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Quantification of arginine requirements of juvenile marine shrimp, *Penaeus monodon*, using microencapsulated arginine

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Abstract. Using microencapsulated L-arginine, the quantitative requirement for amino acid has been determined for the first time for a shrimp species, which can not effectively utilize crystalline amino acids. In an 8 wk feeding trial (1990), juvenile *Penaeus monodon* were fed casein-based purified diets containing one of six levels (13.1, 17.7, 22.3, 26.9, 31.5 and 36.1 g/kg diet) of arginine. In addition to the protein-bound arginine already present in the casein of the test diets, pure arginine was supplemented by L-arginine microencapsulated in cellulose acetate phthalate, which is easily assimilated. The arginine level required for optimal growth was determined by broken-line model analysis of weight gain data to be 25.0 g/kg diet (=54.7 g/kg protein). Examination of the hemolymph 3 h after feeding revealed that the free arginine level in the hemolymph had not increased as a result of increasing levels of the dietary arginine. There was an abrupt increase of urea in the hemolymph when the arginine requirement of the shrimp had been met.

Introduction

Attempts to substitute or supplement dietary protein with individual (or mixtures of) crystalline amino acids (Cowey and Forster 1971, Deshimaru and Kuroki 1974, Deshimaru 1982) or with protein hydrolysates (Andrews et al. 1972, Deshimaru and Kuroki 1975) have resulted in poor growth and survival of penaeid shrimp. Their inability to utilize crystalline amino acids has made it practically impossible to quantify the essential amino acid requirements of shrimp and other crustaceans. Although various approaches have been tried (Mason and Castell 1980, Divakaran and Lim 1990), the quantitative requirements of shrimp for essential amino acids have remained unresolved (Akiyama and Dominy 1989). In a recent study on juvenile *Penaeus monodon*, Chen et al. (1992) demonstrated that L-arginine microencapsulated within either cellulose acetate phthalate (CAP) or glycerol monostearate (GM) could effectively supplement the diet of the shrimp. Test diets containing supplementary CAP- or GM-arginine resulted in a significantly better growth and food conversion than diets supplemented with crystalline-arginine alone. These results indicate that a microencapsulated amino acid may be an effective means of studying the quantitative requirements of shrimp for essential amino acids.

The present study was designed to assess the quantitative arginine requirement of juvenile *Penaeus monodon* (the most widely cultured shrimp species) using CAP-encapsulated arginine. The shrimp were fed casein-based diets supplemented with different levels of CAP-arginine. Arginine is an essential amino acid which functions as a phosphagen (Hird 1986) in crustaceans, whose requirement for this amino acid would appear to be high (Dall et al. 1990). Arginine requirement was monitored by means of weight gain, food conversion and survival. Variations in the contents of free arginine and other amino acids, urea and ammonia in the hemolymph of the test shrimp were measured and the biochemical parameters were compared to growth indices.

Materials and methods

Experimental design

Penaeus monodon were obtained during the summer of 1990 from the hatchery facility of the Tungkang Marine Laboratory of the Taiwan Fisheries Research Institute (Tungkang, Pingtung, Taiwan). A total of 270 apparently healthy and well-acclimated juveniles (inital mean weight $\pm$ SEM = 0.32 ± 0.05 g) were divided into 6 experimental groups, each consisting of 3 replicates of 15 shrimps. Each of the 18 replicates was randomly assigned to a $60 \times 60 \times 46.5$ cm rectangular glass aquarium fitted with undergravel filters. The filter bed consisted of crushed oyster shell and sand. Aquariums were placed under fluorescent light with a 12 h light:12 h dark cycle.

Deionized water was used to adjust filtered natural sea water to 25‰ S. Approximately one-tenth of the culture water in each aquar-

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ium was replaced every other day. Ammonia-N, pH and dissolved oxygen were measured daily; they remained at acceptable levels throughout the trial period. Water temperature was not controlled but was monitored daily, and ranged between 23.0 and 28.0 °C.

The shrimp were fed twice daily (at 09.00 and 17.00 hrs) for 8 wk. Food levels provided were 10% of the shrimp body weight during the first 4 wk and 8% during the subsequent weeks. Uneaten food and exuviae were removed each morning before feeding. All test shrimp were blotted dry with tissue paper and weighed individually every 2 wk in the morning before the first feeding.

Preparation of experimental diets

Six purified experimental diets were designed to provide graded levels of arginine. The basic diet (Table 1) was based on a modified formula (Chen et al. 1991) recommended for penaeid shrimp by the National Research Council (1983). It provided ~15 080 kJ/kg diet (~3607 kcal/kg diet) gross energy, contained 450 g crude protein/kg diet, and had a protein energy ratio of 29.84 mg protein/kJ (124.8 mg protein/kcal). The basic diet contained vitamin-free casein (457.0 g/kg diet) as the sole protein source; this supplied 13.1 g/kg diet protein-bound arginine to the diets (analysis had revealed that casein contained 28.7 g arginine/kg casein). Microencapsulated L-arginine (CAP-arginine) was used to enrich the arginine contents in the test diets by replacing casein with equal amounts of CAP-arginine; the final arginine concentrations were 13.1, 17.7, 22.3, 26.9, 31.5 and 36.1 g/kg diet. The diets were processed into pellets (2 mm diam; 3 to 5 mm long) and freeze-dried. Each diet was sealed in a plastic bag and stored at −20 °C until use.

The preparation of CAP-arginine was based on the method described by Deasy (1984). Two grams of cellulose acetate phthalate (Serva Feinbiochemica, Heidelberg, Germany) were dissolved in 10 ml acetone, and 14 g of L-arginine HCl (Tanabe Seiyaku Co., Osaka, Japan) were added to the solution, which was mixed well. Acetone was finally removed in a rotary evaporator and the CAP-arginine residue was collected. Each gram of CAP-arginine contained 0.875 g L-arginine HCl.

Analysis of free amino acid content of hemolymph

A second batch of 60 shrimp (initial mean weight 1.19±0.31 g) from the same hatchery was divided into 6 groups of 10 shrimps each. Each group was fed one of the six test diets for 1 wk. Cultivation conditions were the same as in the feeding experiment described in the foregoing section. All shrimp were sacrificed and examined 3.0 h after the morning meal on the final day of the 1 wk feeding period. Approximately 1 ml of hemolymph was withdrawn from the ostium of the heart of each shrimp. In each group, the samples from all shrimp were pooled and a volume equal to the pooled hemolymph sample of 50 g/l sulfo-salicylic acid was added to precipitate protein, according to the method of Cross et al. (1975). The supernatants derived from 15 min centrifugation (Model Himac, RPR20-3 rotor, Hitachi Instruments, Tokyo, Japan) at 1 °C and 13 300 $\times g$ were collected and frozen in liquid nitrogen until analysis. Amino acids were analyzed with an amino acid analyzer (Model 6300, Beckman Instruments, Palo Alto, California). The frozen supernatant was thawed, and a subsample of 100 µl was withdrawn and mixed with 500 µl lithium citrate. The mixture was injected into the analyzer (Findley and Stickle 1978). The amino acids were detected as their ninhydrin derivatives, using a fluorescence detector fitted with 440 and 570 nm filters.

Growth (expressed as average percentage weight gain), survival and food-conversion ratios (ratio = amount of food fed:wet weight increment of each replicate) were analyzed for statistical significance ($P<0.05$) by ANOVA, and individual differences between treatments were determined by Duncan's new multiple-range test. The broken-line analysis technique (Robbins 1986) which examined the relationships of dietary arginine levels and shrimp weight gains was used to estimate the arginine requirements. The breakpoint of the regression equations, which gave the least mean-square error, was regarded as the requirement level.

Table 1. Composition of arginine test diets fed to juvenile *Penaeus monodon*

Ingredient	g/kg diet
Casein (vitamin free)[a]	457
Amino acid mixture[b]	30
Corn starch[a]	200
Fish oil[c]	50
Soy lecithin[d]	50
Cholesterol[a]	10
Glucosamine HCl[a]	8
Sodium succinate[a]	3
Sodium citrate[e]	3
Mineral mixture[f]	86
Vitamin mixture[g]	27
Cellulose[a]	41
Sodium alginate (high viscosity)[h]	25
Sodium hexametaphosphate[i]	10

[a] Sigma Chemical, St. Louis, Missouri, USA
[b] Served as attractant and contained alanine:glycine:glutamic acid: betaine (1:1:1:2) (Sigma Chemical)
[c] Hanaqua Feed Co., Kaohsiung, Taiwan
[d] Great Wall Enterprise Co. Ltd., Tainan, Taiwan
[e] Merck, Darmstadt, Germany
[f] Mineral mixture (Merck) supplying the following (g/kg diet): K_2HPO_4, 20; $Ca_3(PO_4)_2$, 27.2; $MgSO_4 \cdot 7H_2O$, 30.4; $NaH_2PO_4 \cdot 2H_2O$, 7.9
[g] Vitamin mixture (Hoffman La Roche, Basel, Switzerland) supplied the following (mg/kg diet): *p*-amino benzoic acid, 100; biotin, 4; inositol, 4000; nicotinic acid, 400; Ca-pantothenate, 600; pyridoxine · HCl, 120; menadione, 40; dl-α-tocopherol, 200; β-carotene, 96; vitamin B_{12}, 0.8; cholecalciferol (D_3), 12; sodium ascorbate, 20 000; folic acid, 8; choline chloride, 1200
[h] Kelco, San Diego, California, USA
[i] Yakuri Chemicals, Osaka, Japan

Table 2. *Penaeus monodon*. Effects of dietary arginine levels on growth, food conversion and survival rates of juvenile shrimp during 8 wk period. Initial average shrimp weight was 0.32±0.05 g. Values are means±SEM (3 replicates of 15 shrimp). Means within a given column followed by same superscript letter are *not* significantly different ($P>0.05$, Duncan's new multiple-range test)

Diet No.	Arginine level (g/kg diet)	Weight gain (%)	Food conversion ratio (g food:g gain)	Survival rate (%)
1	13.1	209.8±35.6[a]	3.67±0.40[a]	48.9±8.3[a]
2	17.7	277.2±23.0[b]	3.15±0.27[ab]	51.1±6.3[a]
3	22.3	327.9±20.0[bc]	2.72±0.24[b]	55.6±8.3[ab]
4	26.9	370.4±25.0[bc]	2.03±0.31[c]	62.2±1.3[ab]
5	31.5	376.2±34.8[c]	1.89±0.17[c]	71.1±3.1[b]
6	36.1	371.9±20.9[c]	2.06±0.25[c]	60.0±5.5[ab]

Results

Growth, food conversion ratio and survival of juvenile *Penaeus monodon* were significantly ($P<0.05$) improved by supplementary arginine (Table 2). There was a gradual

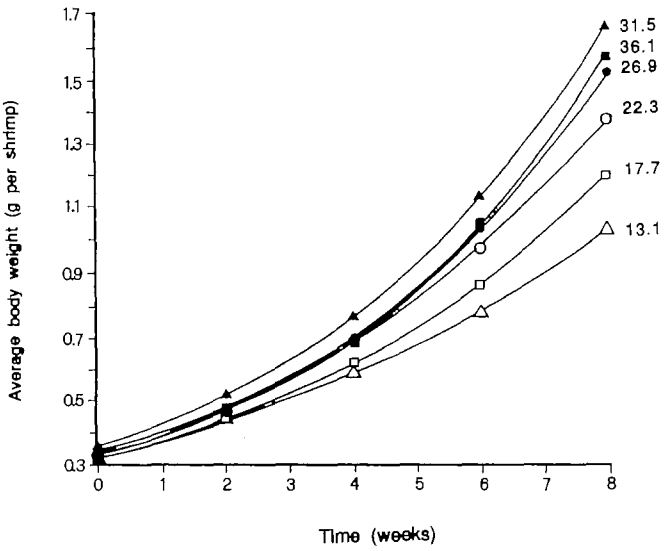

Fig. 1. *Penaeus monodon.* Growth of shrimp fed test diets containing graded levels of arginine (g/kg diet)

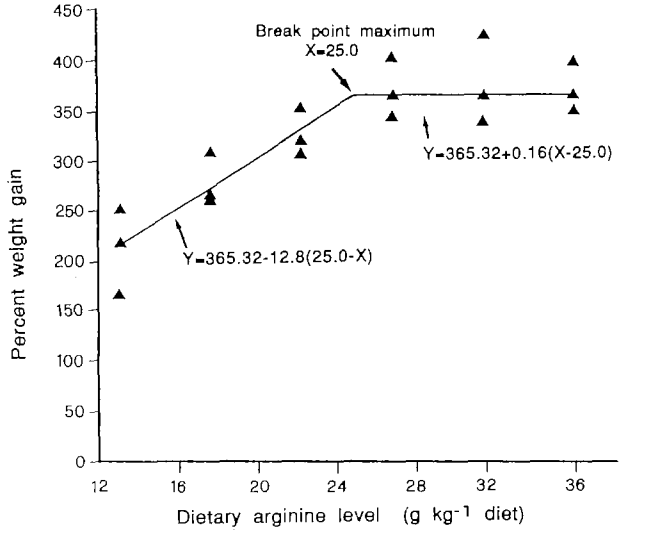

Fig. 2. *Penaeus monodon.* Mean percent weight gain of juveniles fed graded levels of arginine. Break point is point at which a least-mean-square error was obtained (regarded as optimal dietary requirement level)

improvement in shrimp growth as the dietary arginine level increased. Highest levels of growth, food conversion and survival were obtained with a diet containing 31.5 g arginine/kg diet, and lowest levels were recorded in the group fed an unsupplemented diet (Table 2: Diet 1; arginine level = 13.1 g/kg diet). Fig. 1 shows growth of the shrimp during the 8 wk feeding experiment. Although the survival of all experimental shrimp was generally unsatisfactory, the effect of arginine supplementation in reducing mortality was obvious. There was a significant difference in survival rates between the unsupplemented group and the supplemented group displaying the greatest growth (Table 2: Diet 1 vs Diet 5).

Based on the broken-line analyses of growth (as percentage weight gain), the optimal dietary level of arginine for juvenile *Penaeus monodon* was estimated to be

Table 3. *Penaeus monodon.* Effect of dietary arginine level on concentration (μmol/ml) of free amino acids (AA), ammonia and urea in hemolymph of juvenile shrimp fed purified diets

AA	Dietary arginine level (g/kg diet)					
	13.1	17.7	22.3	26.9	31.5	36.1
Essential AA						
Arginine	0.58	0.54	0.56	0.59	0.58	0.61
Histidine	0.15	0.13	0.14	0.14	0.15	0.13
Isoleucine	0.23	0.27	0.26	0.26	0.28	0.28
Leucine	0.57	0.53	0.55	0.54	0.56	0.53
Lysine	0.70	0.63	0.76	0.72	1.34	1.24
Methionine	0.17	0.15	0.17	0.17	0.18	0.17
Phenylalanine	0.33	0.29	0.34	0.29	0.35	0.29
Threonine	0.21	0.19	0.19	0.19	0.22	0.19
Valine	0.30	0.37	0.31	0.38	0.39	0.38
Total	3.28	3.11	3.27	3.29	4.05	6.83
No-essential AA						
Alanine	0.66	0.63	0.67	0.66	1.09	0.84
Asparatic acid	0.20	0.17	0.18	0.19	0.64	0.51
Glutamic acid	0.27	0.25	0.31	0.28	0.89	0.78
Glycine	0.20	0.18	0.16	0.17	0.19	0.20
Ornithine	0.02	0.02	0.02	0.02	0.03	0.04
Proline	0.89	0.73	0.84	0.95	0.79	0.74
Serine	0.30	0.29	0.34	0.27	0.36	0.30
Tyrosine	0.29	0.27	0.32	0.29	0.30	0.28
Total	5.37	4.82	5.43	5.49	7.37	6.83
Ammonia	0.36	0.34	0.23	0.23	0.20	0.18
Urea	10.33	12.96	19.88	60.26	50.22	43.46

~25.0 g/kg diet. The regression line fitted to the percent weight gain displayed a break point (R) at 25.0 g/kg diet (Fig. 2). The relationship between percent weight gain (Y) and dietary arginine level (X) is: $Y=365.32-12.84\ (R-X)$ if $X<R$, and $Y=365.32+0.16\ (X-R)$ if $X>R$. This level is equivalent to 54.7 g/kg protein.

Increasing amounts of arginine in the diet did not produce any increase in the free arginine content of the hemolymph (Table 3), which remained relatively constant over the dietary range. There was an abrupt rise in free lysine in the hemolymph when the dietary level of arginine rose to 31.5 g/kg. This level of supplementation is close to that estimated as being optimal to meet the dietary requirements for arginine (25.0 g/kg diet). Levels of ammonia in the hemolymph decreased with increasing dietary arginine levels, while the level of urea in the hemolymph rose sharply at the optimal arginine supplementation of ~25.0 g/kg (26.9 g/kg diet).

Discussion

Based on the growth data, the dietary requirement of juvenile *Penaeus monodon* for arginine appears to be 25.0 g/kg diet, which corresponds to 5.47% of the dietary protein required to ensure optimal weight gain. This is less than the percentage dietary arginine requirements previously reported for many salmon species (6.0%) and chicks (6.1%), but higher than those for the channel catfish (4.3%), common carp (4.2%), Japanese eel (4.5%)

and swine (1.2%) (National Research Council 1983). It is also less than the arginine contents of whole zoea (5.92%) and whole juvenile (6.57%) and adult (8.28%) muscle reported for *P. monodon* (Penaflorida 1989).

The arginine levels recorded in the hemolymph in the present study provide little insight into the arginine requirement of juvenile shrimp. No classic dose-response curve indicating a large accumulation of free arginine after the shrimps' requirement had been met was observed. In early studies on laying hens (Chiu and Speers 1976), channel catfish (Wilson et al. 1978, 1980, Robinson et al. 1980) and other animals (McLaughlan and Illman 1967), a deficiency of a dietary amino acid resulted in a low concentration of that amino acid in the blood serum with a subsequent increase as the dietary requirement of that amino acid was met. A lack of dose-response in arginine levels has also been observed in rainbow trout (Kaushik et al. 1988) and channel catfish (Robinson et al. 1981). Robinson et al. (1981) suggested that the levels of free amino acids in the blood serum of catfish may depend not only on the dietary concentration of any one individual amino acid, but also on its interrelations with other dietary amino acids. Such interactions could mean that the dose-response rate of an amino acid would be useless in predicting requirement levels. Similar interrelationships may have contributed to the arginine levels observed in the hemolymph of *Penaeus monodon* in the present study.

Another factor that could be involved in the apparent lack of dose-response is the time of blood sampling and the use of CAP-encapsulated arginine in the supplementary diets. In studies with channel catfish (Wilson et al. 1980, Robinson et al. 1981), blood samples were collected regularly 15 to 20 h after the final feeding at the end of the 8 wk feeding experiments, and Kaushik et al. (1988) took blood samples of rainbow trout for analyses 6 h after the morning meal. In the present study, shrimp hemolymph was sampled 3 h after the morning meal. Deshimaru (1976) reported that the level of total free amino acids in the hemolymph of *Penaeus japonicus* reached its peak 3 h after a meal, as did the concentration of free arginine; the elevated free amino acid concentrations returned to pre-feeding levels 12 h after feeding. Following ingestion of ^{14}C-proline-labelled food by *P. esculentus*, the radioactivity in the proventriculus decreased sharply, reaching almost baseline levels within 3 h (Smith and Dall 1991), and there was a peak in the blood radioactivity between 1 and 3 h after ingestion. If the pattern of free arginine levels in the hemolymph of *P. monodon* after a meal did not follow those of *P. japonicus* or *P. esculentus*, the timing of the hemolymph sampling in the present study might have contributed to the lack of significance of arginine levels in the hemolymph.

On the other hand, because the arginine supplement was microencapsulated in the test diets, this may have delayed the release of arginine in the gastrointestinal tract of the shrimp. The delayed release, and thus absorption, may have prevented concentrations of arginine in the tissues from being temporarily elevated and then being immediately catabolized rather than used for protein synthesis (Chen et al. 1992), and thus have increased the effective provision of arginine to shrimp juveniles. Enhanced growth as a result of supplementary arginine was also demonstrated earlier in a study with larval *Penaeus japonicus* fed a crystalline-arginine-supplemented microencapsulated diet (Teshima et al. 1986). Slightly more than half of the ingested pure proline in zein-coated microcapsules was found in the abdominal muscle of subadult *P. esculentus* 3 h after feeding (Smith and Dall 1991). Microencapsulation seems to be an effective technique for adding pure amino acids to diets of shrimp. Cowey and Sargent (1979) suggested that when crystalline amino acids are used to supplement a protein, they are assimilated much more rapidly than those amino acids linked by peptide bonds in the food protein. The tissue concentration of these supplementary acids may thus be transiently elevated, and they may be catabolized rather than utilized for protein synthesis. The controlled release of arginine from microcapsules, on the other hand, could delay the appearance of free arginine in the hemolymph; this requires further investigation.

A number of investigators have shown that an arginine-deficient diet causes several metabolic changes, including decreased urea levels in the hemolymph, in various animals (see Thomas and Deshmukh 1986). Cho and Woodward (1985) and Kaushik et al. (1988) found that urea levels in the plasma of rainbow trout were related to arginine intake and that these levels increased when plasma-free arginine rose above a given threshold. In the present study, the free arginine levels in the shrimp hemolymph showed little change as a result of increased arginine uptake throughout the dietary range, but an abrupt rise in the urea level of the hemolymph occurred when the arginine requirement was met. These elevated urea levels were clearly caused by the increased arginine intake of the shrimp. In contrast, Kaushik et al. suggested that urea in rainbow trout is produced mainly by the catabolism of endogenous metabolites and degradation of nucleotides. There was no significant change in the in vitro activity of liver arginase with increased arginine levels in the diets of rainbow trout (Walton et al. 1986).

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