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Chitosan–Alginate Microcapsules for Oral Delivery of Egg Yolk Immunoglobulin (IgY)

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Chitosan–alginate microcapsules were evaluated as a method of oral delivery of IgY antibodies. Physical characteristics, encapsulation efficiency (EE%), the loading capacity for IgY (IgY loading percentage, %, w/w of microcapsules), gastro-resistance, and release characteristics of these microcapsules in vitro under varying pH were investigated. Optimum physical factors were established for preparation of homogeneous, spherical, and smooth microcapsules. IgY loading% was not significantly altered by pH of the encapsulation medium. Encapsulation efficiency was highest (73.93%) at a pH of 3.5, above which EE% decreased significantly ($p < 0.05$). IgY was released from microcapsules upon exposure to simulated intestinal fluid (SIF, pH 6.8), and decreasing pH increased significantly IgY release ($p < 0.05$). The stability of IgY in simulated gastric fluid (SGF, pH 1.2) was greatly improved by encapsulation in chitosan–alginate microcapsules, and the residual activity was not affected by pH of the encapsulation medium. Moreover, microencapsulated IgY was significantly resistant to pepsin hydrolysis. This approach may enable intact IgY to reach target microorganisms within the lower digestive tract.

KEYWORDS: Alginate; chitosan; egg yolk immunoglobulin (IgY); gastro-resistance; pH of the encapsulation medium

INTRODUCTION

Chicken egg yolk immunoglobulin, referred to as immunoglobulin Y (IgY), has recently attracted considerable attention, as it possesses a large number of advantages compared with mammalian IgG including cost-effectiveness, convenience, and high yield (*1*). Oral administration of specific IgY antibodies has been proven effective against a variety of intestinal pathogens, such as bovine and human rotaviruses, enterotoxigenic *Escherichia coli* (ETEC), bovine coronavirus, *Salmonella* spp., *Edwardsiella tarda*, *Yersinia ruckeri*, *Staphylococcus*, and *Pseudomonas* (2). Therefore, IgY is a promising alternative to antibiotics for passive immunotherapy application.

However, the activity of orally administered IgY may be reduced rapidly, even destroyed completely, under gastric conditions since IgY is sensitive to pepsin and low pH (*3*). On the other hand, IgY is found to be fairly stable against the intestinal proteases digestion (*3*, *4*). Since the primary target site of IgY is in the small intestine, it is necessary to find an effective method to protect IgY against peptic digestion and acidity in the stomach.

The microencapsulation of IgY using liposomes (*5*) and multiple emulsions (*6*, *7*) has been previously described. However, these approaches have limited effectiveness, in some cases destroying antibody activity by the encapsulation procedure itself. The macroencapsulation of IgY, using enteric-coated gelatin capsules, was found to significantly improve antibody stability (*8*). The microencapsulation of IgY using an anionic methacrylic acid copolymer may also be an effective method of protecting IgY from gastrointestinal inactivation (*9*).

Here, we reported on the microencapsulation of IgY using nature polysaccharides such as alginate and chitosan. These two materials have been used extensively as carrier materials in microencapsulation as they are nontoxic, biodegradable, and do not cause undesirable side effects (*10*, *11*). They are both polyelectrolytes and contain opposite charges: Sodium alginate contains a negative charge, while chitosan contains a positive charge. These characteristics enable these compounds to form polyelectrolyte complex membranes through electrostatic attraction (*12*–*14*). Moreover, microencapsulation in chitosan–alginate microcapsules is attractive because of the mild encapsulation conditions that allow preservation of the activity of

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biological macromolecules (*15*) and even that of cells (*16*, *17*). More recently, a great number of publications report that chitosan−alginate microcapsules have been successfully used to orally deliver a wide range of encapsulated materials including proteins (*18*−*20*), peptides (*21*), and drugs (*22*, *23*) past the stomach to intestinal sites.

The objective of this research was to evaluate chitosan−alginate microcapsules as a method of oral delivery of IgY antibodies. This study focused on the effect of encapsulation medium pH on the properties of IgY-loaded chitosan−alginate microcapsules (IgY-CAM). Physical characteristics, encapsulation efficiency (EE%), the loading capacity for IgY (expressed as the IgY loading percentage, %, w/w of microcapsules), gastric stability, and release characteristics of these microcapsules were investigated.

MATERIALS AND METHODS

Materials. A standard strain of ETEC C83903 (O141:K85, K88ab) was obtained from China Institute of Veterinary Drug Control (Beijing, China). Freund's adjuvant, bovine serum albumin (BSA), rabbit anti-chicken IgG conjugated with horseradish peroxidase (HRP), and pepsin were purchased from Sigma (St. Louis, MO). 3,3′,5,5′-Tetramethylbenzidine (TMB) was purchased from Biomol (Hamburg, Germany). Standard chicken IgY was purchased from Promega (Madison, WI). Molecular weight marker was purchased from Amersham Bioscience (Uppsala, Sweden). Sodium alginate (chemically pure grade, 1.05−1.15 × 10^3 cps) was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Chitosan was purchased from Dalian Xindie Chitin Co., Ltd (deacetylation degree ≥ 90%; viscosity ≥ 10 cps; Dalian, China).

Immunization of Chickens. k88 + ETEC strain was grown overnight in Luria−Bertani culture medium at 37 °C using a 1% inoculum from stocks. Cells were harvested by centrifugation at 8000*g* at 4 °C for 15 min, were washed with PBS (pH 7.4, 0.01 M), and were resuspended in PBS. The suspension was concentrated to approximately 10^{10} cfu/mL and was inactivated with 0.3% formol for 24 h at 37 °C.

Lohmann laying hens (16-weeks-old; $n = 15$) were immunized and used for egg production. The hens were initially injected with ETEC whole cells (10^{10} cfu/ mL) emulsified with an equal volume of complete Freund's adjuvant. Each hen was injected with 1 mL (first) dosage at five different sites (200 μL per site) including breast muscles (two sites per left or right) and neck hypodermia (one site). The second and third injections, with incomplete Freund's adjuvant, were given at 2-week intervals following the first injection. Each hen was injected with 1.5 mL (second injection) and 2 mL (third injection) dosage, respectively. Eggs were collected daily after the second booster injection and were stored at 4 °C before use.

Separation and Purification of IgY (*24*). IgY was purified from the water-soluble fraction by a combination of several purification techniques including salt precipitation (precipitation with 50% saturated ammonium sulfate followed by precipitating with 14% (w/v) sodium sulfate) and ultrafiltration using a Vivaflow 50 tangential flow ultrafilter (Vivascience, Hannover, Germany) with a 100 kDa cutoff membrane. The purity of IgY was monitored by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Lab-Image software. IgY powder was obtained with ultrafiltration followed by freeze-drying.

Preparation of Blank Chitosan−Alginate Microcapsules (BCAM) and Studies on Size and Morphology. BCAM was prepared by the one-step procedure described by Vandenberg et al. (*19*). Aqueous sodium alginate solution was prepared at 2% (w/v). Chitosan (1%, w/v) was dissolved in 1% (v/v) acetic acid with gentle warming at room temperature and was filtered through a nylon cloth to remove any undissolved particles. The encapsulation medium was prepared by diluting the chitosan stock solution to 0.2% (w/v) with 1.5% (w/v) (final concentration) $CaCl_2$ solution. The encapsulation pH was adjusted to 4.0 with 4 M NaOH. Sodium alginate solution (10 mL) was pumped at a rate (mL/min) through a needle (an outer diameter of 0.7 mm) with a coaxial air compressor and was extruded into 200 mL encapsulation medium under mechanical stirring for 30 min. The physical factors were tested in single factorial experiments and orthogonal design experiments, including coaxial air volume (0.2, 0.3, 0.4 m^3/h), mechanical stirring (50, 100, 200 rpm), pump rate (4, 6, 8 mL/min), and the distance between the needle and the encapsulation medium (6, 8, 10 cm). The particle size and the size distribution were measured by ImageJ 1.34s and analysis of each sample on digital photographs. The microcapsules obtained were filtered and rinsed with 50 mL of distilled water and were freeze-dried. The surface morphology of the microcapsules was examined using a scanning electron microscope (SEM) (JEM-1200Ex, JEOL Ltd., Tokyo, Japan). Samples were mounted on metal stubs, using double-sided adhesive tape, were gold coated under vacuum, and then were examined.

Preparation of IgY-Loaded Chitosan−Alginate Microcapsules (IgY-CAM) and Studies on Size and Morphology. IgY (purified describe above) was added into 2% (w/v) sodium alginate at 25% loading rate (w/w). The encapsulation medium was prepared as described above. The encapsulation pH was adjusted at a given value (3.0, 3.5, 4.0, 5.0, or 6.0) with 4 M NaOH. According to the optimum physical factors, sodium alginate/IgY solution (10 mL) was pumped at a rate of 4 mL/min through a needle (an outer diameter of 0.7 mm) with a coaxial air volume 0.3 m^3/h and the distance between the needle and the encapsulation medium at 8 cm and was extruded into 200 mL of encapsulation medium under mechanical stirring at 200 rpm. The following procedure was basically the same as that for BCAM formation.

IgY Loading Percentage and Encapsulation Efficiency (EE%). IgY content incorporated into microcapsules was assayed by dissolving dried IgY-CAM (10 mg) in 5 mL of a mixture of 0.2 M $NaHCO_3$ and 0.06 M $Na_3C_6H_5O_7 \cdot 2H_2O$ at pH 8.0 (*25*). Using BCAM as control, protein concentration was assayed by BCA protein assay reagent kit (Pierce Inc., New York, NY) in 96-well ELISA plates. IgY loading% was obtained by the equation

$$\text{IgY loading\%} = C_{\text{IgY}} \times V/W_{\text{IgY-CAM}} \times 100\% \quad (1)$$

where C_{IgY} is the concentration of IgY in sample, V is the volume of the sample, and $W_{\text{IgY-CAM}}$ is the weight of IgY-CAM used for IgY loading percentage determination.

Encapsulation efficiency was expressed as the percentage of total available IgY that actually is incorporated into microcapsules. The EE% of IgY-CAM was calculated from the total IgY amount initially and the IgY content of dried microcapsules (*22*).

In Vitro Stability of IgY to Simulated Gastric Conditions. The stability of IgY to gastric conditions was evaluated using simulated gastric fluid (SGF). Simulated gastric fluid consisted of 3.2 mg/mL pepsin in 0.03 M NaCl, at pH 1.2 (USP 27). The SGF was added to the IgY to give an enzyme-to-substrate ratio of 1:20 (*3*) and was incubated at 37 °C with shaking. At intervals of 0, 0.5, 1, 2, 3, and 4 h, non-encapsulated IgY was neutralized with 2 M Tris-HCl (pH 8.0). Microencapsulated IgY was measured for antibody activity after 2 h of incubation in SGF. The microcapsules were filtered, and IgY was released as described above. Antibody activity was defined as the ability of the anti-K88 + ETEC IgY to bind to K88 fimbriae and was determined by ELISA. Activities were expressed as a percent relative to an untreated positive control (100% activity). The intact IgY was visualized by SDS−PAGE.

In Vitro IgY Release (*20*, *26*). The release of IgY from the microcapsules was studied by incubating 50 mg of IgY-CAM in 50 mL of SGF without pepsin, at 37 °C, with shaking. After 2 h, the microcapsules were filtered and transferred to 50 mL of simulated intestinal fluid (SIF, 0.05 M KH_2PO_4, pH 6.8) without pancreatin (USP 27) and were incubated at 37 °C with shaking. At desired intervals of time, 200-μL aliquots were removed and replaced with the same amount of fresh medium. Protein concentration was assayed using BCA method as described above. The accumulative release percentage (Q%) was calculated by eq 2

$$Q\% = (C_n \times V + V_i \sum_{i=0}^{n-1} C_i)/(W_{\text{IgY-CAM}} \times \text{IgY loading\%}) \times 100\% \quad (2)$$

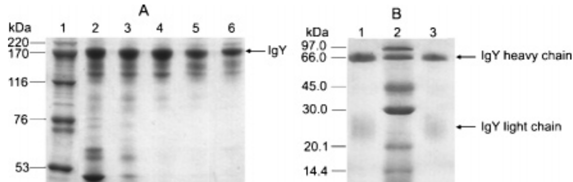

Figure 1. SDS–PAGE patterns of IgY at various phases of purification. (**A**) SDS–PAGE (nonreducing) on 7.5% gel. 1, high molecular weight marker; 2, water-soluble fraction; 3, IgY obtained after ammonium sulfate precipitation; 4, IgY obtained after salting precipitation; 5, final IgY obtained by ultrafiltration; 6, standard chicken IgY. (**B**) SDS–PAGE (reducing) on 15% gel. 1, final IgY obtained by ultrafiltration; 2, low molecular weight marker; 3, standard chicken IgY. On each lane, 10 μg of protein was applied.

where C_n is the sample concentration at T_n, V is the total volume of release medium, V_i is the sampling volume at T_i, and C_i is the sample concentration at T_i (both V_0 and C_0 were equal to zero).

Determination of IgY Acitivity (*9, 27*). The activity of IgY in samples was tested by indirect ELISA. Ninety-six-well ELISA plates were coated by adding 100 μL of K88 fimbrial antigen (0.3 μg/well in 0.05 M bicarbonate buffer, pH 9.6) and were incubated overnight at 4 °C. The plate was washed three times with PBS (pH 7.4, 0.01 M) containing 0.05% Tween 20 (PBST) and was blocked with 100 μL/well of PBS containing 1% (w/v) BSA, at 37 °C for 2 h. After three times rinsing with PBST, samples (100 μL) in which the IgY concentration was adjusted to 1 μg/mL were added to each well and were incubated at 37 °C for 2 h. The plate was washed again, and 100 μL/well of rabbit antichicken IgG conjugated with HRP (1:30 000) was added, and the plate was incubated at 37 °C for 2 h. The plate was washed five times with PBST, and 100 μL of TMB substrate solution (2 mg/mL TMB in buffer, pH 5.0) was added to each well. The plate was incubated at room temperature to allow chromophore development, after which the reaction was stopped by the addition of 50 μL of 2 M H_2SO_4 to each well. The optical density of the wells was determined at 450/630 nm with a plate reader (model Sunrise, Tecan Austria GmbH, Grödig/Salzburg, Austria).

SDS–PAGE. SDS–PAGE was done under nonreducing or reducing conditions on Pharmacia Phast System using a gradient PhastGel and Coomassie brilliant blue staining according to the manufacturer's (Liuyi Instrument Factory, Beijing, China) recommendations.

Statistical Analysis. Statistical analysis was performed using SPSS 11.5 for Windows. All values are expressed as their mean ± standard deviation (SD) and levels of significance were evaluated using one-way ANOVA with the Student–Neuman–Keuls (SNK) test for multiple comparisons. The differences were considered significant at the level of $p < 0.05$.

RESULTS

Separation and Purification of IgY. Molecular weight of IgY is about 180 kDa and is composed of two subunits, a heavy chain of 60–70 kDa and a light chain of 22–30 kDa, as visualized in **Figure 1**. The purity of the IgY finally purified was over 82% as determined by SDS–PAGE and Lab-Image software.

Studies on BCAM Size and Morphology. According to the optimal formulation obtained from single factorial tests and orthogonal design experiments, smooth, spherical, and homogeneous chitosan–alginate microcapsules with a narrow size distribution were prepared (**Figure 2A**,**B**). The mean diameter of BCAM was approximately 1000 μm. However, upon freeze-drying, the spherical structure of microcapsules was disrupted as visualized using SEM (**Figure 2C**,**D**). SEM micrographs of fine surface structures of freeze-dried BCAM formed at various pH values are shown in **Figure 3** (**1A–1D**). It appeared that the surface structure of BCAM was altered under varying

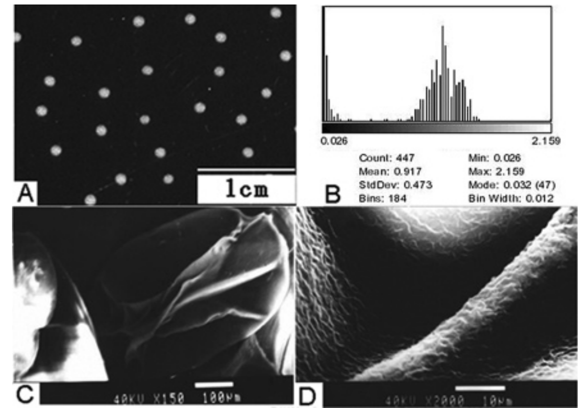

Figure 2. Morphology and size of BCAM formed at pH 4.0. (**A**) Morphology of wet BCAM microcapsules observed by Nikon digital camera. (**B**) The size distribution of wet BCAM microcapsules measured by ImageJ (the *x*-axis represents the possible gray values and the *y*-axis shows the number of pixels found for each gray value in the selected image). (**C**) SEM micrograph of freeze-dried BCAM. (**D**) SEM micrograph of surface structure of freeze-dried BCAM.

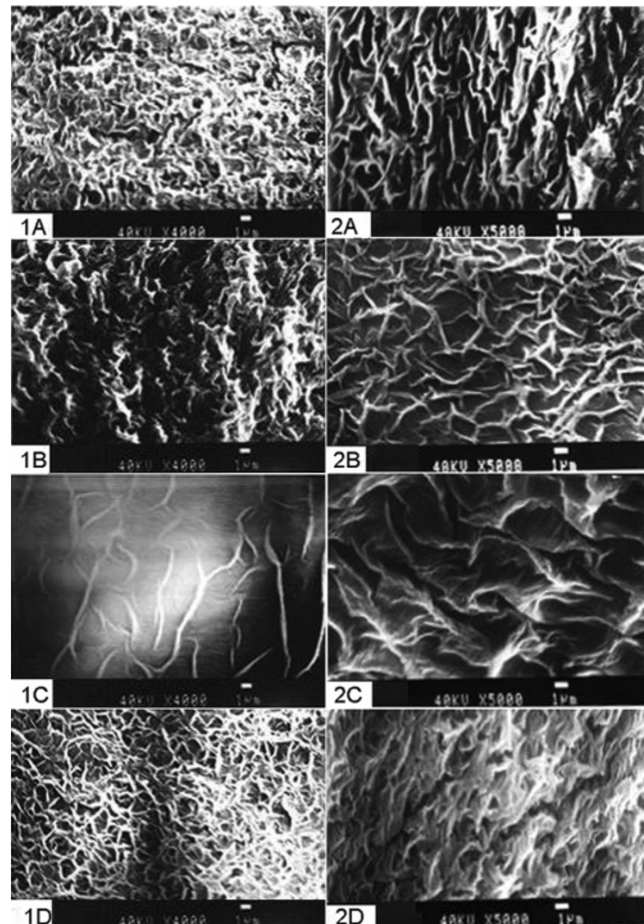

Figure 3. SEM micrographs of fine surface structures of freeze-dried chitosan–alginate microcapsules (**1**, BCAM; **2**, IgY-CAM) formed at different pH values (**A**, 3.0; **B**, 4.0; **C**, 5.0; **D**, 6.0).

encapsulation pH. Disruption of the outer surface of microcapsules formed at some pH values (i.e., 3.0, 4.0, and 6.0) was apparent, where micropores and wrinkles were visible. In the case of pH 5.0, the membrane is denser than that formed at any other pH, which showed a smooth and wrinkled surface. These surface changes could be explained by the variation of the

Table 1. Effect of Encapsulation Medium pH on IgY Loading% and EE%[a]

pH	IgY loading%	EE%
3.0	18.52 ± 0.65 a	65.44 ± 0.90 c
3.5	19.69 ± 0.94 a	73.93 ± 0.86 a
4.0	18.39 ± 0.60 a	68.06 ± 1.71 b
5.0	16.72 ± 1.84 a	60.50 ± 1.05 d
6.0	17.52 ± 1.18 a	61.04 ± 0.77 d

[a] Data are presented as mean ± SD ($n = 3$). Means in a column without the same letter are significantly different ($p < 0.05$).

encapsulation pH which affected the degree of ionization of chitosan and alginate during microcapsule formation.

Studies on IgY-CAM Size and Morphology. After loading with IgY, the size distribution of the microcapsules retained was unchanged as compared to BCAM. SEM micrographs of fine surface structures of IgY-CAM formed at various pH values are shown in **Figure 3** (**2A−2D**). It was observed that the presence of IgY greatly altered the surface of chitosan−alginate microcapsules as compared to corresponding BCAM. This may be due to the loss of encapsulated IgY during microcapsule formation.

IgY Loading Percentage and Encapsulation Efficiency (EE%). Table 1 demonstrates the effect of encapsulation medium pH on IgY loading% and EE%. Increasing pH (from pH 3 to 6) did not affect IgY loading%, which was observed to be 16.72−19.69%. It did affect encapsulation efficiency ($p < 0.05$). Encapsulation efficiency was highest (73.93%) at a pH of 3.5, above which EE% decreased significantly. Moreover, qualitative observations during microcapsule formation revealed that at lower pH values (i.e., 3 and 3.5), microcapsules tended to aggregate and were opaque rather than translucent, as was observed at higher pH values, which agree well with the findings of Vandenberg et al. (*19*).

In Vitro Stability of IgY to Simulated Gastric Conditions. The effect of encapsulation medium pH on the stability of the microencapsulated IgY in SGF was assessed by measuring IgY activity after 2 h of incubation in SGF. Non-encapsulated IgY was rapidly hydrolyzed, with the antibody activity being almost completely lost after 1 h in SGF (**Figure 4A**). The stability of IgY in SGF was improved by encapsulation in chitosan−alginate microcapsules, and the residual activity after incubation in SGF was unaffected by pH of the encapsulation medium. Microencapsulated IgY retained 61.36−74.61% activity after 2 h in SGF under varying pH (from pH 3 to 6) (**Figure 4B**). Intact IgY was readily visualized by SDS−PAGE as a band with an approximate molecular weight of 220 kDa in all samples (**Figure 5A**). To investigate the reason why the increase in molecular weight of target protein occurred, the intact IgY corresponding to IgY-CAM before exposure to SGF was visualized by SDS−PAGE. It can be seen as a band around 220 kDa in the samples from lower encapsulation pH values (i.e., 3.0 and 3.5) and 180 kDa in the samples from higher pH values (i.e., ≥4.0) (**Figure 5B**).

In Vitro IgY Release. The release characteristics of IgY-CAM formed at various pH values were investigated by incubating the encapsulated IgY in SGF and subsequently in SIF. The accumulative release percent of IgY is depicted in **Figure 6**. Over the 2-h SGF incubation, release of IgY from all different formulations was negligible. After transfer to SIF, IgY-CAM began to disintegrate and had a sustained release following a burst effect. IgY release from chitosan−alginate microcapsules was significantly affected by pH of the encapsulation medium ($p < 0.05$), and the release rate increased as pH decreased. At a low encapsulation pH (i.e., 3.0), IgY release was accelerated as the encapsulation pH declined with a release of almost 90% of the IgY after 1-h incubation. In the case of pH 5.0, microcapsules showed a minimum release rate of IgY, and only about 50% of the IgY was released within 6 h in SIF.

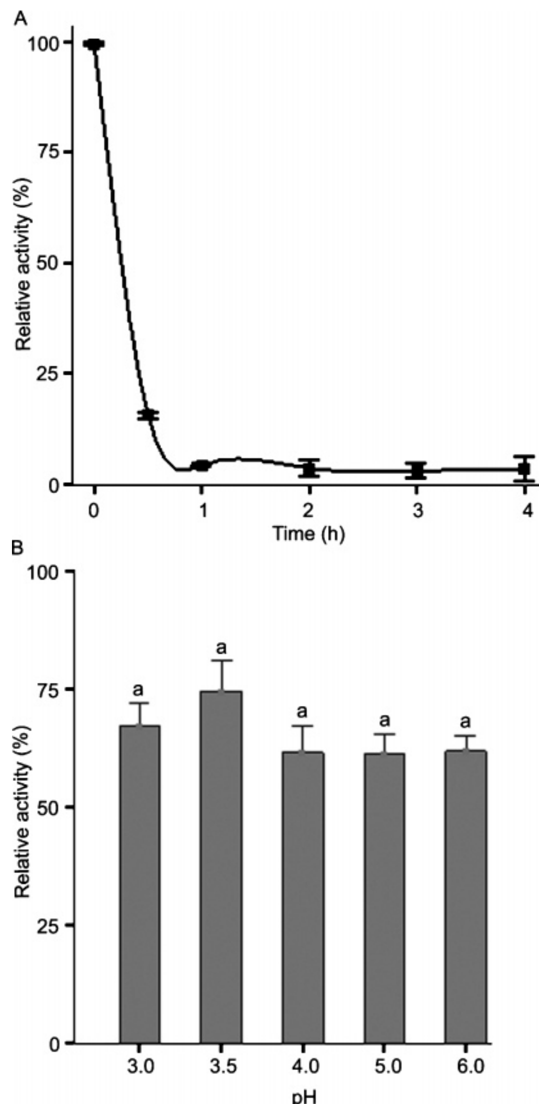

Figure 4. In vitro stability of nonencapsulated (**A**) and microencapsulated IgY (**B**) to simulated gastric conditions. The retaining IgY activity was measured by ELISA and was expressed as % activity relative to an untreated sample. Data are presented as mean ± SD ($n = 3$). (**A**) Nonencapsulated IgY activity retaining over 4 h incubation in SGF. (**B**) Effect of encapsulation medium pH on microcapsules protecting IgY from gastric inactivation. Samples were incubated in SGF for 2 h. Means without the same letter are significantly different ($p < 0.05$).

DISCUSSION

Oral administration of IgY antibodies is an effective means of treating and preventing enteric infections of both viral and bacterial origin (*2*). However, inactivation of IgY with the stomach does occur and may reduce the therapeutic value of IgY antibodies. For IgY, a bioactive protein of interest, an ideal oral delivery system would be characterized as having a high EE%, protecting IgY from gastric inactivation, and providing negligible release in the stomach and rapid, sustain release in

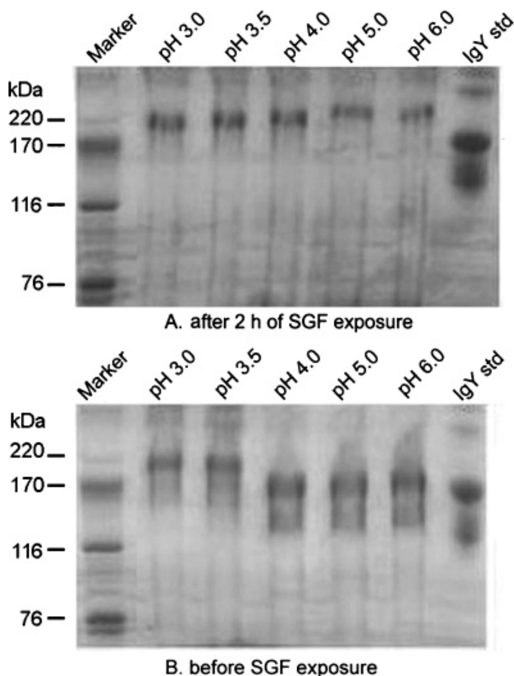

Figure 5. Intact IgY released from IgY-CAM formed at various pH values was visualized by SDS–PAGE (nonreducing, 7.5% gel). On each lane, 10 μg of protein was applied.

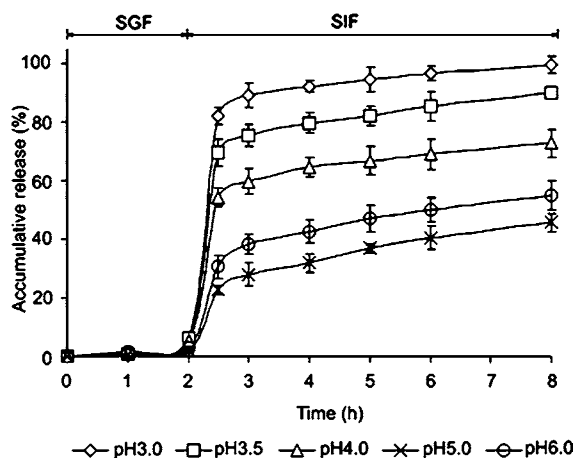

Figure 6. Effect of encapsulation medium pH on IgY release from IgY-CAM; samples were first incubated in SGF and then were transferred to SIF. Data are presented as mean ± SD ($n = 3$).

the small intestine. In the present study, chitosan−alginate microcapsules were evaluated as a method of oral delivery of IgY antibodies.

With the extrusion of alginate into a chitosan solution containing a suitable divalent cation like Ca^{2+}, the interphasic membrane is formed by complexation between two polyelectrolytes of opposite charge through electrostatic interactions. The rate of diffusion of calcium ion toward the alginate core is more rapid than that of chitosan because calcium ion is of low molecular weight and forms a gel core. Subsequently, a microcapsule of a calcium−alginate gel core coated with chitosan−alginate interphasic membrane is produced (*28*).

The disintegration of chitosan−alginate microcapsules is pH-dependent (*20*, *23*). In acid medium, microcapsule matrix material remains intact as a result of the ionic bonds in the microcapsules. Once the microcapsules are exposed to neutral pH, the anionic alginate in the Ca−alginate−chitosan complex can be displaced by hydroxyl ions. Even more important, the chitosan will lose its positive charge. Therefore, the complex disintegrates, the matrix erodes, and the encapsulated material is released in the surrounding fluid. This is in agreement with the observation of the present work where microcapsules remained intact with negligible release of IgY in SGF, while upon transferring to SIF, the microcapsules started to disintegrate.

A simulated gastric solution was used to assess the stability of miceoencapsulated IgY in vitro. It was known from the results that after 2 h of SGF exposure, IgY activity remaining in microencapsulated samples was not affected by the encapsulation pH. Similar to previous reports (*3*), the non-encapsulated IgY was extremely sensitive to gastric conditions, and the antibody activity was rapidly lost. Microencapsulated IgY retained the majority of its activity after 2 h in SGF. This suggests that the chitosan−alginate interphasic membrane can act as a barrier against hydrogen ions. Moreover, chitosan−alginate microcapsules showed a significant effect on the protection of IgY from peptic hydrolysis. This finding is supported by **Figure 5A**. However, it was interesting to find that at lower encapsulation pH values (i.e., 3.0 and 3.5), molecular weight of the corresponding target protein remained unchanged (around 220 kDa) compared with before exposure to SGF. On the other hand, at higher encapsulation pH values (i.e., ≥4.0), molecular weight of the corresponding target protein increased from 180 kDa (before exposure to SGF) to around 220 kDa. These changes in molecular weight of target protein could be attributed to possible association of other unrecognized factors with IgY. In this study, a mixture solution of $NaHCO_3$ and $Na_3C_6H_5O_7{\cdot}2H_2O$ (pH 8.0) was used to completely release IgY from the microcapsules, which can disrupt the interphasic membrane and completely dissolve the fragments of the microcapsules in a short time (*25*). IgY has an isoelectric point (pI) of 5.7−7.6 (*29*), which is negatively charged at pH 8.0. It can be speculated that when IgY-CAM formed at lower pH values (i.e., 3.0 and 3.5) were dissolved directly in the mixture solution, positive sites on chitosan ($pK_a = 6.3$) in sufficient numbers could interact with negative IgY. For IgY-CAM formed at higher pH values (i.e., ≥4.0), there were probably no redundant positive sites on chitosan to induce interaction with IgY. Hence, it can be seen as a band around 180 kDa in **Figure 5B**. However, after exposure to SGF, the degree of ionization of chitosan (corresponding to IgY-CAM formed at higher pH values) increases; therefore, positive sites on chitosan could establish ionic interactions with IgY during the complete release of IgY. This could result in the increase in molecular weight of target protein.

Previous work has revealed that encapsulation medium pH plays an important role in regulating the release of encapsulated materials (*14*, *30*). Huguet et al. (*30*) reported that the hemoglobin release during the microcapsules storage in water was able to be controlled by the encapsulation pH. Similarly, Lee et al. (*14*) observed that the guaifensin release from microcapsules stored in saline depended on the encapsulation pH. In these reports, the release rates varied with the pH dominantly dependent on the compactness of the membrane because of the loop formation of backbone chains of polyelectrolytes. In the present study, in vitro release of IgY from chitosan−alginate microcapsules formed at various pH values was investigated under simulated gastrointestinal conditions. The results showed that encapsulation pH significantly influenced IgY release under simulated intestinal conditions. The release changes observed during SIF incubation, when microcapsules

were formed at various pH values, could be explained by the changes of the membrane compactness (*14*, *30*). The pK_a of alginate is 3.65 and 3.38 for guluronic and mannuronic acids, respectively (*31*). Thus, depending on the pH of the encapsulation medium, in which the sodium alginate/IgY solution is extruded, the degree of ionization of alginate at the microcapsule surface changes and increases with the increase of the encapsulation pH. In this pH range (between 3 and 5), the degree of ionization of chitosan does not change appreciably (*30*). In the case of pH 5.0, there is about 70−80% of the degree of ionization for both polyelectrolytes, and thus each polysaccharide may sustain the rigid and linear conformation to result in the formation of a dense membrane. Above pH 5.0, the degree of ionization of chitosan is suppressed and the chitosan may form some kinds of loops (*14*). This loop formation makes chitosan−alginate membranes less dense and increases the rate of release. Similarly, at lower encapsulation pH values (i.e., 3.0 and 3.5), chitosan is in full ionization form, while alginate at the surface of the microcapsules has a very low content of negative charges because of low ionization and cannot strongly interact with chitosan. The membrane thus formed is less dense and fragile since it is not stabilized by ionic interactions.

Moreover, the results showed that IgY-CAM formed at various pH values released IgY, with burst effect at 0.5 h in SIF, with a subsequent sustained release. This fact could be because freeze-drying has an importance on release behavior of microcapsules. Upon freeze-drying, the larger surface micropores would also contribute to an increased release rate. Similar results were reported previously for dextran and timolol maleate (*15*, *32*). The present study suggests that the lower pH encapsulation medium is beneficial to the release of IgY from IgY-CAM in SIF.

As expected, the present study demonstrates that chitosan−alginate microcapsules can protect IgY from gastric inactivation and can result in a controlled and sustained release of IgY in SIF. Chitosan−alginate microcapsules could, therefore, be an effective vehicle for the oral delivery of IgY. The encapsulation pH was found to have a significant effect on EE% of IgY-CAM as well as on the release of IgY during SIF incubation.

ABBREVIATIONS USED

BCAM, blank chitosan−alginate microcapsules; BSA, bovine serum albumin; EE%, encapsulation efficiency; ELISA, enzyme-linked immunosorbent assay; ETEC, enterotoxigenic *Escherichia coli*; HRP, horseradish peroxidase; IgY, egg yolk immunoglobulin; IgY-CAM, IgY-loaded chitosan−alginate microcapsules; IgY loading%, IgY loading percentage (w/w of microcapsules); PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing Tween-20; pI, isoelectric point; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEM, scanning electron microscope; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; TMB, 3,3′,5,5′-tetramethylbenzidine.

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