ABSTRACT

Objective: Oftentimes, the recombinant antigen for the use of vaccines is less immunogenic than live attenuated or inactivated vaccines. Hence, a potent adjuvant is needed to enhance the immune response. Moreover, the role of vector design is also important to facilitate the improvement of the immune response. The aim of this research was to develop hepatitis B surface antigen (HBsAg)-loaded nanoparticles and *Moringa oleifera* aqueous leaf extracts as an adjuvant using chitosan polymer.

Methods: Chitosan nanoparticles were prepared by the ionic gelation method using sodium tripolyphosphate as the cross-linking agent. A system was composed of chitosan core in which HBsAg and *M. oleifera* extracts were incorporated. The concentration of HBsAg used in this combination was 10 µg/ml and the concentrations of extracts were 10, 50, and 100 µg/ml, respectively. In this study, three types of nanoparticles were produced: HBsAg-loaded nanoparticles, *M. oleifera*-loaded nanoparticles, and combination of HBsAg-*M. oleifera*-loaded nanoparticles. The nanoparticles formed were characterized by the particle size, HBsAg entrapment efficiency using sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the entrapment efficiency of extracts using the total flavonoid method.

Results: The results showed that the particle size was between 111 and 245 nm. The entrapment efficiency of HBsAg in the separate formula was 79%, while that in the combined formula was approximately 96–98%. Furthermore, the entrapment efficiency of the extracts in the separate formula was around 64–91%, while that in the combined formula was 55–82.5%. Particularly, HBsAg-*M. oleifera*-loaded chitosan nanoparticles with the extract concentrations of 50 µg/ml showed the highest entrapment efficiencies of HBsAg and *M. oleifera* extracts of approximately 98 and 82.5%, respectively.

Conclusion: Collectively, the system has been successfully developed, so it is then plausible to determine the function of the devices to enhance the immune response in the future.

Keywords: Nanoparticle, Chitosan, Hepatitis B surface antigen, Adjuvant, *Moringa oleifera*.

INTRODUCTION

Hepatitis B is a type of deadly viral disease caused by the hepatitis B virus (HBV). Approximately 4.5 million new HBV infections occur every year in the world. In Indonesia, the results of basic medical research in 2007 showed the prevalence of hepatitis B at 9.4%, which means that at least one in ten of the Indonesian population had been infected with hepatitis B or approximately 23 million people from the total population of Indonesia. After an acute infection, infants and adults, who become infected with the HBV carrier that has been carrying the disease for years and at a later stage, referred to the occurrence of cirrhosis and hepatocellular carcinoma [1].

The prevention of chronic viral hepatitis B has become a top priority for the global community. An approach was prepared by activating the appropriate immune response during chronic viral infection. Immunization with hepatitis B vaccine is the best preventive measure against infection. However, the hepatitis B vaccine can be available in parenteral dosage forms. It is not very effective because parenteral vaccines only stimulate the systemic immune response and the produced antibody cannot reach the mucosal surface, which is the main entry point of most infectious pathogens [2]. Oral vaccine delivery has a distinct advantage over traditional injected vaccines because it induces both a systemic and mucosal immune response, whereas the injected vaccine only leads to serum antibody production. Stimulating an immune response at the mucosal site is very desirable because many pathogens enter the body at this site. If an immune response occurs at the mucosal site, the pathogen can be prevented from even entering the body [3]. However, the vaccine administered orally has low bioavailability. Therefore, to boost the immune response and to administer oral vaccines, adjuvants and suitable delivery systems are required.

It has been reported that the ethanol extracts of *Moringa oleifera* had the immunostimulatory activity. Extracts of 250 mg of weight increased the activity of the macrophage phagocytic index by approximately 2.85±0.81 [4]. Meanwhile, another study reported that *M. oleifera* leaves have immunomodulatory activity. The aqueous extracts of *M. oleifera* at a concentration of 0.1 mg/ml increased the number of cluster of differentiation 4+ (CD4+) cells and Cluster of differentiation 8+ (CD8+) cells by approximately 54.49% and 15.57%, respectively. It is then believed that *M. oleifera* plays an important role in immune stimulation [5]. Recently, it was reported that *M. oleifera* extracts were loaded into gelatin nanoparticles [6]. However, there is still no report regarding the encapsulation of hepatitis B surface antigen (HBsAg) and *M. oleifera* extracts in nanoparticles. This research aimed to develop nanoparticle hepatitis B (HBsAg) vaccine using the chitosan polymer and to use plant-derived adjuvant for the vaccine delivery systems. Particularly, in the first stage, the ability of HBsAg and *M. oleifera* extracts to be packaged in the nanostructured vesicles was studied.

MATERIALS AND METHODS

Materials

*M. oleifera* extracts were purchased from Megasetia, Indonesia. Toluene, ethanol, nitrobenzine, sodium tripolyphosphate (STPP), acetic
acid, sodium hydroxide, methanol, aluminum chloride ([AlCl₄]⁻), acetic sodium, quercetin, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma-Aldrich. The total percentage of acrylamide (T-acrylamide) 30%, Temed, and silver staining reagent was purchased from Thermo Fisher Scientific. HBsAg was kindly received from Bio Farma, Indonesia.

Methods

Examination of water content

The level of water examination was performed with the toluene distillation method. 200 ml of toluene was added in a distillation flask, and 7 ml of water was also added into this flask and heated until the toluene was saturated. The toluene number was recorded. 2 g of simplisia was put into the flask containing the saturated toluene and heated until the water was completely evaporated. The change in toluene numbers was recorded. In the examination of the water content of M. oleifera extracts, approximately 100 mg of the extracts was weighed and analyzed by the Karl Fischer titrator using reagents of hydridan methanol dry and Combifitritant.

Drying losses examination

The examination of the losses by drying was done by means of the Moisture Balance. Simplisia or extracts were weighed and put into the apparatus and heated at a temperature of 105°C. The weight of the simplisia was recorded before and after the examination. Then, the level of drying losses was then calculated.

Inspection of chromatogram pattern

The mobile phase consisted of chloroform and methanol that was prepared in a ratio of 9:1. 10 ml of mobile phase was put into the chamber and saturated. Simplisia was dissolved in ethanol followed by a filtration process. Quercetin was independently dissolved in ethanol. 10 µl of simplisia solution as well as quercetin solution was applied on the thin-layer chromatography (TLC) plate in the chamber and eluted by the mobile phase. The TLC plate was dried and sprayed with Borocitric solution, followed by heating at 100°C for 2 min. Spots of chromatogram pattern were detected under ultraviolet (UV) light at a wavelength of 366 nm. To check the chromatogram pattern of M. oleifera extracts, the procedure was similar to that of simplisia as described above, except that the ratio of mobile phase of chloroform to methanol was approximately 8:2.

Determination of total flavonoid content

About 50 mg of simplisia was dissolved in methanol to obtain a concentration of 5000 µg/ml and then filtered to remove the remaining undissolved simplisia. 0.5 ml of this solution was added with 1.5 ml of methanol, 0.1 ml of 10% AlCl₃, and 0.1 ml of 1 M sodium acetate and then incubated in a dark room for 30 min. The sample was then analyzed by an UV-visible spectrophotometer at a maximum wavelength of 432 nm.

Preparation of chitosan nanoparticles carrying HBsAg and M. oleifera extracts

Chitosan was dissolved in 1% acetic acid (v/v) to form a concentration of 1.4 mg/ml with a pH of 5. STPP was dissolved in distilled water to form a concentration of 1.47 mg/ml. HBsAg concentration of 10 µg/ml along with extract concentrations of 10, 50, and 100 µg/ml was incubated with a concentration of 0.001%–40% STPP solution was added dropwise to a 750 µl of chitosan solution containing HBsAg and extracts using a 1 ml syringe. It was then stirred at room temperature using a magnetic stirrer. The suspension was centrifuged at 13,000 rpm for 10 min, and the supernatant was collected to determine the entrapment efficiencies of HBsAg and the extracts. The precipitates were redispersed in 100 µl of deionized water and sonicated at 60 Hz for 10 s. Then, the final nanoparticles suspension was characterized.

Characterization of particle size and polydispersity index

Particle size analysis was performed using DelsaTM NanoC (Beckman Coulter). The instrument can measure particle sizes in the range of 0.6 nm–7 µm, with an optimum concentration of 0.001%–40%.

Determination of the entrapment efficiency of HBsAg

The entrapment efficiency was determined through an indirect method by measuring the amount of free HBsAg that was unencapsulated in the nanoparticles. The HBsAg assay was done using silver staining on SDS-PAGE gel with the silver PROTEO kit and observed by a densitometer.

RESULTS AND DISCUSSION

This study presented the packaging of HBsAg and M. oleifera into nanoparticles in which the chitosan polymer was used. It has recently been demonstrated that silver nanoparticles (AgNPs) were synthesized from the leaf extracts of M. oleifera [7]. However, the toxicity of particularly AgNPs to various organisms, such as Pseudomonas putida, Escherichia coli, Daphnia magna, and Chlamydomonas reinhardtii, is undeniable [7]. It has also been suggested that the three major mechanisms of AgNPs toxicity appear to directly damage cell membranes, generate reactive oxygen species, and release Ag ions [8]. Therefore, in this study, we focused on the use of biodegradable polymers such as chitosan to encapsulate HBsAg and M. oleifera extracts, whose degradation polymers cannot be harmful and ensure removal from the body.

Characterization of simplisia and extracts of M. oleifera

This study aimed to investigate the ability of nanoparticles to encapsulate HBsAg and M. oleifera extracts in the same structure to accommodate HBsAg vaccine for enhancing its immune response. Previously, we examined the standardization of M. oleifera simplisia according to the requirements of the Indonesian Herbal Pharmacopeia. As a result, the simplisia have met the requirements based on test parameters and microscopic studies, and thus, it can be extracted for its use as an adjuvant (Table 1).

In the case of the standardization of M. oleifera extracts, it was based on a chromatogram pattern of the ethanol extracts of its leaves. On the basis of the results of the chromatogram pattern, it is likely that the pattern of the extracts is similar to that of simplisia (Fig. 1). The results

Table 1: Simplisia Characteristic Data

| Test parameters | Results (%) | Requirements |
|-----------------|-------------|--------------|
| Drying losses   | 7.0±0.4     | Not >12%     |
| Water content   | 5.2±0.35    | Not >10%     |
| Total flavonoid | 1.4±0.1     | Not <0.5%    |

Values represent mean±SD, n=3
The preparation of chitosan nanoparticles as an antigen carrier matrix is important in this study to package HBsAg and M. oleifera extracts. Particle size plays an important role in the nanoparticle delivery system. Magnocellular cells (M cells) in the follicle-associated epithelium (FAE) of the gut play a role in increasing the immune response. As for the large-sized particles, they will remain in the M cells. If the particle size is <1 µm, it is internalized and systemically transported to the M cells, which potentially generates a systemic immune response. Therefore, it is likely that the role of stirring rate and STPP addition technique is important in this study to package HBsAg and M. oleifera extracts.

Additionally, we added STPP dropwise to the chitosan solution in the preparation of nanoparticles. This method prevented intermolecular crosslinking that leads to a larger particle size. It has been explained that the formation of aggregates leading to the formation of larger particles depends on the contact time between the particles when they collide [19,20]. Time is also essential to obtain a permanent chain formation from 0.05 to 0.39. The results reveal that the particle size distribution was significantly narrowed by increasing the stirring rate from 200 to 800 rpm. Furthermore, the increase in the stirring rate at 1000 rpm leads to an aggregate formation [18]. It is hypothesized that an adequate stirring rate accelerates the dispersion of STPP in the chitosan solution and the increase in shear forces improves the monodispersity, while an intense stirring rate may destroy the repulsive force between the particles and lead to aggregation of the particles.

Table 2: Characteristics of nanoparticles

| No | Formula                | Particle size (nm) | Polydispersity index |
|----|------------------------|--------------------|----------------------|
| 1  | Empty nanoparticles    | 111.1±6.5          | 0.051–0.277          |
| 2  | Nano-HBsAg 10 µg/ml    | 143.8±12.7         | 0.172–0.346          |
| 3  | Nano-M. oleifera 10 µg/ml | 221.9±13.1     | 0.251–0.277          |
| 4  | Nano-M. oleifera 50 µg/ml | 239.7±25.0     | 0.160–0.392          |
| 5  | Nano-M. oleifera 100 µg/ml | 244.6±49.1    | 0.052–0.278          |
| 6  | Nano-HBsAg+ M. oleifera 10 µg/ml | 181.8±33.1 | 0.173–0.356          |
| 7  | Nano-HBsAg+ M. oleifera 50 µg/ml | 219.8±50.5 | 0.195–0.327          |
| 8  | Nano-HBsAg+ M. oleifera 100 µg/ml | 219.2±52.4 | 0.182–0.224          |

Values of particle size represent mean±SD; n=3 and data of polydispersity index represents range. M. oleifera: Moringa oleifera, HbsAg: Hepatitis B surface antigen.
polydispersity index is from 0 (monodisperse particles) to 0.5 (wide particle size distribution). The low polydispersity index indicates that the formed dispersion systems are more stable in the long term [22].

Entrapment efficiency of HBsAg

The entrapment efficiency of HBsAg was determined using an indirect method by measuring HBsAg unencapsulated in the nanoparticles. It was examined using silver staining on SDS-PAGE. In this method, the gel was stained with silver dye and scanned with a densitometer to capture the density in the image. As a result, nanoparticles containing a combination of HBsAg and M. oleifera extracts with the same concentration of 10 µg/ml provided the highest entrapment efficiency of 98.59% (Table 3). The lowest one was given by the 10 µg/ml HBsAg nanoparticles. It is possible that the small size of the HBsAg-loaded nanoparticles of approximately 144 nm limits the entrapment of HBsAg, where it shapes VLP with a size of 20–33 nm. In addition, the results of the captured image from SDS-PAGE showed that there was a light monomer band that appeared in the supernatant samples (Fig. 2, lanes 3–6). Those were having sizes of 26 kDa, which have the same size as HBsAg standard marker. Thus, it is likely that it reflected a minor unencapsulated HBsAg. Since the encapsulation efficiency of HBsAg was high of a minimum of 96%, the packaging of HBsAg was considered successful. In nanoparticles encapsulating the combination of HBsAg and M. oleifera extracts, the entrapment efficiency of HBsAg was concomitant with an increase in the concentration of the extracts. This suggests that there is a possibility of competition between the HBsAg and the extracts to be located in the chitosan-based nanoparticles.

Table 3: Entrapment efficiencies of HBsAg and M. oleifera extracts in nanoparticles

| No | Formula                        | Attachment (%) | HBsAg     | M. oleifera extracts |
|----|--------------------------------|----------------|-----------|---------------------|
| 1  | Nano-HBsAg 10 µg/ml            | 79.47±5.62    | -         |                     |
| 2  | Nano-M. oleifera 10 µg/ml      | -             | 63.91±3.27|                     |
| 3  | Nano-M. oleifera 50 µg/ml      | -             | 90.86±7.14|                     |
| 4  | Nano-M. oleifera 100 µg/ml     | -             | 88.69±7.67|                     |
| 5  | Nano-HBsAg+M. oleifera 10 µg/ml| 98.59±6.48    | 55.41±39.98|                   |
| 6  | Nano-HBsAg+M. oleifera 50 µg/ml| 97.85±7.85    | 82.56±26.02|                   |
| 7  | Nano-HBsAg+M. oleifera 100 µg/ml| 95.95±6.43   | 76.51±24.12|                   |

Values represent mean±SD, n=3, M. oleifera: Moringa oleifera, Hepatitis B surface antigen

Fig. 2: Results of SDS-PAGE of chitosan nanoparticle compared to standard HBsAg. (1) Ladder, (2) Standard HBsAg, (3) Nano HBsAg 10 µg/ml, (4) Nano HBsAg 10 µg/ml + M. oleifera extracts 10 µg/ml, (5) Nano HBsAg 10 µg/ml + M. oleifera extracts 50 µg/ml, (6) Nano HBsAg 10 µg/ml + M. oleifera extracts 100 µg/ml.

ENTRAPMENT EFFICIENCY OF M. OLEIFERA EXTRACTS

The aqueous extract of M. oleifera was highly entrapped in the chitosan-based nanoparticles in a range of 64–91%. It seemed that the highest entrapment efficiency of 91% was provided by the use of the concentration of M. oleifera extracts of 50 µg/ml. Furthermore, in the case of the entrapment efficiency of M. oleifera extracts in the nanoparticles loaded with a combination of HBsAg and the extracts, it showed a high level of approximately a range of 55–82.5%. The highest entrapment efficiency was also obtained using the concentration of extracts of 50 µg/ml. Thus, the maximum entrapment capacity of the extracts having a concentration of 50 µg/ml was shown. Basically, the competition between HBsAg and M. oleifera extracts occurs in the nanoparticles so that, particularly in the case of combination, the entrapment efficiency was lower than that of a single compound. Our results show that the high entrapment efficiency of M. oleifera extracts is 91%, which is better than previous studies. It was reported that the gelatin nanoparticles encapsulated with M. oleifera extracts had an entrapment efficiency of around 83% [5]. Meanwhile, our results showed a comparable entrapment efficiency of M. oleifera of approximately 82.5%, and especially, it is in combination with HBsAg, which also showed a high entrapment efficiency of 98%. To the best of our knowledge, this is the first report of packaging two compounds of HBsAg vaccine and M. oleifera extracts in the same nanostructure. The potential function of the produced chitosan-based nanoparticles loaded with HBsAg-M. oleifera extracts in enhancing the immune response of HBsAg needs to be clarified in the future.

CONCLUSION

The results of the present study concluded that the HBsAg vaccine along with the aqueous extracts of M. oleifera was successfully packaged in the chitosan-based nanostructured particles with highly entrapment efficiencies of HBsAg and M. oleifera extracts.

ACKNOWLEDGMENT

This work was supported by the Research Grant of the Institut Teknologi Bandung Development Project (III) of the Japan International Cooperation Agency, Japan.

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