Soy Protein Isolate (SPI) Based Delivery System as Promising Mastitis Vaccine Carrier Candidate

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ABSTRACT
An orally administered vaccine application is a part of strategies on veterinary vaccine development. Soy protein isolate (SPI) has been scientifically tested in various studies as a potent drug carrier. Mastitis is one of the livestock diseases mainly caused by *Streptococcus agalactiae*, which can be controlled through vaccination programs. This study aimed to determine loading efficiency (LE) and loading capacity (LC) in various formulations of SPI, sodium alginate loaded with inactivated group B *streptococcus* trehalose (GBS-*trehalose*) combination as oral vaccine carrier candidates. Based on the SPI solubility test, we obtained a high percentage of solubility at pH 6-10, ranging from 83.40% to 99.39%. The prepared GBS-*trehalose* loaded SPI-alginate microparticles (pH 6) with a ratio of 1.03:1 yielded LE of 47.37 % and 3.79% LC. The lower concentration of GBS-*trehalose* loaded to SPI-alginate with ratios 0.876:1 and 0.657:1 yields a higher LE of 70.77% to 92.08% and 6.66% to 10.52% LC, confirming the valuable microencapsulation properties of SPI-alginate and the suitability of the ratio formulations. These results demonstrate that SPI-alginate is relevant for developing an inactivated bacterial vaccine carrier for the oral route. More research is needed for microparticles characterization, release capability and *in vitro* stability under various environmental conditions.

Keywords: *Loading Efficiency, Loading Capacity, Soy protein isolates, Vaccine Carrier.*

1. INTRODUCTION

Research into innovative drug delivery systems has increased rapidly over the last two decades, mostly to meet the need for novel drug delivery systems to overcome the many newly developed chemical entities with certain solubility or permeation characteristics [1][2]. Microencapsulation is one of the most effective methods for drug delivery systems, because allows saving the pharmaceutical ingredient in a suitable form in a desired therapeutic concentration range, and for optimizing drug release [3]. Microparticles are polymer particles with a size range of micrometers, which are commonly used in pharmaceutical encapsulation in which the ingredients can be introduced in the form of a solid solution or solid dispersion or where the material can be adsorbed or chemically bonded [4]. Microencapsulation has also been investigated for biopharmaceutical ingredients applications including vaccine antigens, probiotics and others for controlled release in the intestine [5]. There are many types of microparticles, one of which is protein microparticles which with certain treatments have shown great promise in drug/cell delivery and tissue engineering, as well as diagnosis and treatment of diseases [6]. Soy protein isolate (SPI) is one of the promising protein-based microparticles and has been investigated as a bacterial delivery system (*Lactobacillus casei*) which has been confirmed for its nutritional and health benefits, availability, biodegradability, low immunogenicity and similarity with the components of the tissue extracellular matrix [7].

Mastitis is a disease in dairy cattle that causes economic losses due to decreased milk production, premature culling or removal from the herd, unsalable because of poor quality milk, cost of veterinary care and medicines [8]. Mastitis is caused by microbial infection with symptoms categorized into clinical mastitis and subclinical mastitis. In general, the prevention of mastitis is done by maintaining the hygiene and sanitation of the cage, while the treatment is done using antibiotics at the appropriate dose and type [9]. Many therapeutic and preventive approaches to mastitis have been applied to raise the quality of health, welfare and productivity of dairy cattle [10]. Vaccine development against mastitis pathogens has been advancing in the past few decades. Both commercial vaccines and cattle-specific autovaccines using killed whole bacterial cells are commonly used in dairy cattle with poor results in many cases. [11]. Commercial vaccines as well as experimental vaccines utilize mostly the intramuscular, subcutaneous and intramammary routes of administration [11]. Though numerous studies examined experimental mastitis vaccines, not much research is done on how to deliver mastitis vaccine ingredients through the oral route. This study aimed to determine loading efficiency (LE) and loading capacity (LC) in various formulations of SPI, sodium alginate loaded with inactivated group B *streptococcus trehalose* (GBS-*trehalose*) combination as oral mastitis subclinical vaccine carrier candidates.

2. MATERIALS AND METHODS

2.1 Materials

Soy Protein Isolate was purchased from Shandong Crown Soy Technologies Co. LTD (China), by proximate analysis, as follows: protein 90.1%, pH 7.48, fat 0.30%, moisture 6.30%, ash 5.50%, sulphites <10 mg/kg. GBS was characterized by Streptococcus agalactiae isolated from cow's milk in a dairy farm in Bogor City. Sodium alginate (food/halal-grade) and trehalose (pharmaceutical standard grade) were purchased from Sigma-Aldrich.

2.2 Protein Solubility Profile

The protein solubility profile was determined as described by Castro *et.al* [12] with certain modifications. Soy Protein Isolate was prepared in deionized water (3% w/v) and the pH of the mixture was adjusted to 1.0–10.0 with 4 M NaOH or 4 M HCl, as appropriate. Each sample was stirred for 1 hour at room temperature and then filtered using filter paper; the residue left on the filter paper was dried and weighed. Protein solubility was calculated as follows:

$$\text{protein solubility (\%)} = \frac{\text{total protein} - \text{dry residue}}{\text{total protein}} \text{ x } 100$$

2.3 Microencapsulation

2.3.1 Sample Preparation

Soy Protein Isolate (50, 60 and 70 mg) was added into 100 ml deionized water according to the formulations under magnetic stirring at 2000 rpm (C-MAG HS-7, IKA, Germany) at room temperature for 30 minutes. Loading mixtures were prepared by mixing trehalose with 13 X 10^8 cells/ml of inactivated GBS (adjusting with McFarland standard) then mixed well using vortex mixer (Maxi Mix II, Thermolyne, USA). In addition, GBS-trehalose mixture was added dropwise into SPI solution under magnetic stirring at 2000 rpm. Subsequently, SPI GBS-trehalose solution was adjusted to pH 6.0 with 0.1 M HCl solution.

2.3.2 Microparticles Preparation

Sodium alginate (20, 25 and 30 mg) were added each by 15 ml deionized water (pH 4) under constant stirring (2000 rpm) at room temperature. Soy Protein Isolate GBS-trehalose solution was added dropwise into alginate solution under constant stirring (2000 rpm) at room temperature for 45 minutes; thus, GBS-trehalose loaded SPI-alginate weight ratios of 0.876:1, 0.657:1 and 1.03:1 obtained in the total solution volume of 115 ml. Then, the mixture was stirred at 600 rpm for 1 h at room temperature, followed by centrifugation at 14000 rpm for 8 minutes to separate the unbound GBS.

2.3.3 Loading GBS to SPI-alginate Microparticles

Loading efficiency (LE) and loading capacity (LC) were determined as described by Li *et al.* [13]. LE and LC of GBS-trehalose on SPI-alginate microparticles were detected in an indirect way by determining the unbound GBS remained in the supernatant after the performance of centrifuge, and the method was shown as follows. One ml of GBS-trehalose loaded SPI-trehalose microparticles suspension was centrifuged (Centrifuge TGL-20 M, China) at 14000 rpm for 10 min and the amount of GBS in the supernatant was measured by UV-Vis spectrophotometer (Genesys-10S, Thermo Scientific, USA). The supernatant of blank SPI-alginate microparticles was adopted as the blank to correct the absorbance reading value of the GBS-trehalose-SPI-alginate microparticles. The corrected optical density (OD) value was then used to calculate the concentration of GBS in the supernatant. The LE and LC were determined using the following calculations:

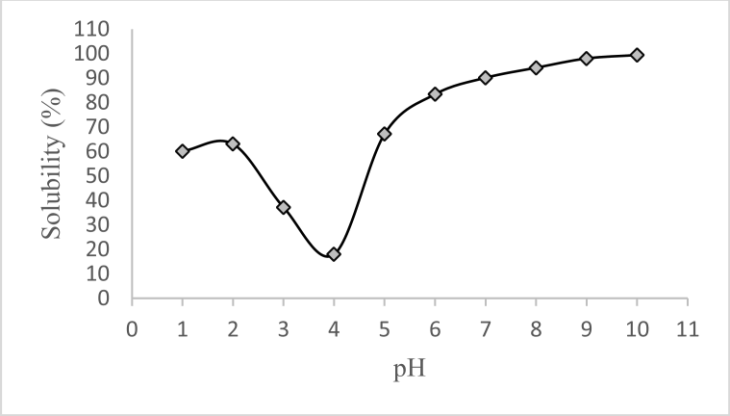

Figure 1. SPI solubility profiles based on various pH treatment.

Table 1. Properties of loaded SPI-alginate microparticles formulations

Formulation	SPI (100 ml)	Sodium Alginate (15 ml)	GBS:Trehalose	LE (%)	LC (%)
F1	70 mg	30 mg	13 X 10^8 cells/ml: 95 mg	47.37±2.72	3.79±0.22
F2	60 mg	25 mg	13 X 10^8 cells/ml: 95 mg	70.77±5.48	6.66±0.18
F3	50 mg	20 mg	13 X 10^8 cells/ml: 38 mg	92.08±1.56	10.52±0.52

$$\text{LE (\%)} = \frac{\text{total amount of GBS trehalose} - \text{unbound GBS trehalose}}{\text{total amount of GBS trehalose}} \text{ x } 100$$

$$\text{LC (\%)} = \frac{\text{total amount of GBS trehalose} - \text{unbound GBS trehalose}}{\text{dried microparticle weight}} \text{ x } 100$$

3. RESULTS AND DISCUSSION

As it can be observed in Figure 1, SPI solubility decreased at pH 3 and 4 (37.17% and 17.95%) then increased at pH 6 up to alkaline conditions (>83.4%). In a study conducted by Ali *et al.* [14], SPI generally had low solubility (<50%) at pH 4 and 5 either without or with additional treatment. The solubility curve of SPI was U-shaped, like those of most plant proteins, the minimum solubility corresponding to their isoelectric point [12]. The higher SPI solubility at alkaline pH values corresponds to the functionality of protein which is closely related to the conformational state, influenced by processing conditions (temperature, pH, solvent) and affected by the equilibrium between protein–solvent and protein–protein interactions [15][16]. In this study, the solubility characteristics for the SPI at pH 6 were improved by as much as 2 times and 4 times higher, when compared to the SPI produced by the acidic isoelectric precipitation process at pH 3 and pH 4, respectively. In a study conducted by Charve and Reineccius [17], higher solubility of SPI in the water improved its ability to form a dried matrix around dispersed compounds in dehydration processes, which entraps them inside the matrix and protects them from air oxidation and UV degradation. Thus, the determination of pH in SPI-based microencapsulation will be relevant starting from pH 6 or higher to obtain good solubility.

Loading efficiency refers to the mass of drug as a percentage of the total mass of the delivery system which can be determined by extracting the drug using solvents, followed by quantification tests, while loading capacity indicates the percentage of the mass of the microparticles that is due to the encapsulated drug [18]. In this study, we observed three formulations with different ratios of SPI-alginate and GBS-trehalose as shown in Table 1. With a fixed amount of GBS, the lower amount of SPI-alginate and trehalose showed greater values of the LE and LC. In addition, the F3 composition showed the highest value for LE and LC with 92.08% and 10.52% respectively, this is in accordance with Hadzieva *et al.* [7] who have suggested that a balanced ratio of constituent materials is essential to provide available functional groups for binding to bacterial cells. Related to this, the study conducted by Burgain *et al.* [19] suggests the possibility role of SPI-alginate serving as a cell adhesion ligand, considering its ability in electrostatic interactions and short-range forces such as Van der Waals, acid-base, hydrogen bonding and biospecific interactions with alginates and cells.

In this study, we also utilize trehalose as an important part of formulation properties. As shown in Table 1 and Figure 2, the involvement and balance of the ratio of trehalose with other microparticle constituents had an impact on the improvement of the LE and LC values along with the decrease of its amount,

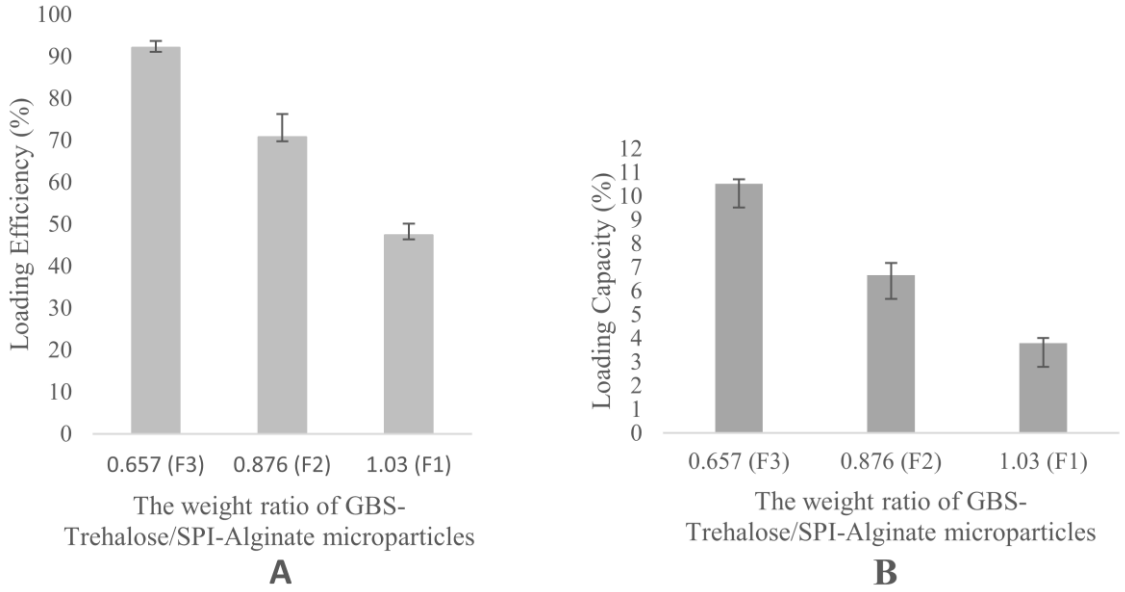

Figure 2. Effect of GBS-Trehalose/SPI-Alginate microparticles weight ratio on loading efficiency (A) and loading capacity (B). Bar expressed standard deviation.

which was indicated by a decrease in the unbound GBS in the supernatant. F2 and F3 with a lower ratios of GBS-trehalose/SPI-alginate (0.876 and 0.657) resulted in higher LE and LC values compared to high ratio properties on F1 (1.03), which indicates an increase in the efficiency of GBS encapsulation by microparticles. According to Domian *et al.* [20], the combination of trehalose-load ingredients in a microparticle encapsulation method yielded efficiencies exceeding 90%, the addition of trehalose was the factor that a greater impact on the properties of microcapsules compared with the factor of other stabilizers in the preparation. According to Nunes *et al.* [21] in their study, the use of trehalose in bacterial microencapsulation plays a major role in heat protection, anticold shock, storage stability and also showed the fewest viability losses during a long term of storage, this provides a profitable value to the method.

In the recent study, we also employ alginate to strengthen the mechanical stability of microparticles by ionic cross-linking. According to Hadzieva [7], alginate with SPI plays a role in the swelling process of microparticles with encapsulated bacteria and affects the bacteria release profile consistently; this shows that biological ingredient release is regulated by a combination of diffusion, dissolution and degradation processes. Solanki *et al.* [22] through their study reported an increase in the adhesion of micro-encapsulated bacteria based on alginate layers; however, mucoadhesion can increase significantly when alginate is mixed with a mucoadhesive polymer. Literally, SPI-based microparticles will interact strongly with mucin in the gastrointestinal tract due to the protonation of NH^{3+} ions and their interaction with the functional groups of the mucin, thus the containing bacteria can be released on the surface of the mucus in a controlled manner [7]. These results indicate that SPI-alginate is relevant for the further development of an inactivated bacterial vaccine delivery system for the oral route.

4. CONCLUSION

Based on the result, we found that the combination of *microparticle* constituents in the form of SPI-alginate and GBS-*trehalose* resulted in high loading efficiency and loading capacity, especially in the formulation with a ratio of 0.657:1. Based on the SPI solubility test, we obtained a high percentage of solubility at pH 6-10, ranging from 83.40% to 99.39%. These results demonstrate that SPI-alginate is relevant for developing an inactivated bacterial vaccine carrier for the oral route. More research is needed for *microparticles* spray dry processing, characterization, release capability and *in vitro* stability under various environmental conditions.

AUTHORS' CONTRIBUTIONS

Dadang Priyoatmojo designed and performed the experiment, prepared materials, collected the data and arranged the first-draft of the manuscript. Tri Handayani and Afi Candra Trinugraha performed and checked data analysis. Teguh Wahyono and Nina Herlina supervised the experiment and revised the manuscript.

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