ENCAPSULATION OF dsRNA GIH (GONAD INHIBITING HORMONE) USING CHITOSAN NANOPARTICLE WITH SEVERAL PHYSIOCHEMICAL OPTIMIZATIONS

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ABSTRACT

Spawning process in black tiger shrimp (P. monodon) is negatively regulated by GIH hormone produced by X organ in eyestalk. Cutting the eyestalk (ablation) is the conventional way to fasten the spawning process. However, this method may causes physiological problems in shrimp. The new way to fasten spawning process is through RNAi. RNAi acts by repressing the GIH expression through postranscription regulation. Injection of naked dsRNA to induce RNAi mechanism is not considered effective since it may cause dsRNA degradation during delivery. Nanoparticle encapsulation is expected to protect the dsRNA from degradation. Chitosan is good source of polymer for nanoparticle formation particularly for biological application since it is non-toxic, biodegradable and biocompatible. This research was aimed to improve chitosan nanoparticle formation using ionic gelation method with natrium tripolyphosphate as cross-linker agents. Optimization of methods were conducted on four parameters that affect the formation of nanoparticle; ratio of polikation/polianion, pH, speed of mechanical stirring and the presence of the surfactant. Characteristic of nanoparticles such as the size, polydispersity index, encapsulation efficiency, shape, and dispersity were analyzed. The result of the experiment found that ratio 4.75:1 for polikation/polianion was the best ratio for nanoparticle formation since it produce stable nanoparticle suspension. Lowering pH of the solution affected the size of nanoparticle with the size < 100 nm obtained at the pH below 4.5. Usage of high-speed homogenizer instead of vortex caused significant size reductions. Presence of the 0.2 % surfactant Tween 80 reduced the polydispersity index which indicate the enhancement of the
nanoparticle stability. Transmission Electron Microscopy (TEM) showed the presence of spherical nanoparticle with size < 100 nm. Gel electrophoresis also confirmed the formation of the nanoparticle with dsRNA undergo migratory shift compared to non-encapsulated dsRNA. Result of this experiment showed that GIH dsRNA can be encapsulated in chitosan nanoparticles using ionic gelation method with ratio of polikation/polianion: 4.75/1, pH below 4.5 and 0.2 % surfactant Tween 80.

Keywords: Chitosan Nanoparticles, Ionic Gelation, dsRNA, RNA Interference, Gonad Inhibiting Hormone.

INTRODUCTION
Shrimp contributes to the most of aquaculture product coming from Indonesia (account for more than 400 metrics ton of shrimp, with value of USD 2.5 billion); however the production still can be increased by reducing the bottleneck problems (Ministry of Marine Affairs and Fisheries, Indonesia, 2013). One of the bottleneck problems of shrimp aquaculture development is the slow reproductive cycle of the shrimp broodstock. The ovarian maturation in shrimp is negatively regulated by secretion of GIH (Gonad Inhibiting Hormone) by X organ in eyestalk (Tsukimura, 2001). Conventionally, shrimp farmer fasten the reproductive cycle by cutting the eyestalk. Although it has been proven as effective method, it also shorten the life of the broodstock, causing the need of continuous broodstock supply from the wildlife (Treyerattrakooot et al, 2008).

RNAi technology is an interesting option to replace the conventional method. On the other hand, previous research has shown that the injection of naked dsRNA only able to reduce the level of GIH up to 30% and the effect only last for five days (Pancoro et al, 2011). This might be due to the degradation of naked dsRNA by RNAse-III enzyme which found abundant in the hemolymph of shrimp (Ruo, 2012).

RNAi delivery system using nanoparticle has been investigated to overcome the problem (Katas et al, 2006). This can ensure that dsRNAis safely protected until reaching the target organ from the injection site. Chitosan is suitable material for this purpose. Derived from the exoskeleton of the shrimp, this material currently popularly used for the template of nanoparticle. This material has several supportive characteristics for biological application such as biodegradable, biocompatible and the low toxicity (Nimesh, 2013).

Chitosan is obtained by deacetylation of chitin. The molecule is a polymer which consists of D-glucosamine and N-acetyl-D-glucosamine chains. When
chitosan (pKa : 5.6) dissolved in acid solution, the amine group will be protonated. Therefore, numerous positive charges are possessed by single chitosan molecule, which can interact electrostatically with dsRNA.

Ionic gelation is the simplest way to create nanoparticles using chitosan as starting material. In this method, chitosan electrostatically interacts with polyanion such as NatriumTripoliphosphate and dsRNA. Polyanion acts as cross-linker agents which induce the formation of 3D matrix with active molecule entrapped inside (Heish, 2008).

The ionic gelation method can be easily controlled and the processes do not involve usage of toxic material, hence it suitable for in vivo usage (Agnihotriet. al, 2004). Nevertheless, according to Etiket. al (2012), this method often produces nanoparticle with board range of size (high polydispersity) and the short-lived nanoparticle because there is tendency of nanoparticle to aggregate overtime.

Thus, chemical and physical parameters must be optimized to produce stable nanoparticle. Type and speed of mechanical treatment are two physical factor which affect the characteristic of nanoparticle, whereas the pH solution, ratio of polycation/polyanion, degree of deacetylation and molecular weight of chitosan and surfactant are the several chemical factors contributed to the size and stability of nanoparticle (Mao et. al, 2009).

**RESEARCH METHODOLOGY**

**Materials**

Chitosan (>80% deacetylation), low molecular weight obtained from Wako, Japan NatriumTripoliphosphate (Na-TPP) obtained from Sigma. dsRNA was over-expressed in *E. coli* and extracted using modified Saksmerprome method.

**Methods**

**Preparation of Nanoparticles**

Nanoparticles were prepared using Calvo-modified methods (Calvoet. al, 1997). Polycation (NatriumTripolyphospate&dsRNA) was slowly added into chitosan mixture with constant mechanical stirring. The different ranges of chitosan/polykation ratio from 1:1 to 6:1 were tested. The pH of Chitosan also was varied ranging from 3, 3,5, 4,4,5 to 5. Nanoparticles were created using two different of mechanical treatment, stirred using vortex
and stirred using high-speed homogenizer. Effect the presence of surfactant was also observed by addition of 0.2% Tween 80 to the chitosan mixture.

**Characterization of Nanoparticles**

**Visual appearance**
Nanoparticle was observed one hour after preparation. The turbidity and the presence of precipitate was compared with the control.

**Gel Electrophoresis Mobility Shift**
Encapsulated dsRNA and non-encapsulated dsRNA were analyzed using electrophoresis in 1% agarose gel for 20 minutes. Subsequently, the dsRNA was stained using ethidium bromide and the dsRNA fluorescence observed using UV. Successfully encapsulated dsRNA show migration inhibition compared to the control.

**Size and Polydispersity Index**
A disposable cell was filled with 1 mL of nanoparticle suspension. Then, the size and polydispersity index of nanoparticle were measured using DLS methods (Delsa Nano C, Beckman Coulter) with scattering angle 165° and measurement temperature 25°C.

**Efficiency of Encapsulation**
Nanoparticles were separated from other materials using centrifugation (13000 x g, 30 minutes). The concentration of non-encapsulated dsRNA left in supernatant was measured using spectrophotometer (wavelength 260 and 280 nm). Encapsulation efficiency was calculated (EE) using formula below:

\[
EE = \frac{\text{dsRNA total} - \text{dsRNA supernatant}}{\text{dsRNA total}} \times 100
\]

**Nanoparticle Size Alteration**
The size of nanoparticle created using surfactant and without surfactant (0.2% Tween 80) were observed after 0, 1, 3 and 5 days storage at 4°C. The change of the size was measured using DLS method (Delsa Nano C, Beckman Coulter).
TEM (Transmission Electron Microscopy) Imaging

100uL of nanoparticle suspension was mixed with 2% (v/v) phospotungstat acid. The droplet of mixture was dropped into copper grid. After dried, the nanoparticle was examined using TEM Jeol 1400.

RESULT & DISCUSSION

In this experiment, several physiochemical parameters were tested to create nanoparticle with best characters in terms of size and stability. Nguyen & Szocka (2010) stated that nanoparticle for drug or nucleic acid delivery should have certain characteristics to facilitate delivery. For instance:  
(i) the size must be below 200 nm  
(ii) none of the material toxic for living organism  
(iii) stable and do not degrade in blood plasm environment  
(iv) phagocytosis and opsonization by immune cell can be prevented  
(v) nanoparticle must be designed so that it has capability to escape from endosomal structure during endocytosis by the cell.

The formation of chitosan nanoparticle indirectly can be observed from the appearance of the suspension. Nanoparticle formed when the solution become turbid compared to the control without any noticeable precipitate formed after incubation (Fan et. al., 2011). This indicates the stable nanoparticle suspension. From all combinations tested, the ratio of polycation / polyanion = 4.75 : 1 was the best ratio for the dsRNA nanoparticle since it is relative turbid compared to the control yet none of precipitate observed.

pH has effect on the size of the nanoparticle and the encapsulation efficiency. From the Figure 2, it can be inferred that the lower pH of chitosan, the nanoparticle formed become smaller. This is as the result of the protonation the amine group of chitosan. Hence, the molecule have a lot of positive charge which facilitate interaction with the negative charge in polyanion (Ruo, 2012). Small nanoparticle enhances the delivery rate, but...
due to the size limitation, the smaller nanoparticle only can load few active materials compared to the larger one (Ruo, 2012). Thereby, it is observed that there are downward trend of the encapsulation efficiency along the decrement of the Ph

Type of the mechanical treatments affects the size of nanoparticle as well. Figure 3 explains the pattern. The usage of the ultrasonic homogenizer instead of the vortex causes the significant reduction in terms of size. Smaller nanoparticles has large ratio of surface area/volume which lead to better diffusion, large permeability in the living tissue and lower probability to be recognized by immune cells (Kou et. al, 2013). Polydispersity index has relation to the distribution of the nanoparticle size in the suspension. Larger value indicates the higher the distribution, hence the nanoparticles become heterogenous. It is obviously seen from the figure 3 that the presence of the surfactant (0.2% Tween 80) lower the value, which means that the nanoparticle formed become more homogenous and more stable.

The result of the stability studies in line with the result of the polydispersity measurement. Nanoparticles created using surfactant remains stable until day 5 storage and did not possess any significant size alteration. In contrast, nanoparticle prepared without involving surfactant was getting larger along the day of the study. This might have to do with the stabilizing effect of the Tween 80. Tween 80 is a sort of anionic surfactant that will be attracted and adsorbed on the surface of chitosan (cationic) nanoparticle. This adsorbtion provides space between nanoparticles, which keep the nanoparticle in distance. Applying Tween 80 as surfactant causes ‘steric stabilization’ effect which enhance the stability of nanoparticle (Napper, 2009).
Nanoparticle prepared using best method (pH 4.5, mechanical treatment using ultrasonic homogeizer and addition of 0.2% Tween 80 as surfactant) was analyzed further using gel electrophoresis and TEM imaging. The gel electrophoresis result showed that the dsRNA band disappeared on the 252 bp (the size of naked dsRNA), and the dsRNA band now observed on the starting point of migration. This indicates that the dsRNA undergo
migratory inhibition because it is entrapped in the structure of the nanoparticle (Katas et al., 2006). TEM imaging also showed the presence of the nanoparticle which is relative homogenous in terms of size (around 40-50 nm) and there is no aggregation observed. The result also indicates that the shape of dsRNA – chitosan nanoparticles is spherical.

Figure 5. Gel Electrophoresis Results (1, 2, 3: Encapsulated dsRNA, K: Control: Nonencapsulated dsRNA) (Left), Result of Transmission Electron Microscopy (Right).

CONCLUSION
The optimal ratio of chitosan to polyanion (dsRNA and NatriumTripolyphospate) was found at 4.75: 1. Lowering the pH of chitosan leads to smaller nanoparticles with diameter below 100 nm achieved at pH lesser than 4.5. On other hand, along with decrement of the pH, there was also declining trend for the amount of dsRNA loaded in the nanoparticle (encapsulation efficiency). Usage of 0.2% Tween 80 as surfactant enhances the stability of the nanoparticle, hence the nanoparticle undergo minimal diameter alteration during storage. TEM imaging also prove the presence of the homogenous spherical-nanoparticles with diameter size approximately 40-50 nm.
REFERENCES


