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Chitosan as a wall material for a microencapsulated delivery system for *Macrobrachium rosenbergii* (de Man) larvae

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Abstract

Chitosan has been widely accepted as a wall material for preparing microcapsules of various purposes in human medicine. The possibility of using chitosan as a wall material for microencapsulating nutrients and drugs for aquaculture purposes, specifically to *Macrobrachium rosenbergii* larvae was evaluated in this study. Two types of chitosan-coated microcapsules were prepared using either acetone (MEC-A) or NaOH (MEC-N) as the cross-linking agents. They were compared with a microbound diet relative to total leaching of nutrients and free amino acids (FAA). Among the microcapsules, MEC-N showed the lowest level of total leaching of nutrients (23.3%) during 5 h of immersion in seawater and released 65% FAA after 60 min. During laboratory trials, 75% larvae had accepted the MEC-N capsule. The results of the study suggest that chitosan can be used as a wall material for preparing microcapsules to deliver drugs and nutrients to *M. rosenbergii* larvae.

Keywords: microencapsulation, *Macrobrahium rosenbergii*, chitosan, drug delivery

Introduction

Development of appropriate vehicles for delivery of therapeutics, immunomodulants and nutrients to finfish and shellfish larvae has been a challenge to aquaculture scientists. Among the strategies being explored for drug/nutrient delivery, the most practical has been administration with feed. However, instability of aquaculture feeds in water leads to leakage of the core material, thereby reducing its availability to larvae (Pedroza-Islas, Alvarez-Ramirez & Vernon-Carter 2000). As a result, microencapsulated particles have gained recognition as potential delivery vehicles with the ability to transport specific substances to the digestive system. Major factors to be considered while designing such microcapsules for crustacean larvae are rate of dissolution of the core material in the culture system, acceptance of the capsule by larvae, digestibility and the cost of production. The primary criterion that considerably Influences the above factors is the wall material used for microencapsulation. Therefore, the most crucial step in microcapsule design is the selection of an appropriate wall material (Sankarikutty, Sreekumar, Narayan & Mathew 1988) as it should facilitate the delivery of drugs within the short span of residence of the diet within the intestine of the larvae (Pedroza-Islas *et al.* 2000).

Microencapsulated drug delivery in aquaculture has so far been accomplished with biopolymers of different origin such as natural gums (Kanazawa 1981), proteins (Petitjean & Csengeri 1995; Yufera, Kolkovski, Fernandez-Diaz, Rinchard, Lee & Dabrowski 2003) and lipids (Lopez-Alvardo, Langdon, Teshima & Kanazawa 1994; Onal & Langdon 2004). However, chitosan has not been evaluated as a wall material so far, despite its favourable characteristics like gelation on contact with counter anions (Bodmeier, Oh & Pramar 1989), formation of films soluble in acidic pH (Remunan-Lopez & Bodmeier 1996) and its susceptibility to digestive enzymes (Remunan-Lopez, Lorenzo-Lamosa, Vila-Jato & Alonso 1998). Being a natural polymer, its degradation products are completely non-toxic to

animals (Kas 1997). These properties of chitosan prompted us to explore the possibilities of using it as a wall material for developing a microencapsulated drug/nutrient delivery system for larvae of *Macrobrachium rosenbergii*.

Materials and methods

Preparation of microcapsules

One microbound and two microencapsulated diets were prepared using potato starch and dried whole shrimp powder as the core material. The nutritional value of the preparations was not considered, because of the exclusive focus on drug delivery. A microbound diet (MBC) was prepared as a precursor of microencapsulated diets for a comparison of the leaching of total nutrients and free amino acids (FAA) and was prepared by mixing dried whole shrimp powder (70% w/w) and potato starch (30% w/w) for 10 min in a food processor with sufficient quantity of 2% potato starch solution as a binder. The preparation was mixed well, dried in a vacuum oven (60 ± 1 °C, 48 h), and sieved to particle sizes ranging from 300 to 700 μm.

The microcapsules were prepared by coating the microbound particles with 1% chitosan prepared in 5% glacial acetic acid. The microbound particles were spread over a plastic tray and coated with chitosan to obtain a 100–200-μm-thick shell using an air gun (Super Mech Engineering Works, Kolkata, India). Force of the air was adjusted to have the particles suspended in air to attain an overall coating. Intermittent manual shaking of the tray was also provided to achieve proper mixing and distribution of particles while coating. The preparation was dried in a vacuum oven (Labline, India) at 40 ± 1 °C for 24 h. The chitosan-coated microbound particles were further divided into two fractions. One fraction was immersed in acetone (Kubota 1993) for 5 min for cross-linking the chitosan wall (MEC-A), whereas the chitosan wall of the other fraction was cross-linked by immersing in 3% (w/v) NaOH (MEC-N) (Chandy & Sharma 1996; Lim, Wan & Thai 1997). Both the preparations were dried in a vacuum oven at 40 ± 1 °C for 24 h.

Morphology of capsules

The surface topography of microencapsulated preparations was analysed by a scanning electron microscope (Leo 435 VP SEM, Welwyn Garden City, UK). The samples were prepared by placing the microspheres on a microscope sample holder and gold sputtering in an argon atmosphere. Adequate care was exercised to obtain a homogenous micro-sphere gold coating.

Leaching of total nutrients

Leaching of total nutrients from microbound and microencapsulated preparations was estimated in seawater following Pedroza-Islas *et al.* (2000). Briefly, 0.3 g each of the preparations was weighed and introduced into five test tubes, to which 25 mL seawater (15 g L^{-1} salinity, pH 8.0) was added. A control of 25 mL seawater (15 g L^{-1} salinity, pH 8.0) was kept for all the experiments. The tubes were incubated at 28 ± 1 °C and one tube was sequentially removed at 1-, 2-, 3-, 4- and 5-h intervals, and the contents were vacuum filtered through Whatman No. 40 filter paper. The filtrate was dried at 60 °C until constant weight. The amount of leached material was calculated based on differences in dry weight between the treatments and the control. All determinations were made in triplicate.

Leaching of total FAA

The initial FAA concentration of the feed preparation was quantified as follows: 15 mg of each preparation was mixed with 30 mL distilled water, homogenized for 15 min, and sonicated for 10 min at 4 °C (500W, 20 KHz, VCX 500, Sonics, Newtown, CT, USA) to disintegrate the particles, filtered through a 0.2 μm pore size cellulose-acetate membrane filter (Sartorius, Goettingen, Germany) and analysed for FAA (Baer, Ryba, Meyer & Butikofer 1996). The leaching experiments were carried out in a 500 mL conical flask containing 250 mL seawater (15 g L^{-1} salinity, pH 8.0) stirred continuously at 60 rpm (Yufera, Kolkovski, Fernandez-Diaz & Dabrowski 2002). Then, 500 mg of each of the feed preparations was added to the flasks and 10 mL aliquots were removed from each flask at 1-, 5-, 15-, 30- and 60-min intervals using a syringe. The samples were filtered through a 0.2 μm pore size cellulose-acetate membrane filter (Sartorius) and analysed for FAA (Baer *et al.* 1996). The leaching rate was calculated as the percentage loss of FAA from the initial concentration.

Feed acceptance by *M. rosenbergii* larvae

Acceptance of microencapsulated diets MEC-A and MEC-N by the larvae of *M. rosenbergii* was assessed

by estimating the frequency of feed intake following Barros and Valenti (2003) over a period of time. Briefly, *M. rosenbergii* larvae at the eighth moult stage were transported to the laboratory and acclimatized. Batches of five larvae were placed in 100 mL beakers containing 80 mL seawater (15 g L^{-1} salinity, pH 8.0) drawn previously from the same larval-culture tank, filtered through a 125 μm pore size nylon mesh screen. Each beaker was provided with aeration to maintain the feed particles in suspension. After 30 min, the digestive tract of each larvae was examined under a light microscope for feed taken in. the number of larvae with any quantity of feed in the stomach was differentially recorded. The experiments were conducted in duplicate and the frequency of feed intake was calculated as follows:

$$\text{Frequency of Food Intake (FFI)}\,\% = (N_1/N_2)\,100$$

where N_1 is the number of larvae with ingested feed and N_2 is the total number of larvae in the beaker.

Statistical analysis

Differences in the leaching rates of total nutrients and FAA at different immersion times and between micro-diets were analysed using one-way ANOVA. A significance level $P<0.05$ was considered (Bailey 1995). Before carrying out the statistical analysis, all data were arc-sine transferred.

Results and discussion

The purpose of this study was to explore the possibility of using chitosan as a wall material for developing an economically practical and easy to prepare microencapsulated drug delivery system for the larvae of *M. rosenbergii*. One microbound and two microencapsulated diets having particle sizes within the acceptable limit of *M. rosenbergii* larvae (Barros & Valenti 2003) were prepared. The microbound diet was aimed as a precursor of microencapsulated diets and for making a comparison for leaching of total nutrients and FAA. Moreover, the preparation of MBC facilitates the incorporation of any nutrient to the microbound core without affecting the formation of the chitosan shell. Scanning electron microscopic studies showed that the size of microencapsulated particles varied between 400 and 900 μm (Figs 1 and 2). The lack of uniformity in the morphology of these particles is due to the mechanical milling operation involved in the feed preparation.

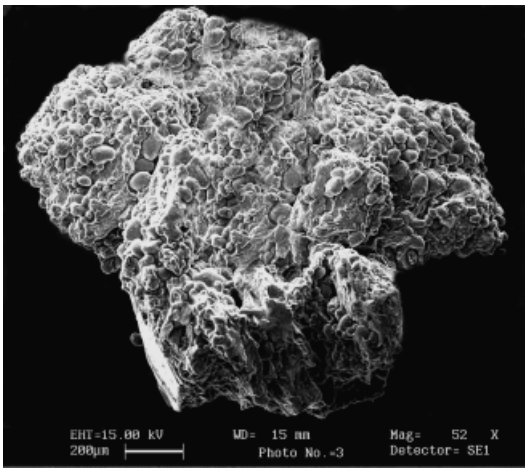

Figure 1 Scanning electron micrograph of MEC-N.

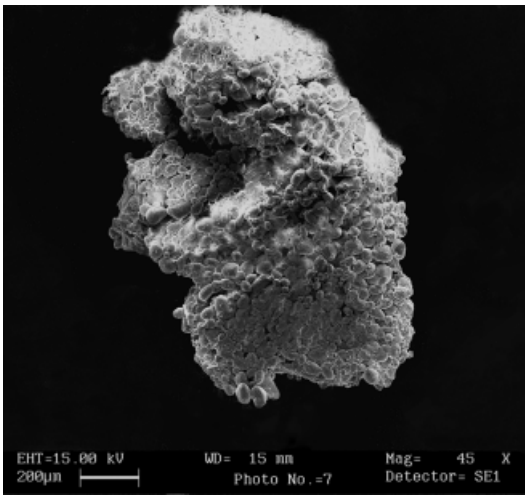

Figure 2 Scanning electron micrograph of MEC-A.

Significant differences could be observed in the leaching pattern of microbound and microencapsulated diets, MEC-A and MEC-N (Fig. 3) ($P<0.05$). Microcapsules cross-linked with NaOH (MEC-N) exhibited 14.43 ± 2.0% leaching of total nutrients during the first hour of immersion in seawater, which subsequently increased to 23.33 ± 3.4% after 5 h. Dissolution of a microcapsule cross-linked with acetone (MEC-A) was also similar, showing 18.9 ± 3.8% by the first hour of immersion and 24.4 ± 2.0% after 5 h. In contrast, a significantly higher leaching of total nutrients was observed from the MBC under the same experimental conditions, with an initial dissolution of 60.32 ± 9.7% during the first hour, which subsequently attained 69.72 ± 5.1% after 5 h.

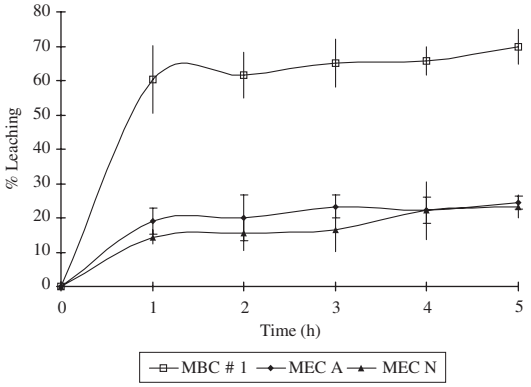

Figure 3 Leaching of total nutrients from microbound (MBC) and microencapsulated preparations (MEC-N and MEC-A) (values are mean ± SD, $P < 0.05$).

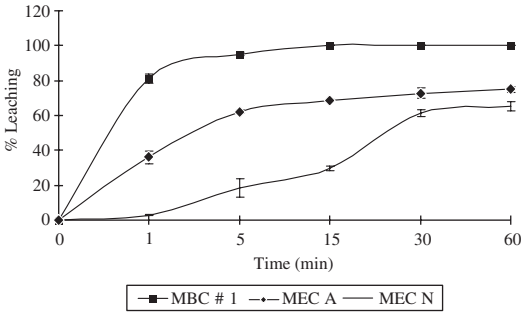

Figure 4 Leaching of total free amino acids from microbound (MBC) and microencapsulated preparations (MEC-N and MEC-A) (values are mean ± SD, $P < 0.05$).

Considering the low percentage of total nutrient leaching observed with microcapsule MEC-N, chitosan could be suggested as a possible wall material for microencapsulating nutrients and drugs for aquaculture animals. Alabi, Cob, Jones and Latchford (1999) measured the leaching rates of protein and total nutrients (by weight) from a commercial microbound diet and the one encapsulated in cross-linked protein wall material. They observed a 50–70% protein loss within 1 h of suspension in seawater for all particle types. The total nutrient loss from the encapsulated diets was 37–39% and that of the microbound diet was 58% during a 6-h period of suspension. Pedroza-Islas, Vernon-Carter, Duran-Dominguez and Trejo-Martinez (1999), after studying the kinetics of leaching of total nutrients in a series of microcapsules with different biopolymer treatments, demonstrated that a microcapsule with a minimum of 60-min residence time in water would provide ample time for shrimp larvae to achieve an adequate intake of the diet. In this background, chitosan microcapsules (MEC-N) would make promising drug/nutrient delivery vehicles.

A substantial difference was observed in the FAA leaching pattern between microbound and each of the microencapsulated preparations ($P < 0.05$) (Fig. 4). During the first minute of immersion, 2.93 ± 0.5% FAA was found to have leached out from MEC-N, 36.0 ± 3.6% from MEC-A and 81.07 ± 2.9% from MBC. However, after 60 min of immersion, 65.2 ± 2.7% of FAA had leached out from MEC-N, 75.03 ± 1.6% from MEC-A and there was complete release from MBC.

Looking at amino acid leaching, only 3% was lost from the MEC-N capsule after 1 min of immersion in seawater and increased to 65% after 60 min of immersion. The amino acid leaching rates reported here are much less than those reported by Lopez-Alvardo *et al.* (1994). They reported that more than 80% of FAA had been lost from alginate, carrageenan and zein microbound particles after 2 min of suspension in aqueous medium and as much as 60% of dietary FAA had been lost from zein-coated and carrageenan- and gelatin-bound diets within 1 min of suspension. Similarly, Ozkizilcik and Chu (1996) reported free lysine losses of 80% in protein-walled microcapsules following 60 min of rehydration. Meanwhile Yufera *et al.* (2002) proposed a complex protein-walled microcapsule prepared by emulsification of dietary compounds dispersed in a basic pH buffered Tris-HCl with soy lecithin and cyclohexane, and cross-linked by using trimesoyl chloride dissolved in diethyl ether. The capsule prepared through this process of interfacial polymerization showed FAA leaching of 17% following a 1-h immersion in distilled water.

However, the high cost of these protein-walled microcapsules, use of organic solvents and highly reactive chemical cross-linking agents diminish their long-term prospects of commercial production and use (Langdon 2003). Moreover, a high bacterial load associated with the breakdown of commercial diets encapsulated within cross-linked, protein-walled capsules has also been reported (Muir & Sutton 1994). The main advantages of chitosan compared with the cross-linked protein wall materials are its non-toxicity, formation of films soluble in acidic pH and well-documented antibacterial property (Allan & Hadwiger 1979; Kendra & Hadwiger 1984; Sudarshan, Hoover & Knorr 1992; Wang 1992; Roller & Covill 1999; Zheng & Zhu 2003). Our previous studies have demonstrated the same against vibrios

associated with *M. rosenbergii* larval rearing systems (Anas, Paul, Jayaprakash, Philip & Singh 2005). These properties further substantiate the potential of using chitosan as a wall material for preparing a microencapsulated drug delivery system for crustacean larvae.

The feed acceptance studies indicated that the frequency of feed intake by the larvae was similar for MEC-N (75%) and MEC-A (73.5%) over a period of 30 min. Disintegration of the microcapsules in the larval intestine could be noticed through microscopic observations. This experiment confirms the acceptance of the microcapsule by the larvae but needs further work to understand the mechanism of breakage of the capsules inside the intestine and its absorption, which is beyond the scope of the present study.

In conclusion, considering the capacity of chitosan wall material to retain the total nutrients and FAA for a considerable length of time in seawater, its acceptance by the larvae of *M. rosenbergii*, together with its inherent characteristics such as non-toxicity, film forming and antimicrobial properties, chitosan – a biocompatible biopolymer – can be suggested as an appropriate wall material for preparing microcapsules for delivering drugs and nutrients to *M. rosenbergii* larvae.

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