Supplementation of ascorbic acid 2-monophosphate during the early postlarval stages of the shrimp *Penaeus vannamei*

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Abstract

A culture trial was performed using five levels of ascorbic acid 2-monophosphate (AmP at 0, 10, 100, 1000 and 2000 mg kg^{-1} diet, expressed as active ascorbic acid, AA) in a semipurified diet for early postlarval *Penaeus vannamei*. The experiment started 10 days after metamorphosis of the mysid larvae into postlarvae (= PL10). Each treatment was run in four replicates. *P. vannamei* postlarvae showed significantly better growth according to dietary AA level after 25 days of feeding, i.e. at PL36 stage. Whereas the dry weight of PL36 in the control treatment (0 mg AA kg^{-1} diet) was only 2 mg, supplementation of 1000 mg AA kg^{-1} increased the shrimp dry weight up to 18 mg. However, the growth in the other treatments was not significantly different from the control. Fitting a broken-line regression to the biomass yield demonstrated an optimal dietary level of 130 mg AA kg^{-1} diet. There were no differences observed among the treatments in stress resistance of postlarval *P. vannamei* to a salinity shock.

KEY WORDS: ascorbic acid 2-monophosphate, larviculture, *Penaeus vannamei*, shrimp, vitamin C

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Introduction

Most aquatic animals, including penaeid shrimp, require a dietary source of vitamin C to prevent the development of deficiency symptoms, e.g. melanized lesions throughout the collagenous tissue underlying the exoskeleton, reduced growth, poor wound repair and, eventually, mortality (Hunter *et al*. 1979; Magarelli *et al*. 1979; He & Lawrence 1993; Shiau & Hsu 1994).

To date, the ascorbic acid (AA) requirements of farmed species of penaeid shrimp have been studied only from the juvenile stage onwards. Little research has been dedicated to the hatchery process, mainly because of its complexity and variable production outputs, the need for large numbers of animals for evaluation and the dependency on live food with varying composition as diet source during the first phases. Moreover, algae, rotifers and *Artemia* contain relatively high levels of AA (Merchie *et al*. 1995a), which impairs determination of the exact larval requirements if lower than these initial dietary levels.

In earlier experiments we evaluated the AA requirements of crustacean larvae using the bioencapsulation technique for live food organisms. No significant differences in production numbers could be identified for larvae of the freshwater prawn *Macrobrachium rosenbergii* (Merchie *et al*. 1995b) or for larval *P. vannamei* (unpublished data) when comparing a control (500 μg AA g^{-1} dry weight, DW) and two AA-enrichment levels in *Artemia* nauplii (1300 and 2750 μg AA g^{-1} DW). This illustrates that, for the last two species, the endogenous AA content of *Artemia* nauplii (500 μg AA/g DW) is sufficient to meet the larval needs.

To study the AA requirements during the postlarval phase, a standard diet which allows the inclusion of exact AA levels

Table 1 Ingredient composition of the basal diet (adopted from Camara 1994)

Ingredient	g kg^{-1} of diet	Product code/supplier
Arginine	10	Sigma A-5131, Sigma NV, Bornem, Belgium
Butylated hydroxyanisole	00.05	Federa NV, Haren, Belgium
Butylated hydroxytoluene	00.05	Federa NV, Belgium
Casein	500	ProtevitK4,3, Dena, Eupen, Belgium
Cellulose	50.05	Sigma C-8002, Sigma NV, Belgium
Cholesterol	5	Sigma C-8503, Sigma NV, Belgium
Ethoxyquin[1]	00.15	Sigma E-8260, Sigma NV, Belgium
Fish oil FO50[2]	75	INVE Aquaculture NV, Baasrode, Belgium
K-carrageenan	40	Sigma C-1013, Sigma NV, Belgium
Mineral mix[3]	100	Sigma Chemical Co., St. Louis, MO, USA
Oil-free lecithin	30	EMULPUR N, Lucas Meyer GmbH & Co., Hamburg, Germany
Soybean oil	30	Vandemoortele NV, Izegem, Belgium
Starch	64.7	Sigma S-5127, Sigma NV, Belgium
Sucrose	50	Sigma S-9378, Sigma NV, Belgium
Vitamin mix[3]	45	Sigma Chemical Co., USA

[1] 1,2-dihydro-6-ethoxy-2,2,4-trimethylquinolin.
[2] Ethyl ester concentrate containing 523 mg *n*-3 highly unsaturated fatty acids (HUFA) g^{-1}, 301 mg 20:5n–3 (EPA) g^{-1}, 189 mg 22:6n–3 (DHA) g^{-1}, DHA/EPA ratio of 0.62.
[3] Teshima *et al*. (1982). Mineral mix (g/100g dry diet): 2 K_2HPO_4; 2.72 $Ca_3(PO_4)_2$; 3.041 $MgSO_4 \cdot 7H_2O$; 0.79 $NaH_2PO_4 \cdot 2H_2O$.
Vitamin mix (mg/100g dry diet): *p*-Aminobenzoic acid, 10.00; biotin, 0.40; inositol, 400.00; nicotinic acid, 40.00 ; Ca-Pantothenate, 60.00; Pyridoxine-HCl, 12.00; Riboflaxin, 8.00; Thiamine-HCl, 4.00; Menadione, 4.00; β-Carotene, 9.60; α-Tocopherol, 20.00; Cyanocobalamine, 0.08; Calciferol, 1.20; Na-Ascorbate, 2000.00; Folic acid, 0.80; Choline chloride, 120.00; Total 2690.08.

over a wide range is needed. Camara (1994) developed a diet using semipurified ingredients to investigate lipid nutrition in postlarval penaeid shrimp, which proved to be suitable for the evaluation of various vitamin C levels (Kontara *et al*. 1997). Since aquaculture feeds are a challenging medium for an unstable component such as AA, the diets used in the present studies were supplied with a stable phosphate ester of vitamin C, ascorbic acid 2-monophosphate (AmP; Gadient & Schai 1994). Bioavailability of this stable AA derivative for shrimp has been demonstrated previously (Shiau & Hsu 1994). This paper reports on the effect of graded levels of AA for the early postlarval stages of penaeid shrimp, *P. vannamei*.

Materials and methods

Diet preparation

Micro-bound diets (Camara 1994) were formulated as shown in Table 1. AmP (PHOSPITAN®C, Showa Denko, Tokyo, Japan) was used as vitamin C source and the various concentrations of the AA phosphate ester were balanced by cellulose. The feed ingredients were bound in a matrix using K-carrageenan and pelleted. The pellets were air-dried, crumbled and sieved to obtain suitable particle size fractions in the ranges of 300–500 and 500–800 μm. Diets were stored at −30°C during the experiment. AmP levels in the diets were verified analytically by phosphatase digestion following the procedure for AA determination (Nelis *et al*. 1997). AmP formulation levels indicated in Table 1 are expressed as actual free ascorbic acid (the commercial AmP product has an activity of 46.5%).

Animals and experimental setup

The experiment was conducted at the Centro Nacional de Acuicultura e Investigaciones Marinas (CENAIM-ESPOL, San Pedro, Ecuador). *P. vannamei* postlarvae were obtained from a commercial hatchery HP (Punta Carnero, Salinas, Ecuador) at an age of 10 days post metamorphosis from the mysid stage (= PL10) and averaged 57 μg body DW. They were stocked in 20 rectangular tanks of 50 L, at a density of 5 PL L^{-1} and kept in a continuous flow-through system. Water exchange ranged from 300% per day at the start of the trial, increasing to 500 and 1000% for PL13–30 and PL31–36, respectively. Temperature and salinity of the oxygen-saturated seawater were kept at 28°C and 35 g L^{-1}, respectively. The photoperiod (12 h light : 12 h dark) was controlled automatically. Five dietary AmP levels (0, 10, 100, 1000 and 2000 mg kg^{-1}, expressed as AA equivalent) were fed over 25 days, each in four replicates. The treatments were assigned at random to the 20 aquaria. Feeding was done three times

Table 2 Ascorbic acid content (mg AA kg^{-1}) incorporated as AmP in the formulated diet[1]

AmP formulated (mg AA kg^{-1} diet)	AmP retention after pelletizing (mg AA kg^{-1} diet)
0	–[2]
10	7 ± 1
100	99 ± 1
1000	990 ± 5
2000	1907 ± 62

[1] AA, ascorbic acid; AmP, ascorbic acid 2-monophosphate.
[2] Under detection limit (< 5 μg AA g^{-1} dry matter).

per day and the daily feed amount calculated as 30% of the body wet weight.

Evaluation

The efficiency of the diets was evaluated at the end of the experiment based on AA incorporation, production results (survival and growth) and stress resistance. AA levels were determined by high-pressure liquid chromatography (HPLC) following Nelis *et al*. (1997). Stress resistance was assessed by means of a salinity test (Tackaert *et al*. 1992): after the experiment 10 postlarvae per tank were immersed in 80 g L^{-1} salinity seawater and subsequent mortality was monitored every 3 min for 1 h. Stress resistance was stated by the cumulative stress index (CSI), calculated as the average value of the replicated treatments obtained after the individual addition of the cumulative mortalities in the consequent time intervals (Dhert *et al*. 1992).

Statistical analysis

Values in the tables represent means and statistical significance of differences in means was determined using one-way analysis of variance. Tukey's multiple range test was applied to detect significant differences among means ($P < 0.05$). Homoscedasticity of variances was controlled by Bartlett's test. In the case of nonhomogeneity of variances, transformations (arcsin √ or log) were applied.

Results

Effective levels of AmP included in the diets after pelletizing are summarized in Table 2. A 95–99% retention was obtained in the range 100–2000 mg AA kg^{-1}, while for the treatment of 10 mg AA kg^{-1} only 70% was registered. No vitamin C was detected in the control diets.

Production results, stress resistance and body AA of the shrimp are presented in Table 3. *P. vannamei* postlarvae initially contained 594 μg AA g^{-1} DW (PL10), a value which declined quickly during the feeding period, i.e. after two weeks of feeding 49–144 μg AA g^{-1} DW was found, further decreasing to 23–126 μg AA g^{-1} DW at the end of the trial. The shrimp fed 2000 mg AA kg^{-1} contained the highest AA levels, although this was not significantly different. AA incorporation (23 μg AA g^{-1} DW) in the treatment fed 100 mg AA kg^{-1} was significantly lower than in the other groups, even when compared with the negative control (99 μg AA g^{-1} DW); this phenomenon was also observed at the intermediate sampling (49 vs. 96 μg AA g^{-1} DW). This lower body AA corresponded with a significantly lower survival, but did not affect the growth rate. From day 15 of feeding onwards, a significantly better growth was obtained for the shrimp receiving 100–2000 mg AA kg^{-1} compared with those fed 0–10 mg AA kg^{-1}. The final biomass of the groups fed 1000 and 2000 mg AA kg^{-1} exceeded by far that of the low-AA treatments, i.e. ≈ 2000 vs. 254–1339 mg DW. The requirement for AA, estimated by fitting a broken-line regression model to the biomass data, was 130 mg kg^{-1} diet. No significant differences in stress resistance were noted among treatments.

Discussion

Losses of AmP were demonstrated to be minor during diet preparation (Table 2), i.e. overall retention generally varied

Table 3 Ascorbic acid incorporation (μg AA g^{-1} DW), survival, individual dry weight, biomass and cumulative stress index of *Penaeus vannamei* postlarvae fed various AmP diets for 25 days[1]

AmP formulated (mg AA kg^{-1} diet)	AA incorporation (μg g^{-1} DW)	Survival (%)	Individual dry weight (mg)	Biomass (mg DW)	Cumulative stress index (–)
0	99 ± 33^{bc}	81.6 ± 6.3^{a}	1.99 ± 0.25^{a}	254 ± 58^{a}	5.43 ± 0.39^{a}
10	82 ± 25^{bc}	81.0 ± 1.8^{a}	2.33 ± 0.22^{a}	291 ± 37^{a}	5.60 ± 0.59^{a}
100	23 ± 6^{a}	64.1 ± 6.1^{b}	16.13 ± 1.46^{b}	1339 ± 348^{b}	4.89 ± 0.95^{a}
1000	62 ± 12^{b}	74.1 ± 1.3^{ab}	17.59 ± 1.88^{bc}	1884 ± 148^{c}	5.72 ± 0.90^{a}
2000	126 ± 14^{c}	76.1 ± 4.0^{a}	19.02 ± 1.08^{c}	2133 ± 187^{c}	5.65 ± 1.20^{a}

Values within the same column with the same superscript are not significantly different ($P > 0.05$).
[1] AA, ascorbic acid; AmP, ascorbic acid 2-monophosphate; DW, dry weight.

from 95 to 100%. Gadient & Schai (1994) showed a similar retention in pelleted shrimp feed of 91 and 98% for AmP and ascorbic acid 2-polyphosphate (ApP), respectively, compared with 65% for free AA. Despite this high retention of the AA derivatives, AA incorporation in *P. vannamei* postlarvae was extremely low, even for the treatments fed 1000 and 2000 mg AA kg^{-1} diet (Table 3). Kontara *et al.* (1997) observed the same low AA concentration in *P. vannamei* postlarvae: only the highest ApP administration (2000 mg AA kg^{-1}) resulted in a body AA of 200 μg AA g^{-1} DW, whereas for the dietary range of 0–100 mg AA kg^{-1} levels remained below 100 μg AA g^{-1} DW. It is likely that leaching of the hydrophilic AA derivatives, once the feed is exposed to the water, caused substantial losses of the vitamin before the pellet was ingested by the shrimp. Kontara *et al.* (1997) reported a 65–75% retention of ApP after processing of the diet, of which another 40–50% was lost during the first 10 min of immersion. Gadient & Schai (1994) demonstrated a 50% retention of both AmP and ApP in shrimp feed immersed for 2 h. Apparently, AmP and ApP, although stable during diet processing and storage, are still prone to leaching, thus influencing greatly the possible AA assimilation by slow-feeding shrimp. To date, however, almost no nutritional studies covering the determination of AA requirements in shrimp mention results of vitamin C leaching. Apart from Kontara *et al.* (1997), only Shigueno & Itoh (1988) report, in the results of their feeding trial with *Penaeus japonicus*, 30% AmP retention after 6 h leaching following a loss of about 50% during diet preparation. Monitoring of the actual vitamin C levels administered to shrimp, taking into account processing but mainly leaching losses, should allow aquaculture nutritionists to determine minimal requirements more accurately.

Significant differences in survival and growth for *P. vannamei* were observed after 10–14 days of feeding, indicating sensitivity of early postlarval shrimp to dietary ascorbate deficiency. 100 mg AA kg^{-1} diet (as AmP) secured optimum growth up to PL25. The final DW and harvested biomass of *P. vannamei* further improved when dietary levels increased up to 1000 mg AA kg^{-1} diet. The significantly lower survival and AA content of this species fed 100 mg AA kg^{-1} (and less pronounced for the ones fed 1000 mg AA kg^{-1}) may be attributed to their rapid initial growth and, consequently, the supply of dietary AA being insufficient to maintain body AA levels. He & Lawrence (1993) estimated an AA requirement for normal survival of juvenile *P. vannamei* (0.5 and 0.1 g initial body weight) of 41 and 120 mg AA kg^{-1} (formulated as ApP), respectively, indicating size-dependent needs decreasing with age. These values correspond to the estimated requirement of 130 mg AA kg^{-1} diet (using AmP) for optimal performance as determined in this study, suggesting a similar AA activity of both AA forms for *P. vannamei*. Both for fish (rainbow trout: Dabrowski *et al.* 1994) and shrimp (*Penaeus monodon*: Kittakoop *et al.* 1996a, 1996b), *in vitro* experiments showed that acid and alkaline phosphatases hydrolyse AmP. Although Dabrowski *et al.* (1994) reported a lower substrate affinity of the fish intestinal phosphatases for ApP compared with AmP, both forms are hydrolysed by the digestive tract phosphatases. Moreover, Giri *et al.* (1994) showed a requirement of 200 mg AA kg^{-1} diet using AmP for *P. monodon* of 0.4 g body weight, which corresponds to the data of Chen & Chang (1994) using ApP levels in a similar range and animals of similar initial body weight. This suggests a similar efficiency of hydrolysis of AmP and ApP in penaeid shrimp juveniles.

Measurement of resistance to salinity shocks has proven a useful tool for quality evaluation of penaeid postlarvae and, moreover, can easily be applied at the hatchery level (Tackaert *et al.* 1992; Rees *et al.* 1994). However, in our test, the transfer of *P. vannamei* postlarvae PL36 to a salinity of 80 g L^{-1} indicated no effect of dietary AA on resistance to osmotic shock. This contrasts with Kontara *et al.* (1997) who reported an improved physiological condition of *P. vannamei* postlarvae fed 2000 mg AA kg^{-1} diet, i.e. 90% of the animals survived the osmotic shock, compared with 33% and 64% for the groups fed 0–20 and 40–100 mg AA kg^{-1}, respectively. Moreover, they demonstrated a significantly positive effect of the highest dietary AA level on the resistance of the shrimp to an infection occurring after 18 days of feeding: the cumulative mortality over 1 week ranged from 63% for the shrimp fed 20–100 mg AA kg^{-1} to 73% for those fed no dietary vitamin C; in the treatment receiving the highest ascorbate level no mortality was recorded. There is no obvious explanation for this difference in physiological conditions between both experiments. Compared with our results, the treatment of 2000 mg AA kg^{-1} diet in the Kontara *et al.* (1997) test resulted in twofold higher levels of AA in the shrimp tissue (215 vs. 126 μg AA g^{-1}) and these elevated stores of vitamin C might explain the significant increase in resistance to an osmotic stress.

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