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Chitosan nanoparticle to carry vitamin C through the gastrointestinal tract and induce the non-specific immunity system of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Chitosan is a polysaccharide that has gained interest in recent to encapsulate active compounds due to its non-toxicity, biodegradability, biocompatibility and immunity system induction. The chitosan nanoparticle was prepared by adding vitamin C to tripolyphosphate (TPP) solution and then blending it stepwise to the chitosan solution under stirring. Nanoparticle characterizations were analyzed by scanning electron microscopy (SEM) and Zetasizer. The release profile of vitamin C into the gastrointestinal and serum of rainbow trout (*Oncorhynchus mykiss*) was portrayed and its effect on the immunity system of the fish was determined as well. The results showed that the chitosan nanoparticle had spherical shape; positive charge with particle size of 185 nm and in vivo controlled release until 48 h. Lysozyme and complement contents in the fish serum increased as long as they were provoked by the nanoparticle. This nanoparticle is a promising technique to boost immunity system of fish.

1. Introduction

Chitosan is a cationic biopolymer obtained from N-deacetylation of chitin, a naturally occurring and the second abundant polysaccharide after cellulose (Jolles & Muzzarelli, 1999). Chitosan has excellent features due to its non-toxicity, biodegradability, biocompatibility and bioadhesion (Dorkoosh, Verhoef, Tehrani, Borchard, & Junginger, 2003; Muzzarelli, 2010). Having encapsulated active compounds, chitosan protects them from harsh conditions into gastrointestinal tract and enhances their absorption (Aranaz et al., 2009).

Vitamin C is one of the most important antioxidants that may reduce the risk of cancer using various mechanisms (Esposito, Cervellati, Menegatti, Nastruzzi, & Cortesi, 2002; Jacobs et al., 2001). In addition, in humans and many animals, vitamin C is not synthesized in digestive tract due to the absence of enzyme L-gulonolactone oxidase that is responsible for the synthesis of vitamin C or ascorbic acid (Wilson, 1973). So, they depend upon exogenous sources of vitamin C.

Overall, the sustainability of vitamin C is labile and most of its functionality loses during processing and storage of food and feeds because of the exposure to high temperature, oxygen and light (Soliman, Jauncey, & Roberts, 1987). Shiau and Hsu (1999) found that approximately 75% of the initial amount of supplemented vitamin C in shrimp feeds was lost during processing at ambient temperature. The utilization of more stable forms of vitamin C is therefore a crucial requirement for human and animal nutrition. Encapsulation is a suitable technique to enhance vitamin C stability (Alishahi et al., 2011).

Rainbow trout culture is an important industry over the world. Regarding intensive culture system for rainbow trout, boosting the immunity system of rainbow trout against stressful circumstances (high density culture) is a good alternative (Cha, Lee, Song, Lee, & Jeon, 2008). Moreover, adequate nutrition has played a crucial role to maintain fish health and its ability to withstand against diseases and immune deficiencies (Amar, Kiron, Satoh, & Watanabe, 2004). In fish, essential nutrients such as proteins, essential fatty acids, vitamin C and E, polysaccharides and some minerals have a pivotal importance to reinforce normal immune functions (Landolt, 1989).

Besides, oral delivery of bioactive compounds in rainbow trout and generally in fish is encountered to the three major barriers, namely the enzymatic barriers sourced from the host luminal and membrane bound enzymes, the immunological cells present within both the enterocytes and underlying connective tissue, and the

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physical barrier of the epithelial cells (Schep, Tucker, Young, Ledger, & Butt, 1999).

Based on the view, the aim of the study was to investigate the encapsulation of vitamin C using chitosan nanoparticle to protect it from harsh conditions through the gastrointestinal tract of rainbow trout (*Oncorhynchus mykiss*) and its controlled release and subsequently its effect on the innate immunity system.

2. Materials and methods

2.1. Materials

Chitosan with the deacetylation degree (DD) of 90% and molecular weight (Mw) of 10 kDa (low molecular weight) was prepared according to a previous study (Abdou, Nagy, & Elsabee, 2008). All other reagents were all commercially available and used without any modification.

2.2. Preparation of chitosan nanoparticle containing vitamin C

A chitosan solution was prepared by dissolving chitosan in 1% (w/v) acetic acid solution until the solution was transparent. Sodium tripolyphosphate (0.5 mg/ml) was dissolved in deionized water. The chitosan solution was flush mixed with an equal volume of TPP solution and the formation of chitosan–TPP nanoparticles began spontaneously via the TPP ionic gelation mechanism. The nanoparticle suspension was gently mixed for 60 min at room temperature before being used for further analysis. Chitosan–TPP/vitamin C nanoparticle was prepared via ionotropic gelation between the positively charged amino groups of chitosan–TPP and the vitamin C. Vitamin C was dissolved with chitosan–TPP solution and then the mixture was subjected to mild magnetic stirring at room temperature for 1 h to promote crosslinking. The nanoparticle was isolated using ultracentrifugation at 10,000 rpm for 30 min at 4 °C. Then the supernatant was discarded and the precipitate was freeze-dried and stored at 4 °C.

2.3. Physiochemical characteristics of chitosan–TPP/vitamin C nanoparticle

The particle size and zeta potential were determined using Zetasizer 3000HSA (Malvern Instrument, London, England). The morphology was observed under a scanning electron microscope (SEM, XL30, Philips, Amsterdam, Netherlands).

2.4. Fish

Healthy rainbow trout (*O. mykiss*), approximately 18–23 cm in body length and approximate 200–250 g in body weight, obtained from a local farming and transported to the facilities at the Fisheries Faculty of Kagoshima University. Subsequently, the fish ($n = 120$) were maintained in four 1000 L tanks supplied with aerated freshwater and the temperature in the water was 14–17 °C. All fish were acclimatized at the site for at least three days before any experimental procedures were carried out.

2.5. Preparation of rainbow trout feed

The commercial rainbow trout feed (Higashimara Co. Ltd. Kagoshima, Japan, 40% crude protein, 11% lipid) was obtained from the market and soaked in water. After 1 h, the feed was crushed and made into a paste. The dried chitosan nanoparticle containing vitamin C (approximately containing 150 mg of chitosan and 75 mg of vitamin C) were added to a 10 g (wet weight) rainbow trout feed paste and mixed thoroughly. Then, this feed mixture was pelletized by pressing through extruders using a die having holes of 5 mm and the pellets were dried at 40 °C in hot air oven for 1 h.

2.6. Oral administration

Rainbow trout was divided into four groups (30 per group), which were kept separately for the experiments. In the first group, the fish were fed with commercial feed (Higashimara Co. Ltd. Kagoshima, Japan, 40% crude protein, 11% lipid) at 2% of the fish body weight per day (twice a day). In the second group, the fish were fed with feeding chitosan/vitamin C nanoparticle mixed feed pellets for three days (twice a day) at 2% of the fish body weight. The third group, was fed with chitosan mixed feed pellets and the forth group, was fed with vitamin C mixed feed pellets. On the 3rd day post-administration, at the predetermined time intervals, stomach, intestine and serum of rainbow trout were collected from each group of all the groups for analysis of vitamin C controlled release profiles. Being finished the vitamin C controlled release profile experiments; the remaining fish in each feeding group were fed with the ordinary commercial feed, as described before, by 20th day to assess immunological parameters, lysozyme and complement, at predetermined time intervals. In each experiment, two samples of the fish in each feeding group were collected and all the experiments were conducted in triplicates.

2.7. In vivo release test

In vivo release test was performed at the predetermined time intervals. Owing to this, the stomach and intestine of rainbow trout of each feeding group (two samples from each feeding group) were isolated and pre-weighted samples were homogenized in 10% cold metaphosphoric acid. The homogenates were ultracentrifuged and the supernatants were analyzed on HPLC, as following, after being filtered through a 0.45 mm pore-size syringe filter (Sartorius, Gottingen, Germany). To analyze vitamin C content in serum, blood collected from caudal fin of the fish (two samples from each feeding group) using non-heparinized syringes, transferred to microcentrifuge tubes, and allowed to clot for 1 h at room temperature and at 4 °C for 5 h. Serum was separated by centrifugation at 3000 rpm for 5 min at 4 °C and maintained at −80 °C for further use (Amar et al., 2004). Then, the amount of vitamin C in the supernatant was determined by HPLC (HP 1100 Series, Waldbronn, Germany). In brief, 1 ml of supernatant were extracted with the same volumes of 4.5% (w/v) metaphosphoric acid solution and filtered through a 0.45 μm Acrodisc filter. An aliquot then was injected into the chromatographic column. The chromatographic system consisted of a quaternary pump, a vacuum degasser, a Rheodyne 20 μl injection loop, a Diode-Array Detector, and it was controlled through a HP Chemostation software. A Hypersil ODS column fitted with a Hypersil ODS guard column was utilized with a mobile phase of HPLC grade water with metaphosphoric acid to pH 2.2 at a flow rate of 0.5 ml/min. The detection was at 245 nm (Qruna-concha, Gonzalez-Castro, Lopez-Hernandea, & Simal-Lozano, 1998). All experiments were performed in triplicate.

2.8. Complement and lysozyme assessment

Hemolytic serum complement activity (alternative pathway, ACP) was examined by a spectrophotometric method based on the lysis of rabbit red blood cells and expressed in ACH 50 units (Yano, 1992). One ACH 50 unit is defined as the activity that causes a 50% lysis of the target cells. To assess serum lysozyme activity, a turbidimetric assay using a Multiskan microtiter plate reader (Labsystems OY, Helsinki, Finland) was employed that evaluates the kinetics of absorbance of a suspension of the lysozyme-sensitive bacterium *Micrococcus lysodeikticus* (Sigma). The lysozyme

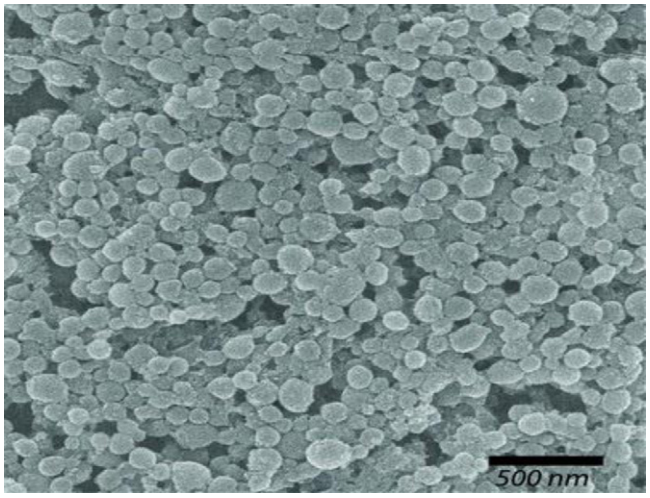

Fig. 1. SEM image of chitosan nanoparticles loaded with vitamin C (at 50 kV and 30,000 magnification).

activity was assessed using hen egg-white lysozyme (HEWL, Sigma) as standard (Ellis, 1992). All the experiments were performed in triplicates.

2.9. Statistical analysis

All experiments were carried out in triplicate and the results were expressed as mean ± S.D. Analysis of variance was performed using SPSS statistical package program (SPSS 13.0 for windows, SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Morphology and size distribution

Fig. 1 shows the morphology of chitosan nanoparticle cross-linked with TPP and loaded with vitamin C. The nanoparticles were spherical. Size distribution of chitosan nanoparticle loaded with vitamin C depicts in Fig. 2. The size and zeta potential of the nanoparticle was 185.4 ± 2.1 nm and 49.3 ± 1.6, respectively.

Moreover, the chitosan nanoparticle could significantly ($p < 0.05$) increase the shelf life and stability of vitamin C (95%) in fish diet within 20 days at ambient temperature compared with vitamin C without the nanoparticles (35%). Further, the obtained nanoparticle in the present study has shown the spherical shape

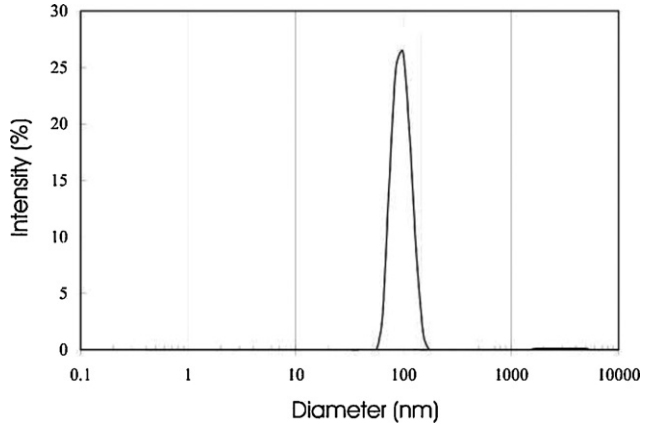

Fig. 2. Particle size distribution of chitosan/vitamin C nanoparticle. The average particle size was 185 nm.

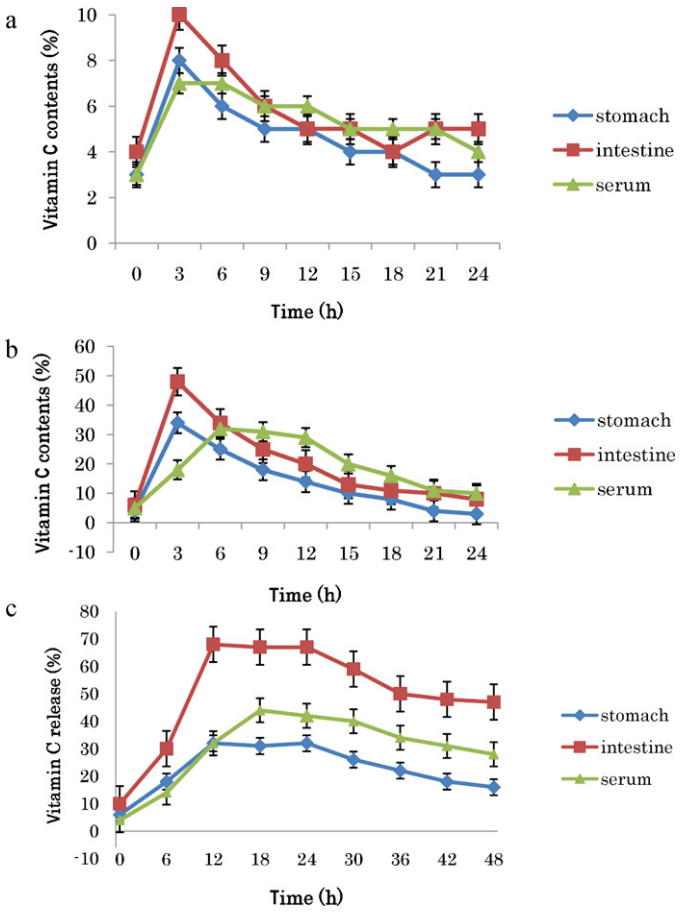

Fig. 3. Controlled release of vitamin C in rainbow trout fed with the different feeding treatments. (a) Control group, (b) rainbow trout fed with feed supplemented by vitamin C and (c) the fish fed with feed supplemented by chitosan nanoparticle loaded with vitamin C.

as well as smooth surface. On the other hand, while the nanoparticle size was 185 at acidic pH (5), the dispersed nanoparticle, when phosphate buffer solution (PBS) was used as medium, was observed that had mean particle size of 722 ± 23 nm at pH 7.4. Owing to these results, the nanoparticle has significantly shown the agglomeration at pH 7.4.

The results are in agreement with other outcomes. Gan, Wang, Cochrane, and MaCarron (2005) illustrated that bovine serum albumin (BSA) incorporated into chitosan–TPP nanoparticles possessed a spherical shape and smooth surface. Xu and Du (2003) and Janes, Calvo, and Alonso (2001) obtained similar results. The zeta potential of the nanoparticle was positive which indicates the presence of amino groups of chitosan on the surface. Dorkoosh et al. (2003) have explained that the positive charge on the surface of chitosan nanoparticles causes its mucoadhesive characteristic.

3.2. In vivo release test

Vitamin C contents in the stomach, intestine and serum of rainbow trout depict in Fig. 3. As shown in Fig. 3a, vitamin C contents in the stomach, intestine and serum of the fish fed with commercial feed as control group were up to 8, 10 and 7%, respectively, just 3 h after feeding and then decreased. Likewise, the vitamin C contents in the stomach, intestine and serum of the ones fed with feed supplemented by vitamin C were 34, 48, and 32%, respectively, just 3 h after feeding and then drastically decreased (Fig. 3b). Conversely, the chitosan nanoparticle loaded with vitamin C has significantly ($p < 0.05$) shown an increasing trend for vitamin C release up to 12 h.

Subsequently; the release trend was almost constant up to 24 h (Fig. 3c) and then gradually decreased. In addition, the release order in the latter group was as follows: intestine > serum > stomach.

The findings significantly ($p < 0.05$) show that the residence time for vitamin C (control and vitamin C supplemented groups) in the gastrointestinal tract of rainbow trout is low as compared with the controlled release profile by the chitosan nanoparticle. This controlled release characteristic of the chitosan nanoparticle is due to its bioadhesion nature (Dorkoosh et al., 2003). The amino moieties on the surface of the chitosan nanoparticle, at pH values below 6.5, have positive charge that they are responsible to attach to negative compounds existing on the epithelial cell membranes. Moreover, this character of the nanoparticle caused that the absorption of vitamin C enhanced through opening of inter and intracellular tight junctions at epithelial cell membranes. Interestingly, the interaction between the chitosan nanoparticle and epithelial cell membranes is revocable. Based on this view, the chitosan nanoparticle exerts no negative effect on vital activities of cell membranes (Dudhani & Kosaraju, 2010; Kean & Thanou, 2010). Further, Dudhani and Kosaraju (2010) have indicated that chitosan nanoparticle could enhance the in vitro stability and controlled release of catechin up to 24 h, thus enhancing in vivo controlled release due to its bioadhesion to epithelial cell membranes. Besides, Park et al. (2007) explained that encapsulation of emulsified lipids with nanoparticle based chitosan could protect biologically active lipid such as fish oil. Further, there are other reports to encapsulate biologically active compounds with chitosan nanoparticles that all of them are in agreement with our findings (Deladino, Anbinder, Navarra, & Martino, 2008; Dudhani & Kosaraju, 2010; Kean & Thanou, 2010; Weerakody, Fagan, & Kosaraju, 2008).

3.3. Complement and lysozyme evaluation

Fig. 4 depicts the complement and lysozyme activities in rainbow trout serum. The order of the complement activity in rainbow trout serum fed with the different feeding treatments is as follows: the fish fed with feed supplemented by chitosan nanoparticle > vitamin C > chitosan > control group (Fig. 4a). As shown in Fig. 4b, the same trend was found for the lysozyme activity in the serum of rainbow trout.

Lysozyme and complement are as the non-specific defense mechanisms in fish (Amar et al., 2004). As shown earlier, the lysozyme and complement activity in the fish fed with feed supplemented by the chitosan nanoparticle loaded with vitamin C was significantly ($p < 0.05$) the highest among the feeding groups because the chitosan nanoparticle containing vitamin C has obtained the synergistic effect (chitosan and vitamin C) to induce the innate immunity system of rainbow trout. Amar et al. (2004) showed that astaxanthin and other bioactive compounds such as vitamin C could induce non-specific defense mechanisms in rainbow trout. In addition, Cha et al. (2008) have explained that chitosan is used as an immunostimulant for salmonids fish against bacterial disease through inducing non-specific defense mechanisms in host body. Likewise, they expressed that immune-inducing characteristic of chitosan is mainly due to its amino moieties. The leukocyte mannose/fucose receptor is involved in the recognition of amino groups, triggering the immune response (Ayyaru & Venkatesan, 2006; Cha et al., 2008). Similarly, Rajesh Kumar et al. (2008) explained that immunization of Asian sea bass (*Lates calcarifer*) with chitosan nanoparticles encapsulated DNA vaccine induced moderate protection against experimental challenge with *Vibrio anguillarum*. Orally DNA vaccinated (chitosan-pVAOMP38) OMP38 gene induced a significant antibody immune response in the fish against OMP38 protein of *V. anguillarum*. In addition, they indicated that the chitosan/pVAOMP38 DNA complexes have higher transfection efficiency in vivo and in vitro studies and lower toxic effect than other cationic polymers.

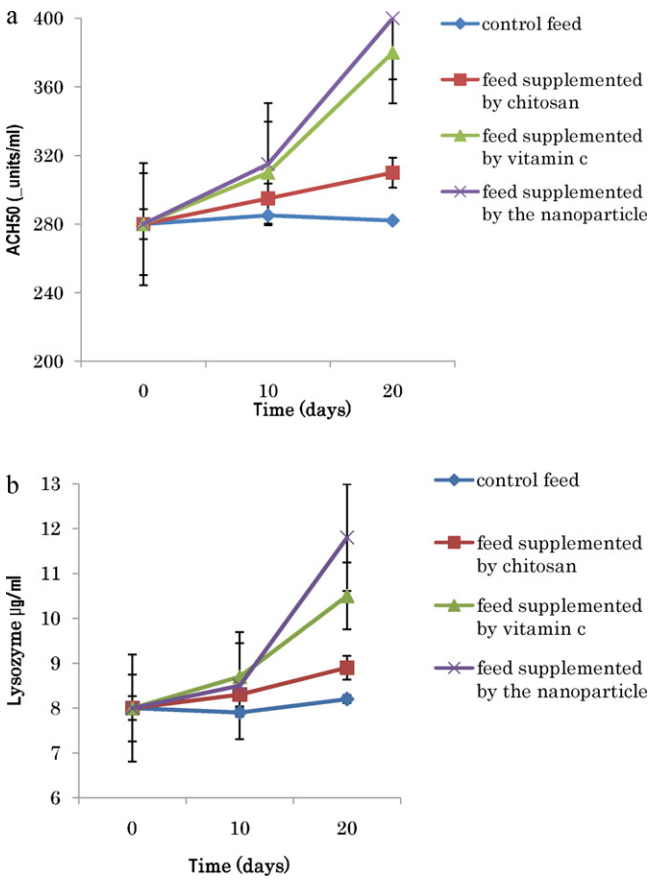

Fig. 4. Complement (a) and lysozyme (b) activities in the rainbow trout serum fed with the different feeding groups.

To our knowledge, there are no reports to explain the effect of controlled release caused by chitosan nanoparticle on the innate immunity system. The nanoparticle has released vitamin C at the longer time compared with the other feeding groups, and it caused the synergistic effect (The integrated effect of chitosan and vitamin C) on the innate immunity system of rainbow trout.

4. Conclusions

Chitosan nanoparticle has shown to be suitable to encapsulate vitamin C in nano size since it maintained the immune-inducing property of vitamin C. On the other hand, the release of vitamin C was regulated by the chitosan encapsulation up to 48 h in the gastrointestinal tract of rainbow trout. The innate immunity indices, lysozyme and complement, have considerably increased in the rainbow trout serum fed with feed supplemented by the chitosan nanoparticle loaded with vitamin C as compared with the other feeding groups. Owing to this, besides vitamin C, chitosan nanoparticle has also shown immune-inducing activity when employed to encapsulate vitamin C as a result of a synergistic effect on inducing the non-specific defense mechanisms of rainbow trout.

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