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The CpG ODNs enriched diets enhance the immuno-protection efficiency and growth rate of Chinese mitten crab, *Eriocheir sinensis*

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ABSTRACT

CpG oligodeoxynucleotides (ODNs), the well-known vaccine adjuvant in mammals, have been proved to mount innate immune responses in crustaceans. In the present study, CpG ODNs was employed as supplements in diets to fed crab *Eriocheir sinensis*, and the changes of immune parameters as well as weight gain were investigated to evaluate its possible application in crab farming. After the crabs were fed with 40 mg/kg and 100 mg/kg CpG ODNs containing diets (designated as C40 and C100 group) for four weeks, the lysozyme activities were significantly enhanced ($p < 0.01$) in both groups, while the catalase activity was only increased ($p < 0.01$) in the C40 group. When those crabs were subsequently challenged with *Aeromonas hydrophila*, the cumulative mortalities in C40 and C100 groups were declined by 10.4% and 10.8% ($p < 0.05$) compared with that of control group, respectively. Interestingly, the final weights of crabs were increased after four weeks' feeding of CpG ODNs, and the percentage of weight gain in C40 group reached 124.5 ± 14.2%, which was significantly higher ($p < 0.05$) than that of control group (78.1 ± 19.2%) and C100 group (107.3 ± 28.2%). The uptake of CpG ODNs by haemocytes and the possible mechanism of CpG ODNs to active the immune response were investigated by using the laser scanning confocal microscope. CpG ODNs (labeled with 5′-end-FAM) could be internalized by the haemocytes after incubation of 20 min, with strong signals detected at the cell membrane and in the cytoplasm. In the cytoplasm, most of the CpG ODNs were localized in lysosome, and some of them escaped from the lysosomal compartments and aggregated around the nuclear. The results clearly demonstrated that CpG ODNs could be internalized directly by crab haemocytes and mostly located in the late endosome. The enhancements of immuno-protection efficiency and growth rate from CpG ODNs as supplements in diets might depend on the uptaking and locating processes, and they could be used as a potential immunostimulant for the crab aquaculture.

1. Introduction

The Chinese mitten crab *Eriocheir sinensis* (Henri Milne Edwards 1854) is one of the most economically important aquaculture species in China and a fashionable table delicacy in East Asia [1]. In recent years, various diseases caused by bacteria [2], viruses [3] and parasites [4] frequently occurred in crab aquaculture and led to drastic decrease in the production and enormous economic losses [5]. The utilization of antibiotics which can eliminate the invaded pathogens may partially contribute to reducing disease outbreak [6,7]. However, the abuse of antibiotics in aquaculture could result in the production of drug-resistant microorganism which is dangerous to human health and damaging to the environment [8]. Therefore, the development of novel and environmentally friendly strategy for the health management and disease control has become an urgent issue in the crab aquaculture industry.

Since the immunostimulants can endow hosts with the enhanced resistance against diseases by directly initiating the activation of non-specific immune system [9], the application of immunostimulants have represented one of the promising orientation of disease control, especially for invertebrate animals who lack of the adaptive immunity. The potential usage of immunostimulants as the substitute of antibiotics has been strongly attracted the exploitation of novel counterparts, such as β-1,3-glucan and other components extracted from bacteria and yeast, and some of them have been tested in the farming of important aquaculture

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species. For instance, the shrimp *Litopenaeus vannamei* fed with β-1,3-glucan enriched diet displayed higher resistance to the invaded pathogen [10]. Among these various immunostimulants, CpG oligodeoxynucleotides (ODNs) are considered as the novel and promising ones which are characterized by a good safety profile and the easy accessibility as well as the apparent immunity boosting functions.

CpG ODNs, also called bacterial DNA or synthetic oligodeoxynucleotides, have been proved to mount innate immune responses in many animal species [11]. In mammals, CpG ODNs can indirectly support the maturation and proliferation of lymphocytes, resulting in various immune responses characterized by the production of Th1-type and proinflammatory cytokines, chemokines and polyreactive IgM [12]. They can be internalized by target cells and locate in the late endosome where they interact with TLR9, finally resulting in the transcription of several important immune factors, such as proinflammatory factors [12]. However, in invertebrates, the possible mechanism of CpG ODNs on the activation of innate immune response is still far from well understood. In the crustacean species, the stimulatory effects of CpG ODNs on the immune system have been reported, and the enhanced lysozyme and phenoloxidase activities were observed in the haemocytes of shrimps injected with multi-copy CpG motifs [13]. The copy number of WSSV in CpG ODNs-pretreated shrimps was significantly decreased after WSSV infection [14], and CpG ODNs could activate the immune response of *E. sinensis* in a dose dependent manner [15]. Although CpG ODNs have been approved to stimulate the immune system in various animals, there are few reports about the immunostimulatory effects of CpG ODNs as an immunostimulant in diet for both vertebrate and invertebrate animals.

In the present study, different proportions of CpG ODNs were added in the basic diets to feed the crabs for four weeks. The immune capability was compared among those crabs and the growth performance was also recorded to evaluate the immunostimulatory effects of CpG ODNs as the dietary supplements. The subcellular localization of CpG ODN in haemocytes was investigated as an attempt to reveal the mechanism of CpG ODNs to active the immune system of crab. The results from the present study may provide a possibility for the application of CpG ODNs as a potential immunostimulant for disease control in aquaculture.

2. Materials and methods

2.1. Crabs, bacteria and CpG ODNs

Crabs *E. sinensis*, weighing approximately 0.12 ± 0.02 g, were collected from a commercial farm in Lianyungang, China. The crabs were maintained in flat-bottomed circulating tanks (75 cm × 100 cm) with aerated tap water at 25–30 °C, pH 7.3–7.5. The water depth in each tank was no more than 15 cm and about 1/3 of the water was replaced everyday. The animals were maintained under these conditions for 21 days to acclimate to the laboratory environment before processing.

In the bacterial challenge experiment, a single colony of *Aeromonas hydrophila* (a gift from Dr. Li Sun) was selected and cultured in LB media for 12 h at 28 °C. The bacteria were collected by centrifuge at 8000 *g* at 4 °C for 5 min, and then washed and resuspended in sterile water for further use.

CpG ODN 2395 labeled by 5′-end-FAM was synthesized by Sangon Biotech Co. (China) for the subcellular localization. For the culture experiment, a set of CpG ODNs (Fig. 1) which were previously found effective to mammalian and aquatic animals [16], were synthesized by Sangon Biotech Co. (China) with the purity above 99.9% and subcloned into the plasmid pUC57. The recombinant plasmids were transformed into *Escherichia coli* and then extracted

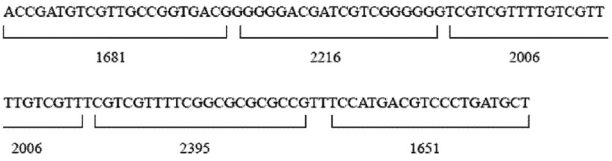

Fig. 1. Description of tandem ODNs sequence. Five CpG ODNs in this table previously used effectively in mammalian and aquatic animals were designed to connect one another in series to form a CpG-rich fragment in the present study.

by using the method as Zhu's description [17]. The purified plasmids were heated at 100 °C for 15 min and cooled down rapidly [13]. The linear and fragment DNA was then freeze-dried and stored at −20 °C for the further experiment.

2.2. Diet preparation

The ingredients for the basic diet of crabs (designated C0) are shown in Table 1. The freeze-dried CpG ODNs were sprayed into the basic diet C0 to give a final proportion of 40 mg/kg (designated C40) or 100 mg/kg (designated C100). Then, the ingredients of the diet were well mixed and extruded by a pellet extruder. The pellets were dried in an oven at 40 °C, then packed and stored at 4 °C for the next step.

2.3. Experimental setup

Totally 1440 healthy crabs were randomly selected and stocked into 9 tanks, and maintained as the description in Section 2.1. The crabs were equally divided into three groups, and each group contained three tanks as three replicates. The 480 crabs equally assigned to three tanks in the first group were fed with the basic diet C0 during the whole experimental period (28 days) and this group was named as C0 group. In the second or third group, 480 crabs were fed with C40 or C100 diets (designated C40 group or C100 group), respectively. Crabs in C40 group and C100 group were fed according to the modified interval feeding method [10], which was 4 days feeding with CpG ODNs containing diets following 3 days feeding with basic diet (Fig. 2). All the crabs were fed with the designated diets twice daily at 8:30 a.m. and 6:30 p.m. to ensure the repletion.

At the beginning of the feeding experiment (0 day), 30 crabs from each tank (90 crabs from each group) were randomly collected to determine the initial weight. After 28-days' feeding, the number of survival crabs was counted in three groups, and the final weight was measured as the description of the initial weight measurement. The percentage of weight gain (WG) and the survival rate (SR) were calculated as below.

$$\mathrm{WG}(\%) = (\text{final weight} - \text{initial weight})/\text{initial weight} \times 100$$
$$\mathrm{SR}(\%) = (\text{final number of survival crabs}/\text{initial number of crabs}) \times 100$$

Table 1
The composition of the basic diet for crab.

Ingredients	Composition (g/kg)	Ingredients	Composition (g/kg)
Soybean meal	250	Phosphatidylcholine	5
Peanut meal	50	Cholesterol	5
Shrimp meal	100	Choline chloride	5
Wheat flour	80	Vegetable oil	20
Corn flour	140	$NaH_2PO_4 \cdot 2H_2O$	2
Fish meal	300	$Na_2HPO_4 \cdot 12H_2O$	3
Multi-vitamin	10	$Ca(H_2PO_4)_2 \cdot 2H_2O$	3
Vitamin C	2	Sodium Alginate	10
Glycine	15		