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ASCORBIC ACID DERIVATIVE REQUIREMENT OF *PENAEUS MONODON*

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ABSTRACT

Two experiments were conducted to determine the Vitamin C requirements of juveniles of *P. monodon*. In experiment 1, practical diet supplemented with different level of ascorbyl phosphate-Mg at 0, 30, 60 and 100 mg/kg of feed, equivalent to 0, 12.6, 25.2 and 42 ppm ascorbic acid, respectively, were fed to *P. monodon* for 10 weeks in 200 l aquarium with a population of 20 shrimps per aquarium. Shrimp fed diet with zero level of ascorbyl phosphate-Mg showed deficiency signs of black spot, erratic swimming, loss of appetite, incomplete molting, soft-shell, sluggishness, flipped gill cover and high mortality. Best growth result was obtained with ascorbyl phosphate-Mg level of 30 to 60 mg/kg. In experiment II, practical diets containing ascorbic acid-glucose at 0, 25, 50 and 100 mg/kg diet, which is equivalent to 0, 13, 26 and 52 mg ascorbic acid/kg diet, respectively were used. In. shrimp fed diet without ascorbic acid glucose supplementation, mortality occurred in week 6 and total mortality recurred by the ninth week. The deficiency signs are soft shell, inactivity, opaque whitish muscle, big head with flipped gill cover, incomplete molting and total mortality. The ascorbic acid glucose required for normal growth and preventing deficiency signs was 25 ppm; 100 ppm was required for maximal feed efficiency.

INTRODUCTION

Ascorbic acid (AsA) is an essential dietary component for most shrimp. It is particularly know for its involvement in hydroxyproline and collagen formation in shrimp (Hunter *et al.*, 1979) as well as reproduction (Alava and Kanazawa, 1995) and stress resistance (Abe *et al.*, 1990; Merchie *et al.*, 1995). Ascorbic acid requirement has been reported in *P. japonicas* (Deshimaru and Kuroki, 1976; Guary *et al.*, 1976; Shigueno, and Itoh 1988; Kanazawa *et al.*, 1993) *P. stylirostris*, *P. californiensis* (Magarelli *et al.*, 1979; Hunter *et al.*, 1979) *P. indicus* (Cahu *et al.*, 1991; Gopal and Raj, 1993). *P. merguiensis* (Boonyaratpalin,1995) and *P. monodon* (Utama and Musa, 1991; Shiau and Jan, 1993; Chen, 1993; Fletcher and Jones, 1993; Catacutan, and Lavilla-Pitogo, 1994; and Shiau and Hsu, 1994).

The ascorbic acid deficiency syndrome observed in *P. californiensis* named "Black Death" was characterized by blackened lesions in the loose connective tissues of the body, especially under the exoskeleton, the abdomen, the carapace, in the gill, and in the foregut and hindgut (Magarelli *et al.*, 1979). Deshimaru and Kuroki (1976) pointed out, deficiency or lack of Vitamin C in feed caused retarded growth, decreased feed efficiency, delayed recovery of injury, white or black lesions under abdominal shell, insufficient ecdysis and high mortality in *P. japonicas*. Catacutan *et al.* (1994) found melanized lesions below the exoskeleton in ascorbic deficient *P. monodon*. This blackened shrimp were very weak and died within a day or two after sampling.

Suggested dietary ascorbic acid levels in *P. monodon* are 2,000 AsA (Shiau and Jan, 1992) or 30 ppm APM (Abe *et al.*, 1990) or 100 to 200 ppm APM (Catacutan and Lavilla-Pitogo, 1994) or 40.25 ppm APM or 156.97 ppm ASTOS (Shiau and Hsu, 1994). The differences in quantitative dietary ascorbic acid requirement were possibly due to form of ascorbic acid used, processing condition and rearing condition. However, the stability, bioavailability and safety of ascorbic acid derivative are not mostly known in shrimps.

The present study determined the stability, bioavailability and adequate level of L-ascorbyl-2-phosphate Mg (APM) and L ascorbic acid glucose (AAG) as Vitamin C sources in practical diet for *P. monodon*.

MATERIALS AND METHODS

Experiment I

The composition of 4 test diets are given in Table 1. The ascorbyl phosphate-Mg (APM) supplemental levels in the test diets were 0, 30, 60 and 100 mg/kg diet. The test diets were processed by commercial shrimp feed pelleting machine at high temperature (95-110 °C), low moisture (16-18%), and dried by vertical dryer to lower the moisture content to about 10%. After drying diets were packed in 3 layer bags and stored at room temperature for feeding trials.

Before the experiment, the hatchery reared *P. monodon* postlavae 15 (PL15) were obtained from the Brackish-water Fisheries Station at Ranot, Songkhla. The shrimps were acclimated to laboratory conditions on commercial pelleted feed until individual weight reached approximately 1.7 grams. Selected individuals were randomly distributed among 24, 200 l aquaria at the rate of 20 individual per aquarium. The aquaria were equipped with running water, aeration and shelter. The shrimp were fed assigned diets to satiation five times daily and bi-weekly quantities consumed were determined. Vitamin C deficiency sign and feeding activity were observed everyday during feeding. Growth and survival were determined every two weeks. Hepatopancreas ascorbic acid was study at the end of the experiment.

Table 1. Composition of the experimental diets containing different levels of Ascorbyl Phosphate Mg for shrimp, *Peneaus monodon* (Experiment I).

Ingredient (g/kg diet)	Diet no.			
	1	2	3	4
Fish meal	280	280	280	280
Shrimp head meal	100	100	100	100
Squid meal	30	30	30	30
Squid liver powder	20	20	20	20
Wheat gluten	60	60	60	60
Wheat flour	200	200	200	200
Soybean meal	100	100	100	100
Rice flour	102	102	102	102
Fish oil	20	20	20	20
Lecithin	20	20	20	20
Zeolite	15	15	15	15
Cholesterol (98%)	5	5	5	5
Vitamin mixture[1]	7.8	7.8	7.8	7.8
Mineral mixture[2]	40	40	40	40
Ethoxyquin	0.2	0.2	0.2	0.2
APM(42 %)	0	0.03	0.06	0.1
Dietary APM (ppm)	0	30	60	100
AsA equivalent (ppm)	0	12.6	25.2	42.0

[1] Vitamin mixture (mg/100 g dry diet) :- thiamin 22.5 ; riboflavin 20.16; choline chloride 150; nicotinic acid 36.7; Ca-pantothenate, 24.0; inositol, 98; biotin, 0.5; folic acid 1.68; Vitamin B_{12}, 0.005; menadione, 13.28; alpha-tocopherol acetate, 148.5; Vitamin A (IU), 1150; Vitamin D_3 (IU), 230;B.H.T., 1; PABA, 20; cellulose, 241.38. TOTAL 780.00

[2] Mineral-mixture (units/100 g diet):- KH_2PO_4 1.0 g; $CaHPO_4\,2H_2O$, 1.0 g; $NaH_2PO_4\,2H_2O$, 1.5 g; KCl 0.5 g.

Stability study: Samples from each diet were stored at room temperature in 3 layer bags and the dietary APM were analyzed immediately after processing and 1, 2, 3 and 4 months after processing. Leaching of dietary APM from test diets were determined by immersion of 30 g of each diet in seawater for 30, 60, 90, 120 min., then placed in deep freezer and followed by freeze drying and APM analysis by HPLC.

Experiment II

Shrimps (*P. monodon*) weighing 1.2 g were randomly distributed in 28 individual chambers (16x25x16 cm). The experimental chamber was fitted with aeration and running seawater. The shrimps were fed to satiation five times a day and tri-weekly quantities consumed were determined. The shrimps were weighed every three weeks and measurement taken of growth rates, feed efficiency and survival. The incidence of deficiency symptoms was also recorded.

Four experimental diets were used in this study (Table 2). The diets were formulated to contain 44.0% crude protein, 10.2% lipid and 16.3% ash and all the nutrients necessary for satisfactory growth of shrimp, except Vitamin C. The amount of ascorbic acid glucose added was 0, 25, 50 and 100 mg/kg diet which is equivalent to 0, 13, 26 and 52 mg ascorbic acid/kg diet, respectively. The diets were processed by mixing all the dry ingredients into a homogenous mixture, then adding oil and 30% of water. The moist mixed feed was passed through a meat grinder and then autoclaved at 120°C for 3 minutes. After that the pellets were dried in an air-flow 60°C oven for 4 to 5 hours to reduce the moisture to approximately 10%. All feeds are packed in plastic bags and stored at room temperature.

Table 2 Composition of the experimental diets containing different levels of Vitamin C-glucose for shrimp, *Peneaus monodon* (Experiment II).

Ingredient (g/kg diet)	Diet no.			
	1	2	3	4
Fish meal	280	280	280	280
Shrimp head meal	100	100	100	100
Squid meal	30	30	30	30
Squid liver powder	20	20	20	20
Wheat gluten	60	60	60	60
Wheat flour	200	200	200	200
Soybean meal	100	100	100	100
Rice flour	102	102	102	102
Fish oil	20	20	20	20
Lecithin	20	20	20	20
Zeolite	15	15	15	15
Cholesterol (98%)	5	5	5	5
Vitamin mixture[1]	7.8	7.8	7.8	7.8
Mineral mixture[2]	40	40	40	40
BHT	0.2	0.2	0.2	0.2
VC-glucose (5.2%)	0	0.25	0.5	1
Dietary AAG (ppm)	0	25	50	100
AsA equivalent (ppm)	0	13	26	52

[1&2] Same as Table 1

RESULTS AND DISCUSSION

Experiment 1

Shrimp growth as determined by average weight, total weight or weight gain are shown in Table 3 and Fig.1. Shrimp fed ascorbyl phosphate-Mg supplemented diets had significantly greater weight gain over the shrimp with no ascorbyl phosphate-Mg supplemented. Analysis of variance showed no significant differences among shrimp fed the 3 treatments of 30 mg, 60 mg and 100 mg/kg ascorbyl phosphate-Mg supplemented diets.

Survival rate at week 6 were 97.5, 97.5, 97.5 and 96.67% for treatment 1 to 4 respectively. By week 8 the survival rate of shrimp fed the diet without ascorbyl phosphate-Mg was significantly lower than the other treatments, there were 67.50, 88.33, 86.67 and 83.33% survival for treatment 1 to 4, respectively. By week 10 the difference had become more pronounced, except shrimp fed diet supplemented with 100 mg ascorbyl phosphate-Mg per kg diet had lower survival and not significantly different from shrimp fed diet without ascorbyl phosphate-Mg supplementation (Fig. 2).

Feeding rates were statistically lower when ascorbyl phosphate-Mg was not supplemented in diet but when the ascorbyl phosphate-Mg supplemented level increased from 30, to 60 and 100 mg/kg no significant difference was detected. The average feeding rates throughout the experimental period were 3.45, 4.24, 4.16 and 4.22% for the ascorbyl phosphate-Mg supplemental levels of 0, 30, 60 and 100 mg/kg respectively.

Feed conversion ratio (FCR) of shrimp fed diet without added ascorbyl phosphate-Mg was significantly higher than shrimp fed diet with ascorbyl phosphate-Mg. There were no significant differences in feed conversion among shrimp fed diet with 30, 60 and 100 mg ascorbyl phosphate-Mg per kilogram diets (Fig. 3).

The ascorbic acid content of the hepatopancreas showed no relationship to dietary ascorbyl phosphate-Mg levels. This result was different from Shiau and Hsu (1994). This indicated that hepatopancreas ascorbic acid concentration might not be a good parameter for ascorbic acid status in *P. monodon*, unless the stability of tissue ascorbic acid during analysis has been investigated.

Table 3. Growth, feeding rate, feed conversion and survival of *Penaeus monodon* fed different levels of Ascorbyl phosphate-Mg for 10 weeks.

	Diet No			
	1	2	3	4
APM (ppm)	0	30	60	100
ASA (ppm)	0	12.6	25.2	42.0
Average wt.				
Initial (g)	1.72 ^{a}	1.70 ^{a}	1.73 ^{a}	1.72 ^{a}
Final (g)	8.10 ^{a}	10.04 ^{b}	10.37 ^{b}	10.72 ^{b}
Gain (%)	371.30^{a}	486.55 ^{b}	500.83 ^{b}	522.90 ^{b}
Total wt.				
Initial (g)	34.35 ^{a}	34.27 ^{a}	34.49 ^{a}	34.37 ^{a}
Final (g)	75.58 ^{a}	155.17 ^{b}	157.1 ^{b}	136.15 ^{b}
Gain (%)	120.24 ^{a}	352.79 ^{b}	355.56 ^{b}	296.37 ^{b}
Feeding rate (%)	3.45 ^{a}	4.24 ^{b}	4.16 ^{b}	4.22 ^{b}
Feed conversion	17.01 ^{b}	2.63 ^{a}	3.09 ^{a}	4.22 ^{a}
Survival (%)	45.83 ^{a}	77.50 ^{b}	76.67 ^{b}	64.17 ^{ab}

Mean within the same row having different superscript (a or b) are significantly different at P<0.01

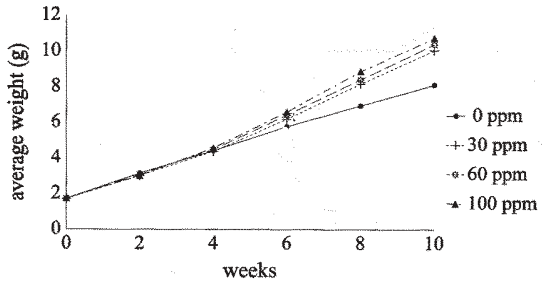

Figure 1. Average weight of shrimp fed diet with different levels of APM.

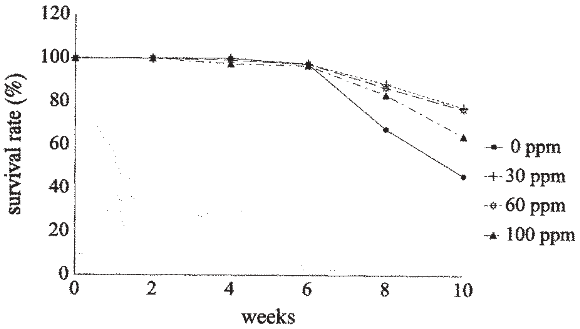

Figure 2. Survival rate of shrimp fed diet with different levels of APM.

Deficiency signs were evident after 6 weeks and shrimps gradually developed black spot, erratic spiral swimming, loss of appetite, incomplete molting, soft shell, sluggishness, flipped gill cover and high mortality. Erratic spiral swimming, incomplete molting and flipped gill cover were found in low percentage. These data indicated that the optimum dietary Ascorbyl phosphate-Mg required for normal growth, feed conversion, survival and good health was 30-60 mg/kg diet, equivalent to 12.6-25.2 mg/kg ascorbic acid/kg diet. This finding is in accordance with the previous observations of Abe *et al.* (1990) and and Hsu (1994).

Stability: After the extrusion process the feeds containing 30, 60 and 100 ppm APM retained around 100, 100 and 90% respectively. APM was practically unaffected by high extrusion temperature. The 10% processing loss might due to mixing or analytical variance. APM was very stable after 4 months. Storing at high temperature and subject to wide temperature range in Thailand (23.2 to 35.4 °C), APM retention were 80, 83.3, 88.9% after 4 months storage, for diets with 30, 60 and 100 ppm APM, respectively. (Fig. 4).

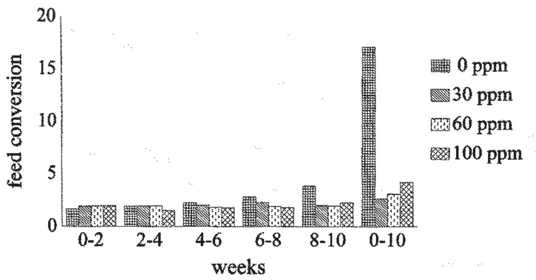

Figure 3. Feed conversion of shrimp fed diet with different levels of APM

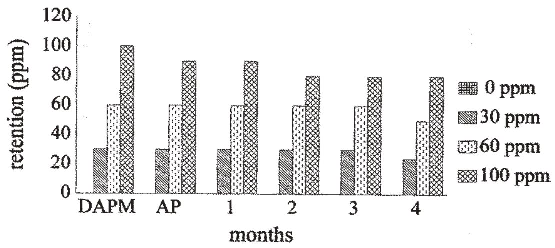

Figure 4. APM retention in shrimp feed after processing and storage DAPM =added amount; AP = after processing.

Leaching (percentage) of dietary APM in flow through seawater system at different time intervals shown in Fig. 5. Leaching percentage increased considerably with immersion time. Leaching percentage was not affected by dietary APM concentration of 30 to 100 ppm. The average leaching percentage at 30, 60, 90 and 120 minute immersion time were 23.3, 42.5, 53.3 and 65.83, respectively.

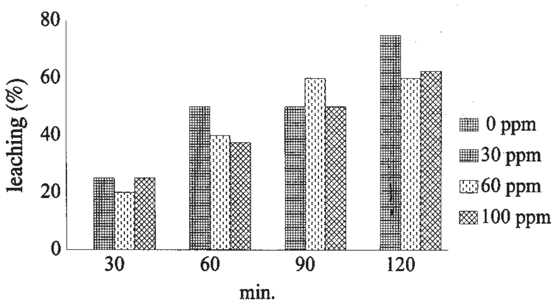

Figure 5. Leaching of APM from shrimp feed in sea water.

The processing loss was minimum (0-10%) which is less than the value reported by Shigueno and Itoh (1988) (47.05%). The differences are possibly due to different processing conditions. Shigueno employed high moisture, low extrusion temperature and 12 hour processing into dry pellets. Under these conditions APM may have decomposed by the action of phosphates, an enzyme the catalyzes phosphoric acid esters which is derived from fish meal in the diet. Storage loss are minimum and similar to finding of Shigueno and Itoh (1988). Substantial amount of APM was leaching out of the shrimp pellet. This is due to the high solubility of APM.

Experiment II

Shrimp fed ascorbic acid glucose supplemented diets had significantly greater total weight and weight gain than the shrimp fed with no ascorbic acid glucose supplement in their diets. This difference was reflected in the survival rate. However, there is no corresponding increase in weight gain with increasing doses of ascorbic acid glucose (AAG) from 25 ppm to 100 ppm (Table 4, Fig. 6 and 7).

Table 4. Effect of ascorbic acid glucose on growth, feed conversion, and survival of *P. monodon* in 9 weeks feeding trial.

Dietary VC		Av. body weight (g)		Gain (%)	Feed Conversion	Survival
AAG (ppm)	AsA (ppm)	Initial	Final			
0	0	1.20	0[a]	-100[a]	-4.28[b]	0
25	13	1.28	9.26[b]	620.67[b]	1.79[a]	100
50	26	1.37	9.30[b]	579.26[b]	1.77[a]	100
100	52	1.30	9.47[b]	634.20[b]	1.42[a]	100

Mean within the same column having different superscript (a or b) are significantly different at $P<0.01$

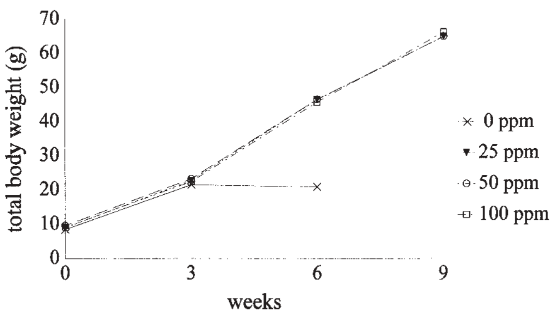

Figure 6. Total weight of *P. monodon* fed diets with different levels of AAG for 9 weeks.

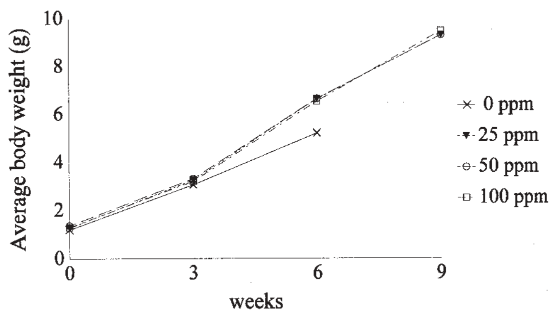

Figure 7. Average body weight of *P. monodon* fed diets with different levels of AAG for 9 weeks.

Feed conversion of diet with no supplementary Vitamin C was significantly higher than that for all other treatments; during the second 3-week period the feed conversion was negative due to weight loss and mass mortality. Feed conversion of 100 ppm AAG diet was significantly lower than the 25 ppm and 50 ppm AAG diets at the third 3-week period, but not significantly lower for the whole 9 weeks feeding period (Fig. 8).

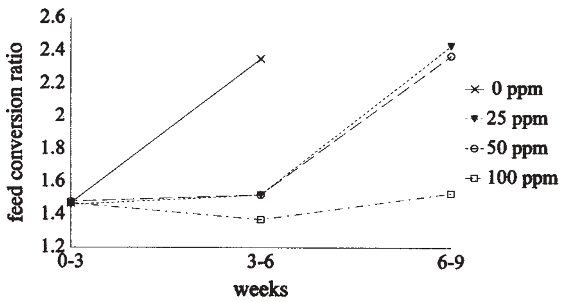

Figure 8. FCR of *P. monodon* fed diets with different levels of AAG for 9 weeks.

Mortality began week 6 in shrimp fed no AAG supplemented diet. Survival rate by the end of 6 weeks trial were 57, 100,100 and 100% for treatment with 0, 25, 50 and 100 ppm ascorbic acid glucose. Total mortality in shrimp fed no AAG supplemented diet occurred in 9 weeks. There was no difference in survival among shrimp fed diet with 25, 50 and 100 ppm AAG in this 9 weeks feeding trial and the survival rate was 100% for all three treatments (Fig. 9).

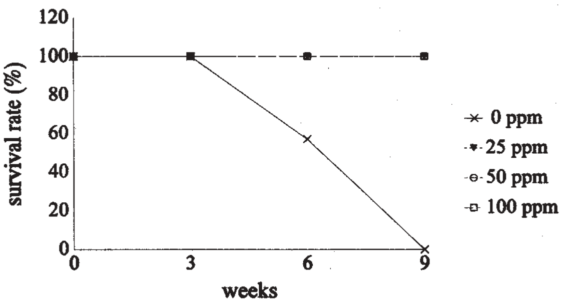

Figure 9. Survival rate of *P. monodon* fed diets with different levels of AAG for 9 weeks.

The feeding rates (% body weight/day) for the first 6-weeks experimental period did not differ significantly from each other in the different treatments. The feeding rate for the third 3 weeks period was significant difference. The overall feeding rate was not significantly different among all AAG supplementary treatments for the 9 weeks trial. In the third 3 week period, feeding rate of shrimp fed diet without supplementary AAG was negligible and feeding rate of shrimp fed 100 ppm AAG supplemental diet was significantly lower than treatments with 25 and 50 ppm AAG.

Shrimp fed on diet without ascorbic acid glucose displayed soft shell, inactive, opaque whitish muscle, big head with flipped gill cover, incomplete molting and high mortality. The most severe deficiency sign was high mortality followed by white muscle and the remaining symptoms were found in lower percentage.

The results indicated that AAG was an effective Vitamin C source for *P. monodon*. The dietary ascorbic acid glucose (AAG) required for normal growth and preventing critical symptoms was 25 ppm, equivalent to 13 ppm ascorbic acid.

This finding was in accordance with the results of our first experiment and results of Shiau and Hsu (1994)or Abe *et al.* (1990), where APM was used as Vitamin C source. The AAG required for high feed efficiency was 100 ppm or equivalent to 52 ppm ascorbic acid. This phenomenon was not found in the ascorbyl-phosphate-Mg experiment. Thus, this indicates that AAG might be more safe to use at high levels. In fact the dietary Vitamin C requirement level has reduced dramatically as stable and bioavailable Vitamin C derivative had been developed. For example, the requirement for ascorbic acid of P. japonicas was about 500-1000 mg/100 g of dry diet (Guary *et al.*, 1976) and the ascorbic phosphate-Mg requirement level was 215 ppm (Shigueno and Itoh, 1988) or 50 ppm (Kanazawa *et al.*, 1993). Therefore, the dietary ascorbic acid requirement level for different species of shrimp and fish are quite similar with in the range of 10-20 mg ascorbic acid equivalent/kg dry diet (Sandiness and Waagba, 1991; Kanazawa *et al.*, 1993). However, the optimal supplemental amount of Vitamin C in diet in fish or shrimp will depend on processing condition, stability, leaching and bioavailability of Vitamin C sources. In general any Vitamin or mineral which is more prone to leaching will be more bioavailable. The difference in dietary ascorbyl phosphate-Mg loss during processing found by Shigueno and Itoh (1988) and results in this study (Experiment I) was due to processing condition which indicated different dietary requirement. The processing condition employed by Shigueno and Itoh involved high moisture, mincing and 12 hr of drying. Then part of the ascorbyl phosphate may have been converted to ascorbic acid by the action of phosphatase enzyme present in animal protein in diet such as fish meal, shrimp head meal, squid meal, during wet processing. This extremely high processing loss of 47% (Shigueno and Itoh, 1988) and also our results led us to conclude that ascorbyl phosphate was an ideal Vitamin C source for high temperature conditions of commercial aquatic feed processing, but not for conditions of moist feed or low temperature, high moisture and long hour drying time. Therefore, the autoclaving of moist pellet employed in this experiment was aimed to subject the diet to high temperature to simulate the commercial processing condition and also to stop any possible enzymatic or bacterial action.

The black dead disease observed among ascorbic acid deficient *P. californiensis*, *P. stylirostris* (Magarelli *et al.*, 1979) and *P. japonicas* (Shigueno and Itoh, 1988) was not seen in *P. monodon* under the present experimental conditions. However, in this study, many other ascorbic acid deficiency signs such as soft shell, incomplete molting, flipped gill and whitish muscle, which have never been reported by any worker for any shrimp, have been observed.

Ascorbic acid glucose is stable to heat, and bioavailable to shrimp as ascorbyl phosphate. The dietary requirement level for normal growth, survival and health was 25 ppm AAG or 30 ppm APM or equivalent to about 13 ppm ascorbic acid.

CONCLUSIONS

Results from the present feeding trials shows that:

1. Both APM and AAG are bioavailable for *Penaeus monodon*.
2. APM is fully stable during processing and storage.
3. The dietary APM or AAG requirement for *P. monodon* are 30 ppm or 25 ppm respectively, or equivalent to about 13 ppm ascorbic acid.
4. Vitamin C deficiency signs observed were: black spot, soft shell, shortened appendix, unclose antenna plate, white muscle, flipped gill cover, incomplete molting, inactivity, and high mortality.
5. APM leaching is greatly increased with immersion time. Therefore study on attractants and feeding technique is necessary for increasing percent retention of all water soluble nutrients and minimizing pollution problems.
6. The results of dietary AAG requirement level led to the conclusion that it is as stable as APM.

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