INTRODUCTION

In nutrition studies of fish, determining the optimum dietary protein level for growth is generally a primary consideration because protein is not only the major constituent of a fish's body, but also it provides critical functions such as enzymes and hormones. Therefore, a continuous supply of protein that is well balanced with amino acids is required for the maintenance and growth of fish. Without satisfying the dietary protein requirement, normal growth cannot be achieved. Many studies have been carried out to determine the protein requirements of commercial fish, and the estimated dietary protein requirements range from 30% to 55%.

Aquaculture production of flounder Paralichthys olivaceus has increased in the past decade in Korea because techniques for the mass larval production of this species have been developed and because it is a fast-growing species compared with other marine fish species such as rockfish Sebastes schlegeli and red seabream Pagrus major. Several studies have been conducted that have investigated the utilization of some plant and animal protein sources as a substitute for fishmeal in the diets of flounder. Although the effects of dietary protein or lipid levels on growth and body composition of flounder have been studied previously, dietary protein requirement was not clearly determined, and the studies showed that increasing levels of dietary lipid had no positive effects on growth. The appropriate dietary protein and lipid levels for this species have also been reported by Lee et al., who have suggested that a diet high in protein (50%) and low in lipids (7%) is suitable for the growth of juvenile flounder from 3.1 g to 12.7 g. Generally, plateus or decreases in the growth of fish fed diets containing protein levels above the requirement have been observed in some species. However, in the study by Lee et al., plateaus or decreases in the growth of fish fed diets containing 30–50% protein levels were not observed. The higher weight gained by flounder fed the higher
protein diet in their study suggested that a protein level of 50% might be lower than that required for maximum growth. Therefore, the present study was conducted to determine the optimum dietary protein level for the growth of flounder.

MATERIALS AND METHODS

Experimental diets

Ingredients and proximate composition of the experimental diets are given in Table 1. Six experimental diets containing protein levels of 40%, 45%, 50%, 55%, 60%, and 65% were prepared. The contents of fishmeal (produced by the steam dry method; Han Chang Fish Meal Co., Pusan, Korea) and casein (Serva; Feinbiochemica GmbH & Co., Heidelberg, Germany) in the diets increased mainly at the expense of dextrin and α-cellulose to increase the protein level. The dietary energy and lipid levels were designed to be isocaloric (4.7 kcal/g diet) and isolipidic (7%), respectively, by adjusting the levels of dextrin and squid liver oil. The ingredients of the experimental diets were mechanically mixed with water at the ratio of 100 g of ingredient mixture to 35–40 g of water and pressure-pelleted. The moist pellet diets were stored at −30°C until required for use.

Fish and feeding trial

Flounder were purchased from a private fish hatchery (Kangnung, Korea). They were fed a commercial feed containing 50% protein for 2 weeks while being acclimated to the experimental conditions. Flounder (average bodyweight 22.7 g) were allocated randomly to 18 green, circular, fiberglass-reinforced plastic tanks (90 cm Φ, 100 cm depth), with 30 fish to each tank. Three replicate groups of fish were hand-fed to visual satiety twice daily at 09.00 hours and 17.00 hours for 9 weeks. Pellet size was adjusted as the fish grew. Filtered seawater (34 ± 0.3‰, mean ± SD) was supplied at a flow rate of 5 L/min to each tank. The water temperature was maintained at 19.2 ± 1.18°C, and natural conditions for the photoperiod were maintained during the feeding trial. Fish in each tank were collectively weighed on the day of initiation and on the day of termination of the experiment after being fasted for 24 h and anesthetized with MS222.

Table 1  Ingredients and proximate composition of the experimental diets

<table>
<thead>
<tr>
<th>Ingredients (g/100 g wet matter)</th>
<th>40</th>
<th>45</th>
<th>50</th>
<th>55</th>
<th>60</th>
<th>65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein, vitamin-free</td>
<td>8.7</td>
<td>9.7</td>
<td>10.8</td>
<td>11.9</td>
<td>13.0</td>
<td>14.1</td>
</tr>
<tr>
<td>White fishmeal</td>
<td>45.0</td>
<td>50.7</td>
<td>56.3</td>
<td>61.9</td>
<td>67.5</td>
<td>73.1</td>
</tr>
<tr>
<td>Dextrin</td>
<td>28.8</td>
<td>23.5</td>
<td>18.2</td>
<td>12.9</td>
<td>7.6</td>
<td>2.3</td>
</tr>
<tr>
<td>Squid liver oil</td>
<td>4.0</td>
<td>3.6</td>
<td>3.2</td>
<td>2.8</td>
<td>2.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Vitamin premix†</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Mineral premix†</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Carboxymethylcellulose‡</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>α-Cellulose‡</td>
<td>5.0</td>
<td>4.0</td>
<td>3.0</td>
<td>2.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Choline salt‡</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Proximate composition

Crude protein (% DM)                                      | 40.4| 46.2| 51.2| 55.2| 61.0| 65.4|
Crude lipid (% DM)                                        | 6.4 | 6.7 | 6.8 | 6.7 | 6.7 | 6.9 |
Crude ash (% DM)                                          | 9.0 | 10.3| 11.8| 13.5| 14.8| 16.4|
Crude fiber (% DM)                                        | 6.5 | 5.4 | 4.4 | 3.3 | 2.2 | 1.1 |
N-free extract (% DM)‡                                     | 37.8| 31.4| 25.9| 21.3| 15.3| 10.1|
Gross energy (kcal/g)                                     | 4.76| 4.70| 4.73| 4.74| 4.76| 4.74|
n-3HUFA (% DM)                                            | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 |

*Vitamin mix contained the following, which were diluted in cellulose (g/kg mix): L-ascorbic acid, 121.2; DL-α-tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; and cyanocobalamin, 0.003.

†Mineral mix contained the following ingredients (g/kg mix): MgSO4·7H2O, 80.0; NaH2PO4·2H2O, 370.0; KCl, 130.0; ferric citrate, 40.0; ZnSO4·7H2O, 20.0; Ca-lactate, 356.5; CuCl, 0.2; AlCl3·6H2O, 0.15; KI, 0.15; Na2SeO3, 0.01; MnSO4·H2O, 2.0; CoCl2·6H2O, 1.0.
†Provided by Sigma Chemical, St Louis, MO, USA.
†Determined by calculating the difference.
n-3HUFA, n-3 highly unsaturated fatty acids (C ≥ 20).
Sample collection and chemical analysis

At the end of the feeding trial, blood samples were obtained from the caudal vein of five fish from each tank using heparinized syringes after they were starved for 24 h and anesthetized with MS222 at a concentration of 100 p.p.m. Blood plasma was collected after centrifugation (3500 × g for 5 min) and stored at −70°C as separate aliquots for analysis of protein, glucose, and cholesterol. Fifteen fish from each tank were randomly sampled at the end of the feeding trial and stored at −70°C for subsequent proximate analysis of the liver and the dorsal muscle. Crude protein content was determined by the Kjeldahl method using an Auto Kjeldahl System (Buchi B-324/435/412; Buchi, Flawil, Switzerland), and crude ash content was determined by the ether extraction method. Moisture content was determined using a dry oven (105°C for 24 h), crude fiber content by an automatic analyzer (Fibertec; Tecator, Hoganas, Sweden), and crude ash content by a furnace muffler (550°C for 4 h). Nitrogen-free extracts (NFE) were determined by calculating the difference. Gross energy contents were analyzed using an adiabatic bomb calorimeter (Parr; Moline, IL, USA). Lipids were extracted by the method of Folch et al., and fatty acid composition of the experimental diets was determined by gas chromatography (HP-5890 II; Hewlett-Packard, Palo Alto, CA, USA) using a capillary column measuring 30 m × 0.32 mm i.d. and a film thickness of 0.5 μm (HP-INNOWax, Palo Alto, CA, USA). The contents of protein, glucose and cholesterol in the plasma were analyzed using commercial clinical investigation kits (Wako Pure Chemical Co., Osaka, Japan).

Statistical analysis

Data were subjected to ANOVA and if significant (P < 0.05) differences were found, Duncan’s multiple range test was used to rank the groups using SPSS version 7.5 (SPSS Inc., Chicago, IL, USA). The data were presented as mean ± SEM of three replicate groups.

RESULTS

The growth performance of fish fed the experimental diets containing various protein levels for 9 weeks are presented in Table 2. Survival was not affected significantly by dietary protein level (P > 0.05). Weight gain and feed efficiency of fish fed diets containing 45% and 50% protein were significantly higher than those of fish fed diets containing 40%, 55%, 60%, and 65% protein (P < 0.05). Fish fed the diet containing 65% protein had the lowest condition factor among all diets (P < 0.05).

Daily feed intake of fish fed the 60% and 65% protein diets was significantly higher than that of fish fed the other diets (P < 0.05). Daily protein intake tended to increase with increasing dietary protein level. The protein efficiency ratio of fish fed the 55%, 60%, and 65% protein diets was significantly lower than that of fish fed the 40%, 45%, and 50% protein diets (P < 0.05).

Plasma constituents of flounder fed diets containing various protein levels are shown in Table 3. Plasma total protein concentration increased with

Table 2  Growth performance of young Japanese flounder fed diets containing various protein levels for 9 weeks

<table>
<thead>
<tr>
<th>Dietary protein level (%)</th>
<th>40</th>
<th>45</th>
<th>50</th>
<th>55</th>
<th>60</th>
<th>65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial wt. (g/fish)</td>
<td>22.9 ± 0.19</td>
<td>22.4 ± 0.23</td>
<td>22.8 ± 0.26</td>
<td>22.1 ± 0.48</td>
<td>23.1 ± 0.17</td>
<td>22.8 ± 0.36</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>97 ± 3.3</td>
<td>98 ± 1.7</td>
<td>97 ± 1.7</td>
<td>97 ± 1.7</td>
<td>97 ± 1.7</td>
<td>97 ± 1.7</td>
</tr>
<tr>
<td>WG (%)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.1 ± 12.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.4 ± 7.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37.5 ± 10.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.2 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.3 ± 9.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.0 ± 14.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FE (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>78 ± 5.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>93 ± 2.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98 ± 5.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79 ± 6.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67 ± 0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>61 ± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CI&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.17 ± 0.031&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.18 ± 0.012&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.18 ± 0.020&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.18 ± 0.012&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.17 ± 0.030&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.10 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DFI (%)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.65 ± 0.120&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.34 ± 0.084&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.21 ± 0.094&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.66 ± 0.230&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.08 ± 0.025&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.31 ± 0.111&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPI (%)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.07 ± 0.049&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.08 ± 0.039&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.13 ± 0.048&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.46 ± 0.127&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.88 ± 0.015&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.17 ± 0.073&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PER&lt;sup&gt;g&lt;/sup&gt;</td>
<td>1.93 ± 0.124&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.02 ± 0.056&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.91 ± 0.105&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.43 ± 0.123&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.09 ± 0.012&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93 ± 0.027&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values (mean ± SEM of three replications) in the same row that do not share a common superscript are significantly different (P < 0.05).

<sup>2</sup>Weight gain = (Final bodyweight − Initial bodyweight) × 100/Initial bodyweight.

<sup>3</sup>Feed efficiency = (Body wet weight gain × 100)/Feed intake.

<sup>4</sup>Condition factor = (Bodyweight × 100)/(Total body length)<sup>2</sup>.

<sup>5</sup>Daily feed (or protein) intake = Feed (or protein) intake × 100/[(Initial fish wt + Final fish wt + Dead fish wt) × No. days fed/2].

<sup>6</sup>Protein efficiency ratio = Body wet weight gain/Protein intake.
dietary protein level up to 50%, after which it plateaued. Plasma total glucose concentration of fish fed the 60% and 65% protein diets was lower than that of fish fed the 40% and 45% protein diets ($P<0.05$). These trends may have resulted from the differences in dietary protein and carbohydrate contents. Plasma total cholesterol concentration was not affected by dietary protein level ($P>0.05$).

Proximate composition and hepatosomatic index (HSI) of fish fed diets containing various protein levels are given in Table 4. Proximate composition of fish dorsal muscle was not affected significantly by dietary protein level ($P>0.05$). Moisture and crude protein contents of liver in fish fed the 60% and 65% protein diets were significantly higher than those of fish fed the other diets ($P<0.05$), but crude lipid contents of liver and HSI of fish fed the 60% and 65% protein diets were significantly lower than those of fish fed the other diets ($P<0.05$).

**DISCUSSION**

When excessive protein is provided, the expensive dietary protein is metabolized as energy without being effectively utilized for growth. These negative growth responses of fish fed diets containing protein levels above the requirement have been observed in other species of fish. However, other studies have shown that the growth response increased linearly up to the minimum required protein level, after which it plateaued. These different responses to excessive dietary protein levels might result from factors such as differences in fish species, dietary protein, and energy levels.

It has been suggested that the decrease in weight gain at protein levels above the optimum is due to a reduction in available dietary energy for growth of fish because of insufficient non-protein energy necessary to de-aminate and excrete the excess amino acids absorbed. Although the carbohydrate requirement of fish has not yet been reported, it is known that the growth rate of fish is reduced when fed carbohydrate-deficient diets because other nutrients such as protein are catabolized for energy and to provide metabolic intermediates for the synthesis of other biologically important compounds. This might explain the result in the present study whereby flounder fed the 55–65% protein diets, which had relatively
low carbohydrate levels, showed lower weight gains than fish fed the 45% and 50% protein diets.

In a previous study, maximum growth of juvenile flounder was observed in fish fed the 50% protein diet. Second order polynomial regression analysis showed that the maximum weight gain response point in the present study also occurred at the 50% dietary protein level ($y = -0.25x^2 + 25.04x - 255.4; r = 0.71$), whereas there were no significant differences in weight gain and feed efficiency between fish fed diets containing 45% and 50% protein. This indicates that, for the growth of young flounder, the optimum dietary protein level could be lowered to 45%. The optimum dietary protein level for flounder determined in the present study was higher than that for herbivorous or omnivorous freshwater fish and lower than that for some marine carnivorous fish. Cowey et al. and Helland and Grisdale-Helland have reported that dietary protein requirements of plaice Pleuronectes platessa and Atlantic halibut Hippoglossus hippoglossus were 50% and 51%, respectively. The best growth rate of turbot Scophthalmus maximus was obtained in a diet containing 69.8% protein.

The different growth responses to dietary protein levels between a previous study and the present study are probably due to feeding and fish conditions, such as water temperature, fish size, and the dietary composition used in the studies. It has been suggested that for some fish species, feed intake and protein utilization decrease with increasing fish size and decreasing water temperature. Although the effect of water temperature on protein requirement of fish is still controversial, some studies have shown recently that water temperature does not affect the protein requirement of fish. The decrease in protein requirement of flounder in the present study compared to that in a previous study may not be because of the differences (19°C in this study and 22°C in the previous one) in water temperature employed.

The difference observed in protein requirement could partly be attributed to the difference in fish size used by the studies. Lee et al. reared smaller flounder with an average bodyweight of 3 g at initiation and 13 g at termination of the experiment, whereas the present study was conducted with larger flounder with an average bodyweight of 23 g at initiation and 110 g at the termination of the experiment. Dietary protein requirements generally decrease with increasing fish size.

Another possible explanation for the different growth responses to dietary protein levels between a previous study and the present study is that the use of protein as an energy source may cause a higher protein requirement in fish. A dietary energy level influences the protein requirement of fish. Studies have shown that adequate levels of non-protein energy sources, such as lipids and carbohydrates, in the diet can minimize the use of protein as a source of energy. The protein-sparing effect obtained by increasing the amount of lipids and/or carbohydrates in diets has been reported in some species of fish. However, negative or no effects on growth and body composition have been reported for flounder when fed a diet containing a high level of lipids. Therefore, the diets of the present study were designed to have low lipid contents (6.4–6.9%). Dietary energy levels in the present study were designed to be isocaloric by adjusting the level of dextrin in accordance with the protein levels and the fixed lipid level, and these values were higher than those of previous diets, which contained approximately 7% of lipids.

The relative better growth and feed efficiency of fish fed the 45–50% protein diets with 24–18% dextrin (31–26% NFE) in the present study may be because of the ratio of carbohydrate to protein in that particular diet was more appropriate. Kikuchi et al. have shown that growth of juvenile flounder (initial average bodyweight 3.5 g) fed a diet containing 44% protein and 19% starch (27% carbohydrate) was significantly higher than that of fish fed either a diet containing 53% protein and 7% starch (14% carbohydrate) or diets containing 33–40% protein and 27–35% starch (34–42% carbohydrate). A recent feeding trial (SM Lee, unpubl. data, 2001) has shown that growth, feed efficiency (54–123%), and protein efficiency ratio (1.0–2.5) of juvenile flounder (initial average bodyweight 4 g) had a positive relationship with dietary dextrin level (0–25%) at a 50% protein level when fed to satiety twice daily. The feed efficiency (73%) and protein efficiency ratio (1.45) of flounder fed a 50% protein diet with 4% dextrin and 7% lipids in a previous study were relatively lower compared to those of fish fed a 50% protein diet with 18% dextrin and 7% lipids in the present study. These results indicate that flounder can utilize carbohydrate more efficiently than lipid as an energy source, and that protein utilization of fish fed a diet containing adequate amounts of carbohydrate (dextrin) as an energy source can be improved. This might explain the decrease in the protein requirement of flounder in the present study compared to that in a previous study. However, carbohydrate utilization by carnivorous fish is generally lower than that by herbivorous or omnivorous fish. Therefore, more detailed studies of carbohydrate utilization are needed to elucidate the protein-sparing effects on flounder.
In the present study, the protein efficiency ratio tended to decrease with increased dietary protein level, which is in agreement with results obtained for other fish species. The low protein efficiency ratio in the 55–65% protein diets indicates that the excessive protein in those diets were used for metabolic purposes other than growth. Conversely, Kikuchi et al. have demonstrated that the protein efficiency ratio of flounder remains unaffected by dietary protein and starch levels. These different protein efficiency ratio responses by flounder fed diets containing different protein levels are probably due to feeding conditions, such as the dietary properties (e.g. diet processing) and the dietary energy levels used in the studies. For example, the experimental diets used in the present study were prepared with a laboratory pellet machine, whereas Kikuchi et al. prepared their diets using a twin extruder.

An increase in liver size proportional to the dietary energy source or level has been reported in some fish. In the present study, lipid contents of liver and HSI of fish fed the 40–55% protein diets were significantly higher than those of fish fed the 60% and 65% protein diets. This difference is probably due to the increase in digestible carbohydrate levels with increasing protein levels in the diets. Lipogenesis from carbohydrate material is a well known biochemical phenomenon, and it has been reported that increasing dietary carbohydrate can be retained as lipid.

When considering these results, a diet containing 45% protein with 7% lipid, 24% dextrin (31% NFE), and 4.7 kcal of gross energy per gram of diet might be recommended for optimum growth and efficient protein utilization by flounder growing between 23 g and 110 g bodyweight.

ACKNOWLEDGMENT

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REFERENCES