

## Influence of Chitosan-Alginate Microcapsules Containing Anti-*Vibrio Harveyi* IgY in the Gastrointestinal Tract Simulation

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### Abstract

Egg yolk immunoglobulin (IgY) is an alternative treatment for the prevention of gastrointestinal infections in pigs, cows, chickens and fish. In our previous report, we preliminarily proved that anti-*V. harveyi* IgY had effective potential to control luminous disease in black tiger shrimp. However, IgY activity may be reduced or destroyed by gastric conditions, particularly low pH and digestive enzymes. Therefore, it is necessary to find an effective method to preserve the therapeutic function of IgY antibodies during gastric passage. Chitosan-alginate microcapsules have been developed to protect IgY from gastric inactivation. The processing factors included different forms of chitosan and alginate, while a CaCl<sub>2</sub> concentration and encapsulation medium was investigated. The optimum results were obtained under the following conditions: High Mw chitosan concentration 0.2% (w/v), medium viscosity alginate concentration 2% (w/v), CaCl<sub>2</sub> concentration 0.5% (w/v). The stability of IgY in simulated gastric fluid (SGF, pH 1.2) was greatly improved by encapsulation in chitosan-alginate microcapsules, and retained greater than 90% activity after 2 h exposure to SGF. Less than 10% IgY was released upon the microcapsules' exposure to SGF for 2 h, and more than 80% IgY was released upon the microcapsules' exposure to simulated intestinal fluid (SIF, pH 6.8) for 16 h.

**Keywords:** chitosan-alginate, microencapsulation, *vibrio harveyi*, IgY

### 1. Introduction

The underlying principle of passive immunization is to employ antibodies taken from other animals to build long-term resistance to disease. This approach has become increasingly popular and its therapeutic properties may represent a viable alternative to other conventional techniques (Hatta, Ozeki, & Tsuda, 1997). There is evidence to suggest that immunoglobulin from the egg yolks of chickens may be particularly effective in the inhibition of infectious disease (Li-Chan, 1999). The benefits of such findings are especially attractive given that chickens are both inexpensive and easily produced on a large scale (Carlander, Kollberg, Wejaker, & Larsson, 2000). Testing has revealed that IgY has a high success rate against several enteric pathogens, including bovine and human rotaviruses, enterotoxigenic *Escherichia coli* (ETEC), *Edwardsiella tarda*, bovine coronavirus, *Yersinia ruckeri*, *Salmonella* spp., *Staphylococcus* and *Pseudomonas* when administered orally. When investigated *in vitro*, IgY was shown to preclude infection and also to limit growth, while in animals it was observed to inhibit the symptoms of infection (Kovacs-Nolan & Mine, 2004).

In our previous study, "The production of anti-*Vibrio harveyi* egg yolk immunoglobulin and evaluation of its

stability and neutralization efficacy” (Srisapoome, Punyokun, Hongprayoon, & Areechon, 2006; Punyokun, Hongprayoon, Srisapoome, & Sirinarumitr, 2013), we found that the activity of IgY was destroyed under gastric conditions which contained pepsin or a low pH value. Otherwise IgY was found to be quite stable in simulated intestinal fluid. 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.

It is possible to control the release of specific active components in foods through a number of techniques based upon microencapsulation. These include extrusion, coacervation, spray cooling or drying, liposome entrapment, nano-encapsulation, and the use of supercritical fluids or spinning disks (Gouin, 2004). Not all of these approaches, however, are equally effective, while some involve the use of prohibitively expensive ingredients. One successful example is the microencapsulation of IgY involving the application of an anionic methacrylic acid copolymer, Eudragit L100-55. Kovacs-Nolan and Mine (2005) reported this particular method to result in significant improvements to the stability of antibodies in simulated gastrointestinal fluid.

Many encapsulated materials contain the polysaccharides, chitosan and alginate, since their properties include biodegradability and biocompatibility, while neither is toxic (Murano, 1998). Both these polysaccharides are polyelectrolytes; as ions, chitosan carries a positive charge while sodium alginate has a negative charge. When alginate and chitosan form a polyelectrolyte complex the binding cannot be reversed, and has greater strength than that between poly-L-lysine and an alginate gel (Thu et al., 1996). The properties of alginate and chitosan have more recently been applied in the production of microcapsules which can be orally administered for the delivery of various encapsulated substances. Peptides, (Hari et al., 1996), proteins (Anal, Bhopatkar, Tokura, Tamura & Stevens, 2003) and drugs (Anal & Stevens, 2005) are among the materials to have been delivered to the intestines using encapsulation to bypass the stomach.

The objective of this study was to evaluate the influence of a number of factors upon the retention of anti-*V. harveyi* IgY under simulate digestive fluid, such as different encapsulation materials, pH of the encapsulation medium, and the concentration of chitosan, alginate and CaCl<sub>2</sub> during microcapsule manufacture. The microcapsules were subsequently determined under simulated gastric fluid and simulated intestinal fluid. These optimized microcapsules will be applied for IgY oral delivery to shrimp.

## 2. Materials and Methods

Commercialized sodium alginate of differing viscosity (high >2000 cP, medium 500–1000 cP and low 100–250 cP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). This alginate was isolated from the strips of *Laminaria hyperborea*. The raw materials of N-acetyl-glucosamine were obtained from Asst. Prof. Dr. Rath Pichyangkura, Head of the Center for Chitin-chitosan Biomaterials, Chulalongkorn University, Bangkok, Thailand. There are 3 types of chitosan of different molecular weights (High Mw, Medium Mw and Low Mw). The samples of chitosan and alginate used in the experiments displayed different properties as shown in Table 1.

### 2.1 Preparation of Water-Soluble Chitosan by N-acetylation

Five grams chitosan was dissolved in 100 mL of 50% NaOH at 37°C for 5 days. The N-acetylated chitosan was filtered, washed with water and dried at 60°C. The samples were carried forward for characterization.

### 2.2 Measurement of Degree of Deacetylation

The methods were modified from Muzzarelli & Rocchetti (1985). Briefly, chitosan solution was prepared in the concentration of 0.01 g in 100 mL of 0.01 M acetic acid and was then measured using a UV-spectrophotometer. The distance between the first derivative of the zero crossing point and that of the sample was measured and the degree of deacetylation calculated.

### 2.3 Measurement of Moisture Content and Solubility of Chitosan

The AOAC method 943.01 was used to measure the moisture content. The sample was dried at 105°C for 24 h or until the weight was constant. The moisture content was then calculated by percentage of weight loss compared to the initial weight of the sample. The solubility of chitosan was modified from Roberts (1992). Chitosan 0.2 g was dissolved in 100 mL of 0.2 M acetic acid and shaken at 100 rpm, at room temperature for 24 h. This solution was filtered through GF/C filter paper and the amount of oven dried insoluble matter was measured. The solubility was calculated from this amount of insoluble matter based on the initial weight of the chitosan after drying at 105°C for 24 h.

### 2.4 Measurement of Chitosan Molecular Weight

Gel permeation chromatography methods were available to evaluate the molecular weight distribution of the chitosan sample where calibration was performed by means of commercial standard methods. The sample was

analyzed at National Metal and Materials Technology, Bangkok, Thailand. Briefly, chitosan solution in 0.2% w/v in 0.5 M acetate buffer was filtrated by 0.45  $\mu\text{m}$  filter. 20  $\mu\text{L}$  of solution was injected to the gel permeation chromatography model Waters 600E equipped with an ultrahydrogel linear 1 column and guard column; 1,000–20,000,000 Mw resolving range. Pullulanase (Mw 5,900–788,000) was set as the polymer standard.

### 2.5 Preparation of Anti-*V. harveyi* IgY

Specific IgY anti-*V. harveyi* strain AQVH001 was prepared from Roman-Brown laying hens and purified with the water dilution method as described previously (Punyokun et al., 2013). IgY powder was obtained and followed by freeze-drying, and its antibody titer was over 250,000 based on indirect ELISA.

### 2.6 Preparation of IgY-Chitosan–Alginate Microcapsules

Microcapsules were prepared by the complex coacervation procedure described by Vandenberg, Drolet, Scott, and de la Noüe (2001). IgY (purified as described above) was added into 2% (w/v) sodium alginate at 25% loading rate (IgY: alginate). Chitosan 0.2% (w/v) was dissolved in 1% (v/v) acetic acid with gentle warming for 1 h. The encapsulation medium was prepared by mixing 0.2% (w/v) chitosan with 1.5% (w/v) CaCl<sub>2</sub> solution. The encapsulation pH was adjusted to 4.0 with 4 M NaOH. Approximately 50 mL of alginate-IgY solution was pumped at a rate 4 mL/min through a 1.4 mm nozzle with a coaxial air compressor. Alginate-IgY was extruded into 500 mL encapsulation medium under mechanical stirring for 15 min. The microcapsules were filtered from the encapsulation medium, rinsed with 0.1 N HCl and dried at 40°C. The surface morphology of the microcapsules was examined using an inverted microscope. The physical factors were tested, including alginate concentration (1%, 1.5%, 2%, 3% (w/v)), chitosan concentration (0%, 0.1%, 0.2%, 0.5% (w/v)), CaCl<sub>2</sub> concentration (0.1%, 0.5%, 1%, 1.5% (w/v)), and pH of encapsulation medium (3.0, 4.0, 5.0, 6.0).

### 2.7 Determination of IgY Retention

The method was modified from Li et al. (2007). IgY retention was expressed as the percentage of total available IgY in the microcapsules. The %IgY retention of IgY capsules was calculated from the IgY content of dried microcapsules after incubation in simulated intestinal fluid (SIF, 0.05 M K<sub>2</sub>HPO<sub>4</sub>, pH 6.8) for 6 h and the IgY content of dried microcapsules in releasing buffer (0.06 M Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>·2H<sub>2</sub>O + 0.2 M NaHCO<sub>3</sub> pH 8). Briefly, 25 mg IgY capsules were incubated in 5 mL SGF (0.03 M NaCl, pH 1.2) for 2 h with a rotating agitator. The IgY capsules were subsequently transferred and incubated in SIF (0.05 M K<sub>2</sub>HPO<sub>4</sub>, pH 6.8) for 6 h. Experiments were performed in triplicate. The samples were determined for protein using a Coomassie Plus Assay Reagent (Thermoscientific, USA). The %IgY retention was obtained by the Equation:

$$\text{IgY Retention (\%)} = \frac{\text{IgY content after SIF incubation}}{\text{IgY content after release buffer incubation}} \times 100 =$$

### 2.8 In Vitro IgY Release from the Microcapsules

IgY release was described by Anal et al. (2003). 20 mg of IgY capsules was dissolved in 5 mL of simulated gastric fluid (SGF, 0.03 M NaCl, pH 1.2) without pepsin, at 37°C, with a rotating agitator for 2 h. The IgY capsules were subsequently transferred and incubated in 5 mL of SIF without pancreatin (United States Pharmacopeial Convention Council of Experts, 2004) with a rotating agitator for 16 h. After incubation 20 $\mu\text{L}$  aliquots were taken at 1, 2, 3, 4, 6 and 16 h and replaced with the same volume of fresh medium. Experiments were performed in triplicate. The protein concentration was analyzed using Coomassie Plus Assay Reagent. The accumulative release percentage (Q%) was calculated by Equation (1):

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

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).

The IgY loading percentage is given by Equation (2) where  $C_{\text{IgY}}$  is the concentration of IgY in the sample,  $V$  is the volume of the sample, and  $W$  is the weight of the IgY microcapsules.

$$\text{IgY loading\%} = C_{\text{IgY}} \times \frac{V}{W} \times 100\% \quad (2)$$

### 2.9 Determination of IgY Activity

The sampling at 1, 2, 3, 4, 6 and 16 h from the *in vitro* IgY release experiment was confirmed in its activity by

indirect ELISA. A polystyrene plate was coated with *V. harveyi*  $10^8$  cells/mL in 0.05 M bicarbonate buffer, pH 9.6, 50  $\mu$ L/well and incubated at 37°C overnight. The plate was washed three times with PBS containing 0.05% Tween 20 (PBST), 200  $\mu$ L/well. A blocking buffer (5% skimmed milk in PBS) was added at 100  $\mu$ L/well and incubated at 37°C for 1 h. The plate was then washed three times with PBST. The IgY sample was diluted 2-fold with blocking buffer then 50  $\mu$ L was added to each well and incubated at 37°C for 1 h. The plate was washed again as above and 50  $\mu$ L of alkaline-phosphatase-conjugated rabbit anti-chicken IgG (Sigma Chemical Co., USA) diluted 5000-fold with PBS was added to each well. After incubation at 37°C for 1 h, the plate was washed and followed by the addition of 100  $\mu$ L of *p*-nitrophenyl phosphate in diethanolamine buffer (pH 8.9) (Sigma Chemical Co., USA). After incubation at 37°C for 30 min, 50  $\mu$ L of 3 N NaOH was applied to arrest the reaction. The color developed was read at 405 nm with an ELISA reader (Labsystem, USA).

### 2.10 Statistical Analysis

The results were constructed to determine one-way analysis of variance (ANOVA) which followed a completely randomized design (CRD), while Duncan's new multiple range test (DMRT) was used to compare the differences of observed average values among groups at the significance level of  $\alpha = 0.05$ .

## 3. Results and Discussion

### 3.1 Chitosan Characterization

The properties of chitosan of different molecular weights are shown in Table 1.

Table 1. Properties of high (HMw), medium (MMw) and low (LMw) molecular weight chitosan

Chitosan	Deacetylation (%)	Moisture content (%)	Solubility (%)	Molecular weight (kDa)
HMw	92.98±0.16	10.53±0.03	95.39±0.97	3.5 x 10 <sup>6</sup>
MMw	92.47±0.12	10.67±0.03	94.42±0.65	2.1 x 10 <sup>5</sup>
LMw	86.25±0.16	15.32±0.13	92.68±0.97	1.3 x 10 <sup>4</sup>

### 3.2 Morphology of IgY Microcapsules

According to the optimal encapsulation condition, IgY-loaded chitosan-alginate microcapsules were obtained. The mean diameter of microcapsules visualized using an inverted microscope was approximately 1,000–1,500  $\mu$ m. (Figure 1A). However, the size was decreased to about 100–200  $\mu$ m under feed air volume 0.3 m<sup>3</sup>/h during the extrusion process (Figure 1B).

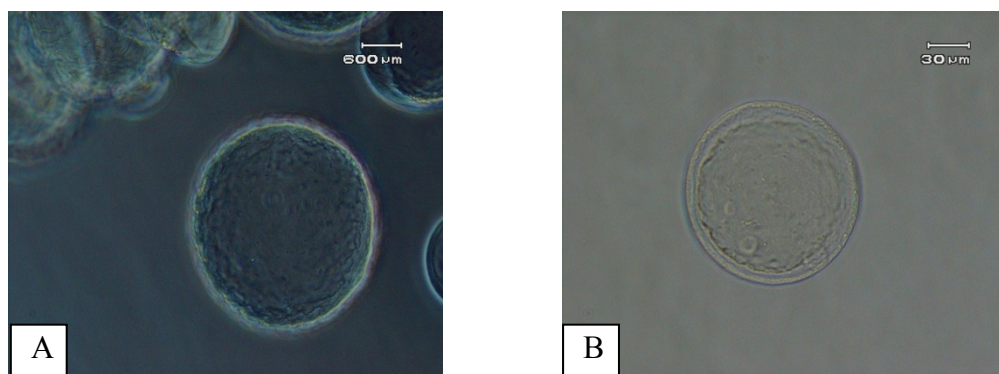


Figure 1. Inverted microscope micrographs of IgY-loaded chitosan-alginate microcapsules. (A) extrusion without air and (B) extrusion with air volume 0.3 m<sup>3</sup>/h

### 3.3 Studies on Optimal Conditions for IgY Microcapsule Preparation

The influence of protein retention in IgY microcapsules during different manufacture processes was investigated. IgY retention was increased significantly when the alginate concentration increased and was maximized at 2.5% (w/v) alginate (Figure 2A). However, increasing the alginate concentration above 2% (w/v) made extrusion extremely difficult, due to the high viscosity of the alginate causing sticking in the nozzle. Bartkowiak and

Hunkeler (2000) described the results of an increase in alginate concentration upon protein retention. Typically the alginate gradient is amplified when microcapsules are externally gelled, hence causing lower permeability of the microcapsule.

The addition of different concentrations of chitosan (0.1%, 0.2%, 0.5% (w/v)) into the encapsulation medium showed about 80% IgY retention but did not differ significantly between treatments. IgY capsules manufactured without chitosan showed only 60% IgY retention (Figure 2B). When polycationic polymers are added to the encapsulation medium, the result is to change the physical characteristics of the microcapsule, so both swelling and permeability are reduced (Thu et al., 1996).

The effect of increasing CaCl<sub>2</sub> concentrations in the encapsulation medium on IgY retention is shown in Figure 2C. IgY retention was higher at lower calcium levels and highest at 0.1% (w/v). The concentration of calcium in the encapsulation medium is an important influence on the level of protein retention. Smidsrød and Skjåk-Bræk (1990) showed that the rate of polymer diffusion determines the degree of alginate in homogeneity, while the rate of inward calcium ion diffusion toward the gelling front also exerts a strong influence. Therefore any reduction in the concentration of external calcium will also reduce the calcium diffusion rate towards the gelling front and thus raise the alginate gradient and cut the permeability of the microcapsule.

The IgY retention of microcapsules during manufacture from various pH levels of the encapsulation medium was demonstrated in Figure 2D. IgY retention was significantly increased when pH was increased from 3.0 to 6.0. This experiment found that pH 4.0 gave the highest IgY retention (81%). It has not yet been precisely determined how pH interacts during the process of encapsulation. The literature is inconclusive, with Vandenberg et al. (2001) finding that the encapsulation pH did not influence the efficiency, while Polk et al. (1994) and Huguet, Groboillot, Neufeld, Poncelet, and Dellacherie (1994) claimed that a lower pH resulted in reduced capsule strength and flexibility.

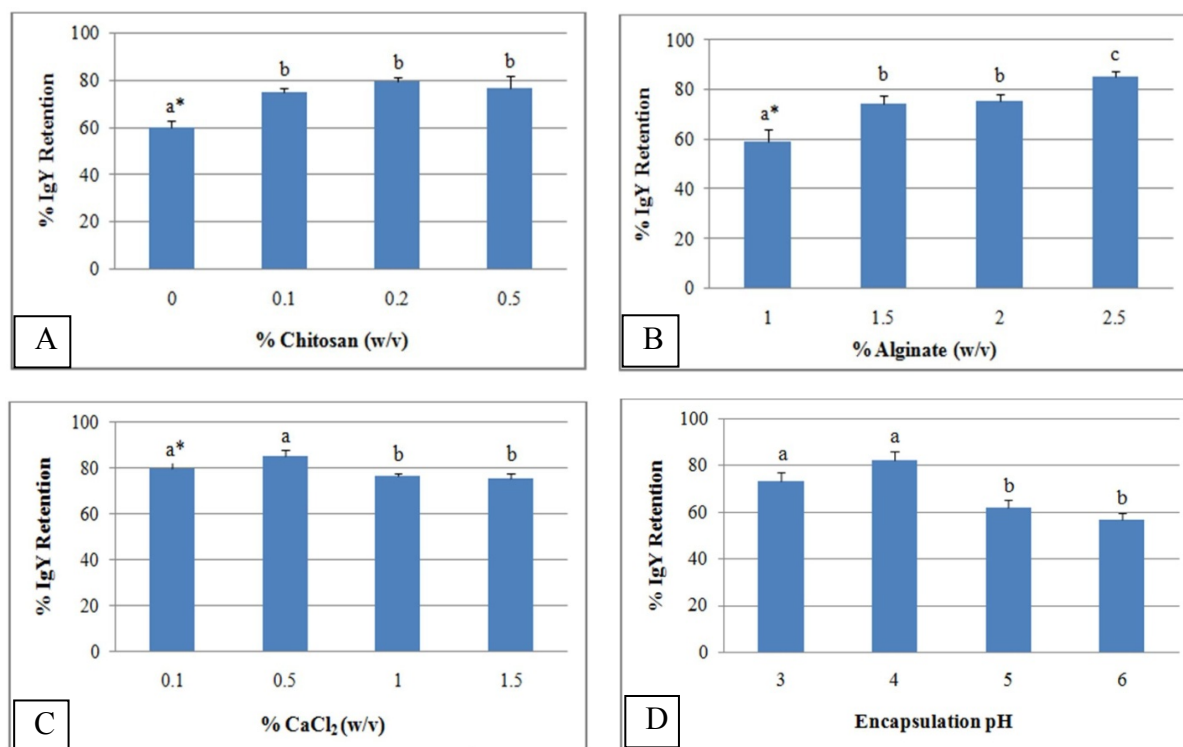


Figure 2. Protein retention during the manufacture of chitosan-alginate microcapsules produced by varying the concentrations of alginate (A), chitosan (B), CaCl<sub>2</sub> (C) and pH of the encapsulation medium (D). Error bars represent the standard error of the mean based on four replicated microcapsule samples

### 3.4 In Vitro IgY Release

The release characteristics of IgY microcapsules, according to the optimal formulation (2% w/v alginate, 0.1% w/v chitosan, 0.5% CaCl<sub>2</sub> and adjusted encapsulation to 4.0), were examined by incubating in the simulated

digestive fluid. IgY microcapsules were first incubated in SGF and subsequently in SIF, and the percentage of protein released over time was measured (Figure 3). About 5% of IgY was released over 2 h of SGF incubation. After transfer to SIF, the granules began to disintegrate, releasing over 60% of their protein within 2 h. The granules continued to disintegrate, reaching 80% release after 16 h incubation (Figure 4). Anal et al. (2003) gave the following description of chitosan-alginate microcapsule disintegration. When the medium is acidic, ionic bonds in the microcapsules prevent the disintegration of the microcapsule matrix material. However, when the pH level becomes neutral, hydroxyl ions displace the anionic alginate in the Ca-alginate-chitosan complex, while the chitosan no longer carries a positive charge. This enables the matrix to swell and the complex to disintegrate, thereby releasing the contents of the capsule.

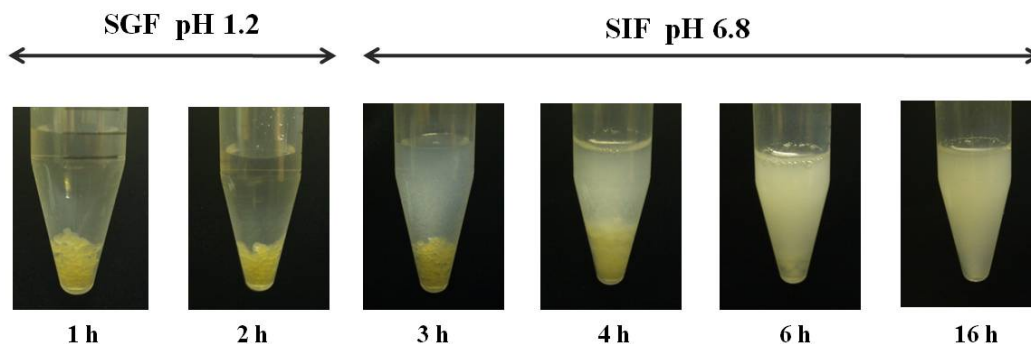


Figure 3. Characterization of IgY loaded microcapsules during incubation. Samples were first incubated in simulated gastric fluid for 2 h, and then transferred to simulated intestinal fluid for 16 h

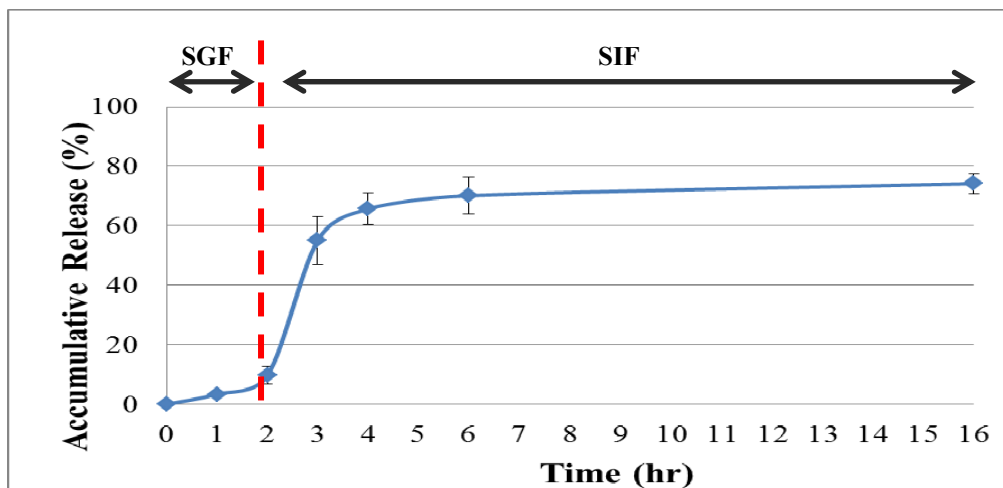


Figure 4. *In vitro* IgY release from IgY loaded microcapsules. Samples were first incubated in simulated gastric fluid for 2 h, and then transferred to simulated intestinal fluid for 16 h. The percentage of accumulative release was calculated. Data are presented as mean  $\pm$  SD (n = 3)

#### 4. Conclusions

The creation of optimized chitosan-alginate microcapsules relies upon the application of the various physical and chemical properties of the materials used. In their optimal form, these microcapsules can prevent gastric inactivation in anti-*V. harveyi* IgY, while demonstrating both quick release in the intestines and limited losses due to gastric fluids. The outcome of this study suggests that IgY may have beneficial applications in the food industry, or as an animal feed supplement since it is able to prevent infections caused by digestive pathogenic bacteria in animals.

Further research on IgY is required to maximize the benefits for the food industry. The effect IgY should be investigated on other shrimp diseases to test its application as an antibody.

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