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Supplementations of DL-Methionine and Methionine Dipeptide in Diets are Effective for the Development and Growth of Larvae and Juvenile Red Sea Bream, *Pagrus major*

Roger Edward P. Mamauag, Jian Gao, Binh Thanh Nguyen, and Janice A. Ragaza

The United Graduate School of Agricultural Sciences, Kagsohima University, Korimoto 1-21-24, Kagoshima 890-0065, Japan

Shunsuke Koshio[1], Manabu Ishikawa, and Saichiro Yokoyama

Laboratory of Aquatic Animal Nutrition, Faculty of Fisheries, Kagoshima University, Shimoarata 4-50-20, Kagoshima 890-0056, Japan

Abstract

Growth trials for larvae and juvenile red sea bream, *Pagrus major*, were conducted to elucidate the efficacy of two molecular forms of methionine; DL-methionine (DL-Met) and methionine dipeptide (Met-Met). For the larvae experiment, five experimental diets were formulated and fed to fish (42 mg) for 30 days. A diet which has 15% soy protein isolate served as the control diet. Similarly, test diets supplemented with DL-Met and Met-Met at 0.5%, which were either precoated by zein or intact, were also formulated. For the juvenile experiment, five experimental diets were formulated wherein the control diet contained 25% soy protein isolate. Test diets were supplemented with DL-Met and Met-Met at 0.75%, which were either coated by carboxymethycellulose or intact and fed to juveniles (0.75 g) for 56 days. The results of two feeding trials showed both DL-Met and Met-Met can be equally utilized by red sea bream larvae and juveniles. Coating the amino acid significantly improved both fish larval and juvenile growth performance. The development of digestive protease activity of larvae was significantly influenced by coating the amino acid, but the type of methionine was not a factor in changing the protease activity of larvae.

The declining supply of fish meal over the years has turned the attention of feed industry operators to plant protein sources. Soybean products such as soybean meal and soy protein isolate are some of the most practical alternative protein source for fish feed ingredients. However, this commonly used alternative protein sources are less suitable for aquafeeds because of the presence of antinutritional factors, high fiber, low palatability, and inferior amino acid profile in comparison to the traditional fish meal protein (Tacon 1994; Alam et al. 2005). Methionine is one of the most limiting essential amino acid in feeds formulated with plant protein ingredients (Ahmed et al. 2003). Several studies showed that methionine supplementation in aquafeeds significantly improved survival, growth, and feed efficiency (Alam et al. 2001; Ahmed et al. 2003). Furthermore, methionine supplementation promoted intestinal growth and enzyme activities by enhancing the proliferation of beneficial bacteria and humoral immunity factor (Tang et al. 2009).

Red sea bream, *Pagrus major*, is one of the most highly valued marine aquaculture fish in Japan (Kato et al. 2002). This species are carnivorous and often fed with trash fish, or formulated aquafeeds containing high levels of fish meal (Huang et al. 2007). The increasing use of plant protein sources such as soybean products in fish feed leads to a common practice of amino acid supplementation to the diet, in order to meet the amino acid requirements of target fish species (Lunger et al. 2007).

[1] Corresponding author.

Although amino acid supplementation has been generally carried out with free-form crystalline amino acids, peptides have been found to show faster and efficient absorption rates in the intestine of fish and mammals (Adibi 1997; Zambonino Infante et al. 1997) which can be attributed to special transport mechanisms for dipeptides (Li et al. 1999). However, a rapid absorption can lead to a transient amino acid imbalance and decreased protein accretion (Tesser et al. 2005). In addition there are evidence that peptide can induce an earlier and greater rise in enzyme activity in the brush border membrane which is essential for larval development (Zambonino Infante et al. 1997). Peptides can be sourced either from hydrolyzed food protein (Kristinsson and Rasco 2000; Wang and De Mejia 2005) or commercially available synthetic dipeptide (Dabrowski et al. 2003). Recently, Mamauag et al. (2011) reported that peptide from soybean hydrolysate can be efficiently utilized by Japanese flounders and can promote growth performance and feed efficiency at a moderate inclusion level of 20% in the diets. Studies addressing a comparison of dietary amino acid sources provided as dipeptides or free amino acid as supplements in fish larvae and juvenile diets are scarce. In addition, amino supplementation studies involve coating procedure of the nutrient which has shown to reduce leaching (López-Alvarado et al. 1994) as well as ensuring the longer residue time in the intestine that will lead to efficiency in absorption (Fox et al. 1995).

The use of soybean products as a protein ingredient have received considerable amount of attention in red seabream diet (Takagi et al. 1999). Therefore, the present aim of this study was to elucidate whether two forms of methionine (DL-methionine [DL-Met] and methionine dipeptide [Met-Met]) which were incorporated in the diet in which a percentage of fish meal was replaced by plant meal can affect the growth performance, protease activity, and blood biochemical parameters of larval and juvenile stage red sea bream. In addition the effect of coating method (coated or uncoated) of methionine in diets was investigated as well.

Materials and Methods

Experimental Diets

The formulation of the larvae experimental diets is presented in Table 1. Five diets were formulated to be isonitrogenous and isolipidic to contain 64% crude protein and 11% crude lipid, respectively. The control diet contains 15% soy protein isolate as a partial source of protein in order for the diet to be deficient with methionine at 0.5% (0.5 g/100 g dry weight). Two test diets based from the control diet formulation were supplemented with 0.5% coated DL-Met or Met-Met. Two similar diet preparations were made; however, the methionine sources were not coated. Methionine was precoated with 1 g of zein dissolved in ethanol and freeze dried which was adapted with modification from the procedure of López-Alvarado and Kanazawa (1994). Microbound diets for larvae were prepared according to the methods of Teshima et al. (1982) and Kanazawa et al. (1989) with a slight modification. Dried powdered ingredients were milled (Retsch ZM 200, Retsch GmbH, Germany) and sieved through a 53 μm mesh to achieve size homogeneity of ingredients. The lipids and the lipid soluble vitamins were blended with 150 mL of 60% ethanol in an electric bender. In sequence, zein and the dry ingredients including DL-Met and Met-Met (coated and uncoated) were blended for about 15 min until the ethanol evaporated and the zein bound together the rest of the ingredients. The mixture was freeze-dried for 48 h and crumbled in an electric motor to make a fine particle and sieved through 125 and 250 μm mesh sizes (Kagaku Kyoeisha Ltd., Tokyo) to obtain desired size of diets. Prepared diets were stored in a cold room prior to the start of the experiment.

In the juvenile red sea bream growth trial five diets were formulated (Table 2) to have a crude protein content of 54% and a lipid content of 13% with a 25% inclusion of soy protein isolate for the diets to be deficient of methionine by 0.75% (0.75 g/100 g). Two diets were formulated to contain 0.75% amino acid of DL-Met and Met-Met that were coated with cooked carboxymethylcellulose (CMC).

TABLE 1. *Ingredient and proximate compositions (%) of the test diets for red sea bream larvae fed for 30 days.*

	DL-Met		Met-Met		
Ingredients	Coated	Uncoated	Coated	Uncoated	Control
Casein[1]	20.0	20.0	20.0	20.0	20.0
Fish meal[2]	44.0	44.0	44.0	44.0	44.0
Soy protein isolate[3]	15.0	15.0	15.0	15.0	15.0
Vitamins[4]	4.8	4.8	4.8	4.8	4.8
Minerals[4]	4.7	4.7	4.7	4.7	4.7
Soybean lecithin[5]	3.0	3.0	3.0	3.0	3.0
n-3 HUFA[6]	2.0	2.0	2.0	2.0	2.0
Methionine dipeptide[7]	—	—	0.5	0.5	—
DL-methionine[8]	0.5	0.5	—	—	—
Zein	6.0	6.0	6.0	6.0	6.5
Proximate composition					
Moisture	12.0	12.1	12.0	12.1	12.1
Protein[9]	63.9	64.0	64.1	63.9	64.1
Lipid[9]	11.8	11.8	11.9	11.8	11.8
Ash[9]	12.2	12.2	12.4	12.3	12.2

[1]Wako Pure Chemicals Industries, Co., Japan.
[2]Nippon Suisan Co., Japan.
[3]Fuji Seiyu Co., Japan.
[4]The same as reported by Kader et al. (2010).
[5]Riken Vitamin Co., Japan.
[6]Oriental Yeast Co., Japan.
[7]Peptide Institute Co., Japan, Compound: Met-Met (M.W. 280.41).
[8]Ajinomoto Co., Japan.
[9]% dry matter basis.

TABLE 2. *Ingredient and proximate compositions (%) of the test diets for red sea bream juvenile fed for 56 days.*

	DL-Met		Met-Met		
Ingredients[1]	Coated	Uncoated	Coated	Uncoated	Control
Casein	10.0	10.0	10.0	10.0	10.0
Fish meal	38.0	38.0	38.0	38.0	38.0
Soy protein isolate	25.5	25.5	25.5	25.5	25.5
Vitamins	4.0	4.0	4.0	4.0	4.0
Minerals	4.0	4.0	4.0	4.0	4.0
Cod liver oil[2]	4.5	4.5	4.5	4.5	4.5
Soybean lecithin	4.5	4.5	4.5	4.5	4.5
Met dipeptide	—	—	0.8	0.8	—
DL-methionine	0.8	0.8	—	—	—
α-cellulose	2.7	2.7	2.7	2.7	3.5
Gluten	1.0	1.0	1.0	1.0	1.0
CMC	3.0	3.0	3.0	3.0	3.0
κ-carrageenan	2.0	2.0	2.0	2.0	2.0
Proximate composition					
Moisture	12.8	12.5	12.4	12.3	12.3
Protein[3]	54.2	54.3	54.3	54.4	54.4
Lipid[3]	13.3	13.4	13.4	13.3	13.3
Ash[3]	11.3	11.2	11.5	11.3	11.4

[1]Suppliers and sources are the same as those in Table 1.
[2]Riken Vitamin Co., Japan.
[3]% dry matter basis.

Two similar formulations were also made; however, the two amino acid forms were not coated with CMC. A control diet was likewise formulated without any supplementation of amino acid. Dried powdered ingredients were milled and sieved through a 710 μm mesh to achieve size homogeneity of ingredients. Coating of DL-Met and Met-Met were carried out according to Millamena et al. (1996) with a slight modification. The dry ingredients were mixed with water to make a paste, and then oil was added to the paste through a mixer. The DL-Met and Met-Met amino acid were carefully precoated with cooked CMC (1 g) and added to the paste mixture. The remaining CMC and *k*-carageenan previously cooked in a water bath (95 C) were also added to the paste to improve diet stability. The dough obtained was passed through a pelletizer using a 1.2–2.2-mm die. Moisture of the diet was then reduced to about 12% in a dry-air mechanical convection oven (DK 400 Yamato Scientific, Tokyo, Japan) at 60 C for 1 h, and the diets were stored in a cold room before the start of the experiment.

Amino Acid Leaching Test

Percentage of methionine amino acid leached from larvae and juvenile test diets were evaluated 2 min after immersion of 50 mg of diet samples into 25 mL of distilled water. Diet suspension was then filtered (Advantec No. 2) and air dried (Ragaza, Kagoshima University, unpublished data). Samples were then prepared for TAA analysis (Teshima et al. 1986). Methionine amino acid percentage lost was calculated as the difference between methionine content in the diet and the methionine amino acid from the filtered solution.

Feeding Trial

The growth experiment was conducted at the Kamoike Marine Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Measured water condition in the entire duration of the study was: temperature (26 ± 2 C), pH (7.6 ± 0.2) and salinity (33.1 ± 0.6 ppt).

Larvae

For the first experiment, fertilized eggs of red seabream (Noguchi-Fuka, Wakayama, Japan) were purchased from the commercial providers and transported to the laboratory. The eggs were incubated at ambient temperature (25–28 C). Rotifers were maintained on bakers yeast and *Chlorella* sp. (Fresh chlorella V-12; Chlorella Industry Co., Ltd., Tokyo, Japan). The rotifers and *Artemia nauplii* were enriched with highly unsaturated fatty acid (HUFA) using AQUALENE (Takeda-Kagakushiryo, Tokyo, Japan). The feeding regimes of the stock larvae were as follows: larvae from day 1–10 were fed exclusively with rotifer, from day 11–13 they were co-fed with rotifer and *Artemia*, and at day 14–25 they were fed solely with Artemia. Control microbound diets were gradually introduced from day 20 prior to the start of the feeding trial to ensure that larvae could easily adjust to the microbound test diets. When larvae reached 25 DAH (days after hatch) stage, the growth experiment was initiated. Larvae with an average initial body weight of 42 mg were stocked in a 100 L tank (80 L water volume) with a flow-through system at a stocking density of 500 larvae/tank, each treatment having three replicates. Feeding was done five times per day at 0800, 1000, 1200, 1400, and 1600 h (*ad libitum*) to ensure that cannibalism would not occur due to underfeeding. Diet particle sizes were adjusted according to fish mouth sizes, based on visual observation. After 30 days, larvae were sampled for weight and length. Body weights were measured by weighing about 100 larvae (wet weight, blotted dry) with electronic microbalance with the same fish measured for total length with digimatic calipher. Samples were taken for proximate body composition, amino acid body composition, and digestive enzyme analysis.

Juvenile

In the second experiment, juvenile red sea bream were obtained from local hatchery (Tawaki Suisan, Kumamoto, Japan), and maintained at the Kamoike Marine Laboratory, Faculty of Fisheries, Kagoshima University, Japan

by feeding commercial feeds until the feeding trial started. Early juveniles (average initial body weight of 0.75 ± 0.2 g) were randomly stocked in 100 L tank (80 L water volume) at 15 fish/tank, with each treatment having three replicates. All fish were fed to apparent satiation with the designated diets twice daily (0800 and 1600 h). Uneaten diets were collected and weighed in the duration of the trial for the adjustment of total feed intake of fish. Initial samples of fish prior to stocking were taken and stored to −20 C for proximate and total amino acid analysis.

After 56 days, fish were starved 24 h before terminal sampling to minimize error in proteolytic enzyme activity analysis and weigh measurement. All fish were anesthetized with iced water for ease in handling during weighing and blood collection. Survival and weight of fish on each tank were measured. Three fish from each replicate tank were randomly taken and stored at −20 C for proximate and amino acid analysis. Using heparinized syringes, blood was collected from the caudal vein of four fish in each replicate tank and pooled. Five fish from each treatment were dissected for liver and digestive tract and were weighed individually and kept in −80 C for further analysis.

Chemical Analysis

Diets and fish whole body were analyzed for moisture (Sartorius MA 35, moisture analyzer), ash, crude protein (Tecator Kjeltec Systems) and total lipid (Soxhlet method) using standard AOAC methods (AOAC 1995). Fish samples were freeze dried (Eyela FDU 1100, Tokyo Rikakikai, Co., Ltd.) prior to proximate composition analysis.

Total amino acid analysis of diets and fish samples were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp.) according to (Teshima et al. 1986). For the analysis of total amino acid, about 2 mg of dry sample was weighed and hydrolyzed with 4 N-methanesulfonic acid for 22 h at 110 C. The pH of the hydrolysate was adjusted to pH 2.2, passed through a 45μm syringe filter (Minisart RC-15 Sartorius, Germany), and stored at 4 C ready for HPLC injection. A known amount of norleucine (0.06 mg) was used as an internal standard.

For the proteolytic enzyme (exopeptidase and endopetidase) activity, liver and digestive tract samples (juvenile) and digestive tract (larvae) of fish was washed with distilled water and blotted dry in a filter paper. Thereafter, 50 mM Tris HCl Buffer solution (pH 7.6) was added at a ratio of 1:4 w/v and homogenized. Samples were then centrifuged at $10{,}000 \times g$, 4 C for 30 min. The supernatant was then removed and was stored in a −80 C freezer for proteolytic and protein content assay.

The proteolytic enzyme activity of liver and digestive tract was determined using a method of Cupp-Enyard (2008) with casein as substrate (0.65% w/v casein solution, 50 mM potassium phosphate buffer). In a 15 mL vial, 5 mL of 0.65% casein solution was equilibrated in a water bath at 37 C for 5 min. One milliliter of the prepared sample was then added to the vial and incubated for 37 C for exactly 10 min. The protease activity and consequential liberation of tyrosine during this incubation time was measured. TCA (5 mL) was then added to stop the reaction. The samples were then incubated for 30 min at 37 C. After the 30 min incubation, the solution was filtered with a 0.45 μm syringe filter. Sodium carbonate and Folin's reagent was then added to the sample and was again incubated for 30 min at 37 C. After the 30 min incubation, 2 mL of the solution were filtered using a 0.45 μm syringe filter into the cuvette and measured in a spectrophotometer at a wavelength of 660 nm.

The calculation to derive the activity of enzyme in units per mL:

$$\text{Units/mL enzyme} = (\mu\text{mole tyrosine equivalents released}) \times 11/(1) \times (10) \times (2)$$

where 11 = total volume (in mL) of assay; 10 = time of assay (in min) as per unit definition; 1 = volume of enzyme (in mL) of enzyme used; 2 = volume (in mL) used in colorimetric determination.

Plasma samples were obtained by centrifugation at $3000 \times g$ for 15 min using a high speed refrigerated microcentrifuge. Blood parameters

TABLE 3. *Percentage of methionine amino acid leached from the experimental diets of larvae and juvenile red seabream after immersion for 2 min in water.*[1]

	DL-Met		Met-Met		
Diets	Coated	Uncoated	Coated	Uncoated	Control
Larvae	11 ± 1.3^{a}	16 ± 1.2^{b}	12 ± 0.99^{a}	15 ± 1.2^{b}	12 ± 1.3^{a}
Juvenile	10 ± 1.4^{a}	15 ± 1.1^{b}	11 ± 1.2^{a}	14 ± 1.4^{b}	10 ± 1^{a}

[1]Each value is the mean ± S.E.M. of data from triplicate groups. Within a row, values with the different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

such as glutamyl oxaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), blood urea nitrogen (BUN), total cholesterol (TCH), and hemoglobin were analyzed spectrophotometrically with an automatic analyzer (SPOTCHEM™ model SP-4430). A portion of blood samples was also analyzed for hematocrit values.

Statistical Analysis

Three replicates were assigned to each dietary treatment, using a completely randomized design. Data were tested Sigma Stat 3.5 (Systat Software Inc., San Jose, CA, USA). Differences between diets were tested by one-way ANOVA on tank means and Tukey–Kramer test if means are significant. In the two-way ANOVA, to isolate the effect of molecular form of amino acid from coating process, only the diets with methionine supplementation were considered. Relative data limited to 0–100% were arcsine transformed prior to testing. Differences were considered significant at $P < 0.05$.

Results

Amino Acid Leaching Test

As shown in Table 3, the test diets in the larvae and juvenile experiment after 2 min of immersion, indicated that methionine amino acid significantly ($P < 0.05$) leached faster when compared to amino acid which was coated by either zein or CMC. In addition, the type of amino acid used did not significantly ($P > 0.05$) affect the leaching rate.

Performance Parameters

Growth performance and feed utilization of the fish are shown in Table 4 (larvae) and Table 5 (juvenile). In the larvae experiment, survival did not differ significantly ($P > 0.05$) in all the dietary treatments. Larvae fed the diets supplemented with either form of methionine showed a significantly ($P < 0.05$) higher growth performance in comparison to the control diet group. Moreover, fish fed the diets supplemented with coated DL-Met and Met-Met showed a significantly ($P < 0.05$) higher final body weight gain compared to the rest of the dietary treatment. The final total length of fish larvae fed the five experimental diets did not differ significantly ($P > 0.05$)

For the juvenile experiment, survival did not differ significantly ($P > 0.05$) in all the dietary treatments. Fish fed the diets supplemented with either form of methionine showed a significantly higher ($P < 0.05$) weight gain and total feed intake when compared to the control diet group. Furthermore, fish fed the diets supplemented with coated DL-Met and Met-Met exhibited a significantly ($P < 0.05$) higher body weight gain and an improved feed conversion ratio (FCR). Total feed intake of fish fed feeds with coated and uncoated methionine were not significantly ($P > 0.05$) different from each other. Digestive tract index (DTI) and hepatosomatic index (HSI) did not vary significantly ($P > 0.05$) in all the dietary treatment groups.

Proximate and Amino Acid Composition of Whole-Body, Proteolytic Enzyme Activity and Blood Plasma Biochemical Parameters

Larvae (Table 4) and juvenile (Table 5) proximate body composition (protein, lipid, ash, and moisture) was not significantly ($P > 0.05$) influenced by the dietary treatment. Similarly, whole-body total amino acid analyzed

TABLE 4. *Performance parameters and whole-body proximate composition (%) of red sea bream larvae fed the experimental diets after 30 days.*[1]

	DL-Met		Met-Met		
	Coated	Uncoated	Coated	Uncoated	Control
Performance parameter					
Initial weight (mg)	42 ± 0.1	42 ± 0.11	41 ± 0.12	42 ± 0.03	41 ± 0.21
Final weight (mg)	283.6 ± 2.16^{c}	256.2 ± 1.16^{b}	286.1 ± 1.26^{c}	258.2 ± 1.29^{b}	235.4 ± 1.68^{a}
Percent weight gain	562 ± 3^{c}	499 ± 3^{b}	571 ± 6^{c}	504 ± 3^{b}	451 ± 4^{a}
Initial TL (mm)	7.8 ± 0.1	7.8 ± 0.08	7.8 ± 0.07	7.8 ± 0.1	7.8 ± 0.01
Final TL (mm)	13.1 ± 0.2	12.3 ± 0.1	12.7 ± 0.1	12.5 ± 0.1	12.3 ± 0.2
SGR[2]	6.30 ± 0.01^{c}	5.96 ± 0.01^{b}	6.34 ± 0.03^{c}	5.99 ± 0.01^{b}	5.69 ± 0.02^{a}
Survival (%)	41.8 ± 1.8	39.6 ± 0.8	39.7 ± 2.9	41.2 ± 5.5	41.4 ± 1.3
Proximate Analysis					
Moisture	73 ± 0.3	74 ± 0.4	73 ± 0.2	75 ± 0.2	74 ± 0.3
Protein[3]	11 ± 0.7	11 ± 0.5	12 ± 0.5	11 ± 0.5	11 ± 0.5
Lipid[3]	1.9 ± 0.2	1.9 ± 0.3	1.8 ± 0.4	1.9 ± 0.2	1.9 ± 0.3
Ash[3]	2.6 ± 0.1	2.6 ± 0.2	2.7 ± 0.2	2.8 ± 0.2	2.7 ± 0.4

[1]Each value is the mean ± S.E.M. of data from triplicate groups. Within a row, values with the different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

[2]Specific growth rate (SGR) = (100 × [ln final fish weight − ln initial fish weight])/days.

[3]Wet weight basis.

TABLE 5. *Performance parameters and whole-body proximate composition (%) of red sea bream juvenile fed the experimental diets after 56 days.*[1]

	DL-Met		Met-Met		
	Coated	Uncoated	Coated	Uncoated	Control
Performance parameters					
Initial weight (g)	0.76 ± 0.02	0.75 ± 0.0	0.74 ± 0.0	0.74 ± 0.0	0.76 ± 0.01
Final weight (g)	25.26 ± 0.6^{c}	21.47 ± 0.33^{b}	25.56 ± 0.45^{c}	21.16 ± 0.31^{b}	17.64 ± 0.21^{a}
Percent weight gain	3233 ± 168^{c}	2816 ± 81^{b}	3419 ± 55^{c}	2770 ± 40^{b}	2273 ± 18^{a}
SGR[2]	6.26 ± 0.08^{c}	6.02 ± 0.05^{b}	6.36 ± 0.02^{c}	5.99 ± 0.02^{b}	5.65 ± 0.01^{a}
Survival (%)	93.3 ± 0.00	93.3 ± 3.8	91.1 ± 2.2	93.3 ± 3.8	91.1 ± 2.2
Feed intake	24.0 ± 0.7^{b}	24.1 ± 0.4^{b}	23.9 ± 0.1^{b}	23.9 ± 0.9^{b}	21.1 ± 0.3^{a}
FCR[3]	0.97 ± 0.0^{c}	1.16 ± 0.04^{b}	0.96 ± 0.0^{c}	1.17 ± 0.02^{b}	1.27 ± 0.01^{a}
DTI[4]	2.53	2.48	2.47	2.49	2.48
HSI[5]	1.11	1.10	1.12	1.12	1.11
Proximate composition					
Moisture	79.3 ± 0.6	80.2 ± 0.7	78.7 ± 0.8	78.5 ± 0.4	80.2 ± 0.3
Protein[6]	14.3 ± 0.2	14.2 ± 0.1	14.7 ± 0.1	14.6 ± 0.1	14.3 ± 0.2
Lipid[6]	6.4 ± 0.3	6.4 ± 0.2	6.5 ± 0.2	6.4 ± 0.1	6.5 ± 0.2
Ash[6]	3.8 ± 0.2	3.5 ± 0.2	3.4 ± 0.2	3.5 ± 0.1	3.5 ± 0.3

[1]Each value is the mean ± S.E.M. of data from triplicate groups. Within a row, values with the different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

[2]Specific growth rate (SGR) = (100 × [ln final fish weight − ln initial fish weight])/days.

[3]Feed conversion ratio (FCR) = dry feed intake/wet weight gain.

[4]Digestive tract index (DTI) = (100 × digestive tract weight)/fish weight.

[5]Hepatosomatic index (HSI) = (100 × liver weight)/fish weight.

[6]Wet weight basis.

in larvae (Table 6) and juvenile (Table 7) was not significantly ($P > 0.05$) different. Larvae proteolytic enzyme activity (Table 8) of fish fed control diet indicated a significantly decreased proteolytic enzyme activity compared with the rest of the dietary treatments. Coating the methionine of either form significantly improved proteolytic enzyme activity.

TABLE 6. *Mean amino acid composition (g/100 g dry sample) of whole-body of red sea bream larvae fed the experimental diets for 30 days.*[1]

	DL-Met		Met-Met		
Amino acid	Coated	Uncoated	Coated	Uncoated	Control
EAA					
Methionine	1.74	1.72	1.71	1.69	1.70
Threonine	2.09	2.08	2.05	2.07	2.05
Valine	2.18	2.17	2.14	2.13	2.12
Isoleucine	1.95	1.93	1.93	1.98	1.97
Leucine	3.86	3.83	3.85	3.82	3.82
Phenylalanine	2.38	2.36	2.38	2.37	2.38
Histidine	1.17	1.18	1.16	1.15	1.14
Lysine	3.81	3.82	3.84	3.82	3.84
Tryptophan	1.10	1.11	1.09	1.07	1.04
Arginine	3.26	3.27	3.31	3.29	3.31
Σ EAA	23.54	23.47	23.46	23.39	23.37
NEAA					
Aspartic acid	4.39	4.41	4.40	4.38	4.37
Serine	2.54	2.52	2.54	2.53	2.53
Glutamic acid	6.47	6.48	6.48	6.49	6.48
Proline	2.28	2.29	2.31	2.28	2.25
Glycine	2.61	2.58	2.59	2.53	2.51
Alanine	2.71	2.70	2.68	2.64	2.64
Cystine	0.03	0.02	0.03	0.02	0.03
Tyrosine	1.73	1.71	1.69	1.68	1.65
Taurine	0.48	0.46	0.45	0.44	0.42
Σ NEAA	23.24	23.17	23.17	22.99	22.88
Σ TAA	46.78	46.64	46.63	46.38	46.25

[1]Each value is the mean of data from triplicate groups. Within a row, values with the different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

However, no significant difference was detected when testing the methionine forms. On the other hand, in the juvenile trial, proteolytic enzyme activities in the liver and digestive tract of juveniles were similar in all dietary treatments (Table 8). Juvenile red seabream blood chemical parameters after the growth trial are shown in Table 9. There were no statistical differences in blood parameters such as GOT, GPT, BUN, TCH, hematocrit, and hemoglobin in fish fed the five experimental diets.

Discussion

The present study indicated that methionine in dipeptide form can be efficiently utilized by larval and juvenile red sea bream. The methionine requirement of larvae and juvenile red seabream was based on the methionine amino acid composition of the body (Table 10) which was analyzed prior to the start of the experiment (Watanabe and Kiron 1994; Wilson 1994; Mambrini and Kaushik 1995). The larval study demonstrated that the incorporation of methionine in the diet, which was insufficient in methionine, significantly improved final body weight by as much as 112% over that of larvae fed a control diet. Similarly, juvenile growth and feed efficiency was positively influenced by supplementation of both DL-Met and Met-Met. This implies that amino acid supplementation plays an important role in the growth and development of the fish. Methionine is one of the amino acids required for normal growth and serves as a precursor of many body components. The result of present study was similar to that of Tesser et al. (2005), in which test diets did not show any differences in growth, feed efficiency and proximate body composition when different molecular forms of arginine were tested in juvenile South American pacu, *Piaractus*

TABLE 7. *Mean amino acid composition (g/100 g dry sample) of red sea bream juvenile fed the experimental diets for 56 days.*[1]

	DL-Met		Met-Met		
Amino acid	Coated	Uncoated	Coated	Uncoated	Control
EAA					
Methionine	1.81	1.79	1.80	1.77	1.80
Threonine	1.79	1.77	1.83	1.80	1.81
Valine	2.68	2.64	2.67	2.65	2.64
Isoleucine	1.89	1.84	1.83	1.81	1.79
Leucine	3.48	3.49	3.45	3.42	3.41
Phenylalanine	2.01	2.03	2.01	2.04	2.02
Histidine	1.22	1.24	1.27	1.27	1.26
Lysine	3.68	3.65	3.63	3.62	3.63
Tryptophan	1.04	1.02	1.03	1.05	1.04
Arginine	3.38	3.39	3.41	3.43	3.45
Σ EAA	22.98	22.86	22.93	22.86	22.85
NEAA					
Aspartic acid	4.01	4.04	4.02	4.01	4.05
Serine	2.08	2.02	2.01	2.05	2.01
Glutamic acid	7.26	7.32	7.24	7.28	7.27
Proline	0.49	0.51	0.49	0.51	0.48
Glycine	3.42	3.42	3.40	3.38	3.37
Alanine	2.59	2.51	2.48	2.47	2.46
Cystine	nd[2]	nd	nd	nd	nd
Tyrosine	2.39	2.35	2.32	2.29	2.26
Taurine	0.49	0.49	0.48	0.47	0.45
Σ NEAA	22.73	22.66	22.44	22.46	22.35
Σ TAA	45.71	45.52	45.37	45.32	45.20

[1]Each value is the mean of data from triplicate groups. Within a row, values with the different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

[2]Not detected.

TABLE 8. *Proteolytic enzyme activity of red sea bream larvae and juvenile fed test diets for 30 and 56 days, respectively.*[1]

Proteolytic activity	DL-Met		Met-Met		
(U mg^{-1} protein)	Coated	Uncoated	Coated	Uncoated	Control
Juvenile					
Intestinal tract	0.2802 ± 0.00	0.2711 ± 0.00	0.2863 ± 0.01	0.2750 ± 0.01	0.2745 ± 0.00
Liver	0.0600 ± 0.00	0.0609 ± 0.00	0.0663 ± 0.00	0.0700 ± 0.01	0.0591 ± 0.00
Larvae					
Digestive system	0.0522 ± 0.00[c]	0.0453 ± 0.00[b]	0.0524 ± 0.00[c]	0.0427 ± 0.00[b]	0.0307 ± 0.00[a]

[1]Each value is the mean ± S.E.M. of data from triplicate groups. Within a row, values with the different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

mesopotamicus. In contrast, juvenile rainbow trout *Oncorhynchus mykiss* growth experiment (Terjesen et al. 2006) showed that amino acid in dipeptide form was efficiently absorbed than an identical amino acid in free form that resulted in higher accumulation rate and postprandial peak time for muscular free amino acid. This may indicate different metabolic handling of amino acids and availability for protein synthesis, which is translated to better growth rate (Dabrowski et al. 2005). Juvenile fish study indicated a faster free amino acid absorption compared with protein bound amino acids for carp, *Cyprinus carpio* (Plakas and Katayama 1981), rainbow trout (Cowey and Walton 1988) and Atlantic cod, *Gadus morhua* (Berge et al.

TABLE 9. *Blood parameters in red sea bream juvenile fed the experimental diets after 56 days.*[1]

	DL-Met		Met-Met		
	Coated	Uncoated	Coated	Uncoated	Control
Hematocrit (%)	38 ± 0.57	38 ± 1.0	37 ± 0.57	38.67 ± 0.88	39.33 ± 0.33
Hemoglobin (g/dL)	6.9 ± 0.05	7.0 ± 0.08	7.0 ± 0.08	7.1 ± 0.15	7.1 ± 0.11
GOT (IU/L)	64.0 ± 0.6	65.7 ± 0.3	65.3 ± 0.9	63.3 ± 0.3	63.2 ± 0.9
GPT (IU/L)	25.6 ± 0.47	26.6 ± 0.37	27.4 ± 0.26	26.4 ± 0.29	26.4 ± 0.57
BUN (mg/dL)	6.5 ± 0.1	6.7 ± 0.1	6.5 ± 0.2	6.5 ± 0.2	6.9 ± 0.1
TCH (mg/dL)	257 ± 2	254 ± 1	258 ± 1	256 ± 2	253 ± 2

BUN = blood urea nitrogen; GOT = glutamyl oxaloacetic transaminase; GPT = glutamic-pyruvate transaminase; TCH = total cholesterol.

[1]Each value is the mean ± S.E.M. of data from triplicate groups. Within a row, values with the different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

TABLE 10. *Amino acid composition of control diets (g/100 g dry diet) and initial whole body (g/100 g dry sample) of red sea bream larvae and juvenile prior to the start of the feeding trials.*

	Larvae		Juvenile	
Amino acids	Control diet	Whole body	Control diet	Whole body
EAA				
Methionine	1.29	1.78	1.04	1.72
Threonine	1.89	2.19	1.61	1.85
Valine	2.71	2.21	2.23	2.77
Isoleucine	2.37	1.98	2.01	1.91
Leucine	4.49	3.84	3.66	3.47
Phenylalanine	2.65	2.46	2.23	2.05
Histidine	1.35	1.21	1.16	1.29
Lysine	3.89	3.85	3.19	3.77
Tryptophan	1.47	1.12	1.17	1.09
Arginine	4.23	3.39	2.50	3.27
Σ EAA	26.34	24.03	20.80	23.19
NEAA				
Aspartic acid	8.92	4.48	3.92	4.05
Serine	3.30	2.66	1.25	2.12
Glutamic acid	2.67	6.98	7.44	7.35
Proline	0.50	2.33	2.15	0.58
Glycine	2.41	2.54	1.54	3.45
Alanine	0.22	3.86	2.34	2.68
Cystine	2.96	0.02	0.48	nd[1]
Tyrosine	1.49	1.65	1.94	2.40
Taurine	1.66	0.54	0.19	0.50
Σ NEAA	24.12	25.06	21.25	23.13
Σ TAA	50.47	49.09	42.05	46.32

[1]Not detected.

1994). However, the rapid amino acid absorption may lead to tissue amino acid imbalance and redirect the excess amino acid to a catabolic instead of an anabolic process, which eventually translated to a poor growth rate (Tesser et al. 2005). Depressed growth have been observed in Jian carp when methionine inclusion was more than 1.2% of the diet (Tang et al. 2009).

The present study indicated that coating technique improved the growth of red sea bream. Coating procedure of amino acid, vitamins and minerals has received positive results in performance parameters in several fish (López-Alvarado et al. 1994; Liu et al. 2002; Segovia-Quintero and Reigh 2004; Alam et al. 2005; Zhou et al. 2007). The mechanism of coating

amino acid in the diet results in a slower rate of breakdown and absorption, forming a more balanced amino acid pool beneficial for protein synthesis (Zhou et al. 2007). Furthermore, coating improves water stability of the diet by reducing leaching loss and increasing availability of the amino acid for fish (López-Alvarado et al. 1994; Watanabe et al. 2001; Alam et al. 2005). The present study showed that the percentage of methionine leached in the water was significantly reduced by as much as 5% (larvae diet) and 3% (juvenile diet) when methionine in the diet was coated.

Improved growth in juveniles fed the diet with DL-Met and Met-Met can likewise be attributed to the improved feed conversion efficiency. Particularly, regardless of types, methionine supplementation increased the feed intake of juvenile red sea bream in this study. This observation was in agreement with the study of rainbow trout, when methionine supplementation to the diets in which fish meal was replaced by plant protein source showed increased feed intake due to the improved palatability (Mambrini et al. 1999). It is noted that some amino acids such as glycine, alanine and valine act as appetite stimulants for red seabream (Ina and Matsui 1980). Poor palatability of diets including 15% soy protein concentrate were also reported for Chinook salmon, *Oncorhynchus tshawytscha* (Hajen et al. 1993). Decreased feed intake can probably be linked to the presence of compounds (Hardy 1996; Alam et al. 2005) and the bitterness properties that are developed during the processing methods (Wang and De Mejia 2005).

The present experiment proved that the dietary treatment used was not a factor to affect body proximate composition and total amino acid profiles of larval and juvenile bodies. The results are in agreement with (Tesser et al. 2005) that dietary arginine supplementation did not affect body protein and body lipid in juvenile South American pacu. Test diets were isonitrogenous and isolipidic thus larvae and juvenile from all dietary treatments received an equal amount of protein and lipid in the diet.

Enzyme data of larvae in the present study showed that there was a significant improvement in the proteolytic activity of larvae fed the diets supplemented with either form of methionine in comparison to the diet that did not have amino acid supplementation. Similarly, (Tang et al. 2009) indicated improved intestinal growth and enzyme activities when methionine was supplemented in the diet of up to 1% of the total ingredient in the diet of juvenile carp. Furthermore, coating of either form of methionine significantly improved the digestive proteolytic activity of larvae. The result was similar to the study of Zhou et al. (2007) when Jian carp, *Cyprinus carpio*, was fed a diet with lysine that was coated wherein increased protease and lipase activity were observed.

In the juvenile trial, neither methionine form nor coating improved proteolytic enzyme activity in liver and intestine of fish. This may be due to the adaptation from the test diets and the developed digestive system of the fish prior to the start of the experiment. Blood parameters of juvenile red seabream are within the normal range as reported in other studies (Takagi et al. 2001; Kader et al. 2010, 2011), suggesting a good physiological condition of the fish after the experiment. Different results among authors on the effect of amino acid molecular form as supplements in diets can be attributed to species type, ontogenetics, proportion of amino acid and the integrated response by fish to amino acid (Tesser et al. 2005).

In summary, the present study demonstrated that Met-Met was similarly effective to DL-Met as a dietary supplement for growth improvement and efficient utilization of amino acid in red sea seabream larvae and juveniles when fed diets containing plant protein. However, when either form of methionine was coated, improved growth performances and proteolytic enzyme activity (in case of larvae) were observed. The effect of methionine and coating procedure in this study suggests that ontogenetic factors elicit different responses in the fish.

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