Effects of Aquanin Plus (Beta-Cyclodextrin Cysteamine Hydrochloride) on the Growth, Survival and Immune Characteristics of Pacific White Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

A study of the effects of Aquanin plus (beta-cyclodextrin cysteamine hydrochloride) on the growth, survival and immune response in Pacific white shrimp (*Litopenaeus vannamei*) was conducted under laboratory conditions. For the determination of the growth-promoting and immunostimulant effects of Aquanin plus administration in the diet, tests were carried out in three treatments (with six replicates per treatment). Each replicate consisted of 25 shrimp (11-12 g) in 500-liter tanks. Shrimp were fed three times daily at 3% body weight per day for 60 days with pelleted feed containing graded levels of Aquanin plus (0, 0.05 and 0.1% of the feed). After 40 days of dietary administration, shrimp fed with 0.1% Aquanin plus had an average body weight (16.76±1.33 g) significantly higher ($P<0.05$) than that of 0.05% Aquanin plus (15.84±0.99 g) and control groups (15.40±1.19 g). There was no significant difference in survival between the two Aquanin plus-treated groups (97.18-97.60%) but their survival rates were significantly higher ($P<0.05$) than those of the control group (83.20%). The shrimp immune responses were measured by total hemocyte count (THC), percentage phagocytosis, phenoloxidase activity and bactericidal activity. The results showed that shrimp which consumed diets containing 0.1% Aquanin plus had significantly higher ($P<0.05$) THC, percentage phagocytosis and phenoloxidase activity than those of the 0.05% Aquanin plus and control groups. Shrimp fed with 0.05 and 0.1% Aquanin plus had bactericidal activity at the serum dilution of 1:16 while the control group was 1:8. In conclusion, the present study indicated that oral administration of 0.1% Aquanin plus for 40 days could increase the growth, survival and immune response of *L.vannamei*.

Key words: Aquanin plus, beta-cyclodextrin cysteamine hydrochloride, *Litopenaeus vannamei*, immune

INTRODUCTION

Currently, Pacific white shrimp, *Litopenaeus vannamei*, native to the Pacific coast of Central and South America, is the major shrimp species cultured in China, Taiwan and Thailand (Lin *et al.*, 1990). Since 2001, shrimp farmers have experienced disease problems associated with production declines in farmed *L. vannamei* (Cheng *et al.*, 2005). Many scientists have attempted to solve the disease problems by enhancing the non-specific immune response, the main internal defense mechanism in shrimp. Use of immunostimulants is another approach to increase shrimp immunity against diseases (Purivirojkul *et al.*, 2006). One immunostimulant product is Aquanin plus (trade name) from Walcom Bio-Chemicals Industrial

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Limited, containing 27% Beta-cyclodextrin cysteamine hydrochloride and 0.1% Vitamin E. Cysteamine hydrochloride succeeded in inducing growth hormone secretion and growth acceleration in juvenile grass carp (*Ctenopharyngodon idellus*) (Xiao and Lin, 2003), as well as increasing IgG, IgA and IgM production. There have been no reports of use of Aquanin plus in shrimp culture, but based on related finding in fish, we hypothesized that cysteamine hydrochloride in Aquanin plus might be useful as a growth promoter and immunostimulant in shrimp culture. The purpose of the present study was to examine the optimum concentration of Aquanin plus to increase growth, survival and immune response which could be recommended for use in shrimp culture.

MATERIALS AND METHODS

Experimental animals

Litopenaeus vannamei (11-12 g) were obtained from a commercial shrimp farm in Chantaburi province, Thailand. A total of 1,500 farm-reared shrimp were transported and acclimated in fiberglass tanks at the Aquaculture Business Research Unit Laboratory, Faculty of Fisheries, Kasetsart University. After 14 days of acclimation, shrimp were used for the experiment. Salinity during the acclimation period and experiment was maintained at 25 ppt.

Experiment 1

Determination of the Aquanin plus effect on growth and survival of white shrimp in the laboratory

For the determination of the growth -promoting and survival effects of Aquanin plus administration in the diet, tests were carried out in three treatments consisting of 0.05% Aquanin plus, 0.1% Aquanin plus and control feed (with six replicates/treatment). Each replicate consisted of 25 shrimp of (11-12 g) in 500-liter tanks. Shrimp were fed four times daily at 3% body weight per day for 60 days with pelleted feed containing graded levels of Aquanin plus (0%, 0.05% and 0.1% of the feed). Feeding rate was adjusted according to shrimp weight throughout the 60-day experiment period. Water quality parameters such as pH, dissolved oxygen (DO), ammonia and nitrite were maintained at suitable levels for shrimp culture throughout the experiment. Growth and survival rates of all treatment groups were recorded every 20 days for 60 days.

Experiment 2

Determination of Aquanin plus effect on the immune characteristics of white shrimp in the laboratory

Three treatments consisting of 0.05% Aquanin plus, 0.1% Aquanin plus and control feed were used in the experiment. A total of 150 shrimp in each treatment (at six replications /treatment) were used. The immune parameters of total hemocytes, percentage phagocytosis, phenoloxidase and bactericidal activity were measured every 10 days for 50 days.

Immune parameters

Preparation of hemolymph samples

Blood sample of 0.5 ml from each shrimp were withdrawn from the base of the 3rd walking leg by a syringe containing 1.5 ml anticoagulant (K-199 + 5% L-cysteine).

Total hemocytes

After collecting hemolymph, hemocytes were counted using a hemocytometer and calculated as the number of blood cells (total hemocytes per cubic millimeter).

Phagocytosis

This method was modified from Itami *et al.* (1994). Two hundred µl of hemolymph were collected from the base of the 3rd walking leg and mixed with 800 µl of sterile anticoagulant. Collected shrimp hemocytes were rinsed

with shrimp saline and the viable cell number adjusted to 1 x 106 cells/ml. The cell suspension (200 µl) was inoculated into a cover slip. After 20 minutes, the cell suspension was removed, and rinsed three times with shrimp saline. Heat-killed yeast (2) was added and incubated for 2 hours. After incubation, heat-killed yeast was removed, rinsed five times with shrimp saline, and fixed with 100% methanol. Then, this cover slip was stained with Giemsa stain and mounted with permount. Two hundred hemocytes were counted. Phagocytic activity, defined as percentage phagocytosis was expressed as:

percentage phagocytosis

$$= \frac{\text{phagocytic hemocytes x 100}}{\text{total hemocytes}}$$

Phenoloxidase activity assay

The method was modified from Supamattaya *et al*. (2000). After the blood was withdrawn, the hemocytes were washed three times with shrimp saline (1,000 rpm 4°C 10 min). Haemocyte lysate (HLS) was prepared from hemocytes in a cacodylate buffer pH 7.4 by using the sonicator at 30 amplitute for 5 seconds and the suspension was then centrifuged at 10,000 rpm., 4°C for 20 min. The supernatant was collected as HLS. Then 200 l of 0.1% trypsin in cacodylate buffer was mixed to the 200 ml HLS followed by 200 l of L-dihydroxyphenylalanine (L-DOPA) at 4 mg/ml as the substrate. Enzyme activity was measured as the absorbance of dopachrome at 490 nm wavelength. Measurement of protein content in HLS was made by using the method of Lowry *et al*. (1951). The phenoloxidase activity was calculated as the increasing of optimum density (OD) per minute per mg of protein as: 1 unit of phenoloxidase = △ OD490 /min/ mg protein

Bactericidal activity

Serum was separated from the blood of each shrimp sample and diluted by 2.6% NaCl at 1:2, 1:4, 1:8, 1:16 and 1:32. Then 0.5 ml of each serum dilution and 0.5 ml of NaCl as the control were used in the study. *Vibrio harveyi* suspension of 0.5 ml (prepared according to the method described in the section *Phenoloxidase activity assay*) was put into each serum dilution and the control. The treatments were incubated at room temperature for 3 h before enumerating the number of bacteria by a spread plate technique. The results were recorded from the dilution that could decrease 50% *V. harveyi* compared with the control.

Statistical analysis

The data were subjected to one-way analysis of variance followed by Duncan's new multiple range test. Differences were considered significant if $P<0.05$.

RESULTS

Experiment 1

Determination of the Aquanin plus effect on the growth and survival of white shrimp in laboratory

After 40 days of administration in diet, shrimp fed with 0.1% Aquanin plus had an average body weight that was significantly higher ($P<0.05$) than those fed with Aquanin plus at 0.05% and the control (Table 1). Considering the percentage survival, there was no significant difference between Aquanin plus -treated shrimps but they were significantly higher ($P<0.05$) than those of the control group (Table 2).

Table 1. Average body weight of *L. vannamei* after 60 days of feeding with Aquanin plus at 0, 0.05% and 0.1%.

Feeding period (day)	Average body weight (g)		
	control	0.05% Aquanin plus	0.1% Aquanin plus
0	11.76 ± 0.42^{a}	11.78 ± 0.46^{a}	11.86 ± 0.48^{a}
20	12.28 ± 0.62^{a}	12.73 ± 0.78^{ab}	12.97 ± 0.78^{b}
40	15.40 ± 1.19^{a}	15.84 ± 0.99^{a}	16.76 ± 1.33^{b}
60	18.04 ± 1.93^{a}	17.48 ± 1.89^{a}	19.52 ± 2.26^{b}

Average values with different superscripts in the same row are significantly different ($P<0.05$).

Table 2. Percentage survival of *L. vannamei* after 60 days of feeding with Aquanin plus at 0, 0.05% and 0.1%.

Feeding period (day)	Percentage survival (%)		
	control	0.05% Aquanin plus	0.1% Aquanin plus
0	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
20	93.60 ± 1.35^{a}	98.68 ± 0.37^{b}	99.20 ± 0.31^{b}
40	83.20 ± 0.11^{a}	97.18 ± 1.66^{b}	97.60 ± 0.15^{b}
60	74.40 ± 6.07^{a}	88.00 ± 6.32^{b}	95.23 ± 1.79^{b}

Average values with different superscripts in the same row are significantly different ($P<0.05$).

Experiment 2

Determination of Aquanin plus effect on the immune characteristics of white shrimp in the laboratory

The immune responses were measured by total hemocytes count (THC), percentage phagocytosis, phenoloxidase activity and bactericidal activity. During the experiments, water quality parameters were maintained as follows: temperature at 28 ± 1^{o}C, pH at 7.8-8.0 and salinity at 25 ppt.

Total hemocytes count

After 20 days of feeding, shrimp fed with diets containing 0.1% Aquanin plus had a total hemocytes count of $13.9 \pm 0.076 \times 10^{6}$ cells/ml which was significantly higher ($P<0.05$) than that of shrimp fed with diets containing 0.05% Aquanin plus and the controls which had total hemocytes count $8.5 \pm 0.41 \times 10^{6}$ and $8.9 \pm 0.23 \times 10^{6}$ cells/ml, respectively. However, after 30-50 days of feeding, both Aquanin plus -treated groups had total hemocytes count significantly higher ($P<0.05$) than those of the control group (Table 3).

Table 3. Total hemocyte count (THC) of *L. vannamei* after 0, 10, 20, 30, 40 and 50 days of feeding with Aquanin plus at 0, 0.05% and 0.1%.

Day	control	Aquanin plus	
	THC (x 10^6 cells/ml)	0.05% THC (x 10^6 cells/ml)	0.1% THC (x 10^6 cells/ml)
0 day	7.05 ± 0.67^a	7.33 ± 0.59^a	7.65 ± 0.16^a
10 day	8.28 ± 3.43^a	8.30 ± 0.86^a	13.50 ± 5.06^a
20 day	8.50 ± 0.41^a	8.90 ± 0.23^a	13.90 ± 0.76^b
30 day	11.00 ± 0.48^a	12.20 ± 0.44^b	14.95 ± 0.52^c
40 day	11.00 ± 0.53^a	12.70 ± 0.53^b	15.65 ± 0.99^c
50 day	11.25 ± 0.51^a	13.38 ± 0.29^b	15.98 ± 0.29^c

Average values with different superscripts in the same row are significantly different ($P<0.05$).

Phagocytic activity

After 20 days of feeding, shrimp fed with diets containing 0.1% Aquanin plus had percentage of phagocytosis significantly higher ($P<0.05$) than those of the 0.05% Aquanin plus and control groups, which had percentage of phagocytosis 24.72 ± 0.62, 23.79 ± 0.36 and 22.11 ± 0.74, respectively (Table 4).

Table 4. Percentage phagocytosis of *L. vannamei* after 0, 10, 20, 30, 40 and 50 days of feeding with Aquanin plus at 0, 0.05% and 0.1%.

Day	control	Aquanin plus	
		0.05%	0.1%
0 day	22.62 ± 0.69^a	21.74 ± 2.50^a	19.88 ± 1.67^a
10 day	22.42 ± 1.75^a	23.68 ± 0.89^a	23.17 ± 1.18^a
20 day	22.11 ± 0.74^a	23.79 ± 0.36^{ab}	24.72 ± 0.62^b
30 day	22.39 ± 0.57^a	24.24 ± 0.41^b	26.31 ± 0.45^c
40 day	23.51 ± 1.20^a	25.73 ± 0.95^{ab}	30.73 ± 2.19^b
50 day	25.75 ± 1.34^a	27.86 ± 1.64^a	33.61 ± 0.85^b

Average values with different superscripts in the same row are significantly different ($P<0.05$).

Phenoloxidase activity

After 10 days of feeding, shrimp fed with diets containing 0.1% Aquanin plus had phenoloxidase activity of 342.82 ± 109.17 unit/min/mg protein which was significantly higher ($P<0.05$) than that of shrimp fed with diets containing 0.05% Aquanin plus and the controls, which had phenoloxidase activity of 315.11 ± 19.90 and 279.27 ± 81.25 unit/min/mg protein, respectively (Table 5).

Table 5. Phenoloxidase activity of *L. vannamei* after 0, 10, 20, 30, 40 and 50 days of feeding with Aquanin plus at 0, 0.05% and 0.1%.

Day	control	Aquanin plus	
		0.05%	0.1%
0 day	255.84 ± 20.53^{a}	246.80 ± 17.20^{a}	251.74 ± 26.18^{a}
10 day	279.27 ± 81.25^{a}	315.11 ± 19.90^{a}	342.82 ± 109.17^{b}
20 day	282.03 ± 26.91^{a}	318.73 ± 14.61^{a}	406.67 ± 26.53^{b}
30 day	290.95 ± 28.83^{a}	301.42 ± 14.41^{ab}	426.37 ± 43.18^{b}
40 day	303.85 ± 25.37^{a}	377.93 ± 39.86^{ab}	471.47 ± 46.82^{b}
50 day	307.08 ± 18.92^{a}	326.65 ± 19.98^{a}	441.98 ± 32.63^{b}

Average values with different superscripts in the same row are significantly different ($P<0.05$).

Bactericidal activity

Shrimp fed with 0.05% and 0.1% Aquanin plus had bactericidal activity at the serum dilution of 1:16 while control occurred at 1:8 after 10 days of experiment (Table 6).

Table 6. Bactericidal activity of *L. vannamei* after 0, 10, 20, 30, 40 and 50 days of feeding with Aquanin plus at 0, 0.05% and 0.1%.

Day	control	Aquanin plus	
		0.05%	0.1%
0 day	1 : 8	1 : 8	1 : 8
10 day	1 : 8	1 : 16	1 : 16
20 day	1 : 8	1 : 16	1 : 16
30 day	1 : 8	1 : 16	1 : 16
40 day	1 : 8	1 : 16	1 : 16
50 day	1 : 8	1 : 16	1 : 16

DISCUSSION

The present study demonstrated that dietary Aquanin plus at 0.1% had a growth -promoting effect on *L. vannamei*, evaluated by measuring the increases in body weight. This growth-promoting effect was due to Beta -cyclodextrin cysteamine hydrochloride which is present in Aquanin plus at 27%. Cysteamine is known to produce a direct or indirect increase

in Growth Hormone (GH) release and growth rates in mammals and chickens (Hall *et al*., 1986). In grass carp (*C. idellus*), cysteamine hydrochloride was applied to promote cultured fish growth in intensive large-scale aquaculture (Xiao and Lin, 2003). Cysteamine and cysteamine hydrochloride have many advantages such as absence of species specificity, simple chemical composition, convenient dietary administration of the drugs to farmed fish and low cost (Xia and Lin, 2003).

Considering the effect of Aquanin plus on immune characteristics, the result from this study suggested that the application of Aquanin plus at 0.1% effectively enhanced the total hemocytes and percentage phagocytosis of *L. vannamei* in 20 days and enhanced the phenoloxidase and bactericidal activities after 10 days. However, 0.05% of Aquanin plus could only increase total hemocytes and bactericidal activity. The immunostimulant ability of cysteamine has not been reported in aquatic animals but in mammals the drug is well known as a relatively specific depletor of tissue somatostatin (a growth hormone inhibitor) and induces a profound, reversible loss of somatostatin -14 biological and immunological activity from somatostatin cells *in vivo* and *in vitro* (Hall *et al*., 1986; Xiao and Lin, 2003). Moreover, Aquanin plus contains 0.1% vitamin E which could enhance cellular and humoral immune response (Reddy *et al*., 1987). These two active components of Aquanin plus could enhance the immunostimulant ability for *L. vannamei* culture.

CONCLUSIONS

The results from this study suggested that in *L. vannamei* dietary Aquanin plus at 0.1% effectively enhanced the growth, survival rate and immune response (including THC, percentage phagocytosis and phenoloxidase activity and bactericidal activity) after 40 days of feeding.

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