starter culture. The pasteurized whey exited the pasteurizer at 7°C. A portion of the whey (2 L) was heated to 79°C for 6 min, then quickly cooled prior to being frozen until fermentation (unhydrolyzed control). The remaining whey was heated to 50°C and the pH of the whey was adjusted with 5 N NaOH to 6.0, the pH of maximum activity for the enzyme. Flavourzyme 1000 L (Novo Nordisk Biochem, Franklinton, NC), a protease/peptidase complex, was added at a concentration of 60 μ L per liter of whey. Samples were taken immediately after addition of the enzyme and every 3 to 6 min to determine the freezing point, which was measured using an Advanced 4D3 Cryoscope (Advanced Instruments, Norwood, MA). Freezing point depression was used to monitor the progress of the reaction and degree of protein hydrolysis (%DH) was calculated as the following:

$$\%DH = \left(\frac{\Delta C}{S\% \times f_{osm}} \right) \times \left(\frac{1}{\omega} \right) \times \left(\frac{1}{h_{tot}} \right) \times 100$$

where C is the increase in osmolality (milli-osmol); $S\% \times f_{osm}$ is the protein concentration (grams of protein per kilogram of water); $1/\omega$ is the calibration factor for the osmometer, representing the reciprocal of the osmotic coefficient of the peptides, which is assumed to be a constant value of 1.04; and h_{tot} is the total number of peptide bonds in the protein (8.8 milliequivalents per gram of whey protein) (Adler-Nissen, 1986).

The freezing point was determined immediately after enzyme addition and periodically to monitor %DH. At various levels of hydrolysis, a 2-L portion of the whey was separated and the enzyme was inactivated (79°C, 6 min) prior to being frozen and stored until fermentation.

Small-Scale Fermentation of Whey

Fermentations of whey and hydrolyzed whey were conducted in a 2.5-L working volume BioFlo III (New Brunswick Scientific, Edison, NJ) fermentation vessel. The whey was thawed, quickly heated in a sterile 4-L flask to 80°C, then immediately cooled to $\leq 40^\circ\text{C}$ to dissolve any lactose that may have crystallized during frozen storage (Holsinger, 1988). Whey was transferred to the autoclaved vessel and adjusted to the desired fermentation temperature (38.0°C). The pH was measured with a calibrated F-615 Fermprobe (Broadley-James, Irvine, CA). The pH was allowed to decline to the setpoint (pH 5.00) due to lactic acid production,

then was maintained at the setpoint throughout fermentation by automatic addition of 5 N NaOH. Sterile Antifoam 289 (Sigma Chemical Co., St. Louis, MO) was added at a level of 150 μ L per liter of whey to control foam development. To minimize the oxygen content of the fermentation vessel, the medium was sparged with sterile filtered (0.2- μ m filter) nitrogen gas for 20 min at a rate of 1 L/min prior to introduction of inoculum. After inoculation, the medium was maintained at the desired conditions (200 rpm agitation, pH 5.0, 38.0°C) described by Kimmel et al. (1998). Fermentations were conducted in duplicate.

Immediately following inoculation, samples were taken for quantification of lactose, lactic acid, cell growth, viable cell numbers, and EPS. Lactose consumption and lactic acid production were monitored using high performance liquid chromatography. Fermentations were terminated when the change in lactose content was $\leq 0.03\%$ (0.3 g/L) per hour for 2 consecutive h after log phase or when the lactose content decreased by 90%. Immediately after the fermentation was terminated, samples were taken for quantification of EPS, cell dry weight, cell numbers, lactose and lactic acid (as below).

Whey Preparation for Scale Up

Whey (820 L) was collected, strained, clarified, and pasteurized as described above. The whey was separated into three streams. The first portion (~15 L) of the whey (unhydrolyzed control) was heated to 79°C for 6 min, and then cooled in an ice bath under constant agitation. Heating to 79°C required less than 4 min, and products were cooled to $< 45^\circ\text{C}$ within 7 min. The remaining whey was divided into 15-L aliquots in sanitized milk cans and heated to 50°C. Flavourzyme 1000 L was added (60 μ L per L of whey) and hydrolysis was monitored as described above. When a 2 to 4% DH was achieved, the milk can was transferred to a boiling water bath and heated as described above. These 15-L volumes of hydrolyzed whey were mixed to create a homogeneous sample. Degree of hydrolysis was reported as the average of these batches. Approximately 25 L of hydrolyzed whey was separated (hydrolyzed control), while 60 L of the hydrolyzed whey was fermented.

Scale Up Fermentations

Fermentations of the 2 to 4% DH-hydrolyzed whey were conducted in an 80-L Mobile Pilot Plant (New Brunswick Scientific) fermentor. The fermentor was steam-sterilized and flushed with nitrogen gas to evacuate the vessel. Whey (60 liters) was pumped from milk

cans into the sterile vessel with a peristaltic pump and was blanketed with nitrogen gas (~5 psi) for the duration of the fermentation to insure anaerobiosis. The whey was heated to 38°C and was adjusted to pH 5.0 prior to inoculation with concentrated HCl.

Agitation was maintained at 100 rpm and the pH was maintained at the setpoint (5.00) through the automatic addition of 15 N NaOH. Samples of the uninoculated, inoculated, and fermented medium were taken to quantify lactose and lactic acid, viable cell numbers and cell dry weight, EPS, total solids, and crude protein. Fermentations were terminated when 90% of the original lactose had been utilized, or after 24 to 28 h, which was the typical time for completion of the small-scale fermentations. Once the fermentation was complete, the spent whey was collected into sanitized milk cans and immediately processed to recover whey protein concentrate.

Processing and Recovery of Whey Protein Concentrates

Whey, hydrolyzed whey, and fermented hydrolyzed whey were pasteurized (74°C, 15 s) to destroy the EPS-producing strain, collected in sanitized milk cans, pumped to a Sharples T-1-P continuous flow Super-Centrifuge (Pennsalt Chemicals, Warminster, PA), and centrifuged at about 40,000 rpm to remove cells. Ultrafiltration and diafiltration with water was performed using a polyethersulfone membrane (MWCO 10,000; Membrane Systems Specialists, Wisconsin Rapids, WI). Diafiltration was performed until the low molecular weight carbohydrate level was less than 0.05% (as determined by the phenol-sulfuric acid method), then the retentate was concentrated to a small volume. Retentate was frozen in trays, freeze-dried, then milled using a Microjet-10J (Quartztech, New York). The resulting whey protein concentrates were stored in double-lined Whirl-pak bags (Nasco, Fort Atkinson, WI) in a freezer (-20°C) until analysis. Prior to weighing a sample for analysis, sample bags were removed from frozen storage and allowed to warm up to room temperature.

Fermentation Analyses and Characterization of Whey Protein Concentrates

Carbohydrate Analysis by HPLC. Lactose and lactic acid were quantified by HPLC as described by Kimmel et al. (1998). Whey samples were treated with 80% (w/v) trichloroacetic acid and centrifuged to remove protein, then diluted with 0.005 M sulfuric acid prior to injection. Lactose, glucose, galactose, and lactic acid were quantified by relating peak area to standard curves.

Measurement of Cell Growth. Appropriate dilutions were plated onto MRS agar (1.5% agar), which was incubated anaerobically in Anaerobic Gas Pak jars (Becton Dickinson, Sparks, MD) at 42°C for 48 h. Cell dry weight was determined in triplicate. A 10-ml sample of fermentation broth was centrifuged ($8000 \times g$) for 10 min and washed twice with 10 ml of distilled water. The pellet was resuspended in 5 ml of distilled water, then transferred to a pre-dried, pre-weighed disposable aluminum weighing dish and dried at 95°C in a convection oven until constant weight was attained. A sample of uninoculated media was prepared in the same manner, and the resulting mass of precipitated protein was subtracted from samples of inoculated fermentation broth.

Quantification of EPS. The procedure used to quantify EPS was based on that described by Gancel and Novel (1994) and modified by Kimmel et al. (1998). Samples were processed in triplicate. Briefly, an accurately-weighed sample of approximately 10 grams of culture medium was heated for 10 min in a boiling water bath. The samples were cooled and proteins were digested with a solution of pronase (Sigma Chemical Co.) and then precipitated with trichloroacetic acid. Cells and protein were removed by centrifugation ($8000 \times g$) for 20 min. Following dialysis (molecular weight cut-off, MWCO, 6000 to 8000) of the supernatant against at least four changes of distilled water for 48 h, the carbohydrate concentration of the retentate was determined in duplicate using the phenol-sulfuric acid method (DuBois et al., 1956). The carbohydrate assay was calibrated by using a mixture of D-galactose, D-glucose, and L-rhamnose (5:1:1) (Gruter et al., 1993). Results were calculated by subtracting the "EPS content" (phenol-sulfuric acid-reacting substances) of the uninoculated media from the final value of the fermentation broth and were reported as mg of carbohydrate per liter.

Compositional analyses. Nitrogen content of the samples was determined by combustion in a nitrogen analyzer (Leco, St. Joseph, MI) according to manufacturer's instructions. A Kjeldahl factor of 6.38 was used to calculate crude protein. Moisture and ash were quantified for each WPC sample according to AOAC methods (1995). Samples were dried to a constant weight overnight in a vacuum oven (65°C) to determine moisture content, and the solids were then incinerated in a Muffle furnace at 550°C for about 6 h to determine ash content. Crude fat was quantified according to the method of Bradley et al. (1993) and AOAC (1995), with the omission of the heating step following addition of ammonium hydroxide. The solvents were completely evaporated using a Soxtec Extraction Unit (Foss Tecator, Eden Prairie, MN) and the recovered fat was dried to constant

weight in an oven at $\sim 102^{\circ}\text{C}$. Results were reported as percentage of fat after subtracting the mean value for blank analyses (10 ml distilled water) from each sample extraction. Total carbohydrate content of the whey protein concentrates was defined as the difference between the mass of the sample and the amount of moisture, lipid, protein, and ash. All compositional analyses were determined in triplicate.

To determine EPS content, 2.5 grams of WPC was placed into a small beaker and rubbed into a smooth paste with 4 ml of distilled water and a glass stirring rod. About 12 ml of additional water was added and the sample was gently stirred for 30 min. The sample was quantitatively transferred to a 25-ml volumetric flask and brought to volume with distilled water. Two 10-ml volumes were taken from the solution, and the EPS analysis was performed as described above. The carbohydrate content of the dialysate, as determined by the phenol-sulfuric acid method, was measured in duplicate.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the whey protein concentrates was performed according to manufacturer's instructions (Bio-Rad Ready Gels Application Guide) using Tris-HCl gradient gels (4 to 20%; Bio-Rad) at 120 volts (Bio-Rad power supply). Gels were stained with a solution of Coomassie Brilliant Blue R-250 (0.1% in 40% methanol, 10% glacial acetic acid) and destained (40% methanol, 10% glacial acetic acid). β -lactoglobulin and α -lactalbumin (Sigma Chemical Co.) and molecular weight standards (Bio-Rad) were used to identify protein bands. Electrophoresis was also performed on a commercially-available whey protein concentrate. Electrophoresis was performed on denatured (with SDS and β -mercaptoethanol) and native (without SDS and β -mercaptoethanol) proteins. WPC samples were prepared on an equal-protein basis.

Solubility of Protein and EPS. Determination of protein solubility was based on the method of Morr et al. (1985) performed at pH 3.0 and 7.0. Briefly, a sample of WPC containing about 500 mg protein was suspended in a solution of 0.1 M NaCl and adjusted to the desired pH, while intermittently monitoring and maintaining the pH over the course of 1 h. The solution was transferred to a 50-ml volumetric flask, filled to volume with 0.1 M NaCl solution, and then mixed by inverting. The protein and EPS contents of this solution were determined in the same manner as above. Determination of EPS solubility was calculated only for fermented whey protein concentrates. A sample was then centrifuged for 30 min at $20,000 \times g$. Half of the supernatant was analyzed for EPS content. The other half of the supernatant was filtered through a Whatman No. 1 filter and the protein content of the filtrate was determined by

combustion; the procedure was performed in triplicate. Solubility was expressed as the relative amount of protein in the filtrate or EPS in the supernatant compared with the original whey suspension prior to centrifugation. Percentage of soluble protein was also determined for the commercial whey protein concentrate. All solubility determinations were performed in triplicate for each WPC sample. Carbohydrate content as measured by the phenol-sulfuric acid method was determined in duplicate.

Statistical Analysis

Minitab (version 11.21, Minitab Inc., State College, PA) was used to conduct statistical analyses. Analysis of variance was used to test for significant differences among mean values of EPS production and cell dry weight for fermentations of whey hydrolyzed to varying degrees and for scale-up fermentations of protein-hydrolyzed whey. Analysis of variance was also used to test for significant differences among mean values of the composition and functional properties for each WPC—unhydrolyzed, hydrolyzed, and fermented (and commercial). Least significant differences among means were evaluated at $p = 0.05$ using Tukey's procedure for multiple means comparisons.

RESULTS AND DISCUSSION

Correlation of Degree of Hydrolysis and EPS Production

Flavourzyme 1000 L was added (60 μL per liter of whey) and hydrolysis (%DH) was monitored. Batches of whey were removed during hydrolysis and immediately heated (79°C , 6 min) to inactivate the enzyme, creating a series of whey samples with varying levels of protein hydrolysis varying from about 2 to 13% DH. Protein hydrolyzed wheys, as well as whey that had not been hydrolyzed but had received the same thermal treatment, were fermented at 38°C and pH 5.00 under anaerobic conditions. Data from these fermentations appear in Table 1. All fermentations were completed between 22 and 28 h.

Unhydrolyzed whey was the only medium that resulted in a decrease in the number of viable cells at the endpoint of the fermentation (Table 1), and only 0.2 g of cell dry weight per liter of whey was produced, which was statistically less than the cell dry weight increase in the hydrolyzed wheys. The lower lactose consumption, viable cell counts, and net cell dry weight for the unhydrolyzed whey indicated whey was a poor fermentation medium for growth of *Lactobacillus bulgaricus* ssp. *delbrueckii* RR. Hydrolysis resulted in more complete ($>90\%$) lactose utilization and a higher level of lactic

Table 1. Results from fermentations with *Lactobacillus delbrueckii* ssp. *bulgaricus* RR of whey hydrolyzed to varying degrees.¹

% DH ²	Time to endpoint (h)	Lactose utilized (%)	5 N NaOH added (g)	Lactic acid quantified (g/L)	Log CFU per ml increase	Net cell dry weight (g/L)
0.00%	27.7	51.6	38.1	9.8	-0.6	0.2 ^A
2.12%	22.7	94.2	108.3	22.8	1.0	1.4 ^B
6.36%	23.3	95.3	108.7	21.2	1.2	1.3 ^B
7.42%	22.6	92.9	95.6	22.0	1.3	1.1 ^C
9.54%	23.8	92.2	107.2	24.1	1.2	1.5 ^B
12.72%	22.4	96.5	103.3	24.2	1.1	1.5 ^B

^{A,BC}Values in the same column with different capital letter superscripts are statistically different (Tukey's family error $P \leq 0.05$).

¹Two-liter volumes of whey were fermented at pH 5.0 and 38.0°C under anaerobic conditions in a 2.5-working volume fermentation vessel (200 rpm). Fermentations were performed in duplicate.

²%DH, degree of hydrolysis.

acid was produced compared to fermenting unhydrolyzed media (Tukey's family error $P \leq 0.05$).

The amount of EPS contained in each whey-based media is shown in Figure 1. Fermentation of the hydrolyzed whey resulted in a significant increase in EPS production (between 313 and 330 mg EPS per liter of whey) compared with the control, which produced no EPS, indicating protein-hydrolyzed whey is a good medium for EPS production. The levels of EPS production in the hydrolyzed media were similar to those obtained by Toba et al. (1992) through fermentation of skim milk by *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 2483 (150 mg EPS per liter), by Cerning et al. (1986) through fermentation of skim milk with 1% casein by *Lactobacil-*

lus delbrueckii ssp. *bulgaricus* CNRZ 416 (285 mg EPS per liter), and by Kimmel et al. (1998) through optimizing EPS production of *Lactobacillus delbrueckii* ssp. *bulgaricus* RR in a semi-defined medium (354 mg EPS per liter).

No significant differences in EPS production were observed among the whey hydrolysates. Although limited protein hydrolysis resulted in increased EPS production through increased low molecular weight nitrogen, increasing the level of hydrolysis did not result in increased EPS production. These experiments also confirmed that unhydrolyzed or unsupplemented whey do not contain sufficient low molecular weight nitrogen for growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* RR and EPS production. Since extensive hydrolysis was not necessary for increased production of EPS, a low level of hydrolysis was selected for scale up and manufacture of EPS-containing whey protein concentrates (2 to 4% DH) to avoid compromising the functionality of the whey proteins.

Figure 1. EPS Production by *Lactobacillus delbrueckii* ssp. *bulgaricus* RR in whey hydrolyzed to varying degrees. Fermentation conditions are described in Table 1. EPS was quantified by method of Kimmel et al. (1998). Error bars represent $\pm$ one standard deviation about the mean sample measurement. Capital letters indicate statistically different values ($P \leq 0.05$).

Manufacture of Whey Protein Concentrates

Relevant data from fermentations of the 60-L volumes of hydrolyzed whey appears in Table 2. Percentage of hydrolysis (%DH) of the whey ranged from 2.4 to 3.4%. Fermentations were terminated after 24 to 28 h, which was the time range for completion of the 2-L fermentations. All three replicates resulted in an increase in the number of viable cells at the end of the fermentation and an increase in cell dry weight per liter. The amount of NaOH added to maintain pH during the 60-L fermentations (between 7 and 9.5 grams NaOH per L whey) was similar to that required during the 2-L fermentations (9.5 grams NaOH per L whey). Lactose utilization varied from 80 to 100% and EPS levels varied from 210 to 330 mg per liter. Since EPS production has been shown to be associated with growth

Table 2. Results from scale up fermentations of hydrolyzed whey with *Lactobacillus delbrueckii* ssp. *bulgaricus* RR for WPC manufacture.¹

Trial	Percent hydrolysis (%DH) ^{2,3}	EPS Yield (mg/L)	Time ⁴ to endpoint (h)	Lactose utilized (%)	15 N NaOH added (L)	Lactic acid quantified (g/L)	Log CFU per ml increase	Net cell dry weight (g/L)
1	3.2 ^A	205 ^A	24.0	79	0.85	18.4	1.1	1.5 ^A
2	2.4 ^A	329 ^B	23.8	96	0.95	23.3	0.7	1.3 ^B
3	2.7 ^A	213 ^A	27.4	81	0.70	17.6	0.9	1.3 ^B
Mean	2.8	249	25.1	85	0.83	19.8	0.9	1.4

¹Sixty-liter volumes of whey were fermented at pH 5.0, 38°C, and 100 rpm.

²%DH, degree of hydrolysis.

³Values in the same column with different capital letter superscripts are statistically different (Tukey's family error $P \leq 0.05$).

⁴Fermentations were terminated between 24 and 28 h.

in this organism (Kimmel et al., 1998), this was not surprising.

Composition of Whey Protein Concentrates

Mean composition of the recovered whey protein concentrates is presented in Table 3. The experimental WPC ranged in protein content from approximately 72 to 74%. Mean values for percentages of moisture, lipid, and ash were below 7.5% for each treatment. The ash content was higher (5 to 6%) in the experimental samples compared to the commercial WPC (3%). Total carbohydrate, as determined by difference, ranged between 7 and 10%. The fermented WPC contained about 6% EPS ("as is" or "wet" basis), demonstrating it was possible to manufacture a WPC containing microbial exopolysaccharide.

Electrophoresis of Whey Protein Concentrates

Electrophoresis was performed on the commercial WPC sample and all manufactured whey protein con-

centrates. Hydrolyzed whey and fermented hydrolyzed whey appeared similar to the unhydrolyzed whey in the number and molecular weight of the protein bands in the gel. For native polyacrylamide gel electrophoresis, bands were compared to standards of α -lactalbumin and β -lactoglobulin. There were no major differences in the movement of the proteins through either type of gel among the whey, hydrolyzed whey, and fermented hydrolyzed whey samples (data not shown).

The absence of additional bands in the hydrolyzed samples confirms that hydrolysis was not extensive (2 to 4% DH), and most of the primary structure of the proteins remained intact. This also indicates that the enzyme behaved primarily as an exopeptidase, where the initial cleavage of peptide bonds is followed by extensive degradation of intermediates to smaller peptides. If endopeptidase activity had been favored, the enzyme attack would have been more random, and a wide range of peptide intermediates should have been visible on the gels.

Table 3. Composition of whey protein concentrates produced in this study.^{1,2}

Mean (%)	WPC ³	HWPC ³	FHWPC ³	CWPC ^{3,4}
Protein	74.3 ^A	71.7 ^B	72.5 ^{AB}	77.7 ^C
Moisture	7.4 ^A	6.1 ^B	5.7 ^B	4.6
Lipid	5.2 ^A	6.2 ^B	7.4 ^C	6.6
Ash	6.4 ^A	5.9 ^{AB}	5.5 ^B	3.3
Total carbohydrate	6.7	9.9	8.8	7.2
EPS	NA ⁵	NA	6.2	NA

¹Values in the same row with different capital letter superscripts are statistically different (Tukey's family error $P \leq 0.05$). Values reported represent the mean of the three trials.

²Protein: combustion; Moisture: vacuum-drying; Lipid: modified Roese-Gottlieb; Ash: incineration; Total carbohydrate: by difference; EPS: as described by Kimmel et al. (1998). Analyses performed on an as is basis.

³WPC = whey protein concentrate; HWPC = hydrolyzed whey protein concentrate; FHWPC = fermented hydrolyzed whey protein concentrate; CWPC = commercial whey protein concentrate.

⁴Composition (except for protein) for the commercial WPC was obtained by sample analysis by the supplier.

⁵Analysis not performed.

Table 4. Solubility of protein and EPS in whey protein concentrates produced in this study.¹

	Mean % soluble protein ²		Mean % soluble EPS ³	
	pH 3.0	pH 7.0	pH 3.0	pH 7.0
WPC ⁴	64 ^A	72 ^A	NA ⁵	NA
HWPC	59 ^A	68 ^A	NA	NA
FHWPC	58 ^A	65 ^A	96	97
CWPC	95 ^B	99 ^B	NA	NA

¹Values in the same column with different capital letter superscripts are statistically different (Tukey's family error $P \leq 0.05$). Values reported represent the mean of the three trials.

²Protein solubility was determined by centrifuging a 1% protein solution (0.1 N NaCl) and filtering the supernatant. Protein solubility is the fraction of original protein quantified by combustion in the filtrate.

³EPS solubility was determined by centrifuging a 1% protein solution. EPS solubility is the fraction of original EPS quantified in the supernatant.

⁴WPC = whey protein concentrate; HWPC = hydrolyzed whey protein concentrate; FHWPC = fermented hydrolyzed whey protein concentrate; CWPC = commercial whey protein concentrate.

⁵Analysis not performed.

Solubility of Whey Protein Concentrates

Protein solubility is often considered a prerequisite for functionality and was therefore determined. Solubilities for the protein and EPS of each sample were determined (pH 3 and pH 7, 0.1 N NaCl) according to the method of Morr et al. (1985) and are summarized in Table 4. Protein solubility at pH 3 and pH 7 ranged from about 60 to 70% for the whey protein concentrate, hydrolyzed whey protein concentrate, and the fermented hydrolyzed whey protein concentrate. The protein in the commercial whey protein concentrate was significantly more soluble ($\geq 95\%$). The EPS was also completely soluble at both pH levels, with no significant differences in EPS concentration in the supernatant and the original solution. The low solubility of the protein in the experimental whey protein concentrates indicated that protein structure had been denatured, which is typical of proteins that have been exposed to high thermal treatments, as occurred during the thermal inactivation of Flavourzyme.

Increased solubility is often a result of proteolysis (Panyam and Kilara, 1996), which does not need to be extensive. Chobert et al. (1988) demonstrated an increase in soluble protein (35 to 45%) when WPC was hydrolyzed to 2.5% DH with trypsin. However the Flavourzyme hydrolysates did not exhibit increased solubility compared with the unhydrolyzed WPC. If Flavourzyme were assumed to have behaved primarily as an exopeptidase, hydrolysis would have resulted in an increased number of small soluble peptides in the whey. Then the intense thermal inactivation of the enzyme could have denatured the remaining native protein structure and caused hydrophobic peptides to aggregate. During ultrafiltration, the soluble peptides would have been removed while the aggregated peptides remained in the retentate, ultimately resulting in the low

solubilities of the whey protein concentrates and no increased solubility in the hydrolysates. In this case, further hydrolysis by Flavourzyme would not be expected to result in increased solubility in the whey protein concentrates.

CONCLUSIONS

Limited protein hydrolysis can be used to produce a fermentation medium from whey suitable for growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* RR and production of EPS. Fermentation of unhydrolyzed whey resulted in lower lactose consumption, lactic acid production, viable cell counts, and net cell dry weight than fermentation of whey hydrolyzed to varying degrees (2 to 12.7% DH). This confirmed that unsupplemented, unhydrolyzed whey is a poor fermentation medium for growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* RR. Fermentation of the hydrolyzed whey resulted in better growth of RR, which resulted in a significant increase in the amount of EPS produced (~325 mg EPS per liter of whey). Increasing levels of protein hydrolysis (up to 12.7% DH) did not result in increased EPS production. As lactose utilization was nearly complete ($> 90\%$), there is no indication that further hydrolysis ($> 12.7\%$ DH) would result in increased EPS production.

Scale up fermentations (60 L) and downstream processing were successfully used to manufacture a WPC containing microbial exopolysaccharide. WPCs prepared in the laboratory contained ~72% protein and fermented WPCs contained ~6% EPS. The WPC ingredients did not exhibit differences among their electrophoretic patterns on native and SDS gels. The whey protein concentrate, hydrolyzed whey protein concentrate, and fermented hydrolyzed whey protein concentrate demonstrated low solubility (60 to 70%) compared to a commercially available WPC ($>95\%$), at both pH 3

and pH 7. The EPS was completely soluble at both pH levels. While it is possible to manufacture an EPS-containing WPC, an alternate means of inactivating the enzyme would be required to minimize the thermal exposure of the proteins.

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