Beneficial effects of dietary supplemented Parachlorella kessleri strain KNK-A001 on pacific white shrimp (Litopenaeus vannamei) and kuruma shrimp (Marsupenaeus japonicus)

Mikinori Ueno¹, Toshiaki Itami², Kenji Yamashita³ and Tatsuya Oda¹.*

Abstract: We studied about supplemental effects of a dry powdered microalga Parachlorella kessleri (KNK-A001) for two shrimp species. As a result it was found that dietary supplementation of 0.005% KNK-A001 showed a tendency to improve growth performance of pacific white shrimp (Litopenaeus vannamei). Furthermore, kuruma shrimp (Marsupenaeus japonicus) fed diet containing 0.05% KNK-A001 showed significantly higher survival rate than control without KNK-A001 in WSSV challenge trial. Increased hemocyte cell density was observed in the hemolymph obtained from the shrimp fed 0.05% KNK-A001, and superoxide anion producing activity of the hemocytes was also increased as compared to the control. These results suggested that KNK-A001 had the beneficial effects on shrimp species.

Key words: Parachlorella kessleri; White spot syndrome virus (WSSV); Marsupenaeus japonicus; Litopenaeus vannamei

Pacific white shrimp (Litopenaeus vannamei) and kuruma shrimp (Marsupenaeus japonicus) are important aquatic organisms as aquacultural food resources in Asian countries including Japan. However, farming of these shrimp species are under various environmental stresses such as changes in water quality, dissolved oxygen, and salinity. To improve health condition of culturing shrimp, development of appropriate rearing procedure including usage of effective supplements or additives is necessary. In addition, culturing shrimp species have often been severely suffered from infection diseases, especially white spot syndrome virus (WSSV) infection, which can cause nearly 100% mortality in the shrimp species within a few days of infection, and result in serious economic losses (Sánchez-Martínez et al. 2007). Since crustaceans do not have acquired immune system, one of the effective strategies for prophylaxis and control of virus infection is immunostimulants which can activate innate immune system. The immune stimulatory effects of various substances such as peptidoglycan, lipopolysaccharides, glucan, and other polysaccharides have been extensively studied in fish and crustaceans (Sakai 1999; Song and Huang 1999; Smith et al. 2003), and protective effects of some of these stimulants have been reported against WSSV infection (Namikoshi et al. 2004).

Parachlorella kessleri strain KNK-A001 is a green microalga taxonomically related to P. kessleri. This microalga is morphologically similar to Chlorella vulgaris, but it has a thick extracellular matrix consisting of polysaccharides instead of a hard cell wall observed in C. vulgaris. We have previously found that dry powdered KNK-A001 has good food value as feed for the Pacific oyster spat and zooplankton rotifer Brachionus plicatilis (Sugiyama et al. 2013). This may be partly due to the easier digestion of KNK-A001 in those organisms as compared to C. vulgaris. Recent studies showed that intraperitoneal injection of KNK-A001 cell suspension into mice resulted in a significant increase in splenic natural killer (NK) cell activity against YAC-1 cells, which was even much higher than that induced by C. vulgaris, suggesting that KNK-A001 has a potent immunostimulating activity (Ueno et al. 2015). To evaluate the practicable usefulness of KNK-A001 as a supplement or immunostimulant, in this study, we investigated the effects of dietary KNK-A001 on the growth performance of pacific white shrimp under usual rearing condition, and on the survival rate of WSSV infected kuruma shrimp.

KNK-A001 was cultured in glucose-based medium originally developed by Kaneka Co. at 30°C. The dry powder of KNK-A001 was prepared with a drum dryer, and used in the experiments. Dry powered KNK-A001 was mixed with basal shrimp diet (EBISTAR from HIGASIMARU Co. for kuruma shrimp and Nanami from Thai Union Feedmill Co., Ltd., Thailand (TUF)
for pacific white shrimp) at varying concentrations at 0, 0.005, 0.01, 0.02, and 0.05% (w/w), and each dry pelleted diets were prepared with an extruder. Healthy pacific white shrimp (Litopenaeus vannamei) weighing 0.5–1.0 g (post larvae 15) were obtained from TUF. Shrimp were randomly divided into five diet groups with four replicates (each replicate contained 20 shrimp/group). Shrimp of each group were fed each test diet for 6 weeks with three to five feeding times per day. Water temperature, salinity, and dissolved oxygen were 25–30°C, around 25‰, and >5 mg/l (with normal aeration), respectively, and feed consumption was recorded daily during the feeding period. Healthy kuruma shrimp (M. japonicus) weighing about 1.5 g were obtained from a local shrimp farm at Kagoshima, Japan, and were kept at 25°C in 200 l rearing tank with continuous flow-through and constant aeration in filtrated seawater during experiments. Similar size of shrimp were selected and divided into four experimental groups (0, 0.01, 0.02, and 0.05% KNK-A001 containing diet groups at 25 shrimp/group). Shrimp of each group were fed each test diet for 6 weeks. During 6 weeks feeding, mean feed consumptions of the groups were monitored, and final mean body weights of shrimp of each group after 6 weeks feeding were measured. The shrimp fed with each diet for 6 weeks were subjected to the virus challenge trial. WSSV suspension was prepared from homogenate of muscle tissue of infected shrimp with typical white spots detected of WSSV by polymerase chain reaction (PCR), and centrifuged 13,000 × g for 10 min in order to harvest supernatant. Based on the preliminary infection experiments at the same condition, the supernatant was 5,000 times diluted with PBS, and then intramuscularly injected to shrimp of each diet group at 0.1 ml/shrimp (6.7 μg/ml, 11–13 shrimp/group). After the infection, shrimp of each group were kept in 200 l tank containing 40 l seawater and continuously fed each test diet (0.1 g/shrimp/day), and the mortality of each group was recorded for 2 weeks. Dead shrimp and live shrimp during 14 days after virus infection were subjected to PCR (Takahashi et al. 1996). Hemolymph was collected from shrimp of each group fed each test diet for 6 weeks, and immediately mixed with 4 times volume of cold K-199 medium (Inada et al 2012) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, and the number of hemocytes was counted with a hemocytometer. To measure superoxide anion producing activity of hemocytes collected from shrimp of each diet group, hemocytes in K-199 medium were plated in 96-well plate (1 × 10^5 cells/100 μl/well). After 2 h incubation at 25°C, nitroblue tetrazolium (NBT) at final concentration of 0.1% and phorbol 12-myristate 13-acetate (PMA) at final concentration of 1 μg/ml were added to each well and incubated for 1 h at 25°C. After incubation, the plate was centrifuged at 2,000 × g for 10 min, and then removed the supernatants. One hundred forty μl of DMSO and 120 μl of 2 M KOH were added to each well to dissolve the formazan formed in cytosol, and absorbance at 620 nm was measured (Castro et al. 2006). To measure phenoloxidase activity, hemocytes (1 × 10^5 cells/ml) were ruptured by ultrasonication, and then centrifuged (15,000 × g for 20 min at 4°C). Fifty μl of the supernatant was mixed with 50 μl of L-DOPA (3 mg/ml of cacodylate buffer, pH7.0), and then incubated for 1 h at 40°C. Phenoloxidase activity was measured at 490 nm (Liu et al. 2004). The absorbance measurement was conducted with microplate reader in triplicate. All the data were analyzed by one-way ANOVA and Dunnett’s multiple comparison tests except for the experiment of WSSV infection against shrimps. Fisher’s exact test was used to compare the survival rate of WSSV infected shrimps between control and each test group. P value of <0.05 was considered statistically significant.

As shown in Table 1, weight gain of 0.05% group and survival rate of 0.005% group tended to be slightly higher than other groups, whereas feed intake and

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Feed intake (g/shrimp)</th>
<th>Weight gain (%/day)</th>
<th>Specific growth rate (%/day)</th>
<th>Feed conversion ratio</th>
<th>Protein efficiency ratio</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.50 ± 0.69</td>
<td>427.6 ± 57.2</td>
<td>2.96 ± 0.19</td>
<td>1.79 ± 0.05</td>
<td>1.49 ± 0.04</td>
<td>76.25 ± 16.01</td>
</tr>
<tr>
<td>0.005%</td>
<td>6.45 ± 0.54</td>
<td>427.1 ± 44.8</td>
<td>2.96 ± 0.15</td>
<td>1.55 ± 0.06</td>
<td>1.76 ± 0.07</td>
<td>88.75 ± 9.46</td>
</tr>
<tr>
<td>0.01%</td>
<td>7.10 ± 0.70</td>
<td>422.7 ± 33.3</td>
<td>2.95 ± 0.18</td>
<td>1.72 ± 0.18</td>
<td>1.59 ± 0.16</td>
<td>81.25 ± 10.31</td>
</tr>
<tr>
<td>0.02%</td>
<td>7.43 ± 0.19</td>
<td>413.8 ± 20.2</td>
<td>2.92 ± 0.07</td>
<td>1.83 ± 0.07</td>
<td>1.48 ± 0.06</td>
<td>78.75 ± 4.79</td>
</tr>
<tr>
<td>0.05%</td>
<td>7.30 ± 0.71</td>
<td>460.2 ± 72.6</td>
<td>3.07 ± 0.23</td>
<td>1.63 ± 0.14</td>
<td>1.67 ± 0.16</td>
<td>80.00 ± 14.72</td>
</tr>
</tbody>
</table>

Shrimp of each group fed diet containing 0 (control), 0.005, 0.01, 0.02, and 0.05% KNK-A001 for 8 weeks. Values are mean ± standard deviation (n=4).

Specific growth rate (%/day) = [100 × (ln(W2) − ln(W1))]/day, in which W1 and W2 is initial and final body weight, respectively.

Feed conversion ratio = dry feed consume (g)/wet weight gain (g).

Protein efficiency ratio = wet weight gain (g)/protein consume (g)

All the data were analyzed by one-way ANOVA and Dunnett’s multiple comparison tests.

* P<0.05, difference between control and 0.005%
Beneficial effect of KNK-A001 on shrimps species

specific growth rate were almost same among the test groups and control. Feed conversion rate and protein efficiency ratio of 0.005% group after 56 days feeding were the best score among the groups. These results suggest the possibility that dietary KNK-A001 shows a tendency to improve the growth performance of pacific white shrimp, and there is no negative or detrimental effect on the shrimp at least up to 0.05% dose. Namely, weight gain of shrimp fed diet containing 0, 0.01, 0.02, and 0.05% KNK-A001 were 324.7, 361.8, 332.9, and 334.1%, respectively. Feed conversion ration of 0, 0.01, 0.02, and 0.05% KNK-A001 groups were 1.47, 1.34, 1.51, and 1.51, respectively.

Fig. 1A shows the survival rates of kuruma shrimp of each group fed each test diet after WSSV challenge. The survival rates of shrimp of the groups fed diets containing 0.02 and 0.05% KNK-A001 were significantly higher than control group fed diet without KNK-A001. The survival rates in 0 (control), 0.01, 0.02, and 0.05% KNK-A001 fed groups were 15, 33, 73, and 91% at 14 days after virus challenge, respectively. These results suggested that dietary KNK-A001 enhanced shrimp resistance against WSSV in a dose-dependent manner. Fig. 1B shows the mean hemocyte cell density of the hemolymph collected from the shrimp of the test groups after 6 weeks fed diet containing varying concentrations of KNK-A001 (0-0.05%). Hemocyte cell density of the 0.01, 0.02, and 0.05% KNK-A001 groups tended to be higher than that of control group, and especially the value of 0.05% group was statistically significant. Because hemocytes are the major immune competent cells in shrimp, increased hemocyte cell density may reflect the potentiation of shrimp immune defense system, which may be responsible for the increased survival rate of 0.05% KNK-A001 group after WSSV infection. As shown in Fig. 1C, PMA-induced superoxide anion producing activity of hemocytes from the shrimps fed diet containing 0.05% KNK-A001 was higher than that of control shrimp. On the other hand, no significant difference in phenoloxidase activity of hemocytes was observed among the diet groups tested (Fig. 1D). Hence, the superoxide anion producing activity but not phenoloxidase activity of hemocytes from 0.05% KNK-A001 group was enhanced relative to control group fed diet without KNK-A001. Table 2 shows PCR diagnosis for WSSV in the 0 (control), 0.01, 0.02, and 0.05% KNK-A001 supplemented diet groups. All the shrimp from control group including survival individuals after 14 days virus infection were WSSV positive. In contrast, no WSSV positive shrimp were detected in the 0.05% KNK-A001 group. Although cannibalism especially between shrimp at...
post-molting stage was observed during virus infection experiment, the percentage of such death was quite low. These results suggest that the increased survival rate of 0.02% and 0.05% KNK-A001 supplemented groups are due to the suppression of the virus infection and propagation. Our results show for the first time that the resistance of kuruma shrimp against WSSV infection was enhanced by oral administration of KNK-A001, and the maximum efficacy was attained at its 0.05% supplementation in diet. Since the increase in hemocyte cell density and their superoxide anion producing activity were observed in 0.05% KNK-A001 producing activity were observed in 0.05% KNK-A001 group, it is suggested that KNK-A001 can activate shrimp immune system, which may lead to increase in survival rate of WSSV infected shrimp. Our recent preliminary experiments suggested that dietary administered KNK-A001 showed survival benefit for juvenile pacific white shrimp in bacterial (Vibrio alginolyticus) and WSSV infection models. Since KNK-A001 can be prepared with relatively low cost at industrial level, and its effective concentrations is quite low, there is no significant cost increase to the diet. The dried cells are easy to handle, and are ready to use without further processing such as isolation of active agents from the cells. Based on these advantages together with the findings obtained in this study, it is considered that KNK-A001 is a promising additive with immunostimulating activity applicable for shrimp farm to prevent WSSV infection. We demonstrated that KNK-A001 had alginate lyase-sensitive uronic acids and other polysaccharides (Ueno et al. 2015). Since alginate (Liu et al. 2006) and other polysaccharides (Sakai 1999) have been reported to stimulate shrimp immune systems, the polysaccharides of KNK-A001 might be possible factors responsible for the protective effect of KNK-A001 against WSSV in kuruma shrimp. Further studies are obviously necessary to clarify the detail action mechanism of KNK-A001 especially the effects on immune systems of shrimp species. To evaluate the practical beneficial effects of KNK-A001, large scale field experiments in shrimp ponds were already started, which may provide further information on KNK-A001 as effective additive or supplement for shrimp species.

Table 2. Detection of WSSV in M. japonicus by PCR assay

<table>
<thead>
<tr>
<th></th>
<th>% of positive</th>
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<tbody>
<tr>
<td></td>
<td>Survival</td>
<td>Death</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.01% KNK-A001</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>0.02% KNK-A001</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.05% KNK-A001</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

During 14 days after infection with WSSV, shrimp of each group fed diet containing 0 (control), 0.01, 0.02, or 0.05% KNK-A001 were subjected to PCR assay. Numbers of individual are shown in parentheses.

References