# Encapsulation of Bromelain in Liposome

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## Abstract

Bromelain has been used as a meat-tenderizing agent in food processing. To increase the availability of bromelain, microencapsulation into liposome was carried out by the dehydration and rehydration method. Small unilamellar vesicles prepared by sonication treatment showed higher encapsulation efficiency (EE) than by the French press method. In the preparation of liposome, the effect of pH and centrifugal force on EE was also investigated and it showed a higher EE at acidic pH than at alkaline pH with centrifugation at $80,000 \times g$. The actual EEs except unencapsulated bromelain which bound on the outside surface of liposome by electrostatic interaction also were investigated, and the optimal EE was at pH 4.6, at 0.6 of a ratio of bromelain to phospholipid (18.2%, w/w). Release of bromelain from liposomes was stimulated as the temperature increased at neutral pH.

Key words: microencapsulation, liposome, bromelain

## INTRODUCTION

Microencapsulation technology presents exciting opportunities for the food technologist in areas such as flavor retention and release, and the enhanced stability and shelf life of sensitive ingredients (1,2). Encapsulation has been used in the food industry for more than 60 years (3). Food ingredients are encapsulated for a variety of reasons including protecting against their environment (water, acid, oxygen, other food ingredients, etc.), stabilization of the ingredients during processing, imparting a controlled release, or simply changing the state of the food ingredients from a liquid to a solid to permit its use in a dry application (3,4).

Liposomal microencapsulation, one of microencapsulation techniques currently used, is a novel approach and has advantages to entrap both within their aqueous compartment and within the hydrophobic bilayer. Kirby et al. (5) reported that the addition of proteolytic enzyme-encapsulated liposomes to cheddar cheese has resulted in halving the normal ripening time in the cheese process. Koide and Karel (6) studied enzyme (lysozyme and pepsin)-loaded liposomes and controlled release by stimuli such as surfactants and $Ca^{2+}$. Kirby et al. (7) and Lee et al. (8) studied the effect of liposome on the stabilization of ascorbic acid. Free ascorbic acid was oxidized at pH 5 in 7 days, however, ascorbic acid encapsulated in liposome remained its activity with a 22.8% after 40 days. Rhim et al. (9) also studied the effect of cholesterol content of liposome on the stability of encapsulated ascorbic acid. With increasing cholesterol content in liposomes, encapsulation efficiency (EE) was decreased, whereas liposome size and the stability of encapsulated ascorbic acid were increased.

Dehydration and rehydration (DR) method for encapsulation of enzyme into liposome is simple and mild, in contrast to the other methods (10-12). It involves no exposure of enzyme to organic solvents and sonication, nor to surfactants other than the vesicle-forming phospholipids. Safety and economy can also be satisfied if lipid vesicles are made of phosphatidylcholine (PC).

This research was carried out to investigate the potential of bromelain-loaded liposome to increase the availability of bromelain. Bromelain, a proteolytic enzyme from pineapple (13), has been widely used as a meat tenderizing agent and as an anti-inflammation drug (14). Liposomes prepared by the DR method were evaluated for their encapsulating conditions, encapsulation efficiency (EE), and stability in the external environment.

## MATERIALS AND METHODS

### Materials

L-$\alpha$-phosphatidylcholine (L-$\alpha$-lecithin, PC) type II-2 from soybean and bromelain were purchased from Sigma Chemical Co. (St Louis, MO, USA), and Bio-Rad protein assay kit from Bio-Rad (Hercules, CA, USA) was used throughout this research. All other reagents were commercially available and of analytical grade quality.

### Preparation of the enzyme solution

Bromelain was solubilized in $KH_2PO_4$-HCl buffered saline solutions (PBS solution). The concentration of phosphate and NaCl was 5 mM and 115 mM, respectively. The pH of PBS solution was adjusted to pH 3.0, 4.6, 7.0, 9.0 and 9.55 for the preparation of liposomes, and to pH 5.0 and 7.0 for the stability test.

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## Preparation of liposomes

The method was based on the dehydration-rehydration procedure of Kirby & Gregoriadis (10) and slightly modified for this research. A 100 mg of PC was dissolved in 4 ml of chloroform:methanol (2:1, v/v) and was dried by a rotary evaporator (at 30°C) to form a thin film round the inside of a round-bottomed flask. Distilled water (4 ml) was added and swirled with 0.5 g of glass beads. The opaque and white suspension formed multilamellar vesicles (MLVs). The suspension was sonicated or pressured to form small unilamellar vesicles (SUVs). The solution of bromelain to be encapsulated (a given concentration by the desired enzyme to PC ratio) was mixed with the suspension and stood for 15 min at room temperature. The suspension was frozen by swirling in a bath of dry ice/ethanol.

To rehydrate the dried PC-bromelain mixture, 0.4 ml of distilled water and glass beads was added, and then diluted with PBS of a given pH (pH 3.0, 4.6, 7.0 and 9.0) to 15 ml of the final volume. The bromelain-loaded liposomes were separated by centrifugation at 80,000×g for an hour. Precipitated liposomes were washed and resuspended with PBS of pH 9.55 (contaning 0.5 M NaCl) for a reversibility test and adjusted to 15 ml, and then centrifuged.

## Preparation of small unilamellar vesicles (SUVs)

SUVs were prepared from MLVs by imparting energy at a high level to liposome suspension. In this research, two methods were compared; sonication and pressure. Liposome suspension was disrupted by sonic treatment for an hour in a bath type sonicator (Mujige, SD-120H, Seongdong ultrasonic Co., Korea), or was extruded in a French press (Amico, 20K, SLM instruments, Inc., USA) at a pressure of 10,000 psi and a flow rate of 5 ml/min, respectively.

## Effect of centrifugal force

Liposome pellets were collected by centrifugation and it was carried out at 12,000×g, 30,000×g and 80,000×g, in order to examine the effect on the encapsulating efficiency (Hitachi preparative ultracentrifuge, SCP 55H, Hitachi Koki Co. Ltd., Japan).

## Effect of pH and bromelain/PC ratio in preparation of liposomes

After freeze drying, PC-bromelain mixture was rehydrated and formed to liposome. The liposome was induced to wash the surface of vesicles that was attached unencapsulated bromelain. The pH of PBS used was pH 3.0, 4.6, 7.0 and 9.0. Liposomes were prepared with various compositions of bromelain to PC such as 0.2:1, 0.4:1, 0.6:1, 0.8:1, 1.0:1 (w/w). Liposomes were prepared with above conditions, respectively and then compared with encapsulating efficiency.

## Reversibility test

This test was carried out to examine an actual EE. Liposomes were washed twice with PBS of a given pH. Finally, unencapsulated bound bromelain on the outside of liposome was induced to detach with PBS adjusted to pH 9.55 (iso-electric point of bromelain) and high salt concentration (0.5 M NaCl). The amount of unencapsulated bromelain was calculated from supernatant, separated by centrifugation. The concentration of bromelain was determined spectrophotometrically, according to the Lowry method (15) improved by Bio-Rad protein assay reagents.

$$\frac{\text{weight of encapsulated bromelain}}{\text{weight of added bromelain}} \times 100(\%)$$

## Determination of liposome size

Liposomes loaded with bromelain were examined their size with particle size analyzer (LS230 Small Volume Module, Coulter Co., USA).

## Release of encapsulated bromelain from liposomes

A release test of encapsulated bromelain was carried out depending on temperature, pH and storage period. Bromelain was measured at an interval of a day for 6 days. A liposome pellet was diluted with PBS solution of pH 5.0 and 7.0 to the concentration of 1 mg/ml of PBS. The suspensions were saturated with air in glass vials at room temperature for an hour, and then stored with capping at 4°C, 20°C and 37°C, respectively. Suspensions stored in glass vials were sampled and centrifuged at 80,000×g for an hour, periodically. The bromelain on the supernatant released from liposomes was measured. The release of bromelain during storage was presented as the cumulative percentage was released.

# RESULTS AND DISCUSSION

## Characterization of liposomes

Kirby and Gregoriadis (10) reported that encapsulation efficiency (EE) of DR method was consistently higher than other types of liposomes tested. EE is affected by preparation methods of liposomes, core to wall ratio and composition of liposomes. Liposomes loaded with bromelain were examined EE and the size distribution. EE value and the size distribution of this study were determined at standard conditions (bromelain/PC = 0.4, a PBS solution of pH 4.6, French press, 12,000×g). Koide and Karel (6) reported that EE of enzyme within liposomes was 24.1% for lysozyme and 22.5% for pepsin, respectively, and their volume mean diameter was 563 nm. Lee et al. (8) reported that EE of ascorbic acid was 46.8%. In this study, the EE was 19.1% and the volume mean diameter was 4.8 μm (Fig. 1). In the same method of preparation of liposome (10), EE was varied with encapsulated material, however, it did not appear to depend on the solute's molecular weight.

## Effect of preparation methods and centrifugal force on small unilamellar vesicles (SUVs)

Liposome structure can be divided into multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs) by the preparation method. The main disadvantage of MLVs is the heterogeneous size distribution and the low efficiency (5~14%) of encapsulation (1),
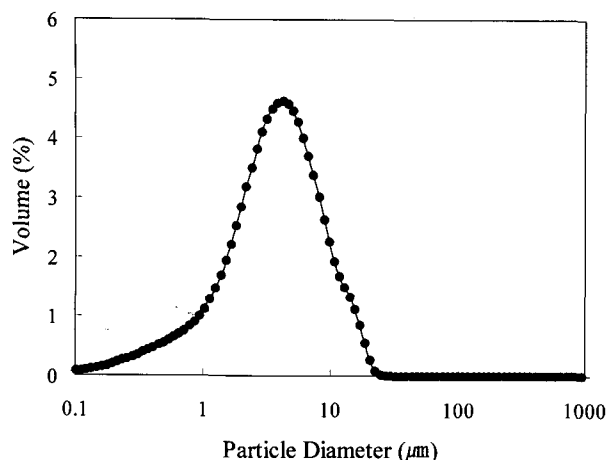
**Fig. 1.** Size distribution of bromelain-loaded liposomes prepared by dehydration-rehydration (DR) method.

thus SUVs are preferred to encapsulate bromelain. In order to reduce hydrated lipid to vesicles of smallest size possible, it is necessary to use a method which imparts energy at a high level to the lipid suspension. This was first achieved by the exposure of MLVs to sonication and it is still the method most widely used for the production of small vesicles. However, because of the problems inherent in subjecting biological materials to sonication (i.e. degradation not only of lipids, but of macromolecules and other sensitive compounds to be entrapped inside liposomes), extrusion of preformed MLVs in a French Press under high pressure method has been developed (16).

Two methods, sonication and pressure, were compared dependent on EE of bromelain in liposomes (Table 1). Additionally, the effect of centrifugal force to purify SUVs from liposome suspension was also investigated at 12,000, 30,000 and 80,000 × g (3,4,8). In the case of the present experiment, the sonication method showed better EE than that of the French press method. SUVs prepared under the sonication method and higher centrifugal force (at 80,000 × g) showed the highest EE with 23.0%. In the method of sonication, EE increased with the increase of centrifugal force, however, a vice versa in the French press method. These facts suggested that SUVs in the sonication method contained a higher proportion of encapsulated bromelain than those in the French

**Table 1.** Encapsulation efficiency (EE) of bromelain in liposomes which depended on preparation methods and centrifugal forces

| Method | Centrifugal force | EE (%) |
|---|---|---|
| Sonication | 12,000 × g | 20.9 |
| | 30,000 × g | 21.5 |
| | 80,000 × g | 23.0 |
| French Press | 12,000 × g | 19.1 |
| | 30,000 × g | 18.3 |
| | 80,000 × g | 17.9 |

*It was carried out at the standard conditions (bromelain/PC = 0.4, a PBS solution of pH 4.6).

press method.

### Effect of pH and bromelain/PC ratio in the preparation of liposomes

To rehydrate the dried PC-bromelain mixture, aqueous solution was added. Table 2 shows EE for bromelain-loaded DR liposomes under several conditions. The observed values of EE were 16.9% at pH 4.6 with 0.4 ratio of bromelain to phosphatidylcholine. This value of EE is lower than those reported by Kirby and Gregoriadis (10,11), in which 40.6% of added albumin was encapsulated from a similar protein/lipid ratio. Koide and Karel (6) also reported that 24.2% and 22.5% of lysozyme and pepsin were encapsulated, respectively.

Each enzyme requires its own optimization to obtain the highest EE values. Practical approaches for improvement of EE seem to lie in optimizing properties of the buffer, which is used for dissolving the enzyme and for suspending vesicles. In present experiments, pH was found to have effects on the EE values.

The effect of ratio of bromelain to PC was also investigated in this study (Table 2). The EE values increased as bromelain increased in amount to 1.0 ratio. If we could make an assumption, the amount of encapsulated enzyme depends only on the capacity of bilayers to accommodate enzyme and on the enzyme concentration in the internal aqueous phase of liposomes, the value of EE should have increased as added amount of enzyme increased. In this study, it was true within the ratios studied, however, it was not true in the other studies about lysozyme and pepsin (6). Koide and Karel (6) discussed these phenomena that excess enzyme weakened the contacts among empty vesicles and hence, decrease the chance for fusion of vesicles, thus, in turn, resulted in the smaller internal aqueous phase for encapsulation. From the economical point of view, the effect of the originally added enzyme quantity on the final value of EE is very important. It is thus appropriate at 0.6 ratio of bromelain to lipid in the commercial aspect. For the sustained release experiment, liposome loaded with bromelain was prepared with this ratio.

### Reversibility test

Since liposome is charged with a negative and lipid chain length of PC is heterogeneous, some of the added enzyme can be bound in the outside of liposomes (17). Bound enzyme is not in an encapsulated state, even though counted into EE.

**Table 2.** Encapsulation efficiency (EE) of bromelain in liposomes under several pHs and several ratio of bromelain to phosphatidylcholine.

| pH | Ratio of bromelain/phosphatidylcholine | | | | |
|---|---|---|---|---|---|
| | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
| 3.0 | 9.9[1] | 16.0 | 22.4 | 20.9 | 24.5 |
| 4.6 | 9.2 | 16.9 | 21.8 | 19.5 | 24.7 |
| 7.0 | 9.3 | 14.4 | 19.1 | 18.1 | 24.6 |
| 9.0 | 11.5 | 13.7 | 18.2 | 17.4 | 23.1 |

[1]Value means EE.

To know the actual EE, bound bromelain was detached from liposomes by a reversibility test. That is, after the encapsulation of bromelain in liposome, precipitated liposome pellets were resuspended in a PBS solution of pH 9.55 (the isoelectric point of bromelain) containing 0.5 M NaCl. It was reported (17) that almost all of the electrostatic interaction could be removed at the isoelectric point of protein with high ionic strength in the outside of liposome, thus bound enzymes could be detached reversibly.

Fig. 2 shows the actual EE after the reversibility test. As the same as Table 2, EE values increased with increasing bromelain concentration. However, about 5% of value was decreased in every case compared to Table 2. This means that 5% of bromelain was bound to the outside of the liposome, not encapsulated.

### Release of bromelain from liposomes

Many different changes can take place in liposomes with time. The phospholipids can undergo chemical and physical degradation in the membrane. Moreover, since the encapsulated enzyme is considered to be located in both the internal aqueous phase and in bilayers, not only the disruption of vesicles but also the perturbation of bilayers may lead to a release of the enzyme. Either as a result of these changes, or otherwise, liposomes maintained in aqueous suspension may aggregate, fuse, or leak their contents.

The release experiments were carried out depending on pH, temperature and time. Fig. 3 and Fig. 4 show the pattern of bromelain release at temperatures of 4°C, 20°C and 37°C at PBS solution of pH 5.0 and 7.0. Suspension incubated at pH 5.0 was released slowly till 5 days. However, after 5 days, the release of encapsulated bromelain was increased substantially through liposome membranes. Fig. 4 presents that encapsulated bromelain incubated at pH 7.0 was released gradually
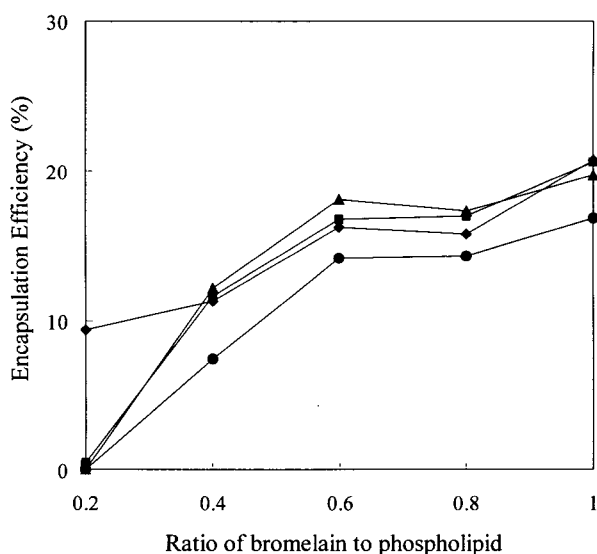


**Fig. 3.** Release of bromelain from bromelain-loaded liposomes during storage at pH 5.0. ●—●, 4°C ; ▲—▲, 20°C ; ■—■, 37°C.
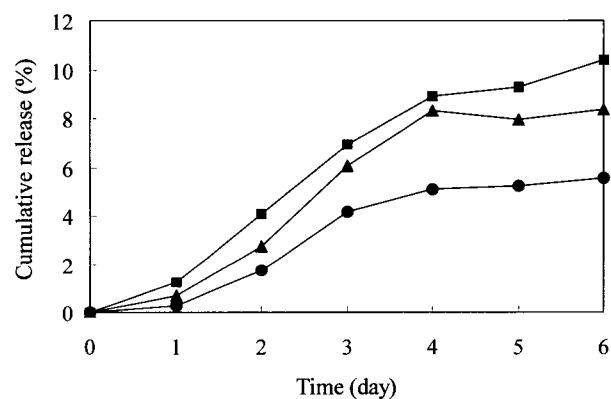


**Fig. 4.** Release of bromelain from bromelain-loaded liposomes during storage at pH 7.0. ●—●, pH 4°C ; ▲—▲, 20°C ; ■—■, 37°C.

from the initial incubation and increased the rate of release slightly more than that in pH 5.0. Lysozyme and pepsin (6) showed about 20% (pH 2.5) or 30% (pH 1.5) of pulse-like release, and at pH 4.2 or higher, only a small percentage (below 10%) of encapsulated enzyme released. Release was accelerated with treating cation or detergents (6). However, the release in the present study was not faster at acidic pH than at neutral pH. It means that pH does not have the same effect as the release of the encapsulated enzyme from liposome.

From the effect of a temperature, higher temperatures were induced to stimulate the release. At a different temperature, PC membranes can exist in different phases, and transitions from one phase to another can be detected by physical techniques as the temperature is increased. In general, release of encapsulated material increased with increasing temperature (5,6), furthermore, protection also was well done with decreasing the temperature (8,9,18).



**Fig. 2.** Actual encapsulation efficiency (EE) of bromelain-loaded liposomes after washing pH 9.55 PBS buffer containing 0.5 M NaCl. ●—●, pH 3.0 ; ▲—▲, pH 4.6 ; ■—■, pH 7.0 ; ◆—◆, pH 9.0.
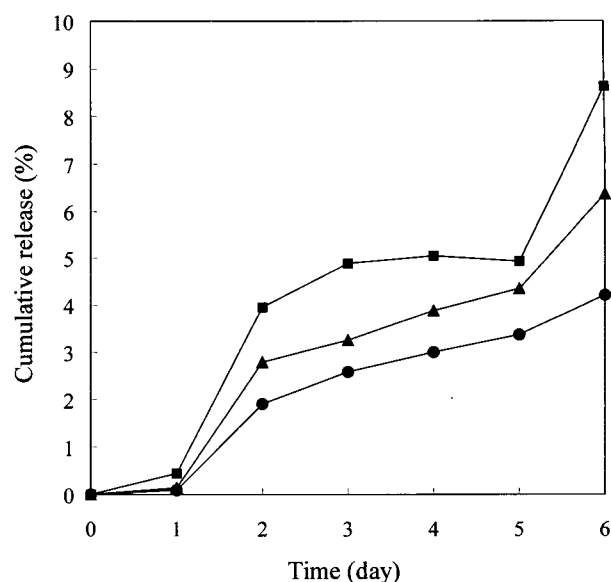
Liposome can protect a food ingredient in a specific environment and expand its shelf-life. Using the properties of a wall material, it also can control that of a core material in desired conditions. Karel and Langer (19) reported the needs and applications of the controlled release of enzyme in food, especially with respect to the protection of viable enzymes during food processing. The release of encapsulated enzyme can be controlled by selecting a carrier according to its stability properties. A low stability will lead to an early release in the food process, whereas a more stable one will postpone the release. The actual mode of release will be determined by both the properties of the carrier and the composition of food. Finally, controlled release needs to look at the basic principles to control the release of encapsulated materials and then consider which technologies can be applied in the food industry (3,20).

## ACKNOWLEDGEMENTS

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