Encapsulation of Chicken Egg Yolk Immunoglobulin G (IgY) by Liposomes

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Encapsulation of antibodies isolated from chicken egg yolk (IgY) in egg lecithin/cholesterol liposomes was attempted. IgY was successfully encapsulated into the liposomes by using the dehydration-rehydration method. Electron microscopic observation demonstrated that the liposomes prepared by this method were large multilamellar vesicles with a diameter of several µm. The encapsulation efficiency was improved by increasing the rehydration temperature to 60°C. The cholesterol/lecithin ratio also affected the efficiency, giving the highest value at a ratio of 1/4 (mol/mol). Some efflux of glucose through the liposomal membranes was observed, particularly for the liposome with a low cholesterol content, but that of IgY was not detected, irrespective of the cholesterol content. Encapsulation reduced the activity loss of the IgY antibodies under acidic conditions. IgY encapsulated in the liposomes was also markedly resistant to pepsin hydrolysis, which usually results in complete loss of activity with unencapsulated IgY, suggesting that liposomal encapsulation is an effective means for protecting IgY under gastric conditions.

Oral administration of antibacterial or antiviral antibody immunoglobulins through infant formulae or other diets is thought to be effective in preventing intestinal infection. The prevention of diarrhoeal diseases by the oral intake of immunoglobulins from immunized cow’s milk has been reported by many researchers.1)  

Chicken egg yolk has recently attracted considerable attention as an inexpensive source of antibodies,2) and the application of egg yolk immunoglobulin (called IgY) to antibody-containing diets has been considered.3) Passive protection of infant mice against murine4) or human5) rotavirus by egg yolk antibodies has already been attempted. Otake et al.7) have reported that protecting rats against dental caries could be achieved by using egg yolk antibodies raised against Str. mutans.

Although the effectiveness of oral administration of IgY is becoming evident, the stability of IgY in the gastrointestinal tract, especially in that of the adult human, is still a subject to be studied. Immunoglobulins are thought to be not very stable under gastric conditions. The exposure of immunoglobulins to acidic solutions at pH 3 or lower will cause denaturation of the protein molecules and reduce the antibody activity.8) Pepsin digestion will also reduce the antibody activity. Our previous studies indicated that the stability of IgY under acidic conditions and toward pepsin digestion was slightly lower than that of bovine IgG.3) On the other hand, IgY has been shown to be fairly stable against digestion by such intestinal proteases as trypsin and chymotrypsin.3) If the stability of IgY to acids and pepsin can be improved by a contrived processing method, the efficiency of protection against infection by IgY-containing diets will increase.

The increased stability of proteins by modifying with polysaccharides or synthetic polymers has been reported. For example, Suzuki et al.9) have reported that polyethylene glycol-modification stabilized human IgG against heating. However, this modification was performed for pharmaceutical use and is not feasible for food processing. The encapsulation of IgY in a natural or biosynthetic material may be an alternative and more feasible solution for food processing.10) In this study, the encapsulation of IgY antibodies in liposomes was attempted to stabilize IgY from peptic hydrolysis under acidic conditions.

Materials and Methods

Materials. Egg yolk immunoglobulin (IgY), which were prepared and purified as described previously,11) were presented by the Central Research Laboratories of Taiyo Kagaku Co. Biotin-conjugated anti-IgY rabbit antibody and streptavidin-horseradish peroxidase conjugate were from Zymed Laboratories. The antisera against chicken IgG (IgY) was purchased from Cappel, while egg yolk lecithin (P-9671) and pepsin (P-6887) were from Sigma Chemical Co. The other reagents were all of analytical grade.

Preparation of liposomes. The dehydration-rehydration method12,13) was used to prepare IgY-encapsulated liposomes. An egg lecithin/cholesterol mixture (100-300 µmol) in chloroform was dried under reduced pressure in a rotary evaporator to leave a thin film on the wall of a 100 ml volume round-bottomed flask. Distilled water (2 ml) was added to the flask, and the mixture was agitated with glass beads at 60°C for 5 min. After standing at room temperature for 10 min, 2 ml of an IgY solution (5 mg/ml of PBS) was added to the suspension and thoroughly mixed. The suspension was then freeze-dried. The freeze-dried material was exposed to humidified nitrogen and then rehydrated by adding 2 ml of water, with subsequent incubation at room or higher temperature for 10 min. The liposomes prepared by this method were precipitated by centrifugation at 30,000 × g for 30 min at 5°C. The content of IgY in the supernatant was determined by the method of Lowry et al.14) or by the enzyme-linked immunosorbent assay described next. The encapsulation efficiency was calculated by the following equation:

Encapsulation efficiency (%)=(weight of added IgY-weight of unencapsulated IgY)/weight of added IgY)×100.

Determination of antibody activity. To the liposomal suspension was added a Triton X-100 solution to give a final detergent concentration of 3%. After incubating at room temperature for 30 min, the mixture was diluted 100-200 times with PBS containing 0.05% Tween 20 (PBS-Tw).

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and served for the enzyme-linked immunosorbent assay (ELISA), which was performed by using the avidin–biotin system. One hundred μl of a 0.01% antigen (bovine serum albumin) solution was added to the wells of a polystyrene microtiter plate (Corning) and incubated at 4°C overnight. After removing this solution, the wells were washed three times with PBS-Tw. Then, 100 μl of an antibody (IgY) solution was added and incubated at room temperature for 2 h. The plate was washed with PBS-Tw and biotin-labelled rabbit anti-IgY antibody dissolved in PBS-Tw was added. After incubating for 2 h, the plate was washed with PBS-Tw prior to adding streptavidin-peroxidase dissolved in PBS-Tw. After incubating for 2 h and then washing, 100 μl of an o-phenylenediamine solution was added to each well. After further incubating at room temperature for 30 min, the reaction was stopped by adding 20 μl of 0N sulfuric acid, and the absorbance of each well was measured at 492 nm.

Acid- and peptin-treatment of liposomes. Liposomes were precipitated by centrifugation at 30,000 × g, and the precipitate was resuspended in PBS. The suspension was then centrifuged again to precipitate the liposomes. Washed liposomes were obtained by repeating this step 3 times total. The washed liposomes containing approximately 1.5 mg of IgY were suspended in 1 ml of PBS acidified with HCl to give a final pH for the mixture of 2.8 or 1.8, and the suspension was incubated at 37°C for 1 to 3 h. In some experiments, peptin was added to this suspension to give a final enzyme concentration of 15 μg/ml. Then, 0.2 ml of the suspension was mixed with 0.6 ml of a PBS solution (pH adjusted to 9.6 with NaOH) containing 4% Triton X-100 to neutralize the solution and to disrupt the lipid membranes. After incubating for 30 min, the mixture was appropriately diluted with PBS-Tw, and the antibody activity was measured by ELISA.

Permeability of the liposomal membrane. Glucose was encapsulated in the liposomes with or without IgY. The concentration of the glucose solution used for encapsulation was 20 mg/ml, and the liposomes were washed twice with PBS. The liposomes were incubated at 37°C for up to 72 h and then centrifuged at 30,000 × g. The glucose concentration of the supernatant was measured by a glucose-test kit (Wako Pure Chemicals), and the IgY concentration of the supernatant was measured by the procedure of Lowry et al.

Agglutination of liposomes. The washed liposomes prepared as just described were suspended in PBS. This suspension was mixed with an appropriately diluted anti-IgY antiserum on a glass microtiter plate. After incubating at room temperature for 1 h, agglutination of the liposomes was observed under a microscope (Olympus BH-2).

Electron microscopy. The washed liposomes were precipitated by centrifugation and fixed for 1 day in a 20 mm phosphate buffer at pH 7.2 containing 1% osmium tetroxide. The fixed material was then dehydrated in ethanol and embedded in Spurr resin. Ultrathin sections were cut and stained in lead tartrate, and these sections were examined by a transmission electron microscope (Akashi LEM-2000).

Results

Encapsulation of IgY by liposomes

The encapsulation of IgY by liposomes was first attempted by such methods as vortex, sonication, and detergent-removal. The encapsulation efficiency of these methods, however, was 5% at the most (data not shown). On the other hand, more than 10% encapsulation efficiency was obtained by the dehydration-rehydration method in our preliminary experiment. To increase the amount of IgY encapsulated by this method, the optimum conditions for encapsulation were investigated.

Figure 1A shows the effect of lipid concentration on the encapsulation of IgY. By increasing the lipid content from 100 to 300 μmol/ml, the amount of encapsulated IgY was increased. The effect of temperature during the rehydration process on the encapsulation efficiency is shown in Fig. 1B. The efficiency was increased to 25% by incubating at 50°C, and to almost 30% at 60°C, indicating that incubating at a high temperature was effective for better encapsulation as described by Jizomoto et al.

The effect of the egg lecithin/cholesterol ratio on the encapsulation efficiency of IgY is shown in Fig. 2. By adding a small amount of cholesterol, the efficiency was markedly increased. However, the efficiency decreased again after increasing the cholesterol ratio to 0.5 or higher.

Permeability of the liposomal membrane

The efflux of encapsulated materials during incubation was monitored to evaluate the stability of the liposomes. A mixture of glucose and IgY was encapsulated into the liposomes, the efflux being shown in Table I. Glucose was slightly permeable through the liposomal membrane (about 0.8% of the encapsulated glucose had leaked after 1 h of incubation), but the permeability was reduced by

![Fig. 1. Effect of Lipid Content (A) and Rehydration Temperature (B) on the Encapsulation Efficiency.](image)

The egg lecithin/cholesterol ratio was 1:1 (mol/mol). The amount of lipid used for (B) was 300 μmol, and the rehydration temperature for (A) was 20°C.

![Fig. 2. Encapsulation of IgY in Liposomes of Different Egg Lecithin/Cholesterol Ratio.](image)

The amount of total lipid was 300 μmol, and the rehydration temperature was 60°C. Bars indicate the standard deviation (n = 4).

<table>
<thead>
<tr>
<th>Table I. Efflux of Encapsulated Materials from the Liposomes</th>
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<td>Liposome</td>
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<td></td>
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<tr>
<td>A</td>
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<td>B</td>
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* Liposome A contained 116 mg of glucose and 18 mg of IgY, and liposome B contained 158 mg of glucose and 13 mg of IgY. They were both suspended in 20 ml of PBS and incubated at 37°C. The concentrations of glucose and IgY in the supernatant were measured periodically. The initial rate of increase in the concentration of glucose or IgY in the supernatant during incubation is used as the efflux rate.
adding cholesterol to the liposomes. No permeation of IgY through the liposomal membrane was observed, even after incubating for 72 h. IgY coexisting in the liposomes did not affect the permeation rate of glucose (data not shown).

Resistance of liposome-encapsulated IgY to acids and pepsin

Liposomes were incubated in an acidic solution in the presence or absence of pepsin, and the antibody activity of IgY encapsulated in the liposomes was measured. Figure 3 shows the effect of the lecithin/cholesterol ratio on the activity of IgY in the liposomes incubated at pH 2.8 for 1 h in the absence of pepsin. The stability of IgY in an acidic solution was improved by encapsulating in liposomes, and the remaining activity was increased by increasing the cholesterol content. Figure 4 shows the results of a similar experiment carried out at pH 1.8 and for 3 h. The reduction in activity was greatest in this experiment because of the longer incubation period in a more acidic solution. However, the activity of IgY encapsulated in liposomes, particularly that in the liposomes with higher cholesterol content, remained to a large extent.

When pepsin was added to the reaction mixture, unencapsulated IgY was rapidly hydrolyzed, the antibody activity being almost completely lost during incubation (Table II). By being encapsulated in the liposomes, however, IgY was protected from pepsin hydrolysis. These results suggest that liposomal encapsulation is an effective means for stabilizing IgY under gastric conditions.

Localization of IgY in liposomes

Figure 5 shows electron microscopic pictures of IgY-entrapped liposomes prepared by the dehydration-rehydration method. Multilamellar lipid vesicles with a size of several μm can be seen. IgY is considered to have been present in the inner aqueous phase or adsorbed to the lipid membrane surfaces.

When the liposomes were incubated with an anti-IgY antiserum, the liposomes agglutinated as shown in Fig. 6, suggesting that some portion of IgY was adsorbed to or incorporated into the outer surface of the liposomes. This also suggests that IgY was adsorbed to the surface of any lipid membranes inside the multilamellar vesicles.

Discussion

Liposome technology has been developed for pharmacological research because liposomes are believed to provide a good delivery system for therapeutic agents to tissues. In most cases, liposomes are administered via systemic injection. The stability of liposomes in the blood stream, and the interaction of liposomes with plasma and its components have been investigated by many researchers.

The effectiveness of orally administrating drug-containing liposomes has also been discussed by several researchers, and the reduction of blood sugar level in diabetic rats by orally administrating liposome-entrapped insulin has been reported. Rowland and Woodley observed that horseradish peroxidase, which is susceptible to degradation in the intestine, was absorbed to the intestinal sacs without degradation when added in a liposome-entrapped form. Matsuura et al. have reported that β-galactosidase encapsulated in liposomes was stable under acidic conditions. These studies suggest that liposomal encapsulation is effective for protecting proteins in the gastrointestinal tract. If IgY antibodies can be efficiently encapsulated in liposomes by a simple preparation method, liposome-encapsulated IgY will act more effectively in preventing intestinal infection.

<table>
<thead>
<tr>
<th>IgY</th>
<th>Relative antibody activity of IgY after incubating with pepsin</th>
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<tr>
<td></td>
<td>At pH 2.8</td>
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<tr>
<td>Unencapsulated</td>
<td>0.007 ± 0.005</td>
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<tr>
<td>(0.539 ± 0.061)</td>
<td>(0.032 ± 0.035)</td>
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<tr>
<td>Encapsulated in liposomes (1:0)</td>
<td>0.605 ± 0.037</td>
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<tr>
<td>Encapsulated in liposomes (1:1)</td>
<td>0.624 ± 0.040</td>
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<tr>
<td>(0.739 ± 0.021)</td>
<td>(0.385 ± 0.061)</td>
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<tr>
<td>(0.803 ± 0.132)</td>
<td>(0.359 ± 0.054)</td>
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* The antibody activity is expressed as the relative ELISA value (the ELISA value for native IgY is taken as 1.0). Each value is an average of 3 replications ± standard deviation. The values in parentheses mean the relative activity of IgY in the absence of pepsin.

b Egg lecithin/cholesterol ratio.
Fig. 5. Electron Microscopic Pictures of IgY-containing Liposomes Prepared by the Dehydration–Rehydration Method. The egg lecithin/cholesterol ratio was 1:1 (mol/mol).

Fig. 6. Agglutination of IgY-containing Liposomes Induced by Adding Anti-IgY antiserum. The lecithin/cholesterol ratio was 1:0 (A) and 1:1 (B). The liposomes were incubated with (+Ab) or without (−Ab) anti-IgY antiserum for 60 min at room temperature.

There are many reports regarding the entrapment of antibodies by liposomes. Most of these studies, however, have aimed to prepare "immunoliposomes," which have specific antibodies coupled to the outer surface of the liposomes. This coupling is, in most cases, performed through the covalent linkages between the immunoglobulin Fc portion and lipid molecules. An immunoliposome is thought to be useful for the immunospecific delivery of drugs or other materials to the antigenic target cells. On the other hand, attempts to encapsulate immunoglobulins inside the liposome vesicles have hardly been reported. Jackson et al. have recently reported the use of liposomes containing anti-idiotypic antibodies for effective oral vaccination. However, the encapsulation efficiency of IgG in the liposomes was not taken into consideration in their study. In the present study, the encapsulation of IgY by
liposomes with high efficiency was attempted.

The dehydration–rehydration method was effective for encapsulating IgY. The high encapsulation efficiency by this method is thought to be due to the concentration of liposomes and IgY by ice nucleation during the freezing process.\(^\text{18}\) Destabilization of the liposomes during this step may also facilitate the incorporation of IgY into the liposomes. Jizomoto et al.\(^\text{18}\) have reported that the resealing process of the destabilized liposomes during rehydration was important for high retention of the encapsulated materials. They also suggested that the resealing process may proceed easily when the lipid bilayer membrane is in a liquid state above the phase transition temperature. The greater encapsulation efficiency at higher rehydration temperatures observed in the present study (Fig. 1) may be ascribable to successful annealing and resealing of the lecithin membranes at such temperatures. Since IgY is stable under pasteurization conditions (62°C for 30 min),\(^\text{3}\) the rehydration–dehydration method operated at 50–60°C is thought to be useful.

IgY molecules inside the liposomes were more stable against acids than were free IgY molecules, although this protective effect was moderate when the cholesterol content was low. This suggests that the liposomal membrane can act as a barrier against hydrogen ions, particularly with a high content of cholesterol, although the barrier function against such small molecular substances as hydrogen ions was not perfect. This finding is supported by the results showing permeability of glucose through the liposomal membrane according to cholesterol content (Table 1). On the other hand, liposomal encapsulation showed a marked effect on the protection of IgY from peptic hydrolysis, indicating that such a high-molecular-weight substance as pepsin cannot pass through the membrane, irrespective of the cholesterol content. These results demonstrate that the liposomal encapsulation of IgY by the dehydration–rehydration method is an effective means for protecting orally administered IgY from gastric conditions, and particularly from peptic hydrolysis. Since the liposomal membrane prepared from egg yolk lecithin is known to be degraded by bile salt,\(^\text{2,25}\) the IgY antibody will be released from the liposomes in the bile salt-rich intestinal tract. IgY, which is fairly stable against digestion by such intestinal enzymes as trypsin and chymotrypsin,\(^\text{3}\) can then be expected to act as a defensive factor in the intestine against infection by pathogenic bacteria or viruses.

It has, so far, been difficult to decide the best lecithin/cholesterol ratio in IgY-encapsulated liposomes for oral use. Liposomes with lower cholesterol content, however, would be more favorable. In spite of the lower protective effect against the acid denaturation of IgY, liposomes with a lower cholesterol content gave much higher encapsulation efficiency (Fig. 2) and showed a marked protective function against pepsin (Table II). Eliminating cholesterol from food is generally thought to be acceptable, and we are now planning to examine the effectiveness of the liposome-encapsulated antibodies against gastrointestinal infection in vivo, using liposomes with varying cholesterol content.

Electron microscopic pictures indicate that the liposomes prepared by the method used in this study were large multilamellar membrane vesicles. The presence of multiple lipid membranes may interrupt the influx of hydrogen ions and protect the encapsulated IgY from the outer acidic conditions. By an agglutination test, the adsorption of IgY on the outer surface of liposomes was suggested. Matsuoka et al.\(^\text{21}\) have reported that 30–50% of the total liposome-entrapped β-galactosidase (on the basis of the enzyme activity) was adsorbed to the outer surface of the vesicles, and that the surface enzymes completely lost their activity when the liposomes were incubated under acidic conditions. In the present study, we were unable to estimate the amount of the surface IgY in our liposomes. However, some portions of the IgY molecules are likely to have been adsorbed to the inner and outer membrane surfaces. Since adsorption to lipid membranes may alter the properties of IgY, the mode of interaction between IgY and liposomal membranes is an important subject for future study.

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References