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Research Article

Physical Characterization and *In Vivo* Study of Ovalbumin Encapsulated in Alginate Microspheres

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ABSTRACT

Microspheres as drug delivery systems have been used widely for oral delivery in the pharmaceuticals formulation. Alginates have received much attention as biodegradable polymer for controlled protein delivery. Ovalbumin was used as model antigen. The aim of the present research was to evaluate physical characteristics of unlyophilized and lyophilized ovalbumin-loaded alginate microspheres produced using aerosolisation technique. Crosslinking agent of $BaCl_2$ and $CaCl_2$ were studied. Physical characteristics of ovalbumin-loaded alginate microspheres were evaluated in terms of encapsulation efficiency, yield, particle size, surface morphology and protein integrity. In vivo immune response was studied using hemagglutination test by measuring antibody titre. High entrapment efficiency of unlyophilized ovalbumin-loaded alginate microspheres using crosslinker $BaCl_2$ and $CaCl_2$ of above 80% and loading of above 65% was produced. Entrapment efficiency of lyophilized ovalbumin-loaded alginate microspheres using 10% lactose lyoprotectant and maltodextrin were 46% and 54% respectively. For protein loading, ovalbumin-loaded alginate microspheres lyophilized using lactose was 28% and using maltodextrin was around 35% respectively. The microspheres size of less than 8µm of all formulas using $BaCl_2$ and $CaCl_2$ both lyophilized and unlyophilized were spherical in shapes and formed smooth surface. Ovalbumin maintained its integrity after release from alginate microspheres showing ovalbumin's 45 kDa molecular weight indicated its stability has been protected by this delivery system after exposure to acid condition. In vivo immune response by hemaglutination test of lyophilized ovalbumin-loaded alginate microspheres with $CaCl_2$ crosslinker consist of 10% lactose or 10% maltodextrin and unlyophilized microspheres using $CaCl_2$ crosslinker showed higher antibody titres than ovalbumin control and blank microspheres. Whereas, unlyophilized ovalbumin-alginate microspheres using Ba^{2+} crosslinking agent resulted antibody titre as low as control and blank microspheres. However, No significant effect of immune response was found between microspheres lyophilized with lactose and maltodextrin. In conclusion, ovalbumin-loaded alginate microspheres crosslinked using $CaCl_2$ are potential as oral delivery systems and results indicated that lyoprotectants were involved in protecting ovalbumin entrapped in alginate microspheres therefore able to deliver antigen to the target site in order to induce antibody response.

Keywords: alginate, microspheres, ovalbumin, integrity, hemaglutination, immune response.

INTRODUCTION

The rapid development of biotechnology, peptide and protein drug substance has been increasingly used for therapeutic[1]. Peptide and protein drug substance has several drawbacks, such as low stability, easy and fast to deactivation, the short half-life, and the difficulty absorbed when given orally. Currently, almost all of protein drugs administered through the parenteral route. This is what causes the patient discomfort and the price is expensive. Many studies were conducted to find a way out to overcome this problem[1]. A major obstacle if the vaccine is given orally is the instability and degradation of the antigen[2]. This degradation is due to a protein antigen that is very easy denatured by stomach acid. The target of antigen uptake is in the Peyer's patches in the small intestine. Peyer's patches is the only place in the gastrointestinal tract which will absorb the antigen or pathogen to be brought to the lymphoid tissue (Gut Associated Lymphoid Tissue). If the antigen is denatured in the stomach, then there will be no antigen to the target so that the immunological response does not appear[3]. One way to avoid denaturation of the protein is to use microspheres of oral delivery systems. Microspheres commonly contain an active ingredient or core material surrounded by a protective layer[4]. Antigen delivery to the carrier in the form of microspheres can prevent degradation by the stomach and stimulates the M-cell to bring vaccine into the core Peyer's patches. M-cell is a cell that can detect the presence of stimulation on payer's patches and carry substances adsorbed on the surface of the core to the payer's patches[5]. Not all ingredients are absorbed on the surface of the payer's patches can be

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Table 1. Formulas of ovalbumin-loaded alginate microspheres

Formula	F1	F2	F3	F4
Ovalbumin 2,5%	V	V	V	V
Alginate 2,5%	V	V	V	V
$CaCl_2$	V	V	-	V
$BaCl_2$	-	-	V	-
Lactose	-	V	-	-
Maltodextrin	-	-	-	V

F1: Formula Microsphere Ca-Alginate; F2: Formula Microsphere -lyoprotectant lactose 10%
F3: Formula Microsphere Ba-Alginate; F4: Formula Microsphere -lyoprotectant maltodextrin 10%

Table 2. Efficiency encapsulation and protein loading of ovalbumin-loaded alginate microspheres

Formula	Encapsulation Efficiency (%)	Protein loading (%)
F 1	82,62 ± 6,81	86,59 ± 6,81
F2	46,29 ± 6,81	28,07 ± 6,58
F3	80,47 ± 9,52	66,86 ± 10,36
F4	54,15 ± 8,82	35,47 ± 1,60

Table 3. Particle size of Ovalbumin-loaded alginate microspheres

Formula	Mean size of particle (μm)
Blank Alginate Microspheres	4,69 μm
F1	7,52 μm
F2	5,84 μm
F3	6,82 μm
F4	4,97 μm

carried by the M-cells but only materials that can penetrate the intestinal lumen as soluble proteins, antigens, bacteria, and viruses[6]. After arriving at the core of the Peyer's patches, these microspheres will rupture and release the antigen carries[5]. Ovalbumin is a protein consisting of 385 amino acid residues[7]. Ovalbumin as an antigen to stimulate the formation of antibodies, but a poor immunogenic therefore ovalbumin is usually used with repeated administration[8]. Repeated administration of the patient causing discomfort, then ovalbumin formulated in preparation with controlled release or delayed[9]. Microencapsulation materials contain one or more drugs covered by the coating. The core may comprise a core material inside and covered by a coating on the walls. Microencapsulation generally has particle size of between 1-2000μm[10]. Polymers commonly used as a coating material in microencapsulated. Biodegradable polymers have demonstrated a high potential to deliver peptides and proteins via the oral route[11]. Alginate is a biodegradable polymer. Sodium alginate is a natural polymer that has good biocompatibility, does not accumulate in the organs of the body, non-toxic, biocompatible, and relatively inexpensive. Alginate can bind selectively with a solution that is generally in the form of a cross junction of multivalent cations (eg Ca^{2+}, Ba^{2+}) and virtually unaffected by temperature[11]. In general, Ca^{2+} is most often used to form a gel, because Ca^{2+} has a low toxicity, the concentration of Ca^{2+} has a significant effect on the stability and pore size of the gel[12]. In addition, ovalbumin-alginate microspheres can be formed successfully from the crosslink $BaCl_2$ solution that produces high encapsulation efficiency and small particles[13]. The number and size of the cation valence compounds determine the continued cross-dimensional shape of the polymer formed. Moreover, differences in the type of cation are also one of the factors determining the selectivity to M cells[6].

Fig. 1. Photographs of freeze-dried ovalbumin loaded alginate microspheres A: maltodextrin as lyoprotectant; B: lactose as lyoprotectant

The encapsulation method used in this study, namely ionotropic gelation, is able to maintain the integrity of the protein. The entire polyelectrolyte used water soluble so that the protein is able to be encapsulated without the use of organic solvents that can destroy the protein. This method is simple, fast, and relatively inexpensive[14].
To further enhance the stability of the protein, ovalbumin is formulated in dry form in oral microspheres formulations. In this study, freeze drying is used as an alternative to drying without heat that can denature proteins and can damage its integrity. In the freeze-drying method generally under pressure varies during freezing and drying processes that require protectant to protect the physical integrity of microparticles formed. Sugar is known to stabilize proteins during the process of freeze drying[15]. Sugar was able to create a physical barrier between the particles and molecules and reduce diffusion and molecular mobility and can prevent protein aggregation and degradation. During the drying process, the sugar water molecules occupy the hydrogen bonding interactions with protein molecules and helps protect the integrity[16]. Therefore, when the lyophilized microspheres and solid when dry, the microspheres plus lyoprotectant, will remain stable because the integrity of the physical form remains sphere, so that the components inside remain intact[15]. Maltodextrin is considered as a potential lyoprotectant in lyophilized proteins[17]. Use lyoprotectant generally 5% to 10% for lactose[18] as well as for maltodextrin[17].

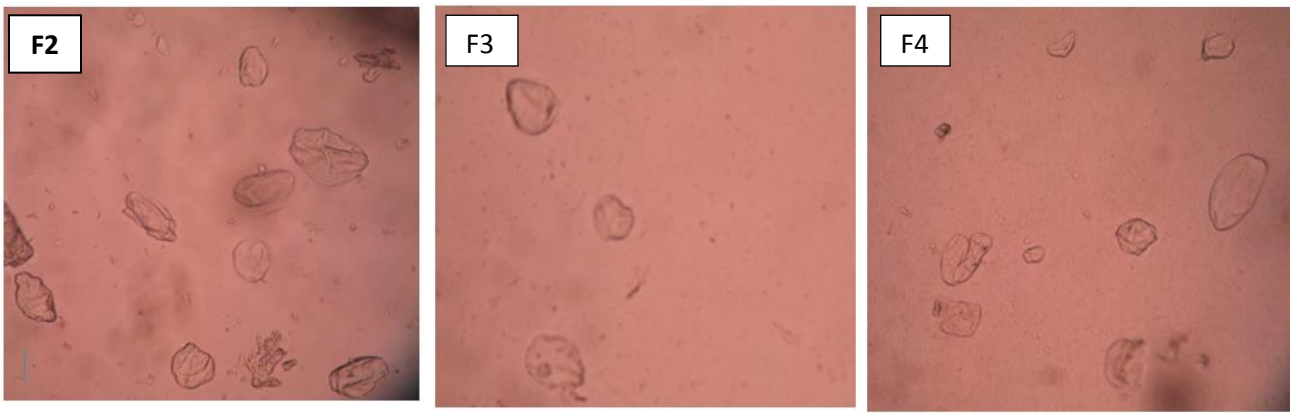

Fig. 2. Morphology of wet microspheres: F2, F3 and F4 at 400x magnification

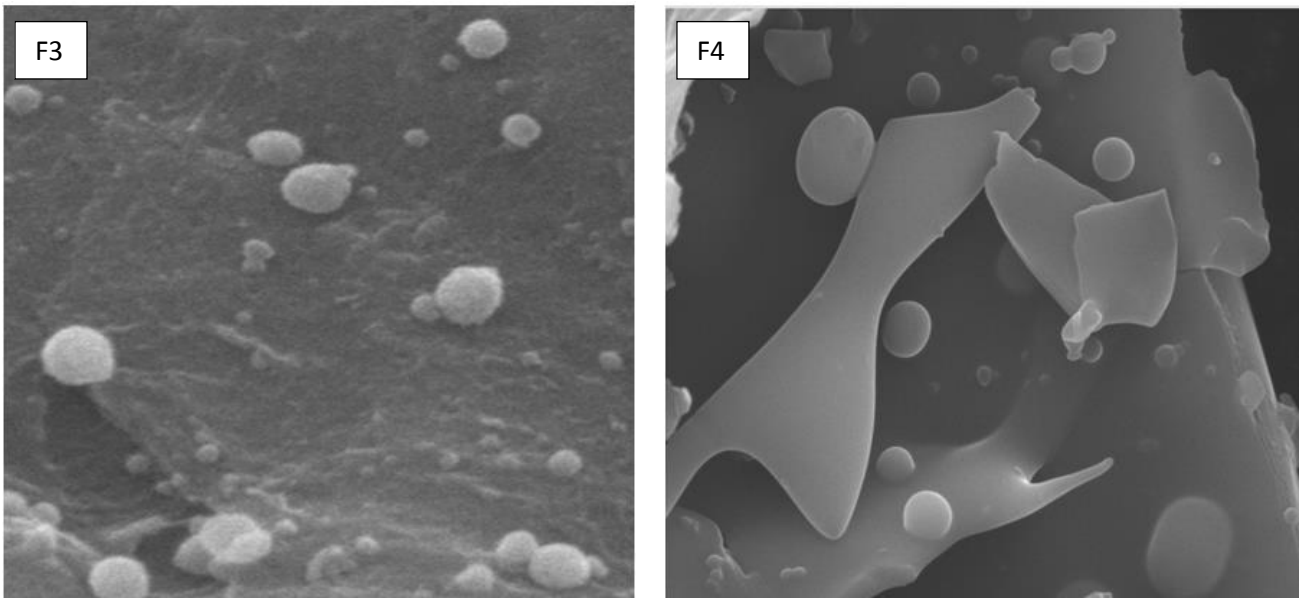

Fig. 3. Scanning Electron Microscope (SEM) of freeze-dried microspheres of formula F3 and F4

In the in vivo study, hemagglutination test is very important to determine the process of formation of antibodies after antigen enters the body, to ascertain whether the antigen can be delivered well and is able to stimulate the proliferation of T-cells and B-cells[19]. If the antigen successfully delivered, it will be detected in serum anti-OVA IgG through agglutination test. Agglutination test is one technique that is often used to determine the bond between the Ag-Ab in the serum[20]. One of the requirements of this technique is to be in the form of particles or antigen-soluble substances, so as to form a clot when bound to antibody. There are two development agglutination test techniques are widely used, namely the indirect hemagglutination and hemagglutination. In hemagglutination, antigen attached to a carrier in the form of erythrocytes. While indirect hemagglutination reacting serum with specific antibodies and then newly added antigen to be checked[21]. Therefore, hemagglutination test is absolutely necessary to prove that the formulation of ovalbumin microspheres prepared to respond immune and whether microspheres delivery is effectively deliver vaccine orally.

This study aims to study the effect of different types of lyoprotectant (lactose and maltodextrin) to the physical characteristics of the microspheres (microspheres size and morphology, encapsulation efficiency and protein loading and protein integrity) and also to study in vivo immune response of ovalbumin-loaded alginate microspheres.

MATERIALS AND METHODS

Materials

Ovalbumin and Sodium alginate (Sigma Aldrich), $CaCl_2$, Lactose and Sodium citrate (Bratachem Chemicals) $BaCl_2$ (Merck), protein quantification kit Sigma; maltodextrin p.g, phospate buffer saline pH 7.2, Na EDTA, aquadest, red gout blood cell, and mus musculus strain Balb C from Pusat Veterenaria Farma (PUSVETMA) Surabaya.

Methods

Preparation of Ovalbumin-loaded Alginate microspheres using aerosolisation technique

Sodium alginate (2.5%) was dissolved in 100 mL of distilled water and ovalbumin (2.5%) was dissolved in it. This solution was then sprayed into 200 mL of 1.5 M $CaCl_2$ or $BaCl_2$ solution with a constant speed at a distance of 8 cm from the surface of the solution, and a pressure of 40 psi. The mixture was stirred at 1000 rpm for 2 hours to complete the crosslink reaction. Microspheres formed were collected and then separated

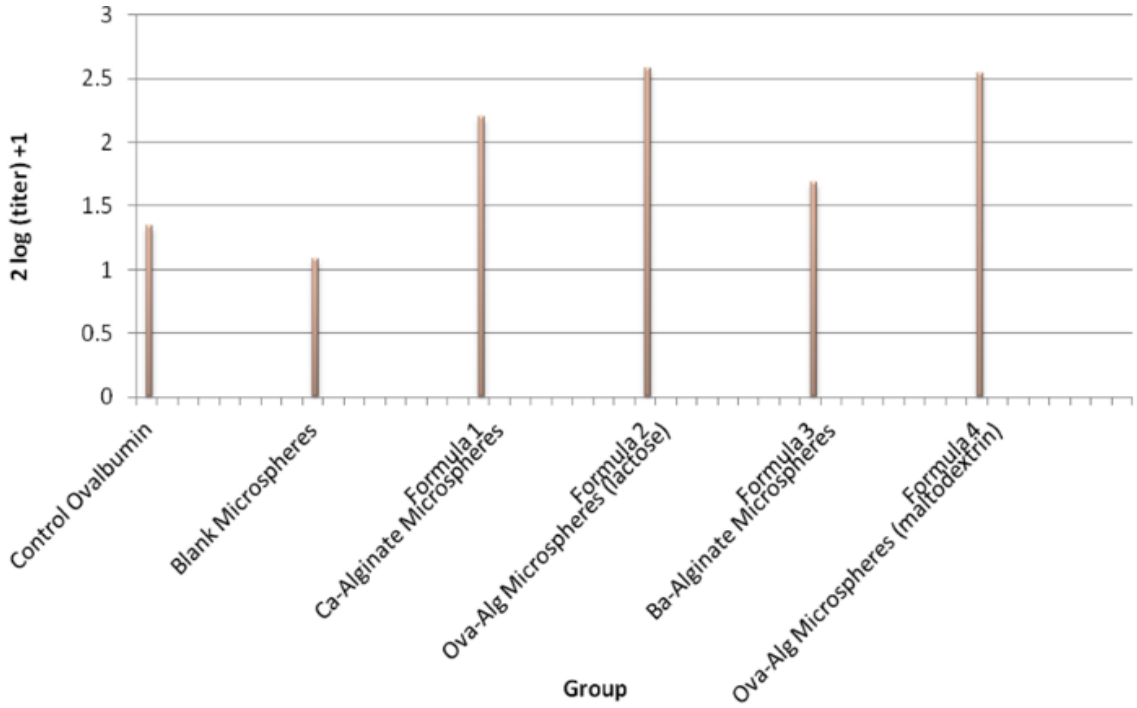

Fig. 4. Hemaglutination titre showing IgG level

by centrifugation at 2,500 rpm for 6 min and washed with distilled water twice. Microspheres resuspended in lyoprotectant solution (1g/10mL) with concentration according to the formula. The suspension was dried by freeze dryer at -80 °C for 29 hours. Formulas of microspheres can be seen in Table 1.

Encapsulation efficiency and protein loadings

The ratio of the actual ovalbumin content in the protein-loaded microspheres to the theoretical ovalbumin content was termed encapsulation efficiency. For protein loadings, accurately 50 mL of sodium citrate pH 8.5 was added in 400 grams of microspheres and was continuously stirred at 1000rpm for 12 hours. The absorbance of ovalbumin was measured using UV Vis Spectrophotometry using protein quantification kit sigma at maximum wavelength of ovalbumin.

Optical Microscope and Scanning Electron Microscope (SEM) study

The morphology of hydrated ovalbumin-loaded alginate microspheres were evaluated using optical microscope, while freeze-dried microsphere was observed after freeze-drying by scanning electron microscope. For SEM analysis, The working distance was 10 mm, beam energy was 20.0 kV, spot size was 5.0, and magnification was 5000. The microspheres were loaded on a double sided carbon tape put on studs before being examined by SEM.

In vivo study

Animals in vivo study has been approved by ethics committee and met national ethic standard by Faculty of Veterinary Airlangga University. Mice were given orally ovalbumin-loaded alginate microspheres or control for 5 days for all groups of mice. At day 7, animals were injected intraperitonially using gout red blood cell suspension. At day 17, bloods were taken intracardially and were analyzed for the serum or supernatan after centrifugation. Hemagglutination study was conducted to analyze imune response by measuring IgG titres.

RESULTS AND DISCUSSION

Ovalbumin-loaded alginate microspheres crosslinked using $BaCl_2$ and $CaCl_2$ were successfully produced using ionotropic gelation aerosolisation technique. The addition of lyoprotectants also used in this research in order to prevent denaturation and increase stability of microspheres. Furthermore, evaluation of different type of lyoprotectants was compared.

The encapsulation efficiency and ovalbumin loading results can be seen in Table 2. Formulas with no lyoprotectants added (F1 and F3) showed the highest encapsulation efficiency and protein loading at above 80% similar to previous alginate microspheres[13]. However, size and morphology of unlyophilized ovalbumin-loaded alginate microspheres was bigger and less smoother compared to lyophilized microspheres.

In terms of formulas using lyoprotectants, F4 produced higher protein encapsulation (54%) and protein loading at about 36% than formulas using 10% lactose (F2) which resulted less encapsulation efficiency and lower loading. This indicated that maltodextrin is a potential lyoprotectants in agreement with other researcher[17].

The microspheres sizes of all formulas were found less than 8 µm (Table 3). The results showed that the incorporation of lactose and maltodextrin as lyoprotectants improved functional properties, due to the better physical characteristics of the ovalbumin-loaded alginate microspheres at concentration 10% w/v at −80 °C freeze drying condition. This indicated by smaller microspheres were formed at F2 and F4.

The addition of lyoprotectant prior to lyophilisation resulted in a homogenous powder in regard to the blank

(Figure 1). For wet microspheres, nearly smooth particles were formed (Figure 2). As expected, hydrogel alginate microspheres obtained with maltodextrin and lactose yielded homogeneous and spherical microspheres during optical microscopy examination (Figure 3).

The SEM micrographs revealed the formation of smooth, small and spherical microspheres with maltodextrin incorporation (Figure 3). The incorporation of maltodextrin might lead to a product with more uniform, smaller and homogeneous pore structures. This fact was in agreement with other authors who indicated that saccharides lyoprotectant stabilized bovine plasma protein. Furthermore, the microspheres size was found to be less than 8µm using optical microscopy and SEM examination.

Possible mechanism of maltodextrin in stabilizes ovalbumin loaded alginate microspheres maybe hydrogen bonding between sugar or alcohol sugar and protein as an important factor during freeze drying. Moreover, the existence of maltodextrin as stabilizer avoided aggregation of particles; therefore smaller microspheres were produced[22].

Integrity structure by SDS PAGE analysis

Ovalbumin was found to be maintained its integrity structure by SDS PAGE analysis. The molecular weight of 45kDa was confirmed when compared to protein standard marker. From literature, molecular weight of ovalbumin was also 45 kDa[23]. The integrity of ovalbumin-loaded alginate microspheres were maintained after incubation in acid pH followed by in pH 7.4 after 8 hours.

In vivo immune response study

In vivo hemaglutination study showed that unlyophilized ovalbumin-alginate microspheres using Ba^{2+} crosslinking agent resulted antibody titre as low as control and blank microspheres (Figure 4). Whereas, lyophilized ovalbumin-loaded alginate microspheres with $CaCl_2$ crosslinker consist of 10% lactose or 10% maltodextrin and unlyophilized microspheres using $CaCl_2$ crosslinker showed higher antibody titres than ovalbumin control and blank microspheres. However, No significant effect of immune response showing by IgG titre level was found between microspheres lyophilized with lactose and maltodextrin.

Ba-alginate microspheres formed two dimensional structures with less empty space provided, this due to smaller size of Ba^{2+} of around 1.74A°, bigger than Ca^{2+} at about 1.14A°. Differences of affinity between Ba^{2+} and Ca^{2+} was also caused the amount of antigen in the M cells, therefore agglutination titre of Ba-alginate microspheres was lower than Ca-alginate microspheres.

Lyophilized alginate microspheres showed the highest titre, this indicated that ovalbumin antigen has arrived at the target site and ovalbumin microspheres was able to across the GI tract barrier in peyer patches produced immune response. O'Hagan (2006) found that vaccine delivery using microparticles was able to enhance high immune response in animal. Results of this research demonstrated the potential of the freeze-dried ovalbumin-alginate microspheres for oral vaccine delivery system.

CONCLUSION

High encapsulation efficiency and loading were produced successfully by aerosolisation technique of alginate polymer and $CaCl_2$ crosslinking agent. Small, smooth and spherical microspheres were also confirmed on the freeze-dried microspheres using 10% maltodextrin. Maltodextrin at concentration 10% were found to be better lyoprotectant agent on the ovalbumin-loaded alginate microspheres than 10% lactose in terms of efficiency, protein loading, size and morphology. However, no significant effect of in vivo IgG titre of ovalbumin-loaded alginate microspheres produced using addition of 10% maltodextrin and lactose lyoprotectant. This indicated that the freeze-dried ovalbumin-alginate microspheres are potential for oral vaccine delivery system.

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DECLARATION OF INTEREST

The authors report no conflicts of interest.

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