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Effect of Additives on the Viability of Bifidobacteria Loaded in Alginate Poly-*l*-lysine Microparticles during the Freeze-drying Process

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Bifidobacteria-loaded alginate poly-*l*-lysine microparticles (bap microparticles) were prepared using an air atomization method and then freeze-dried. The viability of the bap microparticles was investigated as a function of the amount of the bifidobacteria cultures, and the addition of a yeast extract, cryoprotectants, antioxidants and neutralizer. The size of the bap microparticles with and without the bifidobacteria was 84.8±28.5 μm (mean±standard deviation) and 113.1±38.5 μm, respectively. The surface morphology was slightly ellipsoid and wrinkled regardless of the incorporating bifidobacteria. The viability gradually decreased with increasing freeze-drying time. Free-flowing powdered bap microparticles were obtained at least 12 h after freeze-drying the wetted slurry of bap microparticles. However, the particles tended to aggregate when either lactose or ascorbic acid was added. The addition of a yeast extract, cryoprotectants (glycerol and lactose), antioxidants ($NaHSO_3$ and ascorbic acid) and neutralizer ($Mg_3(PO_4)_2$) resulted in a significantly higher viability of the bifidobacteria in the bap microparticles after freeze-drying (0.34-1.84 log) compared with the culture alone.

Key words: Additive, Air atomization, Alginate microparticles, Bifidobacteria, Freeze-drying, Viability

INTRODUCTION

Bifidobacteria are an important species of the human colonic microflora, and are considered to be an indicator of good health (Rasic and Kurmann, 1983). However, these organisms quickly lose their viability when stored in the liquid state in fermented milk (Lee *et al.*, 1999; Cui *et al.*, 2000a, 2000b). In addition, bifidobacteria generally cannot survive in the low pH of the stomach and intestinal bile salts (Cui *et al.*, 2000a; Lee and Heo, 2000). After ingestion, the microorganisms must survive in the gastric environment and reach the colon in quantities sufficiently large (10^6-10^7 cells) to facilitate colonization.

Liquid-type fluid products have a limited shelf-life and occupy large volumes (Lee *et al.*, 1999; Cui *et al.*, 2000b; Wang *et al.*, 2004). Therefore, solid-state preparations containing probiotics are more desirable not only for increasing the stability but also to further process dairy products in the food industry. A novel method for preparing solid-type bap microparticles using an air atomization technique was previously developed (Cui *et al.*, 2000a, 2001). In this process, liquid state alginate microparticles were freeze-dried into solid-state small sized powders. Although the dried alginate beads with a large particle size have been commonly used to incorporate immobilized probiotic bacteria including bifidobacteria (Louis *et al.*, 1990; Lee and Heo 2000), small and controlled size of solid-type microparticles are more desirable in food products due to their easier handling, comfortable taste and higher stability (Cui *et al.*, 2000a, 2001; Picot and Lacroix, 2003).

Freeze-drying has been used commercially for preserving bacterial cells in solid-type food products for more

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than two decades (Kim *et al.*, 1996; Picot and Lacroix, 2003; Wang *et al.*, 2004). However, the freeze-drying process can inactivate the microorganisms. The viability of probiotic microorganisms during lyophilization is strongly dependent on many factors, including the strain organism, age of culture, type and concentration of the drying medium, salt type, residual moisture of the dried product, oxidation, cold shock, change in the cell wall permeability, and lyophilization conditions (de Valdez *et al.*, 1985a, 1985b; Carpenter *et al.,* 1991; Kim et *al.*, 1996; Shah and Ravula, 2000). Because water is removed, the phase changes from a liquid to solid ice crystals. It was suggested that the low water activity and oxygen deficiency of the product's microenvironment are responsible for the altered viability of the immobilized cells. One of the methods used to improve the viability after the freeze-drying process is to add protective additives including L-cysteine, sugars, polyols, amino acids, and certain salts as cryoprotectants (de Valdez *et al.*, 1985b; Louis *et al.*, 1990; Dave and Shah, 1997; Karney *et al.*, 2000). For example, a mixture of adonitol and glycerol with skim milk is a useful suspending medium for increasing the viability of *Lactobacillus plantum* in a free cell culture during freeze-drying (Louis *et al.,* 1990). For these reasons, solid-type bap microparticles need to be guaranteed in order to ensure the particle size and viability of the bifidobacteria. However, the effect of some additives as cryoprotectants on the viability of bifidobacteria in the freeze-dried bap microparticles is unclear.

The aims of this study were to investigate the effect of the different loading amounts of bifidobacteria cultures, yeast extracts, cryoprotectants, antioxidants and neutralizer on the viability of bifidobacteria after freeze-drying the bap microparticles prepared using an air atomization method. The effect of the lyophilization time on the viability of the bap microparticles was examined. In addition, the size and surface morphology of the freeze-dried bap microparticles were examined using a Laser Particle Analyzer and scanning electron microscope (SEM), respectively.

MATERIALS AND METHODS

Materials

Sodium alginate was purchased from Junsei (Tokyo, Japan). L-Ascorbic acid was purchased from Showa (Tokyo, Japan). Calcium chloride was obtained from Shinyo (Osaka, Japan). Poly-*l*-lysine (MW 38,500) was acquired from Sigma (St. Louis, MO. U.S.A.). The freeze dried *Bifidobacterium bifidum* cultures, which are the normal inhabitant species of the human intestine, were purchased from Chr. Hansen's Lab. (Horsholm, Denmark). The lactobacilli Mann Rogosa Sharpe (MRS) broth and yeast extract were obtained from Difco Laboratories (Detroit, MI, USA). The Gaspak™ Anaerobic jar, GasPak™ anaerobic jar system (BBL Microbiology Systems, Cockeysville, MO, U.S.A.), generator envelope, catalyst and indicator strips were purchased from Becton-Dickinson and Microbiology Systems (Spark, MD, U.S.A.). All the other chemicals were of reagent grade and were used without further purification.

Preparation of bap microparticles

The bap microparticles were prepared according to the method reported previously by Cui *et al.* (2000a; 2001). Sodium alginate solution (1.5%) and bifidobacteria culture were mixed and suspended in distilled water. The viability of the bifidobacteria in the bap microparticles was optimized by incorporating the yeast extract, cryoprotectants, anti-oxidants and neutralizer during the freeze-drying process. The amount of the culture load was also varied (0.5-2.5%).

An air atomizing device (Turbotak, Inc., Waterloo, Ontario, Canada) was used under aseptic conditions at a pressure of 0.75 bar and a delivery rate of 8 mL/min using a peristaltic pump to spray the solution into a bath containing 500 mL of a 0.2 M $CaCl_2$ solution. Pressurized air mixed with the liquid was forced into tiny liquid droplets out through the orifice of the atomizing nozzle. The sodium alginate was gelled to form microgel droplets when it came into contact with divalent calcium ions. The microgel droplets were cured for 15 min and filtered through two filter papers in a Buchner funnel. Subsequently, the filtered microgel droplets were washed twice with deionized water and suspended in a filter-sterilized 0.02% poly-*l*-lysine solution for 5 min in order to cross-link the microparticles. The resulting poly-*l*-lysine treated microparticles were again separated by filtration and washed twice with deionized water to remove the additional $CaCl_2$ solution. The particles were then frozen at -37°C for 6 h.

The final free flowing bifidobacteria-loaded alginate poly-*l*-lysine microparticles were obtained by freeze-drying (Ilshin, Seoul, Korea) at -52°C under a pressure of 8 mmTorr. All the solutions, including the sodium alginate and calcium chloride solution, were autoclaved using a steam sterilizer at 121°C for 20 min prior to use. The poly-*l*-lysine solution was sterilized by manually passing it through a 0.22 μm membrane filter. Table I shows the detailed compositions for preparing the bap microparticles.

Size and surface morphology

The size distribution of the freeze-dried microparticles containing the viable bifidobacteria was determined in triplicate using a Laser Particle Analyzer (PAR III, Otsuka Electronics Company, Ltd., Osaka, Japan) according to the natural sedimentation method. SEM (Jeol Ltd., Tokyo, Japan) was used to examine the shape and surface

Table I. Compositions and viability of the bifidobacteria in the bap microparticles after the freeze-drying process

Bifidobacteria	Additives	Survival rate (log cfu g^{-1}) (n=5)
0.5 %	-	7.56 ± 0.11
1.5 %	-	7.91 ± 0.12
2.5 %	-	7.67 ± 0.10
1.5 %	Yeast extract (0.5%)	9.75 ± 0.08[a]
1.5 %	Yeast extract (2.5%)	8.89 ± 0.17[a]
1.5 %	Glycerol (5%)	8.35 ± 0.06[a]
1.5 %	Glycerol (10%)	7.99 ± 0.09
1.5 %	Lactose (2.5%)	8.90 ± 0.02[a]
1.5 %	Lactose (5%)	8.25 ± 0.11[a]
1.5 %	$NaHSO_3$ (0.5 %)	8.73 ± 0.20[a]
1.5 %	$NaHSO_3$ (1%)	8.19 ± 0.14
1.5 %	Ascorbic acid (0.25%)	8.51 ± 0.18[a]
1.5 %	Ascorbic acid (0.5%)	8.61 ± 0.21[a]
1.5 %	$Mg_3(PO_4)_2$ (1%)	8.15 ± 0.19
1.5 %	$Mg_3(PO_4)_2$ (2%)	8.04 ± 0.08

[a]Significantly different from bifidobacteria culture only (1.5 g), $p < 0.05$

morphology of the bap microparticles. An appropriate amount of the freeze-dried bap microparticles was dispersed in ethanol and air-dried onto metal stubs. Each sample was coated with gold using a Joel sputter coater for 6 min.

Effect of lyophilization time

The bap microparticles were prepared by suspending the sodium alginate (1.5 g), bifidobacteria cultures (0.5 g), yeast extracts (0.5 g), glycerol (5.0 g), $NaHSO_3$ (0.5 g) and $Mg_3(PO_4)_2$ (1.0 g) in distilled water (100 mL), and then sprayed using the air atomization method described previously. The resulting slurry was then frozen at -37°C for 6 h and then lyophilized for 12, 14, 18 or 24 h, respectively. The viability of the bifidobacteria in the bap microparticles as a function of the lyophilization time was measured using the plate counting method described previously.

Determination of viability

The viability of the bifidobacteria was assessed using the method reported elsewhere (Lee *et al.*, 1999; Cui *et al.*, 2000a, 2000b). Briefly, the microparticles were incubated in a simulated intestinal fluid (without pancreatin) at pH 6.8, which consisted of a 0.5% yeast extract and 0.05% cysteine HCl and maintained at 37°C for 12 h. One mL of the sample was withdrawn, and diluted aseptically by serial dilutions. The serially diluted solutions were composed of 5.5% MRS and 0.05% L-cysteine HCl. The diluted samples were plated in triplicate in MRS agar, and incubated at 37°C under anaerobic conditions. After 48-72 h, the number of colony forming units (cfu) was counted to determine the viability of the bifidobacteria

RESULTS AND DISCUSSION

Size and morphology

Fig. 1 shows the size distribution of the microparticles with and without the incorporating bifidobacteria. The size of bap microparticles with and without bifidobacteria was 84.8 ± 28.5 μm (mean ± standard deviation) and 113.1 ± 38.5 μm, respectively, showing that the particles without the bifidobacteria were larger. Fig. 2 shows the surface morphology of the bap microparticles indicating that they were ellipsoid in shape. However, it appeared wrinkled regardless of the incorporating bifidobacteria, which was probably due to the loss of water during the freeze-drying process. Nevertheless, this freeze-drying process did not destroy the original structure of the bap microparticles.

Effect of additives on viability

Table I summarizes the effect of additives on the viability of bifidobacteria loaded alginate poly-*l*-lysine microparticles. The viability was maintained at above 10^7 cfu g^{-1} in three different loading amounts of the bifidobacteria cultures. When 1.5% of the loading amount of bifidobacteria cultures was used, the viability of the bifidobacteria was relatively higher. The viability of the bifidobacteria was significantly improved ($p < 0.01$) when 0.5% of the yeast

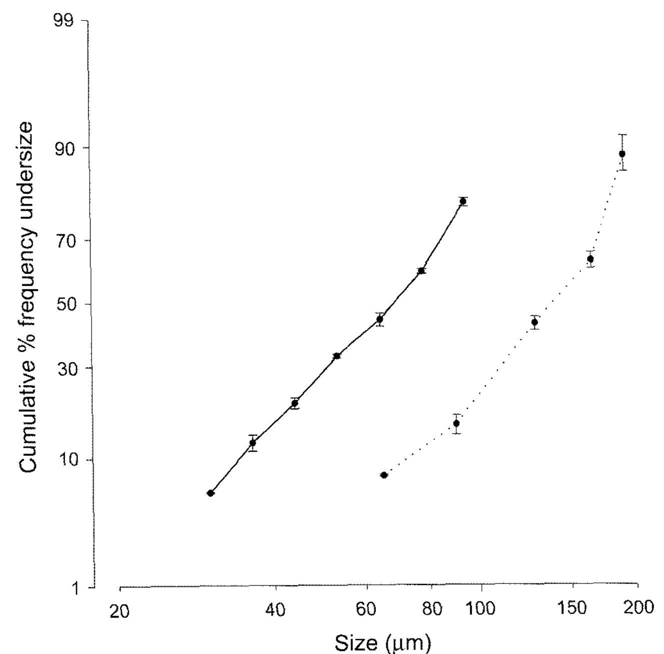

Fig. 1. Size distribution of the alginate poly-*l*-lysine microparticles with (solid line) and without (dotted line) the incorporating bifidobacteria.

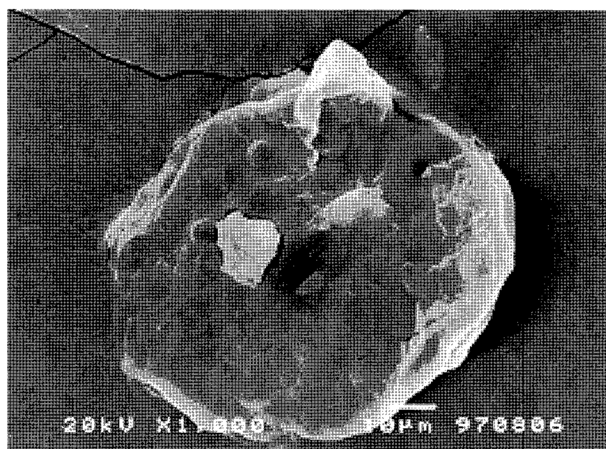

Fig. 2. Surface morphology of the bifidobacteria-loaded alginate poly-*l*-lysine microparticles.

extract was used. In this experiment, the yeast extract appeared to be the most important factor influencing the viability of the bifidobacteria in the bap microparticles.

Low molecular weight glycerol and lactose were also examined as cryoprotectants. The viability of the bifidobacteria with glycerol and lactose was higher than that of the control group. It was reported that the viability of bifidobacteria in calcium alginate beads increased when these cryoprotectants were used due to the immobilization of *Bifidobacterium bifidum* (Kim *et al.*, 1996). The effect of glycerol on protecting lactic acid bacteria varies from strain to strain. These results suggested that the stabilization of dried bifidobacteria was the result of the binding of cryoprotectants to the dried bifidobacteria as a "water-substitute" during the freeze-drying process. The sugars and polyols used as a water substitute were partly used for hydrogen bonding in the dried bifidobacteria (Carpenter *et al.*, 1991). Although the viability of the bifidobacteria was the highest when 5% glycerol and 2.5% lactose were added, 5% glycerol was chosen as the cryoprotectant in the following experiments because lactose caused aggregation on the surface of the $CaCl_2$ solution.

Antioxidants are essential for preventing oxidation because bifidobacteria are anaerobic microorganisms, which rapidly lose their viability upon exposure to air (Dave and Shah, 1997). Antioxidants are often added to the pharmaceutical formulations as a redox system. It is known that antioxidants "soak up" free radicals resulting in decreased oxidation. The viability of bifidobacteria was 5.7×10^8 and 4.4×10^8 cfu g^{-1} when 0.5% $NaHSO_3$ and 0.5% ascorbic acid were added, respectively, which was significantly higher than the control. However, aggregation occurred when ascorbic acid was added during the preparation of bap microparticles. The viability of the bifidobacteria increased slightly when a neutralizer was used. The viability of the bifidobacteria loaded in the alginate poly-*l*-lysine microparticles was 1.5×10^8 and 1.1×10^8 cfu g^{-1} when 1% or 2% $Mg_3(PO_4)_2$ was added, respectively. The mechanism of this effect needs to be further investigated.

The survival of bifidobacteria is affected by various factors such as low pH, the nature of the dissolution media, the presence of nutrients and incubating conditions (Rao, 1989; Louis *et al.*, 1990; Lee *et al.*, 1999; Cui *et al.*, 2000a, 2000b). The MRS broth and yeast extract can act as useful nutrients. L-cysteine is also used as an antioxidant. Therefore, these components were added to the bifidobacteria culture media. The freeze-dried bap microparticles may also contain dead, sublethally injured and viable cells. Under suitable conditions, the injured cells may repair and become viable again i.e. capable of colony formation on a suitable media (de Valdez *et al.*, 1985b).

Effect of lyophilization time

The lyophilization time used to prepare the bap microparticles was one of the most important factors affecting the viability of the bifidobacteria. The amount of remaining moisture as well as the characteristics of the dried microparticles after lyophilization needs to be considered. In order to obtain free-flowing microparticles, it is essential to allow sufficient lyophilization time. However, a long lyophilization time is detrimental to the viability of bifidobacteria, even in alginate poly-*l*-lysine microparticles.

Fig. 3 shows the effect of the lyophilization time on the viability of the bifidobacteria loaded in the alginate poly-*l*-lysine microparticles. The viability decreased with increasing lyophilization time. High numbers of viable bifidobacteria in the products were maintained at levels of approximately 10^9 cfu g^{-1} when the lyophilization time changed from 12 to 18 h. Although the viability was higher at the short lyophilization time, the free flowing properties of the freeze-

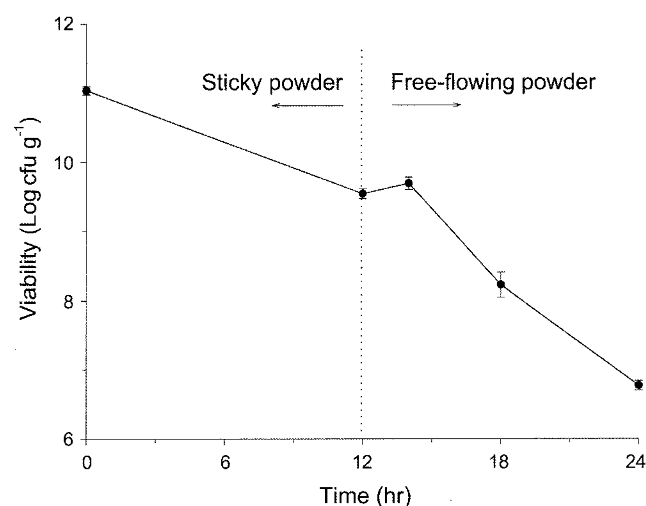

Fig. 3. Effect of the lyophilization time on the viability of the bifidobacteria loaded in the alginate poly-*l*-lysine microparticles.

dried bap microparticles were unsatisfactory. The optimal lyophilization time needed to provide the necessary viability and free-flowing nature and was found to be 12-14 h.

It was reported that the amount of moisture in the bap microparticles strongly influenced the viability of the bifidobacteria during the lyophilization process of preparation or storage (de Valdez *et al.*, 1985a; Wang *et al.*, 2004). Evidence of cell membrane damage in *L. bulgarisus* after the freeze-drying process has also been reported (Castro *et al.*, 1997). However, the injured cells can repair and regain their normal viability under a certain conditions as discussed previously (de Valdez *et al.*, 1985b).

CONCLUSIONS

Solid-type bap microparticles were prepared using an air atomization method followed by a freeze-drying process. The size of the bap microparticles was approximately 84.8 ± 28.5 μm. The morphology of the bap microparticles was ellipsoid in shape but the surface appeared wrinkled. The viability and physical state of the bap microparticles was affected by the lyophilization time. In addition, the viability of the bifidobacteria in the bap microparticles was strongly affected by the incorporating additives.

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