In Vitro Stability of β-galactosidase Microcapsules

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ABSTRACT : The present study was carried out to examine the efficiency of microcapsules and a stability of lactase *in vitro* in the simulated gastric and intestinal conditions. As a coating materials, medium-chain triacylglycerol (MCT) and polyglycerol monostearate (PGMS) were used. The highest efficiency of microencapsulation was found in the ratio of 15:1 as coating to core material with both MCT (91.5%) and PGMS (75.4%). In a subsequent experiment, lactose content was measured to study a microcapsule stability. Lysis of microcapsules made by MCT in simulated gastric fluid was proportionally increased such as 3% in pH 5 and 11% in pH 2 for 20 min incubation. In the case of PGMS microcapsulation, 11-13% of lactose was hydrolyzed at 20 min in all pHs and also very little amount (less than 3%) of lactose was hydrolyzed after 20 min in all pHs. The highest percentages of lactose hydrolysis in MCT and PGMS microcapsules were 68.8 and 60.8% in pHs 7 and 8 during 60 min, respectively. Based on our data, the lactase microcapsules seemed to be stable when they stay in the stomach, and hydrolyzed rapidly in small intestine where the bile acid was excreted. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 12 : 1808-1812)***

Key Words : *In vitro* stability, Microcapsule, Lactase, Milk

INTRODUCTION

Milk is the universal and nutritious food, but a large majority of non-Caucasians and elderly people in Western Europe and U.S., and various ethnic population groups cannot properly digest it because they lack sufficient quantities of lactase (βgalactosidase, EC 3.2.1.23) in their gastric tract, the enzyme that breaks down lactose (Harris, 1972; Kretchmer, 1972; Simmons, 1978). Lactose intolerance is a symptomatic lactose malabsorption which causes the person to quit drinking milk and eating others (Newcomer and McGill, 1984). Researchers who have studied lactose deficiency reported that symptoms appeared more frequently as age increases (Simmons, 1978).

Lactose requires lactase, which is produced in the small intestine in order to hydrolyze lactose into glucose and galactose. In the absence of lactase, lactose maldigestion usually shows symptoms of digestive tract discomfort, cramping and/or diarrhea (Bayless and Rosenberg, 1966; Bayless et al., 1975). To solve this problem, recent approach was tried, which was lactose reduction in milk by lactase prior to consumption (Scrimshaw and Murray, 1988). However, hydrolysis of lactose during process resulted in taste changes in milk, since glucose and galactose were about 4 times sweeter than lactose, and many lactose maldigesters did not like the taste of lactose-hydrolyzed milk (Onwulata et al., 1989; Solomons et al., 1985).

Microencapsulation, which showed potential as carriers of enzymes in food industry, could be a good vehicle for the addition of lactase to milk without off-taste or flavor (Bersen'eve et al., 1990; Jackson and Lee; 1991). Currently, there is a considerable interest in developing encapsulated flavors and enzyme systems. Among several factors to be considered, choice of coating material is the most important aspect, and depends on the chemical and physical properties of the core material, process used to form microcapsules and the ultimate properties desired in microcapsules.

Ideally, oral delivery systems designed to transport a compound of interest through the stomach would be characterized as having a high encapsulation efficiency, provide maximal stability, and therefore, limited release in acidic pH ranges and rapid release in neutral conditions (Vandenberg et al., 2001).

Even though our previous studies showed a possibility of using emulsifier as coating material to coat lactase, the main thing needed to examine was a high stability in the environment of gastrointestinal tract. Because the entrapped lactase may be no longer protected from peptidases present in the gastrointestinal tract. Therefore, the objectives of this study were to examine the efficiency of microencapsulation and a stability of lactase *in vitro* in the simulated gastric and intestinal conditions

MATERIALS AND METHODS

Materials

As coating materials, medium chain triacylglycerol (MCT) and polyglycerol monostearate (PGMS) were used, and purchased from Il-Shin Emulsifier Co., LTD. (Seoul, Korea). As a core material, lactase originated from *Kluyveromyces lactis* in the form of liquid was provided from Culture Systems, Inc. (Mishawaka, IN, USA). The specific activity of the enzyme was 504 units/g. One unit

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hydrolyzed 1.0 μmol of o-nitrophenyl β-D-galactopyranoside to o-nitrophenol and D-galactose per min at the specified pH and temperature, unless otherwise stated.

Microencapsulation

Microcapsules of lactase were made by MCT or PGMS, which were selected as major coating materials from the previous experiment (Kwak et al., 2001). The ratios of coating material to core material were 5:1, 10:1 and 15:1 to maximize lactase content and stability of microcapsules, and mixed at 1,200 rpm for 1 min with a stirrer. An airless paint sprayer (W-300, Wagner Spray Tech. Co., Markdorf, Germany) nebulized a coating material-lactase emulsion at 45°C into a cyclinder containing a 0.05% polyethylene sorbitan monostearate (Tween 60) solution at 5°C. The diameter of the nozzle orifice was 0.4 mm. The chilled fluid was centrifuged at 450×g for 10 min to separate unwashed microcapsule suspension. Microcapsules were formed as lipid solidified in the chilled fluid. To remove residual enzyme adhering to the outside walls of microcapsules, unwashed microcapsules were mixed with 0.05% Tween-60 solution again, centrifuged, and obtained one-time washed microcapsules. The procedure was repeated.

Especially for PGMS microencapsulation, the distilled water was additionally added because PGMS is highly viscous. PGMS and distilled water were mixed with 5:4, w/v), heated to 55°C for 20 min, and stirred with 1,200 rpm for 30 sec for spraying. Microcencapsulation for both MCT and PGMS were done in triplicate.

Efficiency of lactase

The dispersion fluid was assayed for untrapped enzyme according to a modified procedure of Shin et al. (1995). Two mL of the dispersion fluid was filtered by Whatman No. 540, followed by membrane filtration (di. 1.0 μm, Whatman International Limited, Madistone, England). The 2 mL of 5 mM o-nitrophenol βgalactopyranoside (ONPG) (Sigma Chemical Co. St. Louis, MO, USA) heated to 37°C for 15 min was added to 0.5 mL of the dispersion fluid and incubated at 37°C in a water bath for 20 min. The reaction was stopped by adding 0.5 mL of 500 mM Na_2CO_3. The color intensity was read at 420 nm using Beckman DU 650 Spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). Microencapsulation efficiency was calculated as followed:

$$1- \frac{\text{Specific activity of residual in the dispersion fluid}}{\text{Initial specific activity of enzyme in spray solution}} \times 100$$

The dispersion fluid was centrifuged at 200×g to remove the intact capsules from the fluid. Sample measurements were run in triplicate.

***In vitro* study**

To determine the stability in the stomach and intestine, the simulated gastrointestinal solutions were prepared as follows: 1) gastric fluid prepared in sample solution containing pepsin (pH 1.2) and simulated into 4 different fluids with pHs 2, 3, 4 and 5 using 2N HCl and NaOH, and 2) intestinal fluid was prepared in 0.1M PBS buffer (100 mL, pH 7.4) containing 20 mg pancreatin, 10mM cholic acid and 1mM deoxycholic acid, and simulated into 4 different intestinal solutions as pHs 5, 6, 7 and 8 (Rao et al., 1995).

For the gastric and intestinal *in vitro* study, the microcapsules of lactase (2% of milk) added in 2 mL milk (total lactose content: 96 μg), transfered into capped test tubes and incubated at 37°C with 100 rpm mixing for 10 min. During 60 min incubation, each sample was collected at 20 min interval in both simulated gastric and intestinal conditions. The treated samples were centrifuged at 2,490×g and the supernatant was measured for uncapsulated lactase content. All treatments were triplicate.

Lactose content

The content of lactose in milk samples containing microcapsules was determined by Kwak and Jeon (1988). Sample (10 mL) was poured into 25 mL volumetric flask and 2-propanol was filled and mixed thoroughly. It was stood at room temperature for 20 min and centrifuged at 276×g for 10 min, and the supernatant was filtered through Whatman No. 540 and Sep-Pak C_{18} for HPLC determination. Lactose analysis was performed by using a Cosmosil packed column 10_{NH2} (4.6 mm I.D.×25 cm), and HPLC (Waters Corporation, MA, USA). Acetonitril:water (3:1) was used as a mobile phase and propelled at 2 mL/min. Detector was refractive index detector and injection volume was 20 μL. A standard curve was constructed by injecting lactose standards, which yielded a linear curve. All measurements were run in triplicate.

Statistical analysis

Data from each experiment were analyzed by analysis of variance (ANOVA) using a SAS program (20) and differences among treatments were determined by LSD at $p<0.05$, unless otherwise stated.

RESULTS AND DISCUSSION

Microencapsulation by MCT

The efficiency of microencapsulation made by MCT was shown in Table 1. Efficiency of microencapsulation increased proportionally to the increase of coat to core ratio. The efficiency was the greatest (91.5%) when the coat to core ratio was 15:1. Significant differences were found between those of 10:1 (73.9%) and 5:1 (61.4%) ($p<0.05$).

Table 1. Yield of microencapsulation for β-galactosidase with different ratios of coating materials to β-galactosidase[1]

Ratio (w/w)		Yield (%)	
Coating material	β-galactosidase	MCT[2]	PGMS[3]
15	1	91.5[a]	75.4[a]
10	1	73.9[b]	63.7[b]
5	1	61.4[c]	50.2[c]
SEM		0.38	0.53

[1] Means of triplicate. Means in a column without the same letter are not significantly different ($p<0.05$).
[2] Medium chain triacylglycerol.
[3] PGMS (polyglycerol monostearate):distilled water=5:4.

Therefore, the optimum ratio of MCT to lactase was found to be 15:1, even though left over MCT was still found in the upper layer. It seemed that capsules entrapped by MCT remained more stable than that of PGMS.

Microencapsulation by PGMS

Since PGMS is a solid type in room temperature, additional procedure was applied based on the method for MCT microencapsulation; firstly heating process was applied for ease of spraying, and secondly distilled water were added to reduce the viscosity of spray solution for encapsulating lactase. Based on our previous results (Kwak et al., 2001), heating at 55°C for 20 min was revealed as an optimum condition for retaining an enzyme activity.

When the ratio of PGMS to distilled water was 5:4, the optimum ratio of coating material (PGMS+distilled water) to lactase (15:1, 10:1, 5:1) was examined as shown in Table 1. The highest efficiency (75.4%) was formed with 15:1 (coating to core ratio), which was appeared to be lower than that of MCT microcapsules.

Similar studies (Jackson and Lee, 1991; Kim et al., 1996; Magee and Olson, 1981) have reported the optimum ratios of coating (agar, gelatin, soluble starch, milk fat) and core material (ω3fatty acid, iron, flavor etc.) for an efficient microcapsule formation. When ω3 fatty acid was microcapsulated by milk fat, the ratio of coating to core material was 8:2 and the efficiency was 95.6% (Kim et al., 1996). In addition, Sankarikutty et al. (1988) indicated that the 7:3 ratio of cardamon oil to the mixture of gum acacia and maltodextrin showed the highest efficiency among other ratios. Those studies indicated that the optimum conditions including the ratio of coating and core materials, the viscosity of spray solution, the method of microencapsulation varied with kinds of coating, core materials and food to be applied.

***In vitro* study**

A major prerequisite in the use of microcapsules as lactase carriers is that they must contain as much as lactase as possible, and must resist the gastric and intestinal fluids and be captured by the enterocytes before being released into the general circulation (Freund et al., 2000).

In above experiment, we obtained about 70% of encapsulation for lactase with a ratio of MCT or PGMS/lactase=15:1. Therefore, to verify whether the microcapsules were stable in stomach and released effectively in small intestine, we made a simulated gastric and intestinal conditions in a subsequent experiment.

The time-dependent lysis of microcapsules in simulated gastric fluid (pH 1.2) with pepsin was investigated. Lysis of microcapsules made by MCT in simulated gastric fluid was proportionally increased to pH decrease (Figure 1). The percent lactose hydrolysis in a simulated gastric fluid with pepsin was 13% at 60 min incubation. Incubation at pH 5 at 37°C during 20 min caused very little hydrolysis of lactose (3%) from microcapsules. However, incubation at pH 2 during 20 min caused 11% lactose hydrolysis. Less than 4% of the lactose was hydrolyzed after 20 min upto 60 min at 37°C. In the case of PGMS microencapsulation, 11-13% of lactose as hydrolyzed at 20 min in all pHs (2, 3, 4 and 5) and also very little amount (less than 3%) of lactose was hydrolyzed after 20 min (Figure 2). Higher lactose hydrolysis from PGMS microencapsulation in early period than from MCT may be due to the loosely constructed membrane from the mixture of PGMS and water.

To determine how effectively lactose was released in simulated intestinal fluid, a simulated intestinal fluid was prepared with the presence of pancreatin and bile salts, and incubated at 37°C for 60 min (Figures 3 and 4). With both an increase of pH and the duration of incubation, lactose hydrolysis increased dramatically, especially in pHs 7 and 8 in microcapsules made by both MCT and PGMS. Lysis of microcapsules made by MCT was 35% in pH 6, while it was increased upto 58-61% in pHs 7 and 8, respectively.

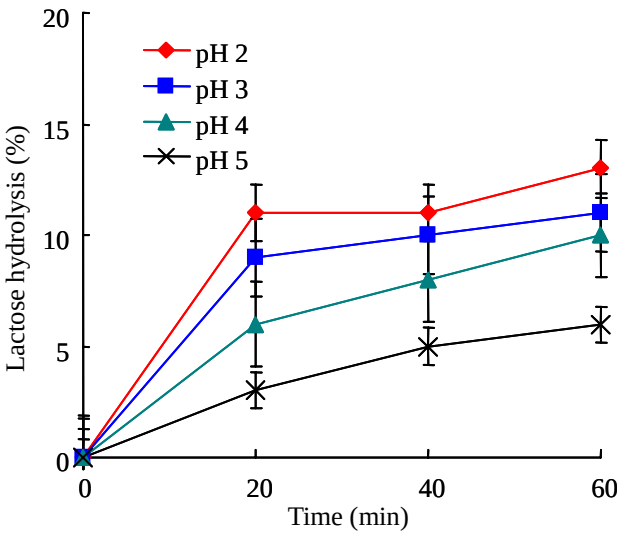

Figure 1. Hydrolysis pattern of lactose from MCT microcapsules as a function of time during incubation in a simulated gastric fluid.

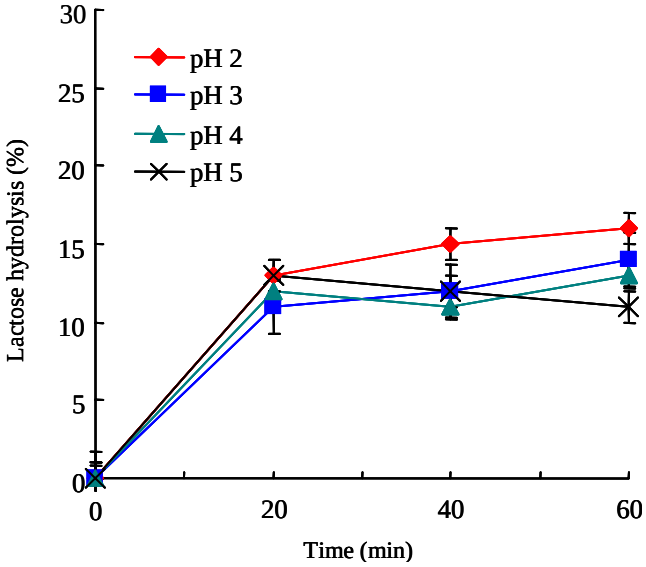

Figure 2. Hydrolysis pattern of lactose form PGMS microcapsules as a function of time during incubation in a simulated gastric fluid.

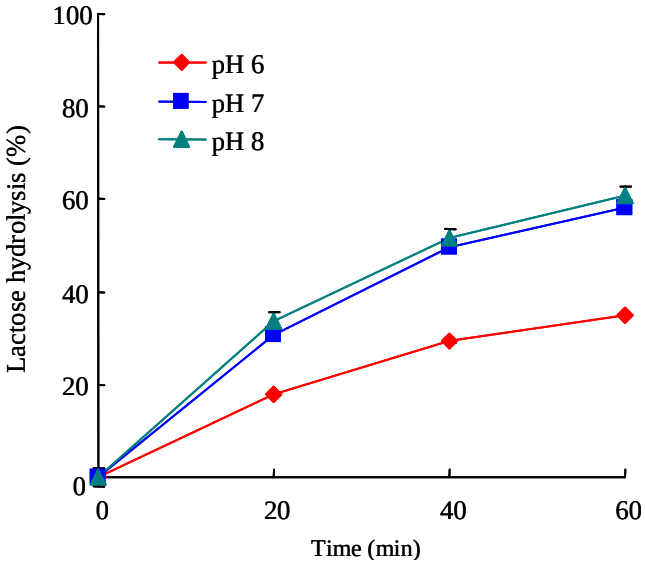

Figure 4. Hydrolysis pattern of lactose from PGMS microcapsules as a function of time during incubation in a simulated intestinal fluid.

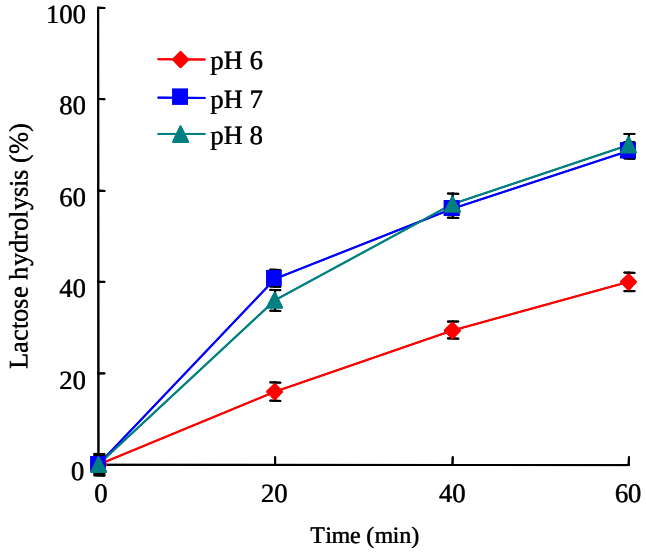

Figure 3. Hydrolysis pattern of lactose from MCT microcapsules as a function of time during incubation in a simulated intestinal fluid.

Similar trend was found in the microcapaules made by PGMS.

Above results indicated that microcapsules were stable in an acidic (pH 2 to 5) condition when they were incubated for 60 min. The microcapsules were not sensitive to action of pepsin. However, the microcapsules were sensitive to incubation in neutral buffer with pancreatin and bile salts. Many authors also obtained a more sensitive for microcapsules to incubation in acidic and basic buffers on pancreatin (Freund, 2000).

The phospholipase, the major enzyme for the lipid digestion in pancreatin, was shown to cause the lysis of microcapsules with action of bile salts (cholic acid and deoxycholic acid) at the concentration of 20 mM after a 30 min incubation and totally disrupted during 60 min. This phenomena can explain an interesting finding in our experiment, which was a profound increase of lactose hydrolysis in pH 6 (35 to 40%), compared to that in pH 5 (6 to 11%). Since the only difference was the addition of pancreatin and bile salts between two samples, the higher hydrolysis of lactose may be influenced from an existence of pancreatin and bile salts in simulated intestinal fluid.

Therefore, the microcapsules may be stable when they stayed in the stomach and hydrolyzed rapidly after they go down to the upper part of small intestine where the bile was excreted. Additionally, since about 90% of the microcapsules was not hydrolyzed in a simulated gastric fluid with pepsin, we expected that β-galactosidase entrapped in the microcapsules may maintain its activity in the stomach, and be released from the microcapsules into the intestine.

Our previous study (Kwak et al., in press) showed a release of iron from microcapsules made by PGMS. *In vitro* study, the rates of iron release from the capsules showed a similar trend to this experiment and significantly affected through the difference of pH in the incubation conditions. A similar study (Freund et al., 2000) was designed to appreciate the stability of liposomes in gastric or intestinal fluid in different incubation. Less than 3% of liposomes was broken in acidic fluid (pHs 3,4,5 and 6) at 37°C for 2 h incubation. In addition, β-galactosidase entrapped in liposome was stable and can digest lactose in milk after the efficient lysis of liposomes in the presence of bile salts

(Kim et al., 1999). These results were in agreement with our *in vitro* results, which demonstrated less than 15% release of microcapsules in an acidic condition.

CONCLUSION

The present study indicated that the ratio of 15:1 as coating materials (MCT and PGMS) to core material (lactase) showed a satisfied efficiency of microencapsulation such as 91.5 and 75.4%, respectively. In a stability study *in vitro*, about 90% of the lactase microcapsules was not hydrolyzed in a simulated gastric fluid with pepsin, lactase entrapped in microcapsules may maintain its activity in the stomach, and be released into the intestine. Therefore, the present study suggested that acceptable milk products could be prepared with microencapsulated lactase. In addition, this study implied that β-galactosidase entrapped in microcapsules may be applied to which milk for lactase-deficient subjects. For this, further experiment *in vivo* level will be necessary for actual use of the microcapsules to human.

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