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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Study on Dietary Essential Amino Acid Requirements by Dipeptides for Marine Fishes

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Department of Marine Life Science

GRADUATE SCHOOL

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Contents

요약문

어류에서 어체 건조중량의 50~60%는 단백질로 구성되어 있다. 육상 가축동물의 경우 주 에너지원으로 탄수화물을 이용하는 반면, 대부분의 양식어류는 탄수화물 이용률이 극히 낮고 상대적으로 단백질의 이용률이 매우 높아 사료 내 단백질 요구량이 육상 가축동물과 비교하여 2~2.5배 가량 높다. 사료의 부적합한 단백질함량 및 아미노산 조성은 어류의 성장을 감소시키거나 사료비용을 증가시키는 결과를 초래한다. 단백질은 어류 사료에 있어서 고려하여야 할 가장 중요한 요소로써 이러한 단백질을 구성하는 각 아미노산의 조성 및 각 요구량 설정은 양식어류의 성장 및 배합사료 비용과 직접적으로 관계된다.

어류의 초기 발달 단계에서 사료로 공급되어지는 아미노산은 조직 단백질과 고분자 화합물의 합성 및 에너지원으로서 중요하게 작용한다 (Terjesen et al., 1997; Wright and Fyhn, 2001; Finn et al., 2002; Ronnestad et al., 2003). 경골어류에 있어서 사료로 공급된 아미노산의 흡수는 단백질의 가수분해에 의한 peptides (mono-, di-peptides) 형태 또는 직접적인 free 아미노산 형태로 이루어진다. Dabrowski et al. (2003) 연구에서 무지개송어를 대상으로 dipeptide 및 free 형태의 이용성을 비교한 결과 dipeptide를 기초로 한 사료가 높은 성장률을 보였고, free 형태를 기초로 한 사료에서는 낮은 성장률을 보였다. 또한 intact protein 및 free 아미노산을 기초로 한 사료로 methionine 연구에서도 free 아미노산 그룹에서 유의적으로 낮은 성장률을 보였다. Free

형태의 아미노산이 dipeptide, intact protein 및 protein-bound amino acids 보다 이용성이 떨어진다는 연구결과들이 최근 많이 발표되고 있다. 그 매커니즘으로 free 형태의 아미노산은 intact protein 보다 빨리 흡수 및 대사가 되어 이용되기 때문에 비효율적이라고 보고되고 있다. 다른 매커니즘으로는 free 형태의 아미노산이 물에서 보다 더 빨리 침출되어 손실되기 때문이라고 보고되고 있다(Zarate and Lovell, 1997). 따라서 free 아미노산의 이용성을 높이기 위해 microencapsulation 및 coating 기술이 개발되어 영양소의 흡수 및 손실을 막기위해 널리 사용되고 있다.

따라서 기존의 어류 사료 내 아미노산 요구량에 관한 연구가 모두 free 아미노산을 사용하여 도출된 결과이고, 설정된 모든 아미노산 요구량 값이 과대평가 된 결과라는 것이 본 연구의 가설이다. 이러한 가설에 착안하여 본 연구는 해산양식 어종인 넙치, 감성돔, 참돔을 대상으로 사료 내 필수아미노산인 루신(leucine)을 free형태와 dipeptide형태로 각각 첨가하여 성장을 비교하였으며, 더 나아가 각각의 형태에 따른 요구량을 비교하여 적정요구량 및 가설을 입증하기 위해 수행되었다.

위에서 언급한 가설을 입증하기 위해 5번의 사양실험을 수행하였으며, 사양실험1(Chapter two)은 참돔을 대상으로 루신(Leucine)을 각각 free와 dipeptide 형태로 첨가(0.7%, 1.4%)하여 비교하였으며, 사양실험2(Chapter three)는 참돔을 대상으로 페닐알라닌(Phenylalanine)을 각각 free와 dipeptide 형태로 첨가(0.7%, 1.4%)하여 이용성 및 성장을 비교하였다.

사양실험3(Chapter four)은 감성돔을 대상으로 각각 free 및 dipeptide 형태로 leucine을 단계별로 첨가(C-0.4%, D-0.7%, D-1.0%, D-1.3% 및 F-0.7%, F-1.0%, F-1.3%)하여 사료 내 적정 요구량을 비교하여 규명하였으며, 사양실험4(Chapter five)는 넙치를 대상으로 각각 free 및 dipeptide 형태로 사료 내 단계별로 첨가(C-0.6%, D-0.9%, D-1.2%, D-1.5% 및 F-0.9%, F-1.2%, F-1.5%)하여 아미노산의 이용성 및 적정 요구량을 규명하였다. 사양실험5(Chapter six)는 넙치를 대상으로 추후 비교연구를 위해 우선적으로 free형태의 phenylalanine을 사료 내 단계적으로 첨가(P-0.4%, P-0.7%, P-1.0%, P-1.3%, P-1.6%, P-1.9%)하여 필수성 및 적정요구량을 규명하였다.

사양실험1의 결과, dipeptide 형태로 루신(leucine)을 첨가한 실험구가 free 형태로 루신을 첨가한 그룹과 비교하여 유의적으로 높은 성장률을 보였다. 일간성장률에서도 dipeptide 그룹에서 free 그룹보다 유의적으로 높은 결과를 보였다. 사료효율에서는 dipeptide 형태로 루신을 첨가한 1.4% 그룹(D-1.4)에서 다른 그룹보다 유의적으로 높은 효율을 보였다. 단백질전환효율 역시 사료전환효율결과와 비슷하게 D-1.4 그룹에서 다른 그룹보다 유의적으로 높은 효율을 보였다. 이 결과는 본 연구의 가설인 기존의 아미노산 요구량이 과대평가되어 도출된 결과라는 것을 확실히 증명하는 결과이다. 이러한 연구결과를 바탕으로 사양실험2에서는 필수아미노산 중 하나인 페닐알라닌(Phenylalanine)을 dipeptide 형태와 free 형태로 첨가하여 참돔을 대상으로 성장실험을 수행하였다. 그 결과, 성장률, 사료섭취율, 일간성장률, 사료전환효율, 단백질전환효율 및 생존율에 있어서 모든 실험구간에 유의적인 차이를 관찰할 수

없었다. 하지만 전어체의 구성아미노산 분석결과에서 0.7%를 첨가한 그룹에서 dipeptide와 free 그룹을 비교한 결과 dipeptide 그룹이 free 그룹 보다 모든 아미노산 함량이 높았다. 하지만 1.4%를 첨가한 그룹에서는 모든 아미노산의 함량에서 dipeptide와 free 그룹간에 아무런 차이를 보이지 않았다. 이 결과는 아마도 참돔의 페닐알라닌 요구량이 0.7% 이하이거나, 다이펩타이드의 결합에 의한 차이로 판단된다. 따라서 사양실험3은 dipeptide와 free 형태의 루신을 사료 내 단계별로 첨가하여 넙치치어를 대상으로 아미노산 형태에 따른 요구량을 비교하기 위해 수행하였다. 그 결과, dipeptide 그룹에서 보다 높은 성장률을 보였으며, 아미노산의 이용률도 높았다. 사료 내 루신의 적정요구량은 free 형태일 때 1.00%, dipeptide 형태일 때 0.88%로, dipeptide 형태의 루신으로 했을 때 적정요구량이 약 0.12% 낮게 분석되었다. 이러한 결과들을 바탕으로 사양실험4는 다른 어종에서는 어떠한 결과를 보이는 알아보기 위해 dipeptide와 free 형태의 루신을 사료 내 단계별로 첨가하여 감성돔 치어를 대상으로 아미노산 형태에 따른 요구량을 비교하기 위해 수행하였다. 그 결과, 사양실험 3의 결과와 유사하게 dipeptide 그룹에서 보다 free 그룹 보다 높은 성장률을 보였으며, 아미노산의 이용률도 높았다. 사료 내 루신의 적정요구량은 free 형태일 때 1.09%, dipeptide 형태일 때 0.99%로, dipeptide 형태의 루신으로 했을 때 적정요구량이 약 0.1% 낮게 분석되었다. 사양실험5는 추후 dipeptide로 도출된 요구량과 비교하기 위해 우선적으로 free 형태의 페닐알라닌(Phenylalanine)을 사료 내 단계별로 첨가하여 넙치치어를 대상으로 수행되었다. 그 결과, 사료 내 페닐알라닌의 함량이 증가함에 따라 성장률이 유의적으로 증가하다가 적정요구량을 넘으면 오히려 감소하는 경향을 보였다. 사료 내 페닐알라닌의 적정요구량은

0.9%로 판단되며, 본 연구결과는 추후 dipeptide 연구결과를 참고하여 보다 더 정확한 요구량이 규명될 것이다.

위 결과들을 종합해 볼 때, 해산어류(넙치, 참돔, 감성돔)는 free 아미노산 보다 dipeptdie형태의 아미노산을 보다 효율적으로 이용하여 성장률을 높일 수 있을 것으로 판단된다. Dipeptide는 해산어류의 아미노산 요구량 연구에 있어서 free 형태의 아미노산을 대체하여 보다 더 정확한 요구량을 알아보기 위한 아미노산 원료로 사용 가능할 것으로 판단된다. 본 연구결과를 비추어 보아 지금까지 보고된 기존의 아미노산 요구량결과는 과대평가될 수 있다는 본 연구의 가설이 확실히 증명되었으며, 이러한 결과들을 바탕으로 하였을 때 넙치에 있어서 배합사료 내 루신의 적정첨가 함량은 0.88%, 감성돔에 있어서 루신의 적정첨가 함량은 0.99%로 판단된다. 또한 나머지 필수아미노산에 대해서도 본 연구의 모델을 기초하여 요구량을 재평가하고 확립하여야 할 것으로 판단된다.

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE: Literature review

1.1. Current world state of fisheries and aquaculture

Aquaculture is one of the most important high protein food producing sectors. The generalization of aquaculture development trends is difficult, and the provided trends are much relevant to its manner in the countries in which it is well founded. World aquaculture has grown dramatically during the last 50 years with rate of less than one million tonnes in 1950 to about 59.4 million tonnes in 2004. The amount of supplied cultured aquatic animals for human consumption reported to be 52.5 million tonnes in 2008. Its contribution to the total production of capture fisheries has increased from 34.5 percent in 2006 to 36.9 percent in 2008; also the world's fish food production for human consumption has reached from 42.6 to 45.7 percent. The mean annual growth rate of aquaculture has been 8.3 (or 6.5 percent except China) percent, higher than the world mean population growth rate, through the years 1970-2008, and its annual per capita supply has increased from 0.7 to 7.8 kg during this period. Except for aquatic plants, the value of the world aquaculture products has been estimated at US$98.4 billion in 2008. The expansion and intensification of aquaculture during the past decades have attributed to research, accordance with consumer demand and improved aquaculture policy and governance.

Different factors are driving the aquaculture to intensification of which the main limiting factor is the unavailability of sites because of restricted non-agricultural land for exploitation. In this regard novel ways of using environment (land and water) for production are needed. By proceeding of intensification, the need for scientific support, services and skilled forces is in

increase.

Aquaculture explores for new species, especially valuable ones, in countries in which the aquaculture is well established. An increasing trend has been reported for facilities of valuable species for mariculture, and in contrary a decrease in facilities for low value species like cyprinids, especially in China. In the future, expansion of mariculture, especially high value species may compensate the reduction in freshwater aquaculture.

Polyculture, especially in marine systems, provides a means for diversified products from a system and reduces the undesirable environmental impacts. Despite of its traditional practice in Asian freshwater and coastal waters, more research and technology transfer on marine combined plant/animal systems, where such systems are less developed, is needed.

In most of the countries, aquaculture aims more on economic sustainability and overall competitiveness rather than high yield per aria unit. In this regard, one of the key factors is the improvement in health management. Because of significant losses in global aquaculture resulting from pathogens and disease, strong emphasis is focused on decreasing the mortality due to disease. Such inclination not only focuses on production and practice, but also on acquiring quality inputs such as clean seed and high quality feed to reduce the risks of production failures. The combination of all these trends can result in an improvement in management and be observed at the individual farm level and specific subsectoral levels, as well. Although such trends have not occurred simultaneously in aquaculture all over the world, it will materialize as different pressures are applied.

1.2. General amino acids metabolism in animals

Amino acids are molecules that include both of amine and carboxylic acid functional group. The main function of amino acids is their use as building blocks for protein synthesis. In addition, amino acids serve as precursors of non-protein nitrogenous high molecular weight compounds (such as nucleic acid, porphyrine and creatine), and substrates for energy. Recently, individual amino acids have been suggested to act as signaling molecules to regulate mRNA translation. For example, leucine, one of the branched-chain amino acids, has been proposed to play a regulatory role in the stimulation of muscle protein synthesis by enhancing availability of specific eukaryotic initiation factors (Anthony et al, 2007).

Twenty of the 80 naturally occurring amino acids are involved in the formation of proteins, in which about one half are considered indispensible or essential for animals. Those must be provided in the diets because their carbon chain cannot be synthesized by the body. The majority of indispensible amino acids (EAAs) are identical in all animals including fish (Dabrowski and Guderley, 2002). They are isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, arginine, and histidine. There are ontogenetical, species-specific and physiological state differences in the EAA requirements in animals, including fish (Dabrowski et al. 2005). Conversely, the amino acids that can be manufactured by the body are classified as dispensable or nonessential, including alanine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine, and tyrosine. Cystine and tyrosine are also termed as “semi-essential” or “conditionally-essential” amino acids. Under certain conditions, methionine will be converted to cystine, and phenylalanine can be converted to tyrosine, but not the other way around. Therefore, when cystine and tyrosine are present in the diet, the requirements for methionine and phenylalanine may be decreased.

1.3. Protein and amino acids

1.3.1. Protein digestion, amino acids absorption and metabolism

The basic mechanism of intestinal absorption of peptides and proteins was begun to be understood by the discovery of free amino acids in gastrointestinal lumen. The understanding of the mechanism was due to discovery of mixed peptidase activity in small intestinal mucosa. The peptides undergo rapid and complete proteolytic degradation in lumen or cells of gastrointestinal tract and then are absorbed only as free AA. However, increasing number of observations of peptides and proteins in the lumen or cells suggested that the absorption of intact peptides is possible (Fricker and Drewe, 1996). Free AAs in fish diets are absorbed more rapidly than AAs digested from intact protein and are not effectively utilized for protein synthesis (Cowey and Walton, 1988; Berge et al., 1994). Crystalline AAs are found to be utilized at a lower efficiency than intact protein or dipeptides (Dabrowski et al., 2003). It was also reported that lower utilization of crystalline AAs can be explained by a finding that more rapid absorption of crystalline AAs can cause AA imbalances in tissues and divert more AAs from anabolic to catabolic processes in juvenile grouper, *Epinephelus coioides* (Luo et al., 2005).

When dietary amino acids are provided in the form of protein, these proteins must be degraded in the digestive tract for the cellular anabolic and catabolic processes in the body tissues. For monogastric mammals and stomach-possessing fish, protein digestion begins in the stomach lumen. Hydrochloric acid secreted by the stomach parietal cells denatures the dietary proteins and decreases stomach lumen pH to approximately 2.0-2.5. The major gastric proteases (two types of pepsin) that are autocatalytically activated from zymogens (pepsinogens) at acidic pH, degrade proteins into large polypeptides, small polypeptides and some free amino acids.

These hydrolytic products and some intact proteins will be passed into the small intestine for further digestion. For most marine fish larvae, gastric pepsin activity and hydrochloric acid secretion seems to be very low or absent (Tanaka et al., 1996, Cahu and Zambonino Infante, 2001). Whether the lack of HCl; and pepsin will hamper enzymatic protein digestion in fish larvae needs to be further addressed (Ronnestad et al. 2003).

The pancreas secretes many types of proteases, including trypsinogen, chymotrypsinogen, elastase (endopeptidase) and carboxypeptidase A and B (exopeptidases). These proteases are secreted as zymogens and are activated in the lumen of the intestine. The starter phase of protein digestion in the small intestine begins when activated pancreatic proteases cleave peptide bonds of amino acid chains. The carboxypeptidases remove a single amino acid from the carboxyl-terminal end of proteins and peptides. The actions of pancreatic proteases reduce proteins into the mixure of oligopeptides (2-6 amino acids) and free amino acids.

Oligopeptides produced by gastric and pancreatic proteases are further digested by the brush border membrane peptidases to free amino acids or di- and tri-peptides before being absorbed. That is the final step of protein digestion. Aminopeptidase N is the most abundance membrane peptidase in enterocytes and cleaves amino acids from the N-terminus of short oligopeptides (Erickson and Kim, 1990) Free amino acids can be absorbed by the intestinal mucosal cells via active transport, simple diffusion, and facilitated diffusion. In respect to mammals, the Na+ - dependent amino acid transport system (the Alanine system (A), large neutral brush border system (LNBB), phenylalanine system (PHY), anionic system (X)), and the Na+ - independent amino acid transport system (leucine system (l) and cationic system (y)) are responsible for transporting amino acids into the cytoplasma (Stipanuk, 2000). Some studies (Smith and Aheava, 1989) suggested that fish have similar amino acid transport systems as

mammals.

Absorbed amino acids are either used for the synthesis of tissue proteins, oxidized for energy production (catabolism), transaminated into other amino acids, used in gluconeogenesis or lipogenesis, or used in the synthesis of other non-protein nitrogenous molecules. The lower *K*m (10-3 − 10mM) or higher affinity of amino acids to the enzymes involved in protein synthesis compared to the enzymes participating in amino acids catabolism (1-10m M) (Walton and Cowey, 1982) assures an efficient use of amino acid resources.

1.3.2. Branched-chain amino acids: leucine, isoleucine and valine

Branched-chain amino acids (BCAAs) are essential amino acids whose carbon structure is marked by a branch point. The BCAAs are valine, isoleucine and leucine having aliphatic side chains that are nonlinear.

These three EAAs play important structural roles and are primarily deposited in body protein, notably in skeletal muscles (Cowey and Walton, 1989). Valine is also involved in the synthesis of the myelin covering of the nerves, and valine deficiency can cause degenerative neurological conditions in mammals. Because of their critical roles in the protein structure, most proteins have a relatively high proportion of BCAAs, and these represent a significant proportion of amino acids consumed by animals.

The increase in circulating BCAAs that occurs after a protein-containing meal is “sensed” by a number of different tissues and has important effects in these tissues (Yang et al., 2008). Thus, the BCAAs serve as important signals to other tissues; among the tissues that respond to

BCAA concentrations are brain and skeletal muscle. Leucine is increasingly recognized as an anabolic nutrient signal, communicating the presence of an ingested protein-containing meal to peripheral tissues, and stimulating insulin secretion by the β-cells of the pancreas and protein synthesis in muscle and adipose tissue through the target of rapamycin signaling pathway (Yang et al., 2008).

The metabolism of BCAAs differs from that of the other amino acids in three important respects. First, rather than being restricted to the liver as for most EAAs, the catabolic enzymes for BCAAs are distributed widely in body tissues, including the kidney, muscle, and even the central nervous system (Cowey and Walton, 1989). Second, all three BCAAs share the same common transporter for intestinal absorption. Finally, the first steps in the oxidation of each of these three amino acids are catalyzed by two common enzymes, and so the organism metabolizes these three amino acids using the same enzymatic system. The first step in BCAA catabolism is transamination catalyzed by BCAT (branched-chain aminotransferase) isozymes. In this reaction, the amino group is transferred from a BCAA to α-ketoglutarate to form glutamate and the respective branched-chain α-keto acid (BCKA). The keto acid products are irreversibly oxidized by the second enzyme in the catabolic pathway, the mitochondrial BCKA dehydrogenase enzyme complex (Brosnan and Brosnan, 2006).

1.3.3. Antagonisms among branched-chain amino acids

Interactions between the BCAAs, leucine, isoleucine and valine are known to produce antagonistic effects in chicks, pigs, rats, and humans (D,Mello, 1994). Reduction of plasma isoleucine and valine concentration after consumption of an excessive amount of leucine has been reported in rats, chicks, pigs, and humans (Block and Harper, 1991). Leucine-induced

changes in plasma levels of isoleucine and valine have mainly been attributed to competitive inhibition during intestinal absorption and increased oxidation through BCKA dehydrogenase activation (Block and Harper, 1991; Langer et al., 2000).

In fish, antagonism involving BCAAs have not been fully assessed, and the results obtained have shown some inconsistencies. No effect of excess leucine on the other BCAAs was observed by Robinson et al. (1984), Choo et al. (1991), and Rodehutscord et al. (1997). However, Chance et al. (1964) observed that the isoleucine requirement of Chinook salmon increased slightly with increasing concentrations of dietary leucine.Hughes et al. (1983) observed changes in concentrations of BCAAs in lake trout given diets containing increasing amounts of valine. Plasma isoleucine and leucine were both elevated in valine-deficient fish, and their concentrations decreased as dietary valine was increased. An antagonist effect of excess leucine on plasma and muscle levels of other BCAAs has also been reported by Hughes et al. (1984) in lake trout and Yamamoto et al. (2004) in rainbow trout. Yamamoto et al. (2004) reported a negative effect of feeding diets formulated to low Ile:Leu and Val:Leu ratios. Choo et al. (1991) and Encarnacao (2005) did not observed any effect of excess dietary leucine on plasma valine and isoleucine concentrations. Rainbow trout showed a high tolerance for dietary leucine; no growth depression occurred with concentrations as high as 9.2% of diet (Choo et al., 1991). Even with excessive dietary leucine concentrations (13.4% of diet), which were overtly toxic, the concentrations of free valine and isoleucine in plasma, liver, and muscle were not depressed (Choo et al., 1991).

1.3.4. Phenylalanine and tyrosine

Phenylalanine is a nonpolar α-amino acid because of the hydrophobic nature of the benzyl

side chain. Tyrosine or 4-hydroxyphenylalanine is synthesized in the body from phenylalanine and is considered a semiessential, or conditionally essential, amino acid. Fish readly convert phenylalanine to tyrosine so that phenylalanine alone can meet requirements for aromatic amino acids (Wilson, 1989; Guillaume et al., 1999). However, the present of tyrosine in the diet will reduce some of the requirement for phenylalanine. Phenylalanine sparing by tyrosine is believed to be between 40-60% in the species studies to date (NRC, 1993; Guillaume et al., 1999).

Aside from being a proteinogenic amino acid, tyrosine has a special role by virtue of the phenol functionality. It occurs in proteins that are part of signal transduction processes and functions as a receiver of phosphate groups that are transferred to the hydroxyl group by protein kinases (so-called receptor tyrosine kinases), with phosphorylation changing the activity of the target protein. L-tyrosine also can be converted into the catecholamines, norepinephrine, and epinephrine, dopamine, and thyroxin (Cowey and Walton, 1989).

The catabolism of L-tyrosine involves a series of reactions that yield fumarate and acetoacetate (3-ketobutyroate). Acetoacetate is a ketone body that can be converted into acetyl-CoA, which in turn can be oxidized by the TCA cycle or be used for fatty acid synthesis (Cowey and Walton, 1989).

1.3.5. Utilization of peptides in formulated diets in fish

The early life stages of fish (larval and juvenile) are characterized by very high growth rates compared to adult stages (Conceicao, 1997). In some species, such as African catfish (Terjesen et al. 1997), larvae growth rate can reach 60% per day during the first days of exogenous feeding. The growth in fish is primarily due to muscle protein deposition (Houlihan

et al. 1995). Consequently, fish have very high dietary amino acids requirements for protein accretion, turnover and major fuel source during this rapid growth period. Utilization of formulated diets is a promising alternative for the live food to rear larval fish since the latter is costly (Zambonino Infante *et al.* 1997) and its nutritional composition difficult to manipulate (Aragao *et al.* 2004). In addition, the crustacean free AA pool varies with species, life stages and culture conditions (Ronnestad *et al.* 1999). Therefore, a great number of studies on young teleost fish amino acid metabolism and requirement have been carried out recently targeting the development of acceptable formulated diets.

Young teleosts can obtain dietary AA in the form of protein-bound, free amino acids (FAA) or peptides. When peptides or FAA are the major amino acid sources in the diets, absorption can be completed in the intestine bypassing the digestion by proteases secreted in the stomach and from pancreas to the intestine. In mammals, amino acids are mainly taken up and released from the gut by the Na+ -dependent amino acid transport system or the Na+ -independent amino acid transport system located in the intestine mucosal cells. Peptides have different transport systems from free amino acids. The mammalian peptide transporter, oligopeptide transporter (Pept-1), is also abundantly expressed in larval zebrafish *(Danio rerio)* (Verri *et al.* 2003).

Studies with larvae of several fish species showed faster absorption of small peptides and FAA compared with protein if injected into the digestive tract prior to their metamorphosis (Rust *et al.* 1993; Ronnestad *et al.* 2003). Therefore, it seems that free amino acids and small peptides are promising dietary amino acids sources in the formulated diets for teleost fish at their early life stages.

Experiments with juvenile rainbow trout *(Oncorhynchus mykiss),* Attllanttiic salmon *(Salmo salar L.)* and Atlantic cod (*Gadus morhua*) (Cowey & Walton, 1988; Espe *et al*., 1993, Berge *et al.* 1994) showed faster FAA absorption rates compared with protein bound AA. The similar observation was also reported in humans (Metges et al., 2000). Ronnestad *et al.* (2000) studied the use of intubation techniques where the authors showed that an FAA suspension (lacking other dietary ingredients) containing L-[35S] methionine was absorbed with a 3.5-times faster absorption rate from the gut than the protein diet containing L-[methylated- 14C]-protein in the Senegal sole. However, this may result from the reduced digestibility of methylated proteins (Keil and Kirchman, 1992). Moreover, numerous studies have also shown that free amino acids are taken up by the intestinal mucosal cells at a slower rate than intact small peptides in rats, hamsters, pigs, humans and fish (Burston *et al.* 1972, Mathews *et al.* 1974, Li *et al.* 1999, Addibi, 1974 and Boge *et al.* 1981). Many studies on the utilization of FAA-based diets that had been carried out with common carp and rainbow trout indicated inferior or even negative utilization of amino acids in comparison to equivalent protein-based diets in respect to fish growth (Murai *et al.* 1982; 1983; Kaushik and Dabrowski 1983, Dabrowski, et al. 2003). The increased deamination at the first feeding stage in rainbow trout alevin (Dabrowski et al., 2003) or higher amounts of total dietary nitrogen excretion through the gill and kidney may be at least partially responsible for the negative or inferior utilization of FAA-based diets by young fish. Murai *et al.* (1984) reported that FAA represented 36% of total nitrogen excretion through the gills and kidney when carp juveniles were fed a diet containing 38.4% free amino acid. The excretion dropped to 12.8% in carp fed with the comparable casein-based diet.

Compared with FAA-based or protein based diets, peptide-based diets may play a positive role in the development of the brush border enzyme expression and gut differentiation in young

fish (Zambonino *et al.* 1997, Cahu *et al.* 2001). Aragao et al. (2004) showed that retention of amino acids was improved when *Artemia*-fed postlarval Senegal sole was supplemented with dipeptides through direct meal intubation. More advantages of peptide inclusion in common carp diets were earlier found by Carvalho *et al.* (1997) who reported that protein hydrolyzates (peptides) can be effectively utilized in this species for growth. A study conducted by Dabrowski et al. (2003) showed that synthetic dipeptides-based diet sustained the high growth rate of rainbow trout alevins and juveniles. However, *in vivo* evidence of the nutritional and metabolic significance of the complete (all EAA) peptide-based diets in larval and juvenile fish remains illusive. Espe *et al.* (1999) showed that muscle protein synthesis in Atlantic salmon decreased 1.5-2.0 folds and the growth rate significantly decreased with an increase in proportion of small peptides. In contract, more recent studies conducted by Aragao *et al*. (2004) showed that dipeptides could improve dietary amino acid balance in comparison to free AA in juvenile Senegal sole. Terjesen et al. (2006) reported that a diet with 1:1 ratio of protein to synthetic dipeptides resulted in better performance by rainbow trout alevins than an entirely dipeptide-based diet, and comparable to a protein-based diet in terms of growth rate, survival and muscle indispensible free amino acid levels.

1.3.6. The influence of amino acids imbalanced diets on animals

All animals including fish must obtain EAAs from diet to maintain protein synthesis and their normal growth. Amino acid balanced diets in monogastric animals must be composed of various protein sources to provide EAAs in the right proportion. In practice, various major dietary proteins are limited in one or more EAA compared to fish meal. For example, soybean protein is limited in leucine, threonine and valine. Oil seeds and wheat gluten are limited in lysine (Giles et al., 1976), whereas meat and bone meal are deficient in methionine. Consuming

one of these proteins as a sole dietary protein source may cause EAA deficiency. Under farming conditions, feed formulations that include plant proteins may result in EAA disproportion or deficiency.

Extensive studies carried out in animals have shown that an amino acid imbalanced diet reduces feed intake and growth of animals (Harper *et al.* 1970; Gietzen 1993). EAA deficient diets affect animals adversely, including depression of their food intake and growth, change of feed intake behavior, development of lesions and low survival rates (Harper *et al.,* 1970). Studies conducted by Koehnle *et al*. (2004) showed that feed intake of rats decreased significantly after they were fed a threonine-deficient diet (threonine is the second most limiting amino acids in soybean protein-based diet for rat (Berry, et al., 1962)) in comparison to the control group. The level of plasma histidine and threonine decreased rapidly after ingesting histidine- or threonine- deficient diets, respectively, while the level of plasma total EAA other than histidine and threonine, increased significantly. There was no significant change in the rat plasma dispensable amino acid (NEAA) concentrations after consumption of histidine- or threonine-deficient diets. Liver and muscle free histidine or threonine concentrations decreased after rats were fed histidine or threonine imbalanced diets (Peng *et al*., 1972). Feurte *et al*. (1999) also reported that plasma threonine concentrations significantly decreased between 30 and 60 minutes after rats ingested a threonine-devoid diet. Furthermore, studies also showed that rats could recover from such deficiency when fed with feed containing the specific EAA they were lacking (Markison *et al.*, 2000). By using human HeLa cells, Ramunas *et al.* (2005) found that proteasomal protein degradation provides amino acids for translation and new protein synthesis before up-regulation of the autophagosomal-lysosomal pathway under the cells external amino acid restriction. The protein degradation through proteasomal or lysosomal pathway is a continuous process for renewal of proteins under normal physiological state in

animals.

The mechanism of interdependence of behavioral response (food rejection) and physiological indicators (concentration of EAA in tissue) is being addressed systematically in mammals. Studies showed that a threonine-imbalanced diet significantly decreased threonine concentrations in the anterior piriform cortex (APC) of the brain (Gietzen, 1993). This correlated with the fact that rats rapidly i.e., within 15 min, reject EAA deficient diet after ingestion of threonine-imbalanced diet. Recent studies by Koehnle *et al*. (2003) confirmed that rats recognized threonine deficient diet within the first meal and reduced the first meal duration and decreased intake of the threonine-deficient diet within 12-16 min. Hao et al. (2005) have argued that mammals recognize dietary EAA deficiency in APC of the brain to signal rejection of EAA deficient diet.

It has been long recognized that animals as ancient as spiders (Greenstone, 1979) optimize EAA proportions in their diets by adapting specific strategy of nutritional polyphagy. By diversification of prey (protein and amino acid composition), predatory insects arrive at selecting nutrients not only by quantity but most importantly also by quality (Mayntz *et al*., 2005).

1.3.7. Effectiveness of crystalline amino acids

Crystalline amino acids (CAA) have been used commercially to meet EAA needs of animals for more than 40 years. Progress in biotechnology allowed reduction in the cost of large-scale production of amino acids, which has been one of the key factors in expanded use of supplemental amino acids in animal feeds. Because of the high cost of fish meal and concurrent

to the increased utilization of more economical protein sourses with “imperfect” EAA profiles, CAA are being increasingly used to meet EAA requirements of the fish. CAA are, as such, becoming key components of cost-effective fish-feed formulations. Nonetheless, some segments of the aquaculture industry have been relatively slow to adopt widespread use of CAA to meet EAA requirements of fish and shrimp, notably because of concerns with the efficiency with which dietary CAA are used by fish and shrimp. Several studies have shown that CAA were utilized as efficiently as those of intact protein origin in meeting EAA requirements of fish (Murai et al., 1987; Kim et al., 1991; Espe and Lied, 1994; Rodehutscord et al., 1995; Rollin, 1999; Williams et al., 2001; Rollin et al., 2003; Espe et al., 2006). Conversely, other studies have indicated that CAA appear to be utilized with a lower efficiency than EAA supplied by intact protein (Yamada et al., 1981; Murai et al., 1987; Espe and Njaa, 1991; Schuhmacher et al., 1997; Zarate and Lovell, 1997; de la Higuera et al., 1998; Refstie et al., 2001; Sveier et al., 2001; Liu et al., 2002; Dabrowski et al., 2003; Peres and Oliva-Teles, 2005; Zhou et al., 2007; Dabrowski et al., 2010).

Several studies in different fish and shrimp species have shown quite convincingly that CAA may be absorbed slightly more rapidly and/or earlier in the gastrointestinal tract than protein-bound amino acids (Deshimaru, 1976; Yamada et al., 1981; Kaushik and Dabrowski, 1983; Murai et al., 1987; Cowey and Walton, 1988; Zarate and Lovell, 1997; Zarate et al., 1999). This faster and/or earlier absorption may result in temporary higher tissue or plasma concentrations of amino acids provided as CAA, because of a slight metabolic dyssynchrony with amino acids derived from protein digestion and a greater proportion of the CAA being catabolized (Zarate et al., 1999; Fox et al., 2006). This hypothesis is supported by evidence of better metabolic utilization of CAA in animals fed more frequently (Tantikitti and March, 1995; Zarate et al., 1999). Reducing the solubility and absorption rate of CAA using coating,

encapsulation, or polymerization techniques reportedly improves the efficiency of utilization of CAA in fish and shrimp (Dabrowski et al., 2003; Alam et al., 2004; Dabrowski et al., 2010).

Experimental evidence also suggests that the dietary ingredient matrix (wheat gluten- vs. corn gluten-based diets) and life stage of the animal may influence the efficiency of CAA utilization in some species (Dabrowski et al., 2003; Nang Thu et al., 2007).

1.4. Essential and nonessential amino acids

Proteins and their building blocks, amino acids, are organic compounds that are essential components of all living organisms. Amino acids can link together by a covalent peptide bond between the α-carboxyl end of one amino acid and the α-amino end of the other (Brody, 1999). Amino acids are molecules containing both amine and carboxyl functional groups, with the general formula $H_2NCHRCOOH$, where R is a side chain. The most naturally abundant and metabolically important amino acids are the L-α-amino acids, in which the amino and carboxylate groups are attached to the same carbon atom.

Twenty primary amino acids are used by cells in protein biosynthesis (Table 1-1). Amino acids are important in many other biological molecules, such as forming parts of coenzymes, precursors for the biosynthesis of structural molecules, metabolic intermediates, and neurotransmitters, hormones, biogenic amines, or numerous other molecules important in the response of the organism to different stimuli. Most microorganisms and plants can biosynthesize all 20 primary amino acids, while animals must obtain some of the amino acids from their diet. The amino acids that an organism cannot synthesize on its own are referred to as "essential amino acids". In contrast, the nonessential amino acids can be synthesized from precursors, for example by addition of an amino group to a tricarboxylic acid (TCA)-cycle intermediate, such as α-ketoglutarate or oxaloacetate (Cowey and Walton, 1989; Zubay, 1993).

The essentiality of various amino acids for fish has been determined either by feeding trials involving the successive deletion of each amino acid in the diet or by isotopic-labeling studies (Wilson, 1989). It is clear from the available evidence published to date that all fish require the same 10 EAAs (Table 1-1) required by most other animals (Ketola, 1982; NRC, 1993).

Table 1-1. Essential and nonessential amino acids[1]

Essential	Nonessential
Arginine	Alanine
Histidine	Asparagine
Isoleucine	Aspartate
Leucine	Cysteine[1]
Lysine	Glycine
Methionine	Glutamate
Phenylalanine	Glutamine
Threonine	Proline
Tryptophan	Serine
Valine	Tyrosine[1]

[1]Conditionally essential.

1.5. Whole-body amino acid profile

Several studies have attested that whole-body EAA profile provides a good estimation of the optimum EAA profile of fish diets (Akiyama et al., 1997; Green and Hardy, 2002). A lot of published papers indicate that for a large majority of fish species fed high-quality diets, deposition of amino acids into body protein represents between 25 and 55% of total amino acids consumed. The deposition of protein is consequently a major determinant of amino acid utilization and requirements of fish (Cowey and Walton, 1989), because there is a very strong association between live weight gain and protein digestibility (Shearer, 1994; Dumas et al., 2007), also there is a very close association between live weight gain and amino acid requirements in absolute terms (g/fish per day). The EAA needs for protein accretion correspond to the amino acid content of tissue protein gain (Kaushik and Seiliez, 2010).

1.6. Blood and muscle amino acid levels

At subrequirement intake levels, the serum or tissue content of the tested EAA should remain low until the requirement for the EAA is met and then increase to high levels when excessive amonnts of the amino acid are fed (Wilson, 1989). This technique has proven to be useful in corroborating the EAA requirements, but only in a few cases. In channel catfish, serum lysine data (Wilson et al., 1977) were useful in confirming the requirement values estimated by weight gain data. However, this technique has not always been reliable for assessing EAA requirements (Kim et al., 1992). Its validity seems to be linked to the nature of the EAA tested, interactions between the different amino acids, and time elapsed between meal and blood sampling (Mambrini and Kaushik, 1995).

1.7. Preferential catabolism

Preferential catabolism refers to catabolism of amino acids to provide energy, when dietary energy intake is limiting protein digestibility, which implies that the animal is portioning EAA away from protein synthesis toward catabolism to meet a specific metabolic need (Moughan, 2003). Distinction between preferential and inevitable catabolism can then be derived from the slope of the relationship between metabolizable energy (ME) intake and protein digestibility (Mohn and de Lange, 1998).

In fish, where amino acids appear to provide a significant proportion of the total energy (ATP) requirement (Ronnestad et al., 2003; Finn et al., 2002), inevitable and preferential catabolism may be difficult to separate. It is not clear to what extent the significant catabolism of amino acids despite adequate ME and net energy intakes is related to inevitable losses of amino acids or catabolism of amino acids that are in excess of requirements, or preferential catabolism of amino acid as energy sources. The determinants of amino acid catabolism in fish deserve to be studied more systematically than it has been the case in the past.

1.8. Catabolism of excess amino acids

Intake of amino acids in excess of the amounts required for protein deposition, maintenance requirements, inevitable catabolism, and preferential catabolism will result in additional catabolisms of these amino acids. Feeding a diet in which the amino acid profile is deficient in one or multiple amino acids compared to dietary requirements will limit protein deposition, limit the retention of the other amino acids, and force their deamination and catabolism.

1.9. Estimated dietary leucine and phenylalanine requirement

Nutrient requirements find their usefulness in their translation into nutritional recommendations for feed formulations. Formulating cost-effective feeds meeting essential amino acid requirements of fish can represent a challenge.

Quantitative estimates have been generated for all 10 essential amino acids in a number of species, including channel catfish, common carp, Indian major carp, Nile tilapia, Pacific salmon, and rainbow trout.

Table 1-2 and 1-3 provide a summary of the experimental conditions and conclusion of studies on leucine and phenylalanine requirements of fish.

Table 1-2. Leucine requirements

Species	IBW (g/fish)	Diet CP (%)	Diet DE (kJ/g)	Leu Levels (% diet DM)	Estimated Requirement
Channel catfish (*Ictalurus punctatus*)	200	24	12.0	0.60-2.00	0.8% of diet 3.5% of CP
Chinook salmon (*Oncorhynchus tshawytscha*)	2.5	41	14.8	1. 0-3.1	1.6% of diet 3.9% of CP
Common carp (*Cyprinus carpio*)	0.5	48	14.7	0-2.50	1.3% of diet 3.3% of CP
Mrigal carp (*Cirrhinus mrigala*)	0.6	40	14.7	0.75-2.00	1.5% of diet 3.9% of CP
Rohu carp (*Labeo rohita*)	0.4	40	14.7	0.75-2.00	1.5-1.6% of diet 3.8-3.9% of CP
Lake trout (*Salvelinus namaycush*)	3.2	35	14.6	0.96-2.24	1.3-1.7% of diet 2.7-3.7% of CP
Nile tilapia (*Oreochromis niloticus*)	0.06	28	8.4	0.60-1.20	1.9% of diet 3.4% of CP
Rainbow trout (*Oncorhynchus mykiss*)	6.0	43	ND	1.10-13.4	3.4% of diet 9.2% of CP
	49.0	34	20.1	1.00-4.20	1.1-1.4% of diet 2.3-2.9% of CP

ND= not determined.

Table 1-3. Phenylalanine requirements

Species	IBW (g/fish)	Diet CP (%)	Diet DE (kJ/g)	Phe Levels (% diet DM)	Estimated Requirement
Channel catfish (*Ictalurus punctatus*)	195	24	9.6	0.20-0.80	0.5% of diet 2.1% of CP
Chinook salmon (*Oncorhynchus tshawytscha*)	2.5	41	14.8	0.96-3.50	1.7% of diet 4.4% of CP
Common carp (*Cyprinus carpio*)	1.5	48	14.7	0-2.75	1.3% of diet(2.9% Tyr) 3.3% of CP(2.9% Tyr)
Rohu carp (*Labeo rohita*)	0.2	40	14.7	0.40-1.65	1.2% of diet(1.0% Tyr) 2.9-3.1% of CP(2.5%)
Mrigal carp (*Cirrhinus mrigala*)	0.6	40	14	0.50-1.75	1.3% of diet(0.1% Tyr) 3.3% of CP
Nile tilapia (*Oreochromis niloticus*)	0.01	28	8.4	0.60-1.80	1.1% of diet(0.5% Tyr) 3.8% of CP
Rainbow trout (*Oncorhynchus mykiss*)	12.7	35	16.0	0.26-1.75	0.7% of diet 2.0% of CP

1.10. Chapter Justification

The purposes of this dissettation are to (1) study the utilization of dietary amino acids (different forms of amino acids) in marine fishes during early life stages (larvae and juvenile), (2) to determine the optimum dietary essential amino acids requirements in marine fishes by free or dipeptide forms of amino acids in terms of their growth performance and concentrations of whole-body amino acids.

In the first set of experiments (including 2 experiments), we evaluated the utilization efficiency of dipeptide and free forms of leucine and phenylalanine in juvenile red seabream. These experiments were listed as Chapter 2 (Comparison of growth performance in red seabream (*Pagrus major*) by free and dipeptide forms of leucine) and Chapter 3 (Comparison of growth performance in red seabream (*Pagrus major*) by free and dipeptide forms of phenylalanine). Our results demonstrated that amino acids are more available to the fish when they are provided in dipeptide form rather than free form.

In another set of experiments (including 2 experiments), we evaluated dietary leucine requirements in juvenile olive flounder and black seabream using dipeptide and free form leucine. These experiments were listed as Chapter 4 (Comparison of leucine requirement in olive flounder (*Paralichthys olivaceus*) by free and dipeptide forms of leucine), and Chapter 5 (Comparison of leucine requirement in black seabream by free and dipeptide forms of leucine).

Finally, we conducted to determine the optimum dietary phenylalanine requirement of juvenile olive flounder (*Paralichthys olivaceus*) was determined by free form of phenylalanine (Chapter 6).

The justification of each chapter is as follows;

Chapter 2 is about the utilization efficiency of dipeptide and free forms of leucine in juvenile red seabream. This study was conducted to investigate the utilization of the amino acid determine the optimum dietary leucine level, and to compare the growth performance by dipeptdie and free forms of leucine.

Chapter 3 is about the utilization efficiency of dipeptide and free forms of phenylalanine in juvenile red seabream. This study was conducted to investigate the utilization of the amino acid, figure out the optimum dietary phenylalanine level, and to compare the growth performance by dipeptdie and free forms of phenylalanine.

Chapter 4 focuses on comparison of leucine requirements in black seabream (*Acanthopagrus schlegeli*) by free and dipeptide forms of leucine. A four-week feeding trial was carried out to provide an innovative experimental model with different forms of leucine (free or dipeptide) for black seabream, and to re-evaluate the previous results on dietary requirement of leucine.

Chapter 5 focuses on the comparison of leucine requirements in olive flounder (*Paralichthys olivaceus*) by free and dipeptide forms of leucine. An eight-week feeding trial was carried out to provide an innovative experimental model with different forms of leucine (free or dipeptide) and to re-evaluate the previous results on dietary requirement leucine.

Chapter 6 focuses on determination of the optimum dietary phenylalanine requirement in juvenile olive flounder (*Paralichthys olivaceus*) by free form of phenylalanine.

CHAPTER TWO

Comparison of growth performance in red seabream (*Pagrus major*) by free and dipeptide forms of leucine

2.1. Introduction

Red seabream (*Pagrus major*) is the most highly valued marine food fish and is prominent for mariculture in Japan and Korea, its culture has increased rapidly (Ikenoue and Kafuku, 1992) and the production follows that of the yellowtail (Koshio, 2002). This fish is carnivorous, and in culture facilities is usually provided by either minced whole fish as feed or prepared feeds containing high levels of fish meal. In recent years, however, the availability of fish feed has been decreased and this situation is not expected to be improved in the near future. As a result, feed manufacturers have become increasingly interested in reducing their reliance on traditional ingredients for use in sea bream feeds. One of the prerequisites for efficiently identifying and developing experimental model for formulating animal feeds is the knowledge of the dietary amino acid requirements.

Leucine is essential for the normal growth and other important physiological functions of fish. Several investigations have been conducted to determine the requirements of various fish species for this amino acid (Chance et al., 1964; Nose, 1979; Wilson et al., 1980; Hughes et al., 1983; Santiago and Lovell, 1988; Choo et al., 1991; Rodehutscord et al., 1997; Millamena et al., 1999; Teshima et al., 2002; Ahmed and Khan, 2006; Abidi and Khan, 2007), and the reported values range from 2.3 to 9.2 % of dietary protein. AA requirements of red seabream have only

been studied for lysine (Forster and Ogata, 1998), taurine and cysteine (Matsunari et al., 2008). Little is known the essential dietary amino acid requirement of red sea bream, and this lack of information may hinder the development of feeds with non-traditional ingredients for this species. Many of the amino acid requirement studies for fish have used diets in which the amino acids have been supplied in crystalline form or are contained in purified proteins.

The growth rate and feed efficiency of the animals in using crystalline or purified proteins are often poor due to the low acceptance of the diets and reduced ability of the fish to utilize free amino acids (Cowey, 1992, 1994). Most amino acid requirement studies involve measuring the growth and feed efficiency response of fish fed a series of diets containing graded levels of the free amino acid. Poor growth due to the factors other than the targeting amino acid level in the diet can reduce the ability of an experiment to distinguish the requirement level of an amino acid (Cowey, 1992). Inclusion of practical feedstuffs, like high quality fish meal, can lead to more distinct patterns of response to increasing levels of amino acid.

Dabrowski et al. (2003) reported that a synthetic dipeptide-based diet can support the growth of rainbow trout, whereas a free AA-based diet could not. Several studies have indicated that CAA appears to be utilized with a lower efficiency than EAA supplied by intact protein (Yamada et al., 1981; Murai et al., 1987; Peres and olive-Teles, 2005; Dabrowski et al., 2010). Also, several stidies in different fish species have shown quite convincingly that CAA may be absorbed slightly more rapidly and/or earlier in the gastrointestinal tract than protein-bound amino acids (Zarate and Lovell, 1997; Zarate et al., 1999).

Essential amino acid requirements of fish are usually determined based on growth rates of fish fed graded levels of the targeted AAs in a free form. The objective of this study was to test

the hypothesis that the EAA requirements in fishes might have been over-estimated in most previous studies which have used a free form of amino acid. Therefore, we conducted a feeding trial in this study with different forms of leucine (free or dipeptide) for utilization of dipeptide in diets for growth performance of red seabream to test this hypothesis, and to provide an experimental model for further re-evaluation of AAs requirement.

2.2. Materials and methods

2.2.1. Experimental design and diets

The compositions and proximate analysis of the experimental diets are shown in Table 2. Four semi-purified experimental diets were prepared containing equivalent of 0.7 or 1.4% (dry dirt) leucine in free or dipeptide form. Leucine-Glycine (Leu-Gly) was used as the leucine source for the dipeptide form and crystalline L-leucine was used as the free form. The dipeptide, Leu-Gly, was supplemented into the experimental diets on the basis of molecular weight of leucine. The experimental diets were kept isonitrogenous and isocaloric by decreasing glycine while increasing the two different forms of leucine levels. Fish meal was included to increase palatability of the experimental semi-purified diets. All experimental diets were formulated to contain 0.3% leucine from fish meal. A mixture of synthetic free amino acids (FAA) without leucine was prepared according to Dabrowski et al. (2003) and used as the main protein source. All diets were well mixed, pelletized and freeze-dried. The dried diets were then prepared as crumble types and sieved to make proper sizes. The size of the diets was gradually increased over the course of feeding trial as fish grew. Amino acid concentrations of the diets and fish meal are provided in Table 2. Leucine concentration in the diets was confirmed to its intended levels.

Table 2-1. Formulation and of the reference diet.

Ingredients	%
Fish meal	7.5
Dextrin	72.5
Vitamin Mix.[1]	2.0
Mineral Mix.[2]	2.0
Choline chloride	1.0
Squid liver oil	14.0
Taurine	1.0

[1]Vitamin premix (g kg^{-1} of mixture): retinyl acetate, 0.667; cholecalciferol, 0.033; menadione, 0.133; thiamine hydrochloride, 2.667; riboflavin, 2.933; d-pantothenic acid hemicalcium, 9.667; pyridoxine hydrochloride, 2.667; cyanocobalamin, 0.007; niacinamide, 20.000; folic acid, 0.320; d-biotin, 0.133; ascorbic acid, 30.000; α-tocopherol, 6.667.

[2] Mineral mixture was based on the composition of Lee et al., 2003.

Table 2-2. Amino acid concentrations of the fish meal (% dry matter)

Amino acids	Content
Arginine	3.65
Histidine	2.60
Isoleucine	2.59
Leucine	4.97
Lysine	5.46
Methionine	1.84
Phenylalanine	2.60
Threonine	3.05
Valine	3.04

[1]White fish meal was kindly provided from Suhyup Feed Co. Ltd., Uiryeong, Korea.

Table 2-3. Composition and proximate analysis of the experimental diets (% dry matter)

Ingredients	Diets (added leucine level %)			
	D-0.7	D-1.4	F-0.7	F-1.4
White fish meal[1]	7.50	7.50	7.50	7.50
FAA mix[2]	38.65	38.65	38.65	38.65
Leucine[3]	0.65	1.85	0.00	0.00
Leu-Gly[4]	0.00	0.00	0.40	1.10
Glycine[5]	1.20	0.00	1.45	0.75
Dextrin[6]	32.00	32.00	32.00	32.00
Mineral mix[7]	2.00	2.00	2.00	2.00
Vitamin mix[8]	2.00	2.00	2.00	2.00
Squid liver oil[9]	14.00	14.00	14.00	14.00
Choline chloride	1.00	1.00	1.00	1.00
Taurine	1.00	1.00	1.00	1.00
Proximate composition				
Dry matter (%)	3.80	3.89	3.89	3.86
Protein (%, DM)	44.0	43.7	44.3	44.3
Lipid (%, DM)	13.2	13.4	13.6	13.1
Ash (%, DM)	1.18	1.13	1.23	1.11

[1]White fish meal was kindly provided from Suhyup Feed Co. Ltd., Uiryeong, Korea.

[2]Free amino acid mixture composition: (g/1384.11 g dry weight mixture; all L-form amino acids unless otherwise indicated): arginine hydrochloride, 37.8; valine, 37.8 (Fluka, Buchs, Japan); lysine, 45.36; D,L-methionine, 31.5 (WooSung, Daejun, Korea); histidine, 22.05; isoleucine, 28.35; phenylalanine; 56.7; threonine, 25.2; tryptophan, 6.3; proline, 365.4; serine, 365.4; alanine, 362.25 (Sigma Chemicals, St. Louis, MO).

[3]Leucine: Sigma Chemicals, L-leucine

[4]Leu-Gly: Sigma Chemicals, Leucine-Glycine

[5]Glycine: Sigma Chemicals, L-Glycine

[6]Dextrin: Daesung Chemicals

[7]Mineral premix (g kg^{-1} of mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[8]Vitamin premix (g kg^{-1} of mixture): 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-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[9]Squid liver oil was purchased from E-Wha oil Co. Ltd., Busan, Korea.

Table 2-4. Amino acid concentrations of the experimental diets (% dry matter)[a]

Amino acids	D-0.7	D-1.4	F-0.7	F-1.4
EAA				
Arginine	1.58	1.57	1.58	1.53
Histidine	0.80	0.76	0.79	0.75
Isoleucine	1.06	1.03	1.02	1.03
Leucine	0.89	1.64	0.83	1.49
Lysine	1.66	1.69	1.67	1.64
Methionine	1.09	1.03	1.08	1.05
Phenylalanine	2.03	2.02	2.01	2.04
Threonine	0.98	0.92	0.94	0.93
Valine	1.60	1.63	1.58	1.59
NEAA				
Alanine	0.74	0.78	0.74	0.78
Aspartic acid	0.48	0.47	0.46	0.47
Glutamic acid	16.78	16.77	16.73	16.76
Glycine	18.74	16.09	19.84	17.23
Serine	1.88	1.83	1.87	1.84

[a]Values are the means of duplication.

2.2.2. Feeding trials

Juvenile red seabreams were transported from a private hatchery to Marine and Environmental Research Institute (Jeju National Univesity, South Korea) acclimated to the experimental facilities and conditions, and fed with a microparticulate diet (Love Larva No. 4, Maruha, Shimonoseki, Japan) for 1 week. Prior to the start of the feeding trial, fish were fed the AAs-free diet (reference diet, Table 1) for 1 week to adjust to the semi-purified diet and deplete possible body reserves of AAs. The conditioned experimental fish averaging at 1.21±0.001 g were then randomly distributed into twenty one 20 L tanks (30 fishes/tank) in a flow through system supplied with sand filtered seawater at a flow rate of 1.5 L/min. Water temperature was between 17 and 20 °C by natural fluctuation in seawater temperature. Dissolved oxygen levels were maintained over 8.0 ppm by aeration and monitored throughout the feeding study. Triplicate groups of fish were hand-fed with the experimental diets to apparent satiation, for 6 weeks. Fish were initially fed six times a day until the 2nd week and then were fed four times a day from 3rd week. The diurnal cycle was 14-h light/10-h dark. Salinity during the experimental period was approximately 33‰. Growth was measured every two weeks. Feeding was stopped 24 h prior to weighing to minimize stress on fish. Experimental protocols followed the guidelines approved by the Animal Care and Use Committee of Jeju National University.

2.2.3. Chemical analysis

At the end of feeding trial, all fishes were sampled for whole-body AA analysis. Diets and whole-body samples were freeze-dried and finely ground using a grinder. Crude protein was determined by Kjeldahl method using an Auto Kjeldahl system (Kejltec System 2300, Sweden). Crude lipid was determined by an ether-extraction method. Moisture was determined by oven drying at 105 for 12 h. Ash was determined by muffle furnace at 550°C for six hours. The AA

compositions of the experimental diets, and whole-body samples were analyzed using an automatic AA analyzer (Biochrom 30, Pharmacia Biotech, Cambridge, England).

2.2.4. Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the mean difference was compared with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type 1 error at 5% ($P \leq 0.05$) for each set of comparisons. Data were presented as means ± SD. Percentage data were arcsine transformed before statistical analysis. Statistical analysis between free AA and dipeptide AA groups were conducted via paired Student's t-test to determine significant differences at 5% ($P \leq 0.05$).

2.3. Results

Growth performance of juvenile red seabream fed the experimental diets with different levels of leucine and two different types of leucine sources, free- or dipeptide, are presented in Table 2-5. The fish fed the leucine in dipeptide form (Leu-gly) had significantly higher weight gain than the fish fed free form, L-leucine in both dietary leucine levels (0.7 and 1.4%) (Fig 2-1). Weight gain and specific growth rate were significaltly increased with increasing dietary leucine level. The highest feed efficiency, feed intake and protein efficiency ratio were found in fish groups fed D-1.4% diet. No significant differences were observed in survival rate among experimental fish groups.

Table 2-6 provides whole-body AA compositions of fish fed the experimental diets. Whole-body histidine, isoleucine, leucine, methionine and valine, were significaltly higher in dipeptide groups than free leucine groups while other essential AA were not significantly different. In the case of whole-body non-essential AA concentrations, alanine, glutamic acid and glycine were significaltly higher in dipeptide groups than free leucine groups while other essential AA were not significantly different. However, there were significant differences between the two fish groups fed free and dipeptide forms of leucine (Fig 2-2 and 2-3). All the essential and non essential amino acid levels in whole-body were significantly higher in dipeptide groups than free groups at 0.7% dietary leucine level. However, no significant differences were observed in 1.4% dietary leucine level.

Table 2-5. Growth performance of juvenile red seabream fed the experimental diets with different levels or molecular forms of leucine for 6 weeks[1]

Diets	D-0.7	D-1.4	F-0.7	F-1.4
IMW (g)[2]	1.21±0.06	1.21±0.04	1.21±0.06	1.21±0.05
FMW (g)[3]	2.41±0.11	2.94±0.10	2.25±0.13	2.64±0.09
Weight gain[4]	99.7±8.7^{a}	143.2±8.6^{c}	85.9±11.0^{a}	118.5±7.4^{b}
FI[5]	85.1±1.3^{ab}	86.7±1.8^{b}	83.4±0.9^{a}	83.9±0.9^{a}
SGR (%)[6]	1.65±0.10^{a}	2.11±0.08^{c}	1.47±0.14^{a}	1.86±0.08^{b}
FCR[7]	2.41±0.23^{bc}	1.71±0.10^{a}	2.70±0.33^{c}	2.15±0.19^{b}
PER[8]	0.89±0.08^{ab}	1.25±0.07^{c}	0.80±0.09^{a}	1.00±0.08^{b}
Survival (%)	95.6±1.9	96.7±3.3	96.7±3.3	90.0±10.0

[1]Means of triplicate groups; values are presented as mean ± SD.

[2]IMW = Initial mean body weight.

[3]FMW = Final mean body weight.

[4]Weight gain (%) = 100 × (final mean body weight - initial mean body weight)/initial mean body weight.

[5]Feed intake (g/g body weight) = dry feed fed (g)/ body weight (g).

[6]Specific growth rate (%) = [(loge final body weight - loge initial body weight)/days] × 100.

[7]Feed conversion ratio = dry feed fed/wet weight gain.

[8]Protein efficiency ratio = wet weight gain/ total protein fed.

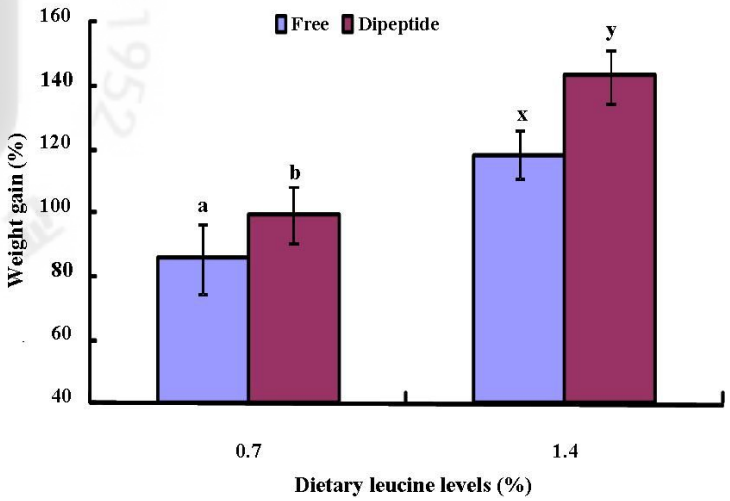

Figure 2-1. Weight gain (%) of juvenile red seabream (*Pagrus major*) fed the experimental diets with different levels or molecular forms of leucine for 6 weeks. Values are means ± presented as different S.D. (n=3). Bars that do not share a common superscript letter are significantly ($P<0.05$).

Table 2-6. Amino acid concentrations in the whole-body of juvenile red seabream fed the experimental diets with different levels or molecular forms of leucine for 6 weeks (% dry matter)[a]

Amino acids	D-0.7	D-1.4	F-0.7	F-1.4
EAA				
Arginine	3.67±0.19	3.54±0.22	2.91±0.47	3.28±0.58
Histidine	1.27±0.06[b]	1.24±0.07[ab]	1.00±0.17[a]	1.15±0.20[ab]
Isoleucine	2.08±0.11[b]	2.10±0.14[b]	1.64±0.27[a]	1.89±0.33[ab]
Leucine	3.81±0.16[ab]	3.85±0.26[b]	3.06±0.48[a]	3.53±0.60[ab]
Lysine	4.34±0.20	4.35±0.26	3.54±0.55	4.01±0.66
Methionine	1.53±0.09[b]	1.51±0.10[ab]	1.20±0.18[a]	1.40±0.25[ab]
Phenylalanine	2.17±0.13	2.16±0.16	1.74±0.32	1.99±0.35
Threonine	2.28±0.11	2.28±0.13	1.79±0.32	2.09±0.40
Valine	2.58±0.15[b]	2.54±0.17[ab]	2.05±0.31[a]	2.33±0.41[ab]
NEAA				
Alanine	3.44±0.20[b]	3.38±0.20[ab]	2.75±0.45[a]	3.07±0.49[ab]
Aspartic acid	4.75±0.25	4.74±0.29	3.72±0.66	4.31±0.81
Glutamic acid	7.19±0.49[b]	7.08±0.71[ab]	5.59±0.84[a]	6.50±1.12[ab]
Glycine	4.62±0.21[b]	4.30±0.24[ab]	3.62±0.65[a]	3.95±0.75[ab]
Proline	1.76±1.54	1.59±1.38	0.63±1.09	1.23±1.29
Serine	2.14±0.10	2.11±0.12	1.71±0.30	1.97±0.37
Tyrosine	1.72±0.09	1.69±0.12	1.35±0.25	1.58±0.31

[a]Values are the means of duplication.

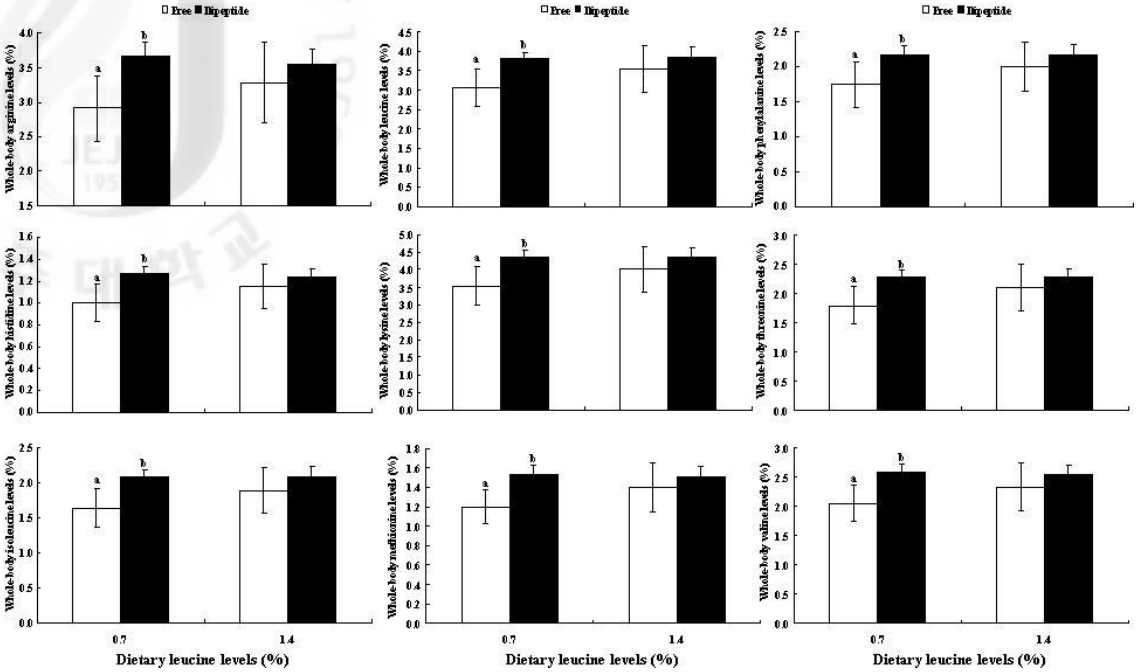

Figure 2-2. Individual essential amino acids (%) in whole-body of juvenile red seabream (*Pagrus major*) fed the experimental diets with different levels or molecular forms of leucine for 6 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different ($P<0.05$).

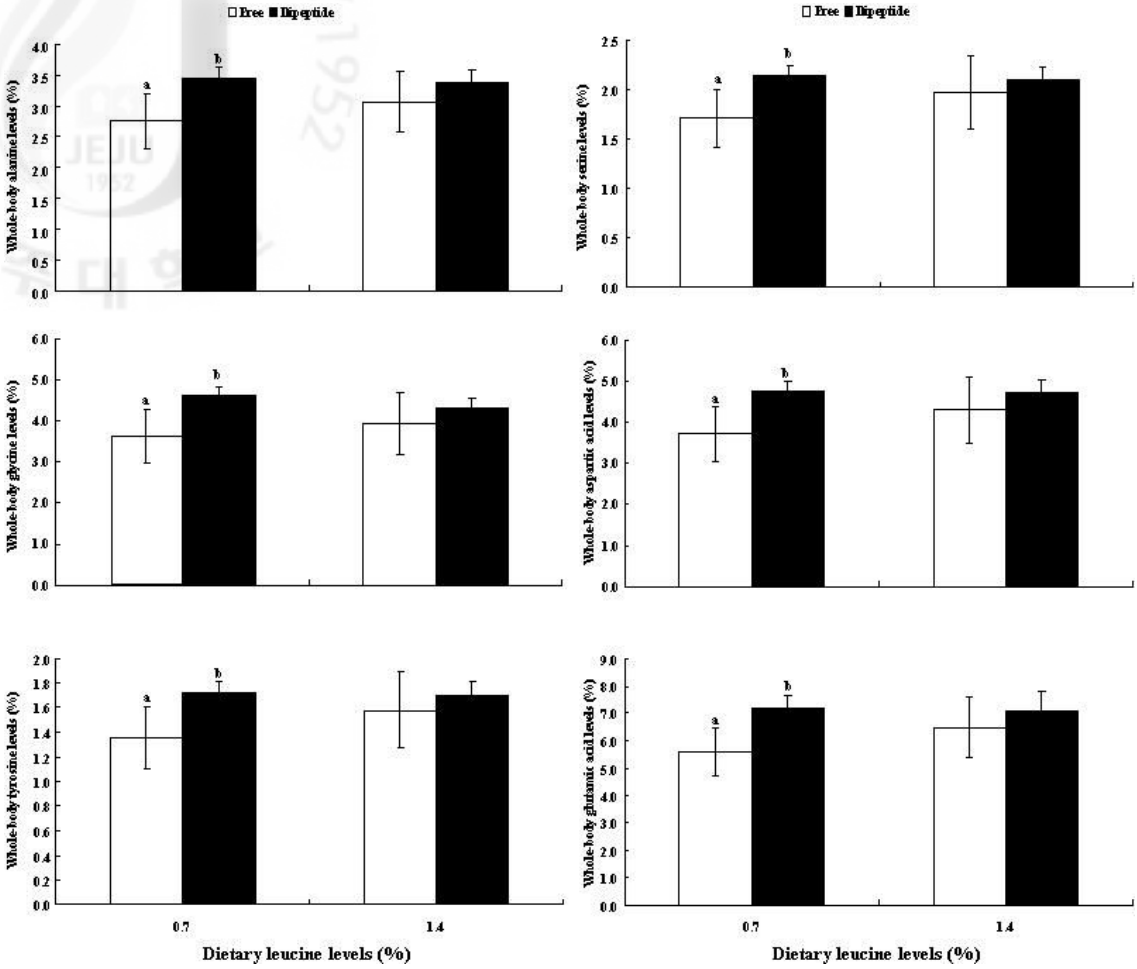

Figure 2-3. Individual non-essential amino acids (%) in whole-body of juvenile red seabream (*Pagrus major*) fed the experimental diets with different levels or molecular forms of leucine for 6 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different ($P<0.05$).

2.4. Discussion

The present study showed that leucine is an essential nutrient for normal growth and improving growth performance of juvenile red seabream. Fish fed the diets supplemented with dipeptide form (Leu-Gly) showed in significantly higher weight gain (WG) than the fish fed the diets supplemented with free form (L-leucine). Free AA in fish diets is absorbed more rapidly than AA digested from intact protein and is not effectively utilized for protein synthesis (Cowey and Walton, 1988; Berge et al., 1994). Crystalline AA is found to be utilized at a lower efficiency than intact protein or dipeptides (Dabrowski et al., 2003). Moreover, several studies in different fish and shrimp species have shown quite convincingly that CAA may be absorbed slightly more rapidly and/or earlier in the gastrointestinal tract than protein-bound amino acids (Murai et al., 1987; Cowey and Walton, 1988; Tantikitti and March, 1995; Zarate and Lovell, 1997; Zarate et al., 1999). The result clearly demonstrated the hypothesis that the previously published data on AA requirements were likely to be over-estimated for many other fish species by the use of crystalline/free AA.

Different dietary leucine levels and molecular forms had significant effect on the whole-body amino acid concentrations of red seabream (Fig 2-1 and 2-2). All the essential and non essential amino acid levels in whole-body were significantly higher in dipeptide groups than free groups at 0.7% dietary leucine level. However, no significant differences were observed in 1.4% dietary leucine level. Several studies have attested that whole-body EAA profile provides a good indicator for estimation of the optimum EAA requirement of fish diets (Akiyama et al., 1997; Green and Hardy, 2002). All the whole-body EAAs composition as well as NEAAs were significantly higher in dipeptide groups at 0.7% levels, indicating that the EAA requirement would be around 0.7%. This result indicates that availability of AAs could be better in fish when

they are fed with dipeptide form instead of free forms.

In conclusion, the present study shows that the availability of AA could be better in fish when AA is provided as dipeptide form rather than free form. Results from the feeding trial clearly demonstrate that AA requirement using free AAs in fish may have been over-estimated in most previous studies. Dieptdies can be used as promising AA source for AA requirement study in fish. The present study indicates that juvenile red seabream requires approximately 0.7-1.4% dietary leucine for optimum growth performance.

CHAPTER THREE

Comparison of growth performance and whole-bldy amino acid compositions in red seabream (*Pagrus major*) fed free or dipeptide forms of phenylalanine

3.1. Introduction

Amino acids (AA) are not only the bulding blocks for protein synthesis but also have regulatory roles in key metabolic functions which are critical for maintenance, growth, feed intake, nutrient utilization, immunity, behavior, larval metamorphosis, reproduction, resistance to environmental stressors and pathogenic organisms in fish (Li et al., 2008). Furthemore, some other beneficial characteristics have been suggested for amino acids, including increment of chemo-attractive properties and nutritional value of low fish meal diets, optimization of metabolic transformation efficiency, suppression of cannibalism, improvement of larval performance and survival (Li et al., 2008).

Fish at their early life stages (Larval and juvenile stages) require higher amino acid levels than older stages, because of both higher growth rate and being amino acids as important source of energy in these stages (Dabrowski, 1986; Ronnestad and Fyhn, 1993; Ronnestad et al., 1999). Also it has been attributed to the underestimated essential amino acid (EAA) requirement in these stages due to the suboptimal growth rates when purified diets were used (Dabrowski, 1986; NRC, 1993).

Amino acids can be supplied either in the forms of protein-bound, free amino acids or

peptides in formulated diets. In mammals it has been proven that absorption of peptides and free amino acids (FAA) are the major transport routes for protein utilization (Abidi, 1997; Ganapathy et al., 1994). In fish the faster absorption of small peptides and FAA, administered through injection to digestive tract prior to metamorphosis, has been shown in comparison to protein (Rust et al., 1993; Ronnestad et al., 2003). Thus, small peptides and FAA can be regarded as promising dietary amino acid resources at early life stages of teleost fish. Moreover, the results of studies on rats, hansters, pigs, humans and fish have revealed the faster absorption of small peptides through the intestinal mucosal cells in comparison to FAAs (Burston et al., 1972; Mathews et al., 1974; Li et al., 1999; Addibi, 1974; Boge et al., 1981).

The results of studies on common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) have revealed the negative utilization of AA when they were fed FAA-based diets in comparison with protein-based diets in term of fish growth (Murai et al., 1982; 1983; Kaushik and Dabrowski, 1983; Dabrowski et al., 2003). It has been suggested that increased deamination or higher total dietary nitrogen excretion through the gill and kidney may be partially the reasons for inferior utilization of FAA-based diets (Dabrowski et al., 2003).

Peptides have different transport systems from free amino acids. It has been shown that tetra- and larger peptides cannot cover the nitrogen requirements in the absence of pancreatic enzymes or lack of brush border peptidase activity (Grimble, 1994; Daniel, 2004), while advantages of di- or tripeptides for specific peptide transporters have been determined on the basis of affinity of PEPT1 transporters (Doring et al., 1998). These transporters were reported to be expressed in teleost larvae prior to exogenous feeding (Verri et al., 2003).

Red seabream (*Pagrus major*) is the most highly valued marine food fish and prominent for

mariculture in Japan and Korea, its culture has increased rapidly (Ikenoue and Kafuku, 1992) and production follows that of yellowtail (Koshio, 2002). This fish is carnivorous, and culture facilities usually provide either minced whole fish as feed, or prepared feeds containing high levels of fish meal. However, with the increasing intensification of culture practices and the increasing demand on fish meal resources, knowledge of the requirement for essential amino acid of the major cultured species becomes imperative for formulation of suitable, cost-effective feeds.

Phenylalanine is an aromatic AA and its requirement is influenced by the level of tyrosine in the diet. Phenylalanine can be converted to tyrosine, however tyrosine cannot be converted back to phenylalanine. Dietary phenylalanine has great impacts on feed intake, growth performance, immunity, and survival of fish in natural environment (Li et al., 2008; Pinto et al., 2008). However, currently a few studies have been conducted to evaluate the effect of phenylalanine supplementation in the diet on aquatic animal.

The present study was done to investigate if red seabream juveniles are able to utilize dipeptide form of phenylalanine as a new protein source, and also to compare the efficacy of dipeptide form with free form in terms of growth performance and whole-body amino acid composition.

3.2. Materials and methods

3.2.1. Experimental design and diets

The compositions and proximate analysis of the experimental diets are shown in Table 2. Four semi-purified experimental diets were prepared containing equivalent of 0.7 or 1.4% phenylalanine per kg diet with different forms of leucine in free or dipeptide. Phenylalanine-Phenylalanine (Phe-Phe) was used as the phenylalanine source for the dipeptide form and crystalline L-phenylalanine was used for the free form. The experimental diets were kept isonitrogenous and isocaloric by decreasing glycine while increasing the two different forms of leucine levels. Fish meal was included to increase palatability of the experimental semi-purified diets. All semi-purified experimental diets were formulated to contain 0.3% leucine from fish meal. A mixture of synthetic free amino acids (FAA) without leucine was prepared according to Dabrowski et al. (2003) and used as the main protein source. All diets were well mixed, pelletized and freeze-dried. The dried diets were then prepared as crumble types and sieved to make proper sizes. The size of the diets was gradually increased over the course of feeding trial as fish grew. Amino acid concentrations of the diets and fish meal are provided in Table 2. Leucine concentration in the diets was confirmed to its intended levels.

Table 3-1. Formulation of the reference diet.

Ingredients	%
Fish meal	7.5
Dextrin	72.5
Vitamin Mix[1]	2.0
Mineral Mix[2]	2.0
Choline chloride	1.0
Squid liver oil	14.0
Taurine	1.0

[1]Vitamin premix (g kg^{-1} of mixture): retinyl acetate, 0.667; cholecalciferol, 0.033; menadione, 0.133; thiamine hydrochloride, 2.667; riboflavin, 2.933; d-pantothenic acid hemicalcium, 9.667; pyridoxine hydrochloride, 2.667; cyanocobalamin, 0.007; niacinamide, 20.000; folic acid, 0.320; d-biotin, 0.133; ascorbic acid, 30.000; α-tocopherol, 6.667.

[2] Mineral mixture was based on the composition of Lee et al., 2003.

Table 3-2. Amino acid concentrations of the fish meal (% dry matter)

Amino acids	Content
Arginine	3.65
Histidine	2.60
Isoleucine	2.59
Leucine	4.97
Lysine	5.46
Methionine	1.84
Phenylalanine	2.60
Threonine	3.05
Valine	3.04

Table 3-3. Composition and proximate analysis of the experimental diets (% dry matter)

Ingredients	Diets (added phenylalanine level %)			
	D-0.7	D-1.4	F-0.7	F-1.4
Casein[1]	8.0	8.0	8.0	8.0
FAA mix[2]	38.0	38.0	38.0	38.0
Phe-Phe[3]	0.3	1.0	0.0	0.0
Phenylalanine[4]	0.0	0.0	0.3	1.0
Glycine[5]	0.7	0.0	0.7	0.0
Dextrin[6]	32.0	32.0	32.0	32.0
Mineral mix[7]	2.0	2.0	2.0	2.0
Vitamin mix[8]	2.0	2.0	2.0	2.0
Squid liver oil[9]	15.0	15.0	15.0	15.0
Choline chloride	1.0	1.0	1.0	1.0
Taurine	1.0	1.0	1.0	1.0
Proximate composition				
Dry matter (%)	6.52	6.59	5.95	5.99
Protein (%, DM)	47.6	46.5	47.0	46.2
Lipid (%, DM)	14.5	14.2	14.6	14.3
Ash (%, DM)	1.14	1.09	1.15	1.11

[1]Casein was purchased from USB Co. Ltd., Cleveland, OH, USA..

[2]Free amino acid mixture composition: (g/1384.11 g dry weight mixture; all L-form amino acids unless otherwise indicated): arginine hydrochloride, 37.8; valine, 37.8 (Fluka, Buchs, Japan); lysine, 45.36; D,L-methionine, 31.5 (WooSung, Daejun, Korea); histidine, 22.05; isoleucine, 28.35; threonine, 25.2; tryptophan, 6.3; proline, 365.4; serine, 365.4; alanine, 362.25 (Sigma Chemicals, St. Louis, MO).

[3]Phe-Phe: Sigma Chemicals, Phenylalanine-Phenylalanine

[4]Phenylalanine: Sigma Chemicals, L-phenylalanine

[5]Glycine: Sigma Chemicals, L-Glycine

[6]Dextrin: Daesung Chemicals

[7]Mineral premix (g kg^{-1}): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[8]Vitamin premix (g kg^{-1}): 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-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[9]Squid liver oil was purchased from E-Wha oil Co. Ltd., Busan, Korea.

Table 3-4. Amino acid concentrations of the experimental diets (% dry matter)

Amino acids	D-0.7	D-1.4	F-0.7	F-1.4
EAA				
Arginine	0.65	0.64	0.64	0.63
Histidine	0.43	0.42	0.43	0.42
Isoleucine	0.58	0.58	0.57	0.58
Leucine	1.01	1.01	0.99	1.02
Lysine	1.28	1.27	1.19	1.20
Methionine	0.50	0.49	0.48	0.49
Phenylalanine	0.64	1.36	0.69	1.39
Threonine	0.44	0.44	0.44	0.44
Valine	0.83	0.83	0.84	0.84
NEAA				
Aspartic acid	0.26	0.27	0.26	0.28
Glutamic acid	7.88	7.74	7.82	7.86
Glycine	8.35	7.57	8.47	7.56
Serine	0.76	0.75	0.74	0.76
Tyrosine	0.18	0.17	0.18	0.18

Values are the means of duplication.

3.2.2. Feeding trials

Juvenile red seabream were transported from a private hatchery to Marine and Environmental Research Institute, Jeju National Univesity, South Korea, acclimated to the experimental facilities and conditions, and fed with a microparticulate diet (Love Larva No. 4, Maruha, Shimonoseki, Japan) for 1 week. Prior to the start of the feeding trial, fish were fed the AAs-free diet (reference diet, Table 1) for 1 week to adjust to the semi-purified diet and deplete possible body reserves of AAs. The conditioned experimental fish averaging at 1.46±0.001 g were then randomly distributed into twenty one 20 L tanks (30 fishes/tank) in a flow through system supplied with sand filtered seawater at a flow rate of 1.5 L/min. Water temperature was between 17 and 20 °C by natural fluctuation in seawater temperature. Dissolved oxygen levels were maintained in excess of 8.0 ppm by aeration and monitored throughout the feeding study. Three replicate groups of fish were hand-fed with the experimental diets to apparent satiation, for 6 weeks. Fish were initially fed six times a day until the 2 week and then were fed four times a day from 3 week. The diurnal cycle was 12-h light/12-h dark. Salinity during the experimental period was approximately 33‰. Growth was measured every two weeks. Feeding was stopped 24 h prior to weighing to minimize stress of the fish. Experimental protocols followed the guidelines approved by the Animal Care and Use Committee of Jeju National University.

3.2.3. Chemical analysis

At the end of feeding trial, all fishes were sampled for whole-body AA analysis. Diets and whole-body samples were freeze-dried and finely ground using a grinder. Crude protein was determined by Kjeldahl method using an Auto Kjeldahl system (Kejltec System 2300, Sweden). Crude lipid was determined by an ether-extraction method. Moisture was determined by oven drying at 105 for 12 h. Ash was determined by muffle furnace at 550°C for six hours. The AA compositions of the experimental diets, and whole-body samples were analyzed using an automatic AA analyzer (Biochrom 30, Pharmacia Biotech, Cambridge, England).

3.2.4. Statical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means were compared with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type 1 error at 5% ($P \leq 0.05$) for each set of comparisons. Data were presented as means ± SD. Percentage data were arcsine transformed before statistical analysis. Statistical analysis between free AA and dipeptide AA groups were conducted via paired Student's t-test to determine significant differences at 5% ($P \leq 0.05$).

3.3. Results

The results of the growth performance are presented in Table 3-5. There were no significant differences among the experimental groups. However, the protein efficiency ratio slightly increased in the groups fed higher phenylalanine levels in both free and dipeptide forms in despite of a decrease in feed intake. Also, feed effiency slightly increased by higher inclusion levels of both free and dipeptide forms. The survival rate was between 90.0 - 97%, without significant differences among treatments.

Total AA concentrations of whole-body are provided in Table 3-6. The obtained results revealed that whole-body amino acid content of fish was not affected by higher inclusion level of phenylalanine. Also similar whole-body AA concentrations were detected when the fish were fed with either free or dipeptide forms of phenylalanine. Interestingly, the fish group fed 0.7 % dipeptide phenylalanine showed higher essential AA concentrations than the fish group fed the same level of phenylalanine in free form (Fig. 3-1 and 3-2). However, no significant differences were detected when they were compared using paired Student's t-test.

Table 3-5. Growth performance of juvenile red seabream fed the experimental diets with different levels of phenylalanine supplement and molecular forms for 6 weeks[1]

Diets	D-0.7	D-1.4	F-0.7	F-1.4
IMW[2] (g)	1.46±0.06	1.46±0.04	1.46±0.06	1.46±0.05
FMW[3] (g)	3.00±0.15	3.00±0.06	2.99±0.19	3.02±0.05
Weight gain[4]	105.8±10.6	105.9±4.0	105.3±13.2	107.0±3.3
FI[5]	95.0±0.5	88.5±2.2	86.8±1.5	84.9±1.3
SGR[6] (%)	1.72±0.12	1.72±0.05	1.71±0.15	1.73±0.04
FCR[7]	2.11±0.17	1.97±0.16	1.96±0.23	1.88±0.12
PER[8]	0.99±0.08	1.10±0.09	1.09±0.14	1.17±0.07
Survival (%)	90.0±3.3	93.3±3.3	96.7	91.1±9.6

[1]Means of triplicate groups; values are presented as mean ± SD.

[2]IMW = Initial mean body weight.

[3]FMW = Final mean body weight.

[4]Weight gain (%) = 100 × (final mean body weight - initial mean body weight)/initial mean body weight.

[5]Feed intake (g/g body weight) = dry feed fed (g)/ body weight (g).

[6]Specific growth rate (%) = [(loge final body weight - loge initial body weight)/days] × 100.

[7]Feed conversion ratio = dry feed fed/wet weight gain.

[8]Protein efficiency ratio = wet weight gain/ total protein fed.

Table 3-6. Amino acid concentrations in the whole-body of juvenile red seabream fed the experimental diets with different levels of phenylalanine supplement and molecular forms for 6 weeks (% dry matter)

Amino acids	D-0.7	D-1.4	F-0.7	F-1.4
EAA				
Arginine	3.70±0.40	3.63±0.72	2.79±0.66	3.66±0.87
Histidine	1.32±0.15	1.30±0.24	0.98±0.24	1.31±0.33
Isoleucine	2.17±0.27	2.10±0.40	1.60±0.37	2.13±0.55
Leucine	3.97±0.46	3.85±0.72	2.96±0.69	3.94±0.96
Lysine	4.49±0.63	4.47±0.82	3.43±0.71	4.53±1.03
Methionine	1.58±0.18	1.56±0.30	1.18±0.26	1.51±0.36
Phenylalanine	2.25±0.28	2.21±0.41	1.71±0.41	2.25±0.54
Threonine	2.29±0.31	2.22±0.47	1.69±0.41	2.31±0.58
Valine	2.65±0.29	2.56±0.46	1.97±0.42	2.58±0.62
NEAA				
Alanine	3.55±0.41	3.60±0.59	2.70±0.63	3.58±0.87
Aspartic acid	4.86±0.56	4.83±0.78	3.59±0.82	4.94±1.04
Glutamic acid	7.17±0.97	7.15±1.30	5.54±1.30	7.37±1.87
Glycine	4.60±0.46	4.68±0.98	3.56±0.83	4.65±1.06
Proline	0.92±1.60	1.78±1.73	1.57±1.39	1.10±1.90
Serine	2.22±0.30	2.14±0.41	1.62±0.38	2.22±0.54
Tyrosine	1.79±0.21	1.75±0.32	1.34±0.32	1.74±0.42

Values are the means of duplication.

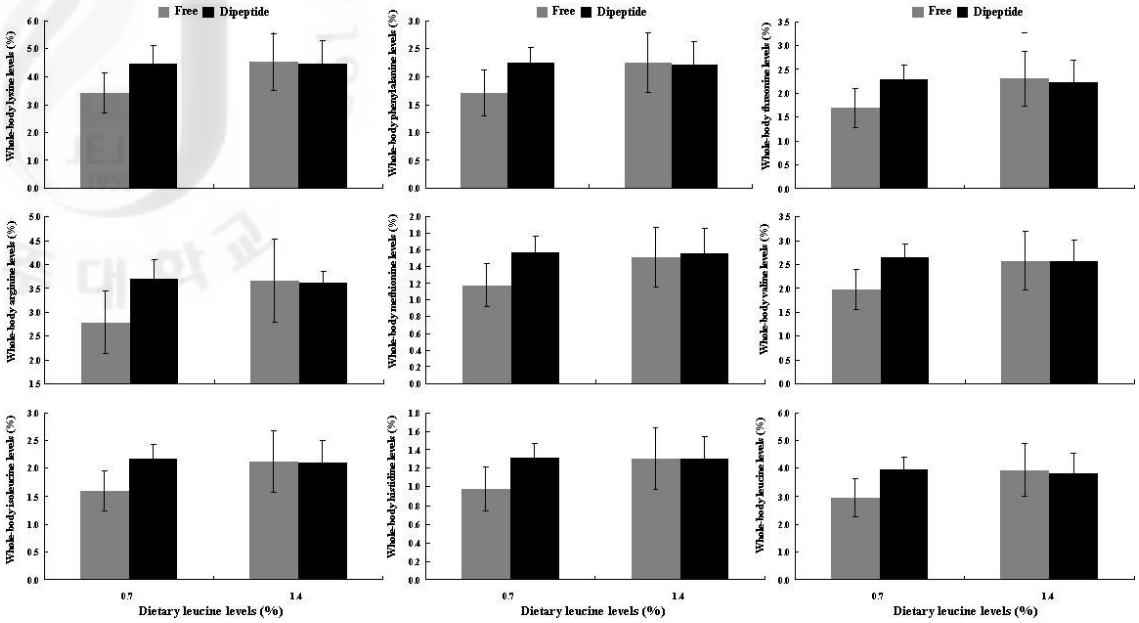

Figure 3-1. Individual essential amino acid (%) in whole-body of juvenile red seabream (*Pagrus major*) fed the experimental diets with different levels of phenylalanine supplement and molecular forms for 6 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different ($P<0.05$) after pared Student's *t*-test.

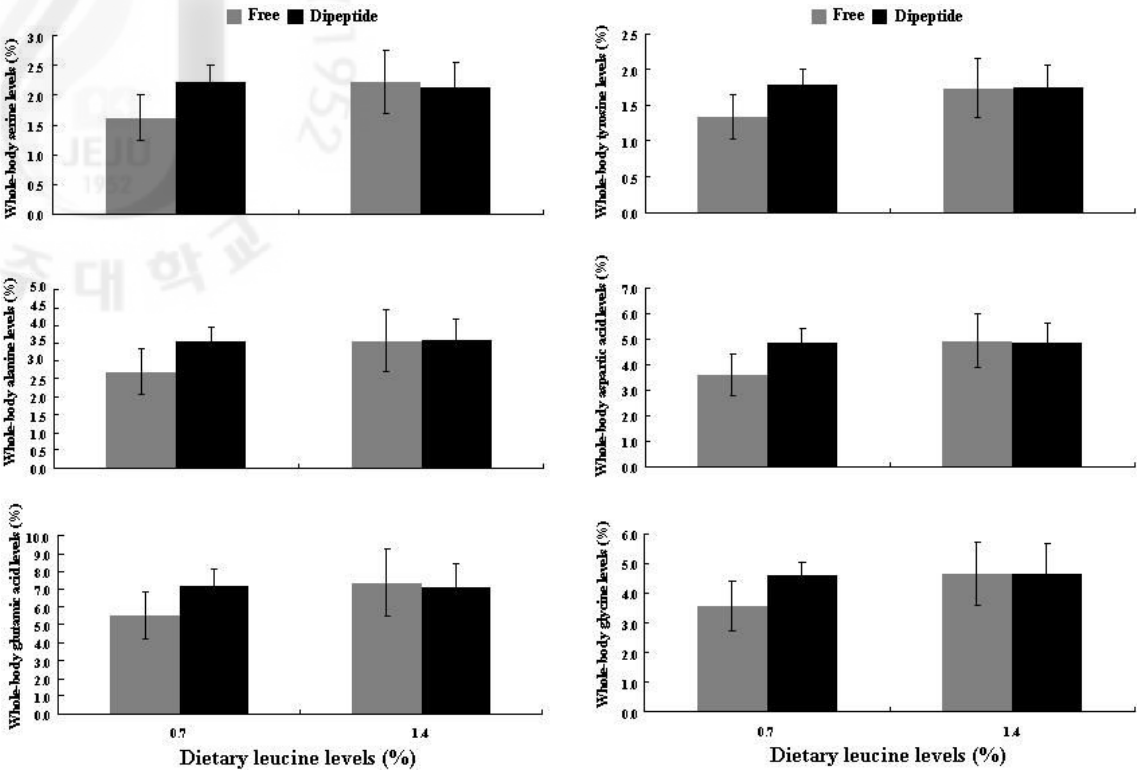

Figure 3-2. Individual non-essential amino acid (%) in whole-body of juvenile red seabream (*Pagrus major*) fed the experimental diets with different levels of phenylalanine supplement and molecular forms for 6 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different ($P<0.05$) after pared Student's *t*-test.

3.4. Discussion

The optimal dietary phenylalanine requirement has been determined in several fish species including Chinook salmon (Chance et al., 1964), common carp (Nose, 1979), channel catfish (Robinson et al., 1984), Nile tilapia (Santiago and Lovell, 1988), rainbow trout (Kim, 1991) and rohu carp (Abidi and Khan, 2007) at the range of 0.5% to 1.7% of diet. In the present study, therefore, only two levels of phenylalanine at 0.7% and 1.4% were used to compare efficiency of different forms of phenylalanine in either free or dipeptide.

Dabrowski et al. (2003) reported for the first time that a dipeptide based diet can support the growth performance of rainbow trout (*Oncorhynchus mykiss*) at early life stages while a FAA based diet could not. In the present study, red seabream juveniles could consume dipeptide phenylalanine with a similar efficiency to its free form but no significant differences were observed in growth performance or feed utilization. Similarly, Tesser et al. (2005) found that juvenile South American pacu (*Piaractus mesopotamicus*) can utilize dipeptide arginine without any adverse effects on growth performance in comparison to free form. Also, Kwasek et al. (2010) have reported the efficient utilization of dipeptides by Koi carp (*Cyprinus carpio*) without any significant differences with FAA in respect to growth performance.

Several studies have attested that whole-body EAA profile can provide a good parameter for the estimation of optimum dietary EAA level in fish (Akiyama et al., 1997). Further, Kwasek et al. (2010) showed that FAA concentrations (especially EAA) in whole-body can be used as an excellent indicator of the availability of dietary AA sources. In the present study, neither essential nor non essential AA levels in whole-body were significantly different among the

experimental groups. In agreement with our results, Zhang et al. (2006) could not find any significant changes in muscle EAA concentrations of common carp (*Cyprinus carpio L.*) larvae fed FAA or dipeptide based diets. But in contrast, Kwasek et al. (2010) found that the concentrations of threonine, arginine, valine, methionine, isoleucine, leucine, phenylalanine and lysine in the whole-body of koi carp fed FAA based diet significantly decreases in comparison to the group fed dipeptide based diet.

Efficiency of EAA utilization is the most important key factor for the evaluation of EAA requirements in fish. The lack of data on the utilization efficiency of the first-limiting EAA is the major weakness in modeling the AA requirements. Available data on the utilization efficiency of the first-limiting EAA in terrestrial animals is still controversial; some authors observed no effect of dietary EAA level on its utilization efficiency at marginal lysine intake (Susenbeth, 1999; Mohn et al., 2000) while others found an increased utilization efficiency of a specific EAA when it was the first-limiting EAA.

In fish, lysine utilization efficiency was estimated only in few studies even though many published studies dealt with the response to variable dietary lysine concentrations (Hauler and Carter, 2001a; Wilson, 2003). In Atlantic salmon fed marginal lysine levels, efficiency of lysine utilization was not affected by lysine intake and was estimated to be between 71 − 78% (Hauler and Carter, 2001b). Hauler and Carter (2001a) further proposed that marginal lysine intake is utilized with a constant efficiency for weight gain in fish. In rainbow trout, however, a decrease in lysine utilization efficiency with an increase in dietary lysine level has been reported (Rodehutscord et al., 1995). A reduction of lysine utilization efficiency with an increase in intake is indeed to be expected due to a reduction of lysine absorption rate or its increased catabolization for energy production or both of them.

In this study, interestingly, the fish fed 0.7% phenylalanine in dipeptide form had higher whole-body AA concentrations than the fish fed 0.7% of free form while no differences were observed in fish fed 1.4% level of both free and dipeptide (Table 3-6). This is very significant result, to our knowledge, because the red seabream fed the marginal level (0.7%) of phenylalanine showed the differences in its whole-body AA compositions accumulating higher AAs from dipeptide than AAs from free form. Therefore, the finding in this study indicates that dipeptide form of AAs can be utilized with higher absorption efficiency compared to free form of AAs in red seabream at the early stages when the AAs were provided with marginal levels.

The first advantage of dietary dipeptides is their attractant properties that can enhance the feed acceptance (Harada, 1989). It is unclear that if vertebrates can grow merely on synthetic dipeptide based diet (Dabrowski et al., 2003). Carvalho et al. (1997) reported that the exclusive use of peptide hydrolysates reduces the fish growth. They found that most of the EAA concentrations in the whole-body of koi carp decreased when they were provided with FAA or dipeptide based diets. This may indicate that EAA provided in the form of FAAs are excreted and/or not used for growth (Murai et al., 1984; Zhang et al., 2006). Absorption of FAAs occurs faster than protein-bound amino acids and may result in AA imbalances resulting in a depression in protein utilization (Rønnestad et al., 2000). Moreover, an excess level of di- and tripeptides can be similarly damaging, due to either the saturation of their transport mechanisms (Verri et al., 2003) or their instant hydrolysis to FAAs (Carvalho et al., 2004). However, better absorption of single peptide compared with a mixture of the equal amino acids has been shown in fish (Boge et al., 1981). It has been shown that partial substitution of protein-bound amino acids by di- and tripeptides of up to 20% enhances the growth performances in European sea bass larvae, while a higher substitution levels resulted in a reduced performance (Zambonino Infante et al., 1997). Also, it has been suggested that the use of different dipeptides by switching

of AA sequence can profoundly affect the absorption characteristics (Daniel, 2004). Dabrowski et al. (2003) declared that intestinal peptide transport and hydrolysis can support growth and protein synthesis in the vertebrates that show poor responses to FAA based diets.

Cytosolic and brush border aminopeptidases are considered to be involved in the utilization of dietary peptides (Cahu and Zambonino, 1995; Kurokawa and Suzuki, 1998). The exact mechanism by which the specific peptide transporters in intestinal brush border epithelium are mediated by composition of dipeptides needs to be elucidated in future studies.

In conclusion, both dipeptide and free form of phenylalanine can be used with similar utilization efficiency. Dipeptide form of AA seems to have higher absorption efficiency than free form of AA when their dietary levels are marginal. The improvement of growth performance with increment of dietary phenylalanine level to an optimum level has been reported in previous studies, while further inclusion level could not result in better growth performance (Ngamsnae et al., 1999). Moreover, the protein source has been regarded as one of key factors in fish phenylalanine requirement (Ngamsnae et al., 1999). According to the better utilization efficiency of dipeptide form in this study, further future studies using different levels of dipeptide are highly recommended for determination of phenylalanine requirement. Also, in the present study dipeptide form of phenylalanine was Phe-Phe. The types of dipeptides, such as Phe-Gly, Phe-Ala and Phe-Pro, can affect the growth performance of the fish. Further studies are needed to determine the optimum dietary phenylalanine requirement in diets for juveline red seabream in details by other types of dipepties.

CHAPTER FOUR

Comparison of leucine requirements in black seabream (*Acanthopagrus schlegeli*) by free and dipeptide forms of leucine

4.1. Introduction

Most microorganisms and plants can synthesize all 20 primary amino acids, while animals should obtain some of the amino acids (AAs) from their diet. It has been reported that fish like other animals require the ten EAAs for growth (Wilson et al., 1980). The quantitative EAA requirement of various fish species has long been studied to attain optimum growth and feed utilization, cost-effective diet formulation, and desirable carcass quality. However, dietary EAA requirement have been quantified for only a few important aquacultured fish species (NRC, 1993).

Crystalline amino acids (CAA) have been used commercially to meet EAA needs of animals for more than 40 years (NRC, 2011). Several studies have shown that CAAs can be utilized as efficiently as those from intact protein in meeting EAA requirements of fish (Murai et al., 1987; Kim et al., 1991; Espe and Lied, 1994; Rodehutscord et al., 1995; Rollin, 1999; Williams et al., 2001; Rollin et al., 2003; Espe et al., 2006). Conversely, other studies have indicated that CAAs appear to be utilized with lower efficiency than EAAs supplied by intact protein (Yamada et al., 1981; Murai et al., 1987; Espe and Njaa, 1991; Schuhmacher et al., 1997; Zarate and Lovell, 1997; de la Higuera et al., 1998; Refstie et al., 2001; Sveier et al., 2001; Liu et al., 2002; Dabrowski et al., 2003; Peres and Oliva-Teles, 2005; Zhou et al., 2007;

Dabrowski et al., 2010).

Several studies in different fish and shrimp species have shown quite convincingly that CAAs may be absorbed slightly more rapidly and/or earlier in the gastrointestinal tract than protein-bound amino acids (Deshimaru, 1976; Yamada et al., 1981; Kaushik and Dabrowski, 1983; Murai et al., 1987; Cowey and Walton, 1988; Zarate and Lovell, 1997; Zarate et al., 1999). This faster and/or earlier absorption may result in temporary higher tissue or plasma concentrations of amino acids provided as CAAs, because of a slight metabolic dyssynchrony with amino acids derived from protein digestion and a greater proportion of the CAA being catabolized (Zarate et al., 1999; Fox et al., 2006). This hypothesis is supported by evidence of better metabolic utilization of CAAs in animals fed more frequently (Tantikitti and March, 1995; Zarate et al., 1999). Reducing the solubility and absorption rate of CAAs using coating, encapsulation, or polymerization techniques reportedly improves the efficiency of utilization of CAAs in fish and shrimp (Dabrowski et al., 2003; Alam et al., 2004; Dabrowski et al., 2010).

Our previous study showed that a synthetic dipeptide leucine (Leu-Gly) based diet producessignificantly higher growth performance than free leucine based diet in olive flounder (not published). Dabrowski et al. (2003) reported that a dipeptide-based diet can support the growth of rainbow trout, whereas a free amino acid-based diet could not. Luo et al. (2005) found that weight gain of the fish fed an intact protein-based control diet was significantly higher than that of the fish fed crystalline AA-based diets in a methionine study, and concluded that crystalline AA is likely to be utilized at a lower efficiency than AA derived from intact protein.

Leucine is essential for the normal growth and important physiological functions of fish. It

is increasingly recognized as an anabolic nutrient signal, communicating the presence of an ingested protein-coating meal to peripheral tissues, and stimulating insulin secretion by the β-cells of the pancreas and protein synthesis in muscle and adipose tissue through the target of rapamycin signaling pathway (Yang et al., 2008). Leucine deficiency resulted in reduced growth and feed efficiency, as well as biochemical malfunctions. The optimal dietary leucine requirement have been reported for some fish speciess including Chinook salmon (Chance et al. 1964), common carp (Nose 1979), channel catfish (Wilson et al. 1980), Japanese eel (Nose, 1979), Nile tilapia (Santiago and Lovell 1988), chum salmon (Akiyama, 1987), lake trout (Hughes et al. 1983) and Indian major carp (Abidi and Khan, 2007) with a range between 1.8% and 3.2% (expressed as percentage of protein).

Black seabream (*Acanthopagrus schlegelii)* (Bleeker) is currently one of the most emerging aquacultured species in Korea because of its desirable taste and high market price. The nutritional value of several plant protein sources have been evaluated for potential use in black seabream feeds (Ngandzali et al., 2011; Zhou et al., 2011). However, to our knowledge, no information has been published concerning the leucine requirements of this species.

EAA requirements of fish are usually determined based on growth rate of fish fed graded levels of the targeted AA in a free form. The objective of this study was to test the hypothesis that the EAA requirement in fishes might have over-estimated in most previous studies which have used a free form of amino acids. Thus, we conducted a feeding trial in this study with different forms of leucine (free or dipeptide) for black seabream to test this hypothesis, and to provide an experimental model for further re-evaluation of AAs requirement.

4.2. Materials and methods

4.2.1. Experimental design and diets

A semi-purified basal diet was formulated to contain 0.4% leucine from fish meal. Fish meal was included to increase palatability of the experimental diets. A mixture of synthetic free amino acids (FAAs) without leucine was prepared according to Dabrowski et al. (2003) and used as the main protein source. Six additional diets were prepared by adding incremental levels (0.3%) of different forms of leucine in free or dipeptide from to the control diet to provide 0.7, 1.0, and 1.3% leucine level in diet. Leucine-Glycine (Leu-Gly) was used as the leucine source for the dipeptide form and crystalline L-leucine was used as free form. The dipeptide, Leu-Gly, was supplemented into the basal diets on the basis of molecular weight of leucine. The experimental diets were kept isonitrogenous and isocaloric by decreasing glycine while increasing the two different forms of leucine levels. All diets were well mixed, pelletized and freeze-dried. The dried diets were then prepared as crumble types and sieved to make proper sizes. The size of the diets was gradually increased over the course of feeding trial as fish grew. The compositions and proximate analysis of the experimental diets are given in Table 4-1. Amino acid concentrations of the fish meal and experimental diets are provided in Tables 4-2 and 4-3. Leucine concentration in the diets was confirmed with its intended levels.

Table 4-1. Composition and proximate analysis of the experimental diets (% dry matter)

Ingredients	Diets (added leucine level %)						
	C-0.4	D-0.7	D-1.0	D-1.3	F-0.7	F-1.0	F-1.3
White fish meal[1]	8.0	8.0	8.0	8.0	8.0	8.0	8.0
FAA mix[2]	42.0	42.0	42.0	42.0	42.0	42.0	42.0
Leu-Gly[3]	0.0	0.5	1.0	1.5	0.0	0.0	0.0
Leucine[4]	0.0	0.0	0.0	0.0	0.3	0.6	0.9
Glycine[5]	1.5	1.0	0.5	0.0	1.2	0.9	0.6
Dextrin[6]	28.0	28.0	28.0	28.0	28.0	28.0	28.0
Taurine[7]	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral mix[8]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin mix[9]	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Squid liver oil[10]	14.0	14.0	14.0	14.0	14.0	14.0	14.0
Choline chloride[11]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Proximate composition							
Dry matter (%)	7.1	7.3	7.2	6.6	7.2	6.8	6.4
Protein (%, DM)	47.3	47.4	47.6	47.3	47.4	47.9	47.6
Lipid (%, DM)	14.5	14.2	14.6	14.3	14.5	14.7	14.9
Ash (%, DM)	2.9	2.7	2.6	2.7	2.2	2.9	2.7

[1]White fish meal was kindly provided from Suhyup Feed Co. Ltd., Uiryeong, Korea.

[2]Free amino acid mixture composition (g/1488.9 g dry weight mixture; all L-form amino acids): arginine, 42; valine, 42; lysine, 50.4; methionine, 35; histidine, 24.5; isoleucine, 31.5; phenylalanine; 63; threonine, 28; tryptophan, 7; proline, 388.5; serine, 388.5; alanine, 388.5 (Sigma Chemicals, St. Louis, MO).

[3]Leu-Gly: Sigma Chemicals, Leucine-Glycine.

[4]Leucine: Sigma Chemicals, L-leucine.

[5]Glycine: Sigma Chemicals, L-Glycine.

[6]Dextrin: Daesung Chemicals

[7]Taurine: Sigma Chemicals.

[8]Mineral premix (g kg^{-1}): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[9]Vitamin premix (g kg^{-1} of mixture): 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-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[10]Squid liver oil was purchased from E-Wha oil Co. Ltd., Busan, Korea.

[11]Choline chloride: Sigma Chemicals.

Table 4-2. Amino acid concentrations of the fish meal (% dry matter)

Amino acids	Content
Arginine	3.65
Histidine	2.60
Isoleucine	2.59
Leucine	4.97
Lysine	5.46
Methionine	1.84
Phenylalanine	2.60
Threonine	3.05
Valine	3.04

Table 4-3. Amino acid concentrationsa of the experimental diets (% dry matter)[a]

Amino acids	C-0.4	D-0.7	D-1.0	D-1.3	F-0.7	F-1.0	F-1.3
EAA							
Arginine	1.59	1.58	1.61	1.54	1.59	1.53	1.61
Histidine	1.01	0.93	1.01	1.01	0.94	0.98	0.97
Isoleucine	1.19	1.10	1.24	1.11	1.29	1.12	1.23
Leucine	0.48	0.72	1.03	1.45	0.74	1.00	1.45
Lysine	2.13	2.08	2.19	2.13	2.04	2.07	2.14
Methionine	1.17	1.13	1.13	1.16	1.16	1.14	1.21
Phenylalanine	2.16	2.13	2.20	2.15	2.16	2.14	2.18
Threonine	0.82	0.80	0.84	0.83	0.82	0.84	0.80
Valine	1.51	1.48	1.50	1.48	1.50	1.52	1.54
NEAA							
Alanine	8.12	7.90	7.92	8.20	8.19	8.15	7.95
Aspartic acid	0.53	0.45	0.56	0.52	0.51	0.49	0.50
Glutamic acid	0.71	0.71	0.77	0.75	0.73	0.70	0.74
Glycine	1.36	1.29	1.34	1.32	1.31	1.20	1.33
Proline	8.74	8.61	8.94	8.68	8.60	8.64	8.50
Serine	8.24	8.42	8.12	8.26	8.51	8.10	8.13
Tyrosine	0.17	0.15	0.16	0.13	0.14	0.12	0.13

[a]Values are the means of triplication.

4.2.2. Feeding trials

Black seabream (*Acanthopagrus schlegeli*) at the early stages were transported from a private hatchery (Muan, Korea) to the Marine and Environmental Research Institute for feeding study (Jeju National University, South Korea). The fish were fed a microparticulate diet (Love Larva No. 1-4, Maruha, Shimonoseki, Japan) for one week to acclimate them to the experimental facilities and conditions. The conditioned experimental fish averaging at 3.23±0.003 g were then randomly distributed into twenty one 20 L tanks (15 fishes/tank) in a flow-through system with sand filtered seawater. The water flow was kept at a rate of approximately 1.5 L/min and water temperature was between 20 and 24 °C by natural fluctuation in seawater temperature. Dissolved oxygen levels were maintained at a proper level over 8.0 ppm by aeration and monitored throughout the feeding study. Three replicate groups of fish were hand-fed the experimental diets to apparent satiation for 4 weeks. Fish were initially fed 5 times per day until 2nd week and then fed 4 times per day from 3rd week. Growth was measured every 2 weeks. Feeding was stopped 24 h prior to weighing to minimize stress on fish. Experimental protocols followed the guidelines approved by the Animal Care and Use Committee of Jeju National University.

4.2.3. Chemical analysis

At the end of feeding trial, all fishes were sampled for whole-body AA analysis. Diets and whole-body samples were freeze-dried and finely ground using a grinder. Crude protein was determined by Kjeldahl method using an Auto Kjeldahl system (Kejltec System 2300, Sweden). Crude lipid was determined by the ether-extraction method. Moisture was determined by oven drying at 105 °C for 24 h. Ash was determined by muffle furnace at 550 °C for 6 h. AA compositions of the experimental diets and whole-body samples were analyzed using an automatic amino acid analyzer (Biochrom 30, Pharmacia Biotech, Cambridge, England).

4.2.4. Statical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means were compared with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type 1 error at 5% ($P \leq 0.05$) for each set of comparisons. Data were presented as means ± SD. Percentage data were arcsine transformed before statistical analysis. Statistical analysis between free AA and dipeptide AA groups were conducted via paired Student's t-test to determine significant differences at 5% ($P \leq 0.05$). The dietary leucine requirement was estimated using the broken-line regression method (Robbins, 1986).

4.3. Results

Growth performance of juvenile black seabream fed the experimental diets with different levels of leucine and two different types of leucine sources, free- or dipeptide, are presented in Table 4-4. The fish fed the leucine in dipeptide form (Leu-Gly) at all the three dietary leucine levels (0.7, 1.0, and 1.3%) had significantly higher weight gain than the fish fed free form (L-leucine) (Fig 4-1). Weight gain and specific growth rate were significantly increased in the fish fed the diets supplemented with free or dipeptide leucine compared to those of the fish fed the control diet containing only the basal level of 0.4% leucine (Fig 4-2). The lowest feed conversion ratio was found in fish fed D-1.0 % diet and highest level was observed in the group fed F-0.7 % diet.Protein efficiency ratio of fish fed the D-1.3% diet was significantly higher than that of the fish fed the C-0.4 and F-0.7% diets. No significant differences were observed in feed intake or survival among experimental groups.

Whole-body protein composition is shown in Fig 4-3. Whole-body protein increased with increasing dietary leucine level. All the dipeptide-fed fish groups had higher whole-body protein than the free groups, even though it was not significant.

The concentrations of total amino acids in the whole-body are given in Table 5. Among the total amino acids, there were no significant differences in any of the essential or non-essential amino acids in the whole-body samples. The free amino acid concentrations of the whole-body are provided in Table 6. Among the free amino acids, the concentrations of leucine and taurine in the whole-body increased with increasing dietary leucine level. However, the concentrations of isoleucine, lysine and valine in the whole-body decreased with increasing dietary leucine level.

The optimum dietary requirement of leucine for olive flounder by broken-line regression analysis was estimated at 1.09 and 0.99% dry diet for free and dipeptide forms, respectively (Fig 4-4 and 4-5).

Table 4-4. Growth performance of juvenile black seabream (Acanthopagrus schlegelii) fed the experimental diets with different levels or molecular forms of leucine for 4 weeks[1]

Diets	C-0.4	D-0.7	D-1.0	D-1.3	F-0.7	F-1.0	F-1.3
IMW (g)[2]	3.18±0.06	3.19±0.04	3.25±0.06	3.22±0.02	3.25±0.03	3.25±0.03	3.25±0.06
FMW (g)[3]	4.40±0.07	5.25±0.03	5.79±0.80	5.71±0.29	4.84±0.22	5.00±0.22	5.24±0.17
Weight gain[4]	38.1±5.0^{a}	64.5±1.4^{bc}	78.3±10.2^{c}	77.4±10.0^{c}	49.0±6.0^{ab}	54.1±8.3^{ab}	61.2±7.7^{bc}
FI[5]	2.84±0.45	2.59±0.57	3.02±1.28	3.68±0.50	4.06±1.90	3.52±0.69	4.05±1.73
SGR (%)[6]	1.24±0.14^{a}	1.91±0.03^{bc}	2.20±0.55^{c}	2.20±0.22^{c}	1.53±0.15^{ab}	1.66±0.21^{ab}	1.83±0.18^{bc}
FCR[7]	2.35±0.38^{c}	1.26±0.29^{a}	1.17±0.22^{a}	1.49±0.18^{ab}	2.54±1.05^{d}	2.05±0.53^{bc}	2.12±1.16^{ab}
PER[8]	0.91±0.16^{a}	1.73±0.35^{bc}	1.84±0.38^{c}	1.43±0.19^{abc}	0.96±0.50^{a}	1.09±0.33^{ab}	1.17±0.49^{abc}
Survival (%)	55.6±13.9	64.4±15.4	62.2±20.4	51.1±10.2	60.0±17.6	66.7±13.3	53.3±23.1

[1]Means of triplicate groups; values are presented as mean ± SD.

[2]IMW = Initial mean body weight.

[3]FMW = Final mean body weight.

[4]Weight gain (%) = 100 × (final mean body weight - initial mean body weight)/initial mean body weight.

[5]Feed intake (g/g body weight) = dry feed fed (g)/ body weight (g).

[6]Specific growth rate (%) = [(loge final body weight - loge initial body weight)/days] × 100.

[7]Feed conversion ratio = dry feed fed/wet weight gain.

[8]Protein efficiency ratio = wet weight gain/ total protein fed.

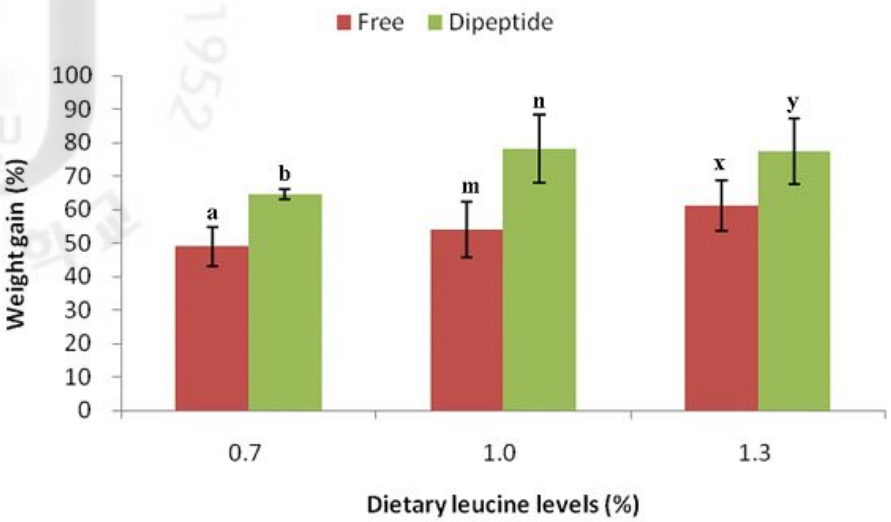

Figure 4-1. Weight gain (%) of juvenile black seabream (*Acanthopagrus schlegelii*) fed the experimental diets with different levels or molecular forms of leucine for 4 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different ($P<0.05$).

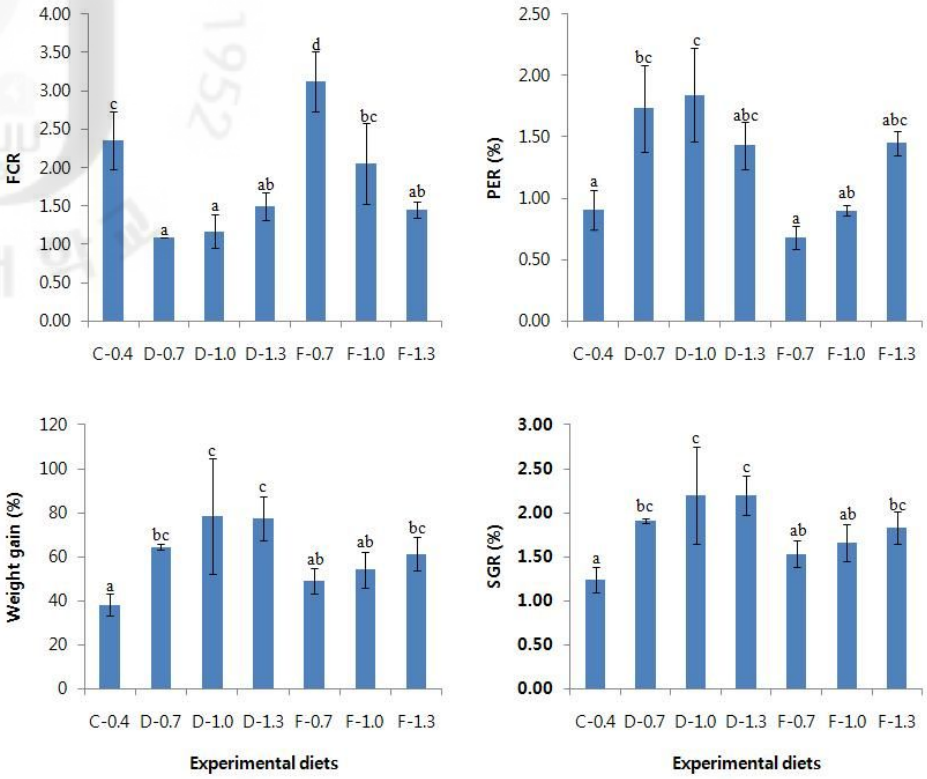

Figure 4-2. Growth performance of juvenile black seabream (*Acanthopagrus schlegelii*) fed the experimental diets with different levels or molecular forms of leucine for 4 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different (P<0.05).

Table 4-5. Amino acid concentrations in the whole-body of juvenile black seabream fed the experimental diets with different levels or molecular forms of leucine for 4 weeks (% of protein)[a]

Amino acids	C-0.4	D-0.7	D-1.0	D-1.3	F-0.7	F-1.0	F-1.3
EAA							
Arginine	6.61±0.13	6.78±0.13	6.86±0.18	6.96±0.22	6.92±0.18	6.99±0.13	6.85±0.05
Histidine	2.29±0.14	2.45±0.02	2.44±0.08	2.49±0.07	2.46±0.09	2.48±0.06	2.45±0.01
Isoleucine	3.38±0.03	4.09±0.09	3.90±0.19	4.10±0.18	4.01±0.16	3.98±0.06	3.95±0.07
Leucine	6.96±0.05	7.40±0.07	7.19±0.27	7.44±0.04	7.30±4.22	7.13±0.06	7.22±0.06
Lysine	7.92±0.06	8.43±0.13	8.18±0.43	8.49±0.34	8.35±0.34	8.39±0.10	8.16±0.09
Methionine	2.58±0.44	2.92±0.02	2.89±0.13	2.99±0.94	2.92±0.06	2.34±0.06	2.88±0.03
Phenylalanine	3.95±0.06	4.17±0.08	4.06±0.15	4.21±0.15	4.15±0.13	4.14±0.06	4.11±0.05
Threonine	4.14±0.15	4.28±0.06	4.24±0.17	4.37±0.21	4.19±0.04	4.33±0.05	4.23±0.06
Valine	4.49±0.09	3.69±1.87	4.55±2.66	4.09±1.57	3.12±0.17	3.66±2.66	3.58±0.07
NEAA							
Alanine	6.78±0.30	6.74±0.21	6.79±0.28	7.00±0.31	6.95±0.18	7.03±0.26	6.83±0.07
Aspartic acid	9.09±0.27	9.35±0.19	9.17±0.40	9.69±0.62	9.24±0.19	9.47±0.26	9.12±0.16
Glutamic acid	13.6±0.16	13.9±0.08	13.7±0.61	14.2±0.71	13.9±0.33	14.2±0.18	13.7±0.09
Glycine	8.32±0.57	7.88±0.26	8.61±0.30	8.41±0.29	8.54±0.11	8.74±0.56	8.45±0.20
Proline	7.37±0.96	8.46±0.54	8.85±0.62	6.47±0.70	7.01±0.97	6.69±1.26	7.46±1.01
Serine	4.02±0.10	3.86±0.22	3.96±0.31	4.05±0.36	3.90±0.19	4.03±0.24	3.74±0.19
Tyrosine	2.94±0.32	3.25±0.03	3.13±0.12	3.27±0.09	3.18±0.07	3.20±0.08	3.19±0.06

[a]Values are the means of triplication.

Table 4-6. The free amino acid concentrations in the whole-body of juvenile black seabream fed the experimental diets with different levels or molecular forms of leucine for 4 weeks (% of protein)[a]

Amino acids	C-0.4	D-0.7	D-1.0	D-1.3	F-0.7	F-1.0	F-1.3
EAA							
Arginine	0.88	0.87	0.99	0.86	0.81	0.79	0.79
Histidine	3.04	3.05	4.12	3.64	3.40	3.44	3.85
Isoleucine	1.17	1.51	0.83	0.66	1.19	0.82	0.75
Leucine	2.32	3.09	3.39	3.50	2.78	2.88	3.12
Lysine	1.90	2.60	1.93	1.68	2.13	1.71	1.50
Methionine	1.48	1.12	1.06	1.09	1.25	1.24	1.28
Threonine	0.91	0.96	1.06	0.84	0.88	0.82	0.97
Tryptophan	1.37	1.26	1.12	1.22	1.42	1.16	0.90
Valine	2.07	2.87	1.73	1.24	2.25	1.58	1.28
NEAA							
Alanine	8.90	7.84	6.15	8.02	7.43	8.01	7.04
Cysteine	0.35	0.29	0.18	0.26	0.24	0.27	0.25
Glutamic acid	2.22	2.43	2.39	2.38	2.24	2.41	2.00
Glycine	4.91	5.34	5.95	6.50	5.01	5.59	5.96
Proline	12.39	17.48	18.70	16.89	16.19	17.71	16.92
Serine	1.52	1.59	1.90	1.37	1.62	2.08	1.38
Tyrosine	1.34	1.07	1.00	0.96	1.11	1.05	1.11
Taurine	29.0	28.3	30.4	34.3	28.0	30.3	32.7

[a]Values are the means of triplication.

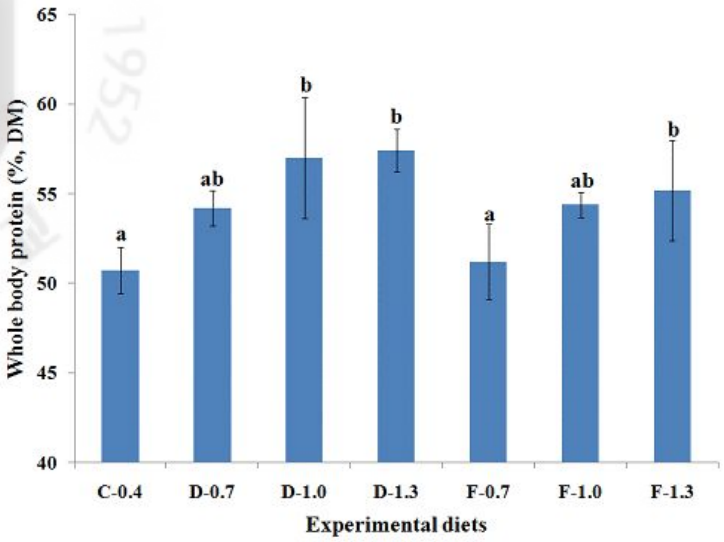

Figure 4-3. Whole-body protein (%, DM) of juvenile black seabream (*Acanthopagrus schlegelii*) fed the experimental diets with different levels or molecular forms of leucine for 4 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different ($P<0.05$).

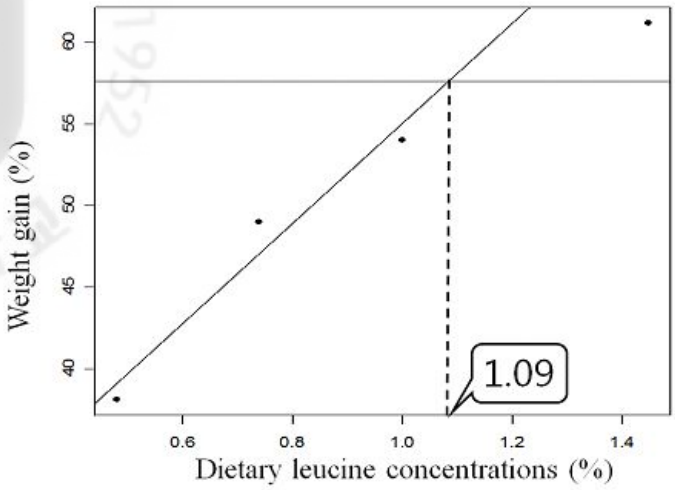

Figure 4-4. Broken-line relationship of weight gain (%) to dietary L-leucine levels as free form. Each point represents the average of three groups of fish.

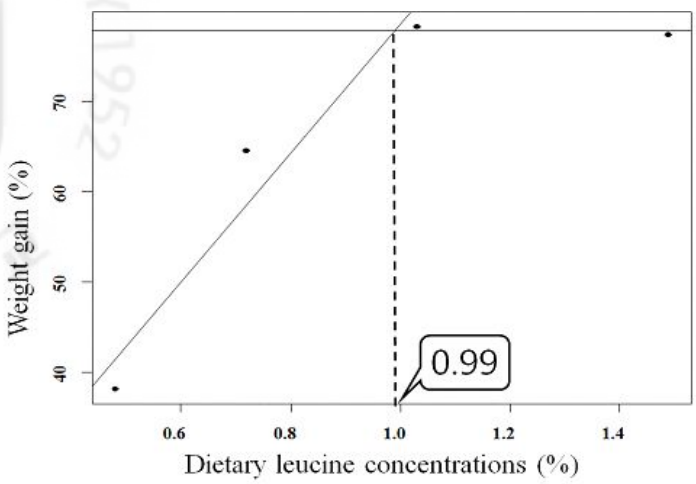

Figure 4-5. Broken-line relationship of weight gain (%) to dietary Leu-Gly levels as dipeptide form. Each point represents the average of three groups of fish.

4.4. Discussion

The results of the present study showed that leucine is an essential nutrient for normal growth and improvement of growth performance in juvenile black seabream. The fish fed the diets supplemented with dipeptide form (Leu-Gly) showed significantly higher weight gain than the fish fed the diets supplemented with free form (L-leucine). There was a positive correlation between WG and increment of leucine supplementation level, regardless of its form. Our previous study showed that a synthetic dipeptide leucine (Leu-Gly) based diet produsedsignificantly higher growth performances than free leucine based diet in olive flounder (not published). Free AA in fish diets is absorbed more rapidly than AA digested from intact protein and is not effectively utilized for protein synthesis (Cowey and Walton, 1988; Berge et al., 1994). Crystalline AAs are found to be utilized at a lower efficiency than intact protein or dipeptides (Dabrowski et al., 2003). Moreover, several studies in different fish and shrimp species have shown quite convincingly that CAA may be absorbed slightly more rapidly and/or earlier in the gastrointestinal tract than protein-bound amino acids (Murai et al., 1987; Cowey and Walton, 1988; Tantikitti and March, 1995; Zarate and Lovell, 1997; Zarate et al., 1999).

Different dietary leucine levels and molecular forms had significant effects on the whole-body protein levels of black seabream (Fig 4-3). Whole-body protein levels increased with increasing dietary leucine level. All the dipeptide-fed fish groups had higher whole-body protein than the free groups, even though the differences were not significant. This result indicates that availability of AAs could be better in the fish when they are fed with dipeptde forms rather than free forms. Also, dipeptides could be used as AA sources in diets for olive flounder juveniles. As noted by the authors (Luo et al., 2007), crystalline amino acids are likely to be utilized at a lower efficiency than amino acids derived from intact protein because more rapid absorption of

the crystalline amino acids results in an amino acid imbalance in the tissue. In turn, this may diver more amino acids away from anabolic to catabolic processes.

Among the free amino acids, the concentrations of leucine and taurine in the whole-body increased with increasing dietary leucine level. On the other hand, the concentrations of isoleucine, lysine and valine in the whole-body decreased with increasing dietary leucine level. Branched-chain amino acids (BCAAs) including isoleucine (Ile), leucine (Leu) and valine (Val) are known to produce antagonistic effects when the proportions of these three amino acids in the diet are imbalanced. Reduction of plasma isoleucine and valine concentration after consumption of an excessive amount of leucine has been reported in rat, chicks, pigs and humans (Block and Harper, 1991; Langer et al., 2000). Leucine-induced changes in plasma levels of isoleucine and valine have mainly been attributed to competitive inhibition during intestinal absorption and increased oxidation through branched-chain α-keto acid (BCKA) dehydrogenase activation (Block and Harper, 1991; D'Mello, 1994; Langer et al., 2000). Chance et al. (1964) observed that excess Leu and Ile in diets depressed growth of Chinook salmon. On the other hand, Hughes et al. (1984) reported that growth depression by excess Leu in lake trout was relieved by Val enrichment. However, Choo et al. (1991) reported that growth depression and abnormal morphology of rainbow trout fed excess Leu diet did not result in antagonistic effects of BCAA but toxicity of the excess Leu itself. Robinson et al. (1984) reported that antagonisms between BCAA did not occur when the three BCAA in diets met the requirements. Fish fed the dipeptide leucine in the present study did not exhibit antagonistic effects such as depressed growth or survival. The results on growth performance in present study indicate that three BCAA in experimental diets probably met the requirements.

The optimum dietary Leu level for the juvenile olive flounder by a broken-line regression

analysis base on weight gain data was estimated to be 1.09 and 0.99% for the free and dipeptide Leu, respectively (Fig. 4-5). The finding in the present study is very significant because, to the best of our knowledge, it is the first report on the essentiality of leucine and its requirement in this species. Lower requirement of leucine for the fish was observed by dipeptide form in comparison to the free form indicating very interesting and important facts that many published data on AA requirements by using crystalline free AA were likely to be overestimated for most fish species. The dietary Leu requirement, 0.99% and 1.09%, by the free or dipeptide form of leucine was quite similar to the values for other fish species such as Nile tilapia (Santiago and Lovell, 1988) and lake trout (Hughes et al. 1983). The lower Leu requirement obtained from dipeptide Leu groups in the present study is mainly attributed to the only difference in molecular forms of Leu. The result clearly demonstrated the hypothesis that the previously published data on AA requirements were likely to be over-estimated for many other fish species by the use of crystalline/free AA.

In conclusion, the present study shows that the availability of AA could be better in fish when AA is provided as dipeptide form rather than free form, and that dipeptides can be used as AA source for AA requirement study in fish. The dietary leucine requirement for black seabream was estimated at 1.09 and 0.99% based on the weight gain for free leucine or dipeptide leucine, respectively, providing the hypothesis that previously published AA requirements by using free AA was likely to be overestimated. It is also suggested that requirements of other nine essential amino acids are needed to be re-evaluated with dipeptides. We conclude that the optimum dietary leucine requirement is 0.99% for optimum growth performance of juvenile black seabream.

CHAPTER FIVE

Comparison of leucine requirements in olive flounder (*Paralichthys olivaceus*) by free and dipeptide forms of leucine

5.1. Introduction

The nutritive value of dietary protein source for fish is mainly influenced by its amino acid (AA) compositions (Wilson and Cowey, 1985; Wilson and Poe, 1985). The quantitative essential AA requirements of various fish species have long been studied to attain the optimum growth and feed utilization, cost-effective diet formulation, and desirable carcass quality (National Research Council, 1993).

Dabrowski et al. (2003) reported that a synthetic dipeptide-based diet can support the growth of rainbow trout during its early stages, whereas a free AA-based diet could not. Luo et al. (2005) found that weight gain of fish fed an intact protein-based control diet was significantly higher than that of the fish fed crystalline AA-based diets in a methionine study, and concluded that crystalline AAs are likely to be utilized at a lower efficiency than AAs derived from intact protein. It is generally thought that the inefficiency of free AAs in fish is due to their faster uptake and subsequent catabolism compared to those from intact protein (Murai et al., 1987; Cowey and Walton, 1988; Dabrowski et al., 2003, Ronnestad et al., 2000; Dabrowski et al., 2007). Another explanation was proposed by higher leaching loss of free AAs than bound AA in aquatic environments (Zarate and Lovell, 1997). Therefore, microencapsulation or coating techniques of nutrients including crystalline AAs have recently been developed and

widely employed to reduce the rate of nutrient uptake (Villamar and Langdon, 1993; De la Higuera et al., 1998), and the leaching rates of free AA from feeds during exposure to water (Segovia-Quintero and Reigh, 2004).

Leucine is an essential AA for optimal growth and health in fish because it stimulates protein synthesis in muscle tissues. It is a keto-genic AA, and is specific among branched-chain AA (BCAA) in its ability to stimulate insulin release from the islet cells of the pancreas (Panten et al., 1974). A leucine deficiency can cause severe nutritional malfunctions including growth retardation. Leucine was proposed as one of the limiting AAs that affects the growth rate of turbot larvae (Conceicao et al., 1997). This might be true for other flatfishes and the requirement has been reported for several fish species including Chinook salmon (Chance et al. 1964), common carp (Nose 1979), channel catfish (Wilson et al. 1980), Japanese eel (Nose, 1979), Nile tilapia (Santiago and Lovell 1988), chum salmon (Akiyama, 1987), lake trout (Hughes et al. 1983) and Indian major carp (Abidi and Khan, 2007).

Olive flounder, *Paralichthys olivaceus*, is one of the most important marine cultured species in Korea, Japan and China. In the past, emphasis was given to verify protein requirements (Lee et al., 2002; Kim et al., 2002; Kim et al., 2003; Kim et al., 2005; Kim et al., 2010) but information regarding AA requirements for this species is still in its infancy. AA requirements of olive flounder have only been studied for lysine (Forster and Ogata, 1998), methionine (Alam et al., 2000; Alam et al., 2001) and arginine (Alam et al., 2002).

Essential amino aicd (EAA) requirements of fish are usually determined based on growth rates of fish fed graded levels of targeted AAs in free form. Dabrowski et al. (2010) hypothesized that the AA requirements in fishes might have been over-estimated in most

previous studies which had used a free form of AAs. Therefore, we conducted a feeding trial with two forms of leucine (free or dipeptide) for olive flounder to test this hypothesis, and to provide an experimental model for further re-evaluation of AAs requirement.

5.2. Materials and methods

5.2.1. Experimental design and diets

A semi-purified basal diet was formulated to contain 0.6% leucine from fish meal. Fish meal was included to increase palatability of the experimental diets. A mixture of synthetic free amino acids (FAA) without leucine was prepared according to Dabrowski et al. (2003) and used as the main protein source. Six additional diets were prepared by adding incremental levels (0.3%) of different forms of leucine in free or dipeptide from to the control diet to obtain 0.9, 1.2, and 1.5% leucine levels in diet. Leucinyl-Glycine (Leu-Gly) was used as the leucine source for the dipeptide form and crystalline L-leucine was used as free form. The dipeptide, Leu-Gly, was supplemented into the basal diets on the basis of molecular weight of leucine. The experimental diets were kept isonitrogenous and isocaloric by decreasing glycine while increasing the two different forms of leucine levels. All diets were well mixed, pelletized and freeze-dried. The dried diets were then prepared as crumble types and sieved to make proper sizes. The size of the diets was gradually increased over the course of feeding trial as fish grew. The compositions and proximate analysis of the experimental diets are given in Table 5-1. Amino acid concentrations of the diets are provided in Table 5-2. Leucine concentration in the diets was confirmed to its intended levels.

Table 5-1. Composition and proximate analysis of the experimental diets (% dry matter)

Ingredients	Diets (added leucine level, %)						
	C-0.6	D-0.9	D-1.2	D-1.5	F-0.9	F-1.2	F-1.5
White fish meal[1]	12.0	12.0	12.0	12.0	12.0	12.0	12.0
FAA mix[2]	39.0	39.0	39.0	39.0	39.0	39.0	39.0
Leu-Gly[3]	0.0	0.5	1.0	1.5	0.0	0.0	0.0
Leucine[4]	0.0	0.0	0.0	0.0	0.3	0.6	0.9
Glycine[5]	1.5	1.0	0.5	0.0	1.2	0.9	0.6
Dextrin[6]	26.0	26.0	26.0	26.0	26.0	26.0	26.0
CMC[7]	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Mineral mix[8]	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin mix[9]	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Squid liver oil[10]	14.0	14.0	14.0	14.0	14.0	14.0	14.0
Proximate composition							
Dry matter (%)	12.3	10.4	10.7	10.9	11.2	11.1	10.8
Protein (%, DM)	43.6	44.0	44.0	43.9	43.8	43.8	43.2
Lipid (%, DM)	8.8	8.6	8.9	8.7	8.9	8.9	8.9
Ash (%, DM)	3.7	3.8	3.7	3.7	3.8	3.8	3.8

[1]White fish meal was kindly provided from Suhyup Feed Co. Ltd., Uiryeong, Korea.

[2]Free amino acid mixture composition: (g/1384.11 g dry weight mixture; all L-form amino acids unless otherwise indicated): arginine hydrochloride, 37.8; valine, 37.8 (Fluka, Buchs, Japan); lysine, 45.36; D,L-methionine, 31.5 (WooSung, Daejun, Korea); histidine, 22.05; isoleucine, 28.35; phenylalanine; 56.7; threonine, 25.2; tryptophan, 6.3; proline, 365.4; serine, 365.4; alanine, 362.25 (Sigma Chemicals, St. Louis, MO).

[3]Leu-Gly: Sigma Chemicals, Leucine-Glycine

[4]Leucine: Sigma Chemicals, L-leucine

[5]Glycine: Sigma Chemicals, L-Glycine

[6]Dextrin: Daesung Chemicals

[7]CMC: Carboxyl methyl cellulose, Sigma Chemicals

[8]Mineral premix (g kg^{-1}): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[9]Vitamin premix (g kg^{-1}): 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-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[10]Squid liver oil was purchased from E-Wha oil Co. Ltd., Busan, Korea.

5.2.2. Feeding trials

Olive flounder at the early stages were transported from a private hatchery (Woodo Fisheries, Jeju, Korea) to a semi-recirculating marine culture system for the feeding study, at Jeju National University, Jeju, South Korea. The fish were fed a microparticulate diet (Love Larva No. 1-4, Maruha, Shimonoseki, Japan) for one week to acclimate them to the experimental facilities and conditions. The conditioned experimental fish averaging at 0.27±0.001 g were then randomly distributed into twenty one 20 L tanks (23 fishes/tank) in line with the semi-recirculation system. The water flow was kept at a rate of approximately 1 L/min and water temperature was maintained at 21°C during the feeding trial by a thermometer (OKE-6422H, Busan, Korea). Dissolved oxygen levels were maintained at over 8.0 ppm by aeration and monitored throughout the feeding study. Triplicate groups of fish were hand-fed with the experimental diets to apparent satiation for eight weeks. Fish were initially fed six times a day until the 5th week and then were fed four times a day from 6th week. Growth was measured every two weeks. Feeding was stopped 24 h prior to weighing to minimize stress on fish. Experimental protocols followed the guidelines approved by the Animal Care and Use Committee of Jeju National University.

5.2.3. Chemical analysis

At the end of feeding trial, all fishes were sampled for whole-body AA analysis. Diets and whole-body samples were freeze-dried and finely ground using a grinder. Crude protein was determined by Kjeldahl method using an Auto Kjeldahl system (Kejltec System 2300, Sweden). Crude lipid was determined by an ether-extraction method. Moisture was determined by oven drying at 105 for 12 h. Ash was determined by muffle furnace at 550°C for six hours. The AA compositions of the experimental diets, and whole-body samples were analyzed using an

automatic AA analyzer (Biochrom 30, Pharmacia Biotech, Cambridge, England).

5.2.4. Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the mean difference was compared with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type 1 error at 5% ($P \leq 0.05$) for each set of comparisons. Data were presented as means ± SD. Percentage data were arcsine transformed before statistical analysis. Statistical analysis between free AA and dipeptide AA groups were conducted via paired Student's t-test to determine significant differences at 5% ($P \leq 0.05$). The dietary leucine requirement was estimated using the broken-line regression method (Robbins, 1986).

5.3. Results

Weight gain of olive flounder at the early stages fed the experimental diets with different levels of leucine and two different types of leucine sources, free- or dipeptide, are presented in Figure 5-1. The fish fed the leucine in dipeptide form (Leu-Gly) had significantly higher growth rate at all the three dietary leucine levels (0.9, 1.2, and 1.5%) than the fish fed free form, L-leucine. Growth rate and survivals were significantly increased in the fish fed the diets supplemented with free or dipeptide leucine compared to those in fish fed the control diet containing only the basal level of 0.63% leucine (Fig.5- 2). All the dipeptide-fed fish groups had significantly higher survival than the control group while the fish fed only the highest level (1.5%) of free leucine was significantly higher than the control.

Table 3 provides whole-body AA compositions of fish fed the experimental diets. The AA compositions of whole-body were not significantly different. However, there were significant differences between the two fish groups fed free and dipeptide forms of leucine (Fig. 5-3). The Student's t-test indicated that all the essential AAs levels in the whole-body were significantly higher in dipeptide groups than free AA groups at 0.9% dietary leucine level. At 1.2% dietary leucine level, whole-body essential AA concentrations were not significantly different except for arginine. At 1.5% dietary leucine level, whole-body leucine, histidine and methioine concentrations were significantly higher in dipeptide groups than free leucine groups while other essential AAs were not significantly different. The fish fed dipeptide diet had significantly higher whole-body non-essential AA concentrations, than the group free AA diet at the level of 0.9%,except for proline. Also the groups fed dipeptide leucine had significantly higher tyrosine than the fish fed free leucine.

The optimum dietary requirement of leucine for olive flounder at the early stages was estimated to be 1.00 and 0.88% (2.27 or 2.0% of dietary protein) based on the broken-line regression analysis for free and dipeptide leucine, respectively (Fig. 5-5).

Table 5-2. Amino acid concentration of the experimental diets (% dry matter)[a]

Amino acids	C-0.6	D-0.9	D-1.2	D-1.5	F-0.9	F-1.2	F-1.5
EAA							
Arginine	1.50	1.34	1.42	1.47	1.41	1.45	1.44
Histidine	0.76	0.69	0.75	0.74	0.72	0.75	0.70
Isoleucine	1.03	0.96	1.07	1.05	1.01	1.04	1.01
Leucine	0.63	0.84	1.20	1.50	0.90	1.15	1.40
Lysine	1.72	1.52	1.69	1.65	1.76	1.68	1.69
Methionine	1.01	0.95	1.01	1.03	0.99	1.04	1.02
Phenylalanine	1.86	1.69	1.77	1.75	1.76	1.79	1.70
Threonine	1.03	0.91	0.96	1.00	0.97	0.98	0.92
Valine	1.46	1.32	1.44	1.43	1.35	1.42	1.34
NEAA							
Alanine	9.80	9.03	9.70	9.67	9.63	10.04	9.67
Aspartic acid	0.83	0.73	0.77	0.78	0.77	0.79	0.76
Cysteine	0.14	0.12	0.13	0.12	0.08	0.13	0.11
Glutamic acid	1.23	1.06	1.17	1.13	1.14	1.14	1.09
Glycine	2.05	1.62	1.44	1.20	1.77	1.48	1.21
Proline	11.63	10.49	10.74	11.30	10.62	9.94	10.30
Serine	8.97	8.07	8.86	9.01	8.38	8.59	8.54
Tyrosine	0.26	0.26	0.24	0.22	0.23	0.25	0.24

[a]Values are the means of duplication.

Table 5-3. Whole-body amino aicd concentration of juvenile olive flounder fed the experimental diets with different levels or molecular forms of leucine for 8 weeks (% dry matter)[a]

Amino acids	C-0.6	D-0.9	D-1.2	D-1.5	F-0.9	F-1.2	F-1.5
EAA							
Arginine	3.47±0.53	4.25±0.74	3.86±0.11	3.91±0.12	3.54±0.17	3.48±0.54	3.67±0.79
Histidine	1.09±0.43	1.45±0.23	1.30±0.02	1.35±0.05	1.14±0.21	1.10±0.50	1.10±0.45
Isoleucine	2.09±0.26	2.54±0.45	2.24±0.02	2.36±0.07	2.19±0.02	2.18±0.29	2.31±0.50
Leucine	3.84±0.51	4.64±0.77	4.12±0.04	4.34±0.18	3.97±0.03	4.00±0.55	4.08±0.63
Lysine	4.18±0.63	5.09±0.79	4.54±0.02	4.73±0.15	4.35±0.04	4.31±0.74	4.57±0.90
Methionine	1.15±0.85	1.85±0.33	1.65±0.02	1.68±0.04	1.33±0.41	1.69±0.12	1.13±0.85
Phenylalanine	2.04±0.43	2.58±0.43	2.27±0.01	2.38±0.10	2.15±0.14	2.09±0.51	2.19±0.60
Threonine	2.20±0.48	2.76±0.44	2.47±0.03	2.55±0.10	2.26±0.13	2.21±0.56	2.35±0.62
Valine	2.64±0.31	3.20±0.54	2.87±0.06	2.99±0.10	2.77±0.03	2.72±0.38	2.90±0.62
NEAA							
Alanine	3.96±0.45	4.77±0.76	4.40±0.12	4.40±0.16	4.08±0.21	4.16±0.47	4.34±0.89
Aspartic acid	4.70±1.25	6.12±1.05	5.44±0.12	5.65±0.23	4.96±0.44	4.77±1.50	5.05±1.48
Cysteine	0.31±0.14	0.48±0.05	0.41±0.01	0.44±0.03	0.32±0.15	0.33±0.19	0.28±0.24
Glutamic acid	7.56±1.34	9.43±1.55	8.32±0.17	8.71±0.35	7.92±0.21	7.68±1.54	8.18±1.95
Glycine	4.76±0.65	5.76±1.11	5.46±0.47	5.11±0.16	4.75±0.31	4.71±0.35	5.00±0.69
Proline	3.18±0.35	3.71±1.00	3.44±0.23	3.36±0.24	3.30±0.25	3.11±0.23	3.33±0.70
Serine	2.63±0.47	3.22±0.51	2.94±0.11	2.99±0.12	2.62±0.31	2.66±0.64	2.74±0.65
Tyrosine	1.31±0.88	2.01±0.33	1.75±0.01	1.85±0.09	1.42±0.52	1.27±0.99	1.24±0.90

[a]Values are the means of duplication.

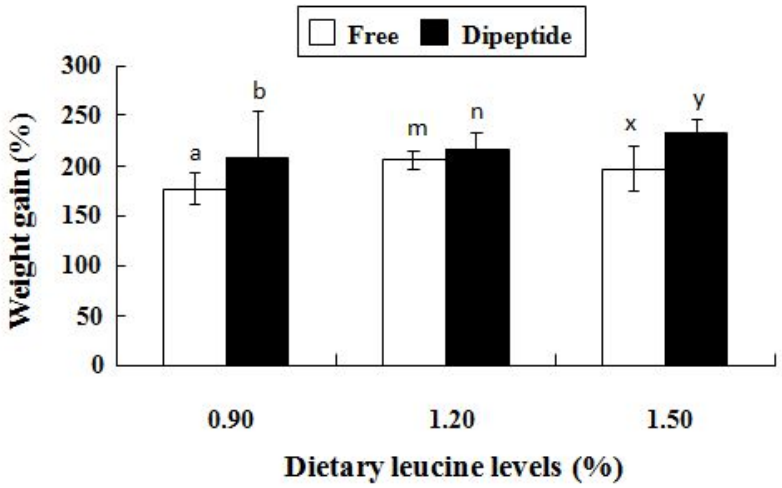

Figure 5-1. Individual weight gain (%) of juvenile olive flounder (*Paralichthys olivaceus*) fed the experimental diets with different levels or molecular forms of leucine for 8 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different (P<0.05).

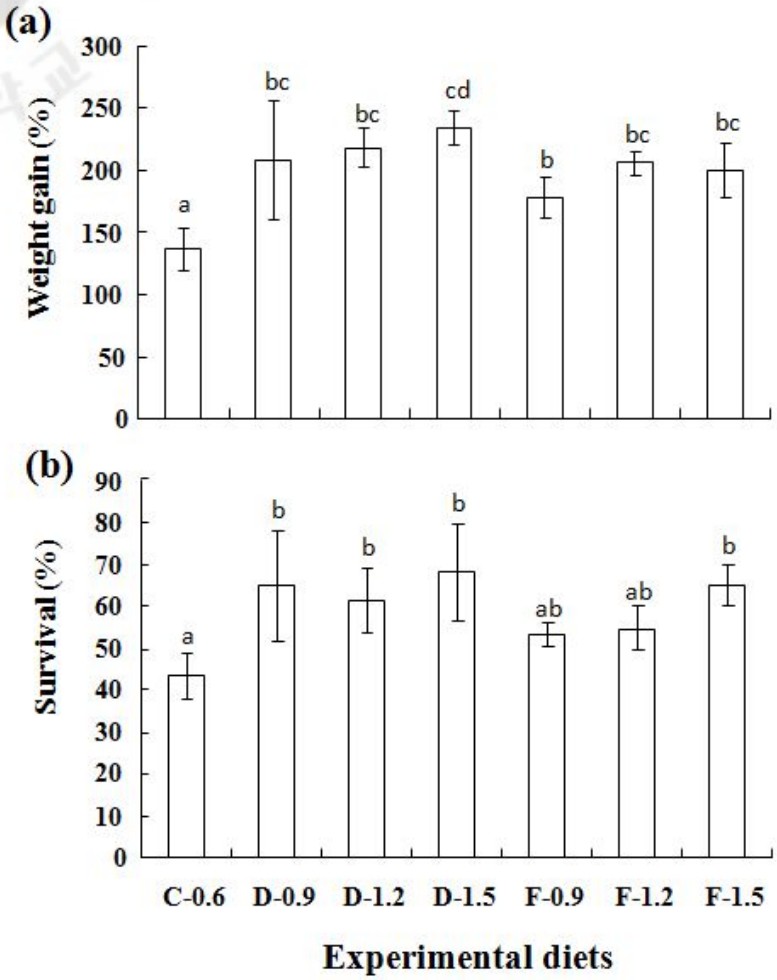

Figure 5-2. Weight gain (a) and Survival (b) of juvenile olive flounder (*Paralichthys olivaceus*) fed the experimental diets with different levels or molecular forms of leucine for 8 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different ($P<0.05$).

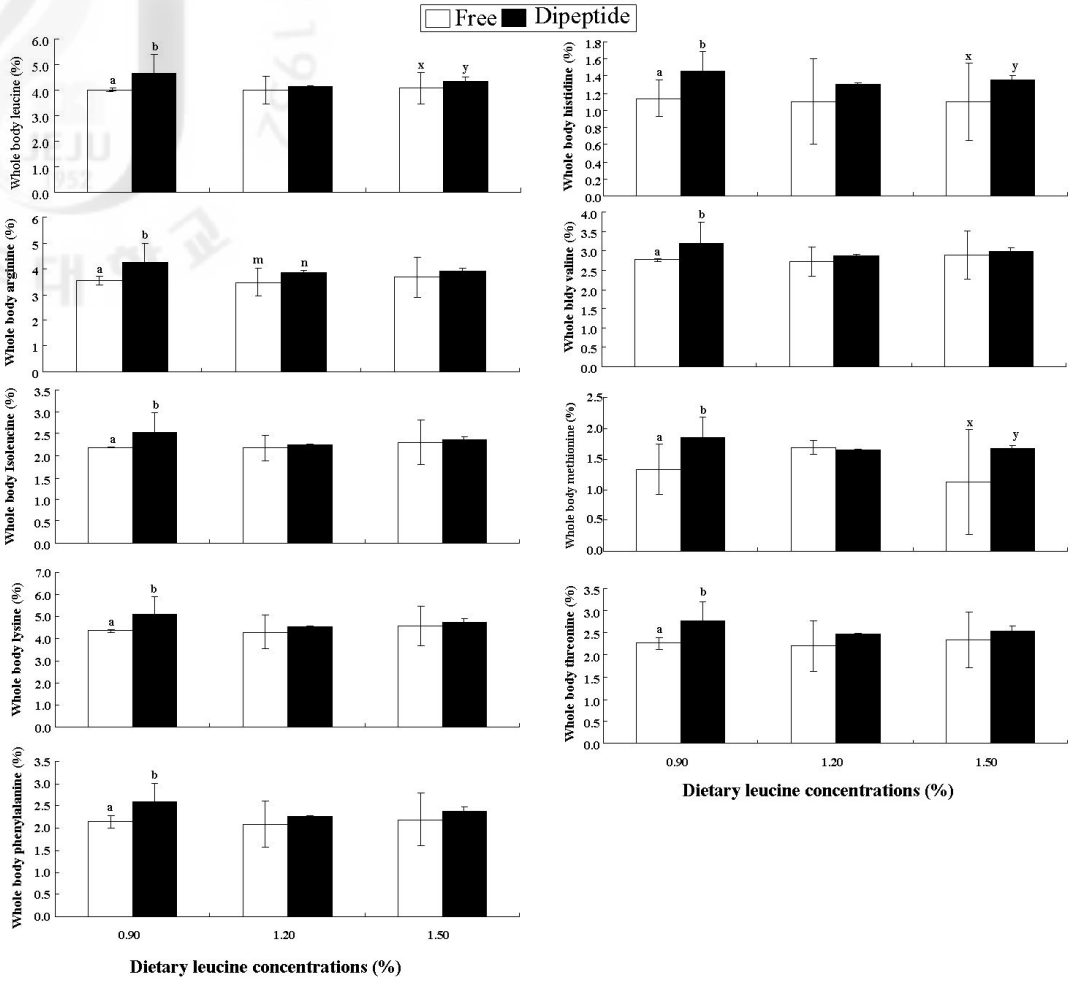

Figure 5-3. Individual essential amino acids (%) in whole-body of juvenile olive flounder (*Paralichthys olivaceus*) fed the experimental diets with different levels or molecular forms of leucine for 8 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different ($P<0.05$).

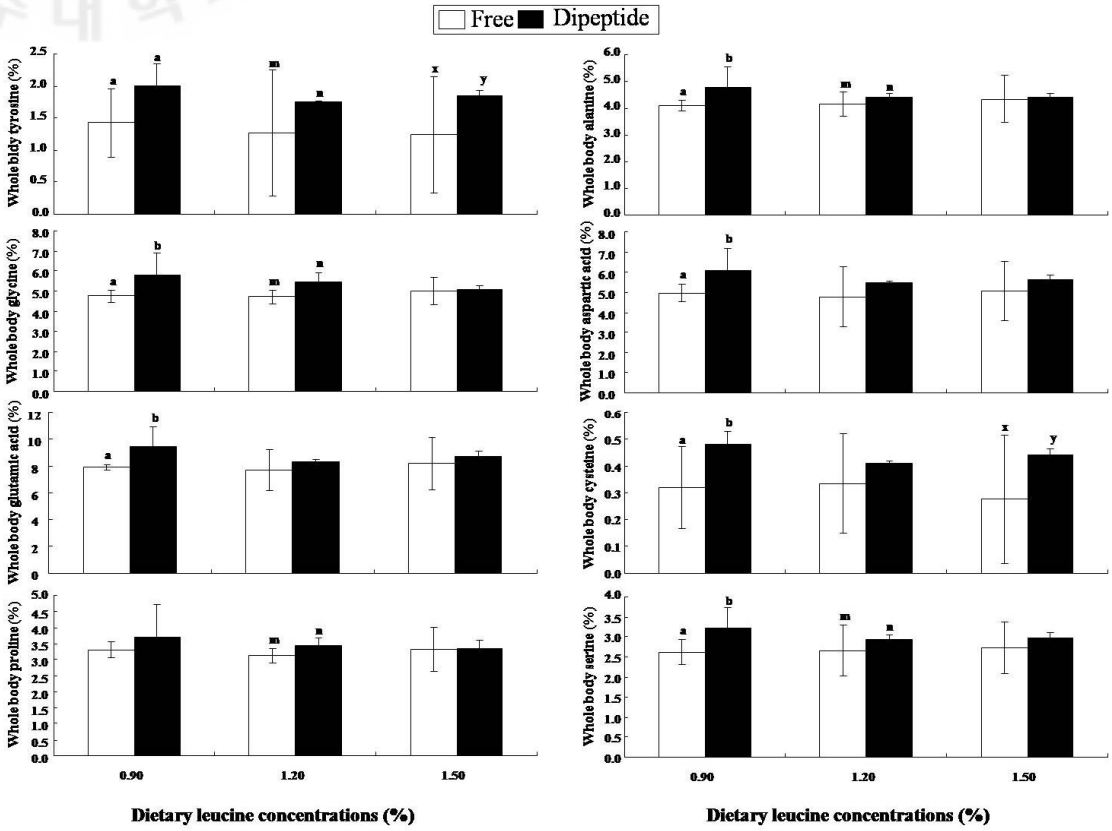

Figure 5-4. Individual non-essential amino acid (%) in whole-body of juvenile olive flounder (*Paralichthys olivaceus*) fed the experimental diets with different levels or molecular forms of leucine for 8 weeks. Values are means ± S.D. (n=3). Bars with different letters are significantly different ($P<0.05$).

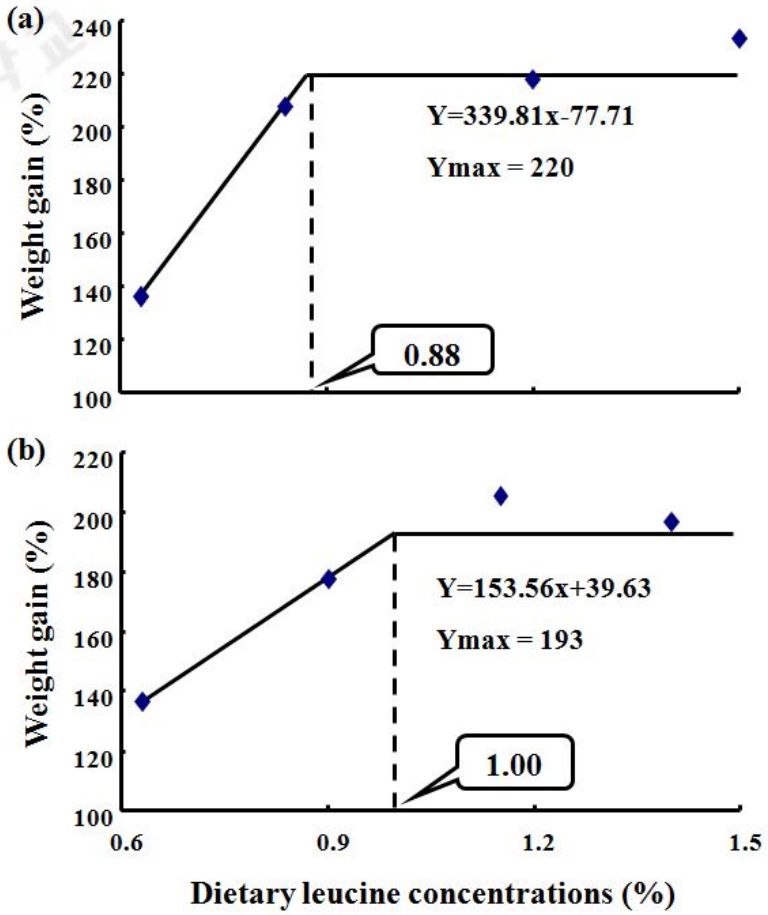

Figure 5-5. Broken-line regression analysis of weight gain (%) against varying levels of dietary Leu-Gly as dipeptide (a) and L-leucine as free form (b). Each point represents the average of three groups of fish.

5.4. Discussion

Fish fed the diets supplemented with dipeptide form (Leu-Gly) showed significantly higher weight gain (WG) than the fish fed the diets supplemented with free form (L-leucine). There was a possitive correlation between WG and increment of leucine supplementation level regardless of its form. Branched-chain amino acids (BCAAs) including isoleucien (Ile), leucine (Leu) and valine (Val) are known to produce antagonistic effects when the proportions of these three amino acids in diets are imbalanced. Chance et al. (1964) observed that excess Leu and Ile concentration in diets depressed growth of Chinook salmon, whereas growth depression by the excess dietary Leu did not occur in lake trout when dietary Val was enriched (Hughes et al., 1984). Also, growth depression or abnormal morphology in channel catfish and rainbow trout by antagonisms between BCAA did not occur when the three BCAAs in diets met the requirements (Robinson et al., 1984; Choo et al., 1991). Fish fed the dipeptide Leu in the present study did not exhibit antagonistic effects such as depressed growth or increased mortality. The results regarding growth performance in this study indicated that the three BCAAs in experimental diets probably met the requirements.

The optimum dietary Leucine level for juvenile olive flounder based on growth data was estimated to be 1.00 and 0.88% for the free and dipeptide Leucine, respectively, by the broken-line regression analysis (Fig. 5-5). Lower requirements of leucine for the fish was determined by dipeptide form compared with free form of AA indicating very interesting and important fact that many published data on AA requirements by using crystalline free AAs were likely to be overestimated for most fish species. The dietary Leucine requirement, 0.88%, in dipeptide form in the present study was lower than the free form of Leucine in other fish species (Chance et al. 1964; Nose, 1979; Akiyama, 1987; Millamena et al. 1999; Abidi and Khan, 2007), while the

requirement, 1.0%, by the free form of Leucine was quite similar to that for other fish species such as Nile tilapia (Santiago and Lovell, 1988) and lake trout (Hughes et al. 1983). The lower Leucine requirement obtained from dipeptide Leucine in the present study is mainly attributed to the only difference in molecular forms of Leucine. The present result clearly demonstrates the hypothesis that the previously published data on AA requirements by the use of free AA were likely to be over-estimated for many other fish species.

The basic mechanism of intestinal absorption of peptides and proteins was begun to be understood by the discovery of free amino acids in gastrointestinal lumen. The understanding of the mechanism was due to discovery of mixed peptidase activity in small intestinal mucosa. The peptides undergo rapid and complete proteolytic degradation in lumen or cells of gastrointestinal tract and then are absorbed only as free AA. However, increasing number of observations of peptides and proteins in the lumen or cells suggested that the absorption of intact peptides is possible (Fricker and Drewe, 1996). Free AAs in fish diets are absorbed more rapidly than AAs digested from intact protein and are not effectively utilized for protein synthesis (Cowey and Walton, 1988; Berge et al., 1994). Crystalline AAs are found to be utilized at a lower efficiency than intact protein or dipeptides (Dabrowski et al., 2003). It was also reported that lower utilization of crystalline AAs can be explained by a finding that more rapid absorption of crystalline AAs can cause AA imbalances in tissues and divert more AAs from anabolic to catabolic processes in juvenile grouper, *Epinephelus coioides* (Luo et al., 2005). Zambonino et al. (1997) reported that partial substitution of whole protein with di- and tripeptides up to 20% appeared to be beneficial for the growth and survival of European sea bass larvae. Williams et al. (2001) found that in Asian seabass, *Lates calcarifer*, fish fed crystalline AAs produced markedly inferior performance than those fed the intact protein diets, and that this was more noticeable when the dietary protein content was sub-optimal. The study by

Williams et al. (2001) could explain that growth difference between free and dipeptide AAs in the present study was more noticeable in sub-optimal level of dietary Leucine (~0.9%). The indispensable AA profile of fish carcass has been commonly used as a good indicator of amino acids requirements (Wilson and Cowey, 1985; Bicudo and Cyrino, 2009). Several studies have attested that whole-body EAA profile provides a good estimation of the optimum EAA profile of fish diets (Akiyama et al., 1997; Green and Hardy, 2002). All the whole-body EAA composition as well as Leucine were significantly higher in dipeptide groups at 0.9% indicating that these EAA requirement would be around 0.9%. Interestingly, this might suggest that a single EAA deficiency can affect other AA compositions in fish regardless of essential or nonessential AAs. Tyrosine of whole-body in all dipeptide groups was significantly higher than in the free groups. The availability of AAs could be better in the fish when they are fed with dipeptide forms than free forms. Also, dipeptide could be used as AA sources in the diet of olive flounder juveniles. As noted by Luo et al. (2007) and Ronnestad et al. (2000), absorption of crystalline amino acids is much faster than protein-bound amino acids, which eventually may lead to AA imbalances and decrease protein utilization.

In conclusion, the results of the present study showed that the availability of AAs could be improved in fish when AAs are provided as dipeptide forms rather than free forms. Dipeptides can be used as an AA source for AA requirement study in fish. The dietary Leucine requirement for olive flounder was estimated at 1.00 or 0.88% (2.27 and 2.00% of dietary protein) for free Leucine or dipeptide Leucine, respectively, proving the hypothesis that previously published AA requirements by using free AA was likely to be overestimated. The optimum dietary Leucine requirement is 0.88% (2.00% of protein) for optimum growth performance of olive flounder at the early stages. It is also suggested that requirements of other nine essential amino acids need be re-evaluated with dipeptides.

CHAPTER SIX

Dietary phenylalanine requirement of juvenile olive flounder (*Paralichthys olivaceus*) by free form of phenylalanine

6.1. Introduction

Ten amino acids are considered essential for normal fish growth (Wilson, 1989). Amino acids are also a major energy source during the larval and juvenile stages of fish (Ronnestad et al., 1999; Ronnestad and Conceicao, 2005). The requirement for individual essential amino acids varies among fish species, often significantly (Wilson, 1991; NRC, 1993). A deficiency of indispensable amino acid results in poor protein utilization and growth retardation, and also lower weight gain and feed efficiency. In severe deficiency cases it leads to lower diseases resistance and efficiency of the immune function. For example, findings have shown that tryptophan-deficient fish becomes scoliotic, showing curvature of the spine (Akiyama et al., 1985) and methionine deficiency produces lens cataracts (Cowey et al., 1992). Thus, there is a direct need to determine the amino acid requirements of the fish species to develop amino acid balanced feed so that adequate amount of essential amino acids could be supplied in the diet for proper growth and reproductive potential.

Phenylalanine is an aromatic indispensableAA, the sole precursor of tyrosine, which is the precursor of thyroxine, required for normal growth and metabolic processes (Khan & Bamji 2007). Tyrosine is an aromatic semi-indispensable AA, the precursor of dopamines and the adrenocortical hormones norepinephrine (NE) and adrenaline. Dopamines regulate central and

peripheric nervous system activity and can therefore be related to the control of stress in fish (Lehnert & Wurtman1993). It has been shown that dietary supplementation of tyrosine reduces acute stress responses such as cold exposure in rodents (Brady, Brown & Thurmond 1980; Lehnert, Reinstein, Strowbridge & Wurtman 1984). Tyrosine is also the precursor of melanin, which plays an important role in fish pigmentation.

The requirements for EAAs is known only for a limited number of cultured fish species including Rainbow trout, Pacific salmon (Chinook, chum, coho), Channel catfish *Ictslurus punctstus*, common carp *Cyprinus carpio* L., Indian major carp (rohu and mrigal), Japanese eel, and tilapia (NRC, 2011). Conversely, requirements for one or more amino acids are known for a number of commonly cultured species (including Cobia, croaker, drum, sea bass, turbot, flounder, and some other species).

Olive flounder, *Paralichthys olivaceus*, is one of the most important marine cultured species in Korea, Japan and China. In previous studies, emphasis has been given to verify its protein requirement (Lee et al., 2002; Kim et al., 2002; Kim et al., 2003; Kim et al., 2005; Kim et al., 2010) but the information on AA requirements of this species is still in its infancy. AA requirement of olive flounder have only been studied for lysine (Forster and Ogata, 1998), methionine (Alam et al., 2000; Alam et al., 2001) and arginine (Alam et al., 2002).

No information is available on the total aromatic amino acid requirement of juvenile olive flounder. Hence, the present study was undertaken to quantify the total aromatic amino acid (Phenylalanine+tyrosine) requirement of juvenile olive flounder.

6.2. Materials and methods

6.2.1. Experimental design and diets

The basal diet (P-0.4) contained the minimum level of phenylalanine, 0.4% of the diet, from the fish meal. Fish meal was included to increase palatability of the experimental semi-purified diets. A mixture of synthetic free amino acids (FAA) without phenylalanine was prepared according to Dabrowski et al. (2003) and used as the main protein source. Incremental levels of L-phenylalanine were added to the basal diet range from 0.4 to 1.9% of the dry diet (designated as P-0.4, P-0.7, P-1.0, P-1.3, P-1.6, and P-1.9, respectively).

The experimental diets were kept isonitrogenous and isocaloric by decreasing glycine while increasing the phenylalanine levels. All diets were well mixed, pelletized and freeze-dried. The dried diets were then prepared as crumble types and sieved to make proper sizes. The size of the diets was gradually increased over the course of feeding trial as fish grew. The compositions and proximate analysis of the experimental diets are given in Table 6-1. Amino acid concentrations of the fish meal and experimental diets are provided in Tables 6-2 and 6-3. Leucine concentration in the diets was confirmed with its intended levels.

Table 6-1. Formulation of the reference diet

Ingredients	%
Fish meal	7.5
Dextrin	72.5
Vitamin Mix.[1]	2.0
Mineral Mix.[2]	2.0
Choline chloride	1.0
Squid liver oil	14.0
Taurine	1.0

[1] Vitamin premix (g kg^{-1} of mixture): retinyl acetate, 0.667; cholecalciferol, 0.033; menadione, 0.133; thiamine hydrochloride, 2.667; riboflavin, 2.933; d-pantothenic acid hemicalcium, 9.667; pyridoxine hydrochloride, 2.667; cyanocobalamin, 0.007; niacinamide, 20.000; folic acid, 0.320; d-biotin, 0.133; ascorbic acid, 30.000; α-tocopherol, 6.667.

[2] Mineral mixture was based on the composition of Lee et al., 2003

Table 6-2. Formulation of the experimental diets (% dry matter)

Ingredients	P-0.4	P-0.7	P-1.0	P-1.3	P-1.6	P-1.9
White fish meal[1]	15.0	15.0	15.0	15.0	15.0	15.0
FAA mix[2]	33.0	33.0	33.0	33.0	33.0	33.0
Phenylalanine[3]	0.0	0.3	0.6	0.9	1.2	1.5
Glycine[4]	1.5	1.2	0.9	0.6	0.3	0.0
Dextrin[5]	32.0	32.0	32.0	32.0	32.0	32.0
Taurine[6]	1.0	1.0	1.0	1.0	1.0	1.0
Mineral mix[7]	2.5	2.5	2.5	2.5	2.5	2.5
Vitamin mix[8]	2.5	2.5	2.5	2.5	2.5	2.5
Squid liver oil[9]	12.5	12.5	12.5	12.5	12.5	12.5
Proximate composition						
Dry matter (%)	12.3	10.4	10.7	10.9	11.2	11.1
Protein (%, DM)	43.6	44.0	44.0	43.9	43.8	43.8
Lipid (%, DM)	8.8	8.6	8.9	8.7	8.9	8.9
Ash (%, DM)	3.7	3.8	3.7	3.7	3.8	3.8

[1]White fish meal was kindly provided from Suhyup Feed Co. Ltd., Uiryeong, Korea.

[2]Free amino acid mixture composition: (g/1384.11 g dry weight mixture; all L-form amino acids unless otherwise indicated): arginine hydrochloride, 37.8; valine, 37.8 (Fluka, Buchs, Japan); lysine, 45.36; D,L-methionine, 31.5 (WooSung, Daejun, Korea); histidine, 22.05; isoleucine, 28.35; threonine, 25.2; tryptophan, 6.3; proline, 365.4; serine, 365.4; alanine, 362.25 (Sigma Chemicals, St. Louis, MO).

[3]Sigma Chemicals, L-Phenylalanine

[4]Glycine: Sigma Chemicals, L-Glycine

[5]Dextrin: Daesung Chemicals

[6]Taurine: Sigma Chemicals

[7]Mineral premix (g kg^{-1}): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[8]Vitamin premix (g kg^{-1}): 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-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

[9]Squid liver oil was purchased from E-Wha oil Co. Ltd., Busan, Korea.

Table 6-4. Amino acid concentrations of the experimental diets (% dry matter)[a]

Amino acids	Content
Arginine	3.65
Histidine	2.60
Isoleucine	2.59
Leucine	4.97
Lysine	5.46
Methionine	1.84
Phenylalanine	2.60
Threonine	3.05
Valine	3.04

[a]Values are the means of duplication.

Table 6-4. Amino acid concentrations of the experimental diets (% dry matter)[a]

Amino acids	P-0.4	P-0.7	P-1.0	P-1.3	P-1.6	P-1.9
EAA						
Arginine	1.78	1.76	1.77	1.79	1.77	1.78
Histidine	0.79	0.77	0.78	0.79	0.79	0.79
Isoleucine	1.03	1.02	1.01	0.99	1.01	1.02
Leucine	2.14	2.18	2.17	2.16	2.15	2.12
Lysine	2.28	2.23	2.24	2.25	2.28	2.26
Methionine	1.12	1.09	1.22	1.14	1.13	1.12
Phenylalanine	0.45	0.68	0.99	1.18	1.57	1.82
Threonine	1.09	1.07	1.06	1.11	1.09	1.08
Valine	1.76	1.73	1.78	1.67	1.76	1.78
NEAA						
Alanine	1.88	1.78	1.86	1.79	1.83	1.84
Aspartic acid	1.17	1.15	1.14	1.18	1.19	1.13
Glutamic acid	8.75	8.77	8.74	8.77	8.76	8.79
Glycine	23.97	22.87	22.25	22.03	21.78	21.69
Serine	0.56	0.53	0.55	0.58	0.52	0.55
Tyrosine	0.37	0.36	0.37	0.36	0.38	0.37

[a]Values are the means of triplication.

6.2.2. Feeding trial

Juvenile olive flounder (*Paralichthys olivaceus*) at the early stages were transported from a private hatchery (Woodo Fisheries, Jeju, Korea) to the Marine and Environmental Research Institute for feeding study, Jeju National University, South Korea. The fish were fed a microparticulate diet (Love Larva No. 1-4, Maruha, Shimonoseki, Japan) for one week to acclimate them to the experimental facilities and conditions. The conditioned experimental fish with mean body weight of 0.81±0.02 g were then randomly distributed into eighteen 20 L tanks (30 fishes/tank) in a flow-through system with sand filtered seawater. The water flow was kept at a rate of approximately 1.5 L/min and water temperature was between 20 and 24 °C by natural fluctuation in seawater temperature. Dissolved oxygen levels were maintained at a proper level over 8.0 ppm by aeration and monitored throughout the feeding study. Three replicate groups of fish were hand-fed the experimental diets to apparent satiation for 6 weeks. Fish were initially fed 5 times per day until 2nd week and then fed 4 times per day from 3rd week. Growth was measured every 2 weeks. Feeding was stopped 24 h prior to weighing to minimize stress on fish. Experimental protocols followed the guidelines approved by the Animal Care and Use Committee of Jeju National University.

6.2.3. Chemical analysis

At the end of feeding trial, all fishes were sampled for whole-body AA analysis. Diets and whole-body samples were freeze-dried and finely ground using a grinder. Crude protein was determined by Kjeldahl method using an Auto Kjeldahl system (Kejltec System 2300, Sweden). Crude lipid was determined by the ether-extraction method. Moisture was determined by oven drying at 105 °C for 24 h. Ash was determined by muffle furnace at 550 °C for 6 h. AA compositions of the experimental diets and whole-body samples were analyzed using an automatic amino acid analyzer (Biochrom 30, Pharmacia Biotech, Cambridge, England).

6.2.4. Statical analysis

All experimental diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means were compared with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type 1 error at 5% ($P \leq 0.05$) for each set of comparisons. Data were presented as means ± SD. Percentage data were arcsine transformed before statistical analysis. The dietary phenylalanine requirement was estimated using the broken-line regression method based on the weight gain (Robbins, 1986).

6.3. Results

Growth performance of juvenile olive flounder were significantly affected by the dietary phenylalanine concentrations (Table 6-5). Weight gain of fish increased with increasing levels of phenylalanine up to 1.0% diet and peaked at 210%, beyond which it showed a declining tendency. Specific growth rate of olive flounder followed the same pattern as WG. The most efficient feed conversion ratio was observed in the group fed 1.0% phenylalanine. Fish fed the diets exceeding 1.0% phenylalanine level did not show any improvement in protein efficiency ratio whereas fish fed the diets with lower phenylalanine level reduced the efficiency of protein utilization. No significant differences were observed in survival among experimental groups.

Brokenline regression model used to determine the relationship between weight gain and dietary phenylalanine levels. The optimum dietary requirement of phenylalanine for olive flounder at the early stages was estimated to be 0.8% based on the weight gain by broken-line regression analysis for free form of phenylalanine (Fig. 6-1).

Table 6-5. Growth performance of juvenile olive flounder (*Paralichthys olivaceus*) fed the experimental diets containing graded levels of phenylalanine for 6 weeks[1]

Diets	P-0.4	P-0.7	P-1.0	P-1.3	P-1.6	P-1.9
IMW (g)[2]	0.82±0.02	0.80±0.01	0.77±0.01	0.81±0.03	0.81±0.02	0.82±0.02
FMW (g)[3]	2.17±0.12	2.29±0.07	2.40±0.12	2.45±0.08	2.34±0.19	2.26±0.25
Weight gain[4]	166±20.5^{a}	186±3.5^{ab}	210±13.7^{b}	204±21.9^{ab}	188±16.2^{ab}	176±36.5^{ab}
FI[5]	84.5±7.9^{ab}	81.0±1.4^{a}	80.1±1.8^{a}	83.7±10.1^{ab}	93.0±6.3^{b}	84.3±3.6^{ab}
SGR (%)[6]	2.32±0.19^{a}	2.50±0.03^{ab}	2.69±0.11^{b}	2.64±0.17^{ab}	2.52±0.13^{ab}	2.40±0.31^{ab}
FCR[7]	2.09±0.19^{b}	1.81±0.07^{ab}	1.65±0.16^{a}	1.71±0.30^{ab}	2.04±0.09^{ab}	1.99±0.27^{ab}
PER[8]	0.97±0.09^{a}	1.11±0.04^{ab}	1.23±0.11^{b}	1.20±0.23^{ab}	0.99±0.04^{ab}	1.02±0.14^{ab}
Survival (%)	56.7±13.4	53.5±14.6	55.7±12.7	58.9±7.64	60.0±17.6	58.9±6.48

[1]Means of triplicate groups; values are presented as mean ± SD.

[2]IMW = Initial mean body weight.

[3]FMW = Final mean body weight.

[4]Weight gain (%) = 100 × (final mean body weight - initial mean body weight)/initial mean body weight.

[5]Feed intake (g/g body weight) = dry feed fed (g)/ body weight (g).

[6]Specific growth rate (%) = [(loge final body weight - loge initial body weight)/days] × 100.

[7]Feed conversion ratio = dry feed fed/wet weight gain.

[8]Protein efficiency ratio = wet weight gain/ total protein fed.

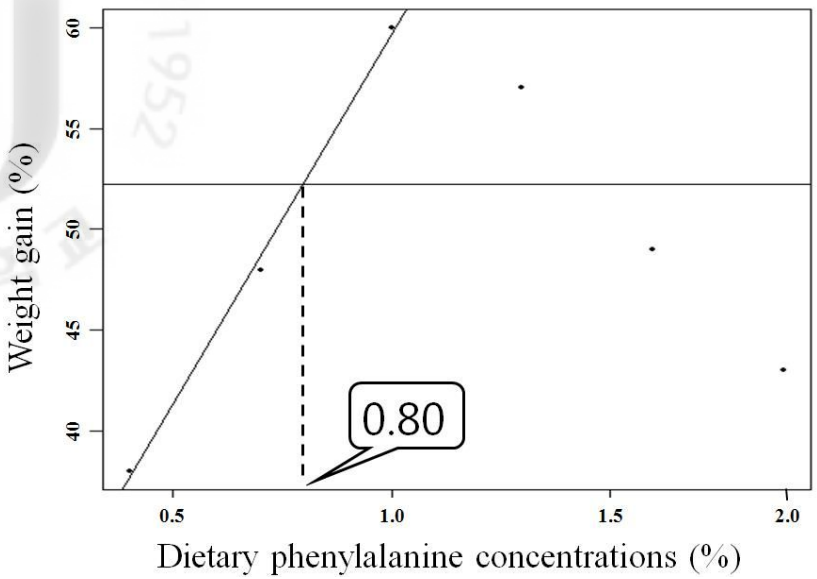

Figure 6-1. Broken-line regression analysis of weight gain (%) against dietary phenylalanine levels. Each point represents the average of three groups of fish.

6.4. Discussion

The present study showed that phenylalanine is an essential nutrient for normal growth and improvment of growth performance in juvenile olive flounder. The optimum dietary requirement of phenylalanine was found to be 0.8% of the dry diet for the species. The finding in the present study is very significant in feed formulation for the species because, to our knowledge, this is the first report on the essentiality and requirement level of phenylalanine in this species. This value is similar to values reported for certain other species, such as rainbow trout (0.7%, Kim, 1993), Channel catfish (0.5%, Robinson et al., 1980), and lower than that for Chinook salmon (1.7%, Chance et al., 1964), Common carp (1.3%, Nose, 1979), Rohu carp (1.2%, Khan and Abidi, 2007), Mrigal carp (1.3%, Ahmed, 2009) and Nile tilapia (1.1%, Santiago and Lovell, 1988). The differences are probably due to differences in fish speices, feeding behavior, feed compositions, rearing conditions and nutritional status of fish.

During the 6-week feeding trial, dietary supplementation of phenylalanine significantly influenced growth performance and feed utilization in the juvenile olive flounder. The growth performance measured as weight gain, specific growth rate, feed conversion ratio and protein efficiency ratio increased linearly with increasing level of dietary phenylalanine up to 1.0% of the diet, after which it decreased as the phenylalanine level increased. High dose of dietary some nutrients could result in negative growth performance in fish. In the study fingering Indian major carp exhibited poor growth performance and feed utilization compared to the fish fed an optimum dietary arginine level (Ahmed and Khan, 2004). The toxic effects by a high or mega dose of dietary some nutrients have been reported with respect to growth performance in other fish species. In the present study, inclusion of phenylalanine at higher level than 1.0% of the diet did not improve growth performance. This may be the result of excessive intake of

phenylalanine resulting in poor growth because disproportionate amount of one amino acid affects the utilization of the other amino acids (Coloso et al., 1999). This result was similar to previous studies on Indian major carp (Ahmed and Khan, 1999), juvenile silver perch (Ngamsnae et al., 1999), and juvenile gilthead seabream (Marcouli et al., 2006).

In conclusion, phenylalanine should be supplemented in the diets for juvenile olive flounder. The findings in the present study suggest that an optimum level of dietary phenylalanine would be approximately 0.8% of the diet for maximum growth performance and feed utilization.

SUMMARY

The purposes of this dissettation are to (1) study the utilization of dietary amino acids (different forms of amino acids) in marine fishes during early life stages (larvae and juvenile), (2) to determine the optimum dietary essential amino acids requirements in marine fishes by free or dipeptide forms of amino acids in terms of their growth performance and concentration of whole-body amino acids.

Most microorganisms and plants can synthesize all 20 primary amino acids, while animals should obtain some of the amino acids (AAs) from their diet. It has been reported that fish require the same ten indispensable amino acids as other animals for growth (Wilson et al., 1980). The quantitative essential amino acid (EAA) requirements of various fish species have long been studied to attain the optimum growth and feed utilization, cost-effective diet formulation, and desirable carcass quality. However, dietary EAA requirements have been quantified for a few number of fish raised in Aquaculture (NRC, 1993).

Experiment 1 (Chapter 2) is about the utilization efficiency of dipeptide and free forms of leucine in the diets of juvenile red seabream. This study was conducted to investigate the utilization of the amino acid, figure out the optimum dietary leucine level, and to compare the growth performance by dipeptdie and free forms of leucine. Juvenile red seabream (BW: 1.21 g) were fed one of four experimental diets for 6 weeks which were formulated to contain 45% crude protein with two levels of leucine levels of 0.7 and 1.4% by different forms of leucine (free and dipeptide, L-leucine or Leu-Gly) (designated as D-0.7, D-1.4, F-0.7 and F-1.4, respectively). At the end of the feeding trial, the fish fed the leucine in dipeptide form (Leu-Gly) had significantly higher weight gain at all the two dietary leucine levels (0.7, and 1.4%) than the

fish fed free form, L-leucine. All the essential and non essential amino acid levels in whole-body were significantly higher in dipeptide groups than free groups at 0.7 and 1.4% dietary leucine levels. Results from the feeding trials clearly demonstrated that AA requirement using free AAs in fish may have been over-estimated in most previous studies. Dieptdies can be used as promising AA source for AA requirement study in fish. The present study indicates that juvenile red seabream requires approximately 0.7-1.4% dietary leucine for optimum growth performance.

Experiment 2 (Chapter 3) is about the utilization of dipeptide and free forms of phenylalanine in diets of juvenile red seabream. This study was conducted to evaluate the efficacy of dipeptide form of phenylalanine as a new source of amino acid in terms of growth performance and whole-body amino acid composition in comparison to free form for red seabream (*Pagrus major*). Fish (1.46±0.001 g) were fed four isonitrogenous and isocaloric experimental diets containing 0.7 or 1.4% phenylalanine either in free or dipeptide form. A feeding trial was carried out in three replicates and the fish were fed to apparent satiation for six weeks. At the end of the experiment all the fish in each tank were counted and weighed for evaluation of survival and growth performances, sampled for whole body amino acid composition analysis. The results showed that growth performance and survivals of red seabream were not significantly affected by the changes in phenylalanine form or inclusion level. Whole-body amino acid compositions revealed no significant changes in concentrations of both essential and non-essential amino acids regardless of the increase in phenylalanine levels or the use of its different forms. The finding in this study indicates that juvenile red seabream can utilize dipeptide phenylalanine as efficiently as free form without any undesirable effects on growth performance or whole body amino acid composition.

Experiment 3 (Chapter 4) focuses on comparison of leucine requirements in black seabream (*Acanthopagrus schlegeli*) by free and dipeptide forms of leucine. A four-week feeding trial was carried out to provide an innovative experimental model with different forms of leucine (free or dipeptide) for black seabream, and to re-evaluate the previous results on dietary requirement ofleucine. Triplicate groups of fish (average weight, 3.23± 0.03 g) were fed seven isonitrogenous and isocaloric experimental diets containing 0.4, 0.7, 1.0 and 1.3% leucine in either free or dipeptide form (designated as C-0.4, D-0.7, D-1.0, D-1.3, F-0.7, F-1.0 and F-1.3, respectively). After four weeks of feeding trial, the fish fed the diets supplemented with dipeptide leucine showed significantly higher growth performance than the fish fed free leucine. Whole-body protein levels increased with increasing dietary leucine level. The concentrations of leucine and taurine in the whole-body increased with increasing dietary leucine level. However, the concentrations of isoleucine, lysine and valine in the whole-body decreased with increasing dietary leucine level. The requirements of leucine in diets for the fish were estimated at 1.09 or 0.99% based on broken-line regression analysis for free or dipeptide leucine, respectively. The results clearly demonstrated that the AA requirement using free AAs in fish may have been over-estimated in most previous studies. Dipeptides can be used as promising AA source for AA requirement study in fish. This study indicates that juvenile black seabream requires 0.99% dietary leucine for optimum growth performance.

Experiment 4 (Chapter 5) focuses on the comparison of leucine requirements in olive flounder (*Paralichthys olivaceus*) by free and dipeptide forms of leucine. We report a promising solution to solve the overestimated essential amino acid (AA) requirements in culture of fish species. An eight-week feeding trial was carried out to provide an innovative experimental model with different forms of leucine (free or dipeptide) and to re-evaluate the previous results on dietary requirements of essential AAs. Triplicate groups of olive flounder at the early stages

(average weight, 0.27± 0.001 g) were fed seven isonitrogenous and isocaloric experimental diets containing 0.6, 0.9, 1.2 and 1.5% leucine in either free or dipeptide form (designated as C-0.6, D-0.9, D-1.2, D-1.5, F-0.9, F-1.2 and F-1.5, respectively). After eight weeks of the feeding trial, the fish fed the diets supplemented with dipeptide leucine showed significantly higher growth performance than the fish fed free leucine. Whole-body AA concentrations and survival rate were significantly higher in the fish fed dipeptide leucine than the fish fed the basal diet without leucine supplementation. The requirements of leucine in diets for the fish were estimated at 1.00 or 0.88% (2.27 or 2.00% of dietary protein) based on broken-line regression analysis for free or dipeptide leucine, respectively. The results demonstrated that the AA requirement using free AAs in fish may have been over-estimated in most previous studies. AAs are more available to the fish when AAs are provided in dipeptide rather than free forms. Dipeptides can be used as promising AA source for AA requirement study in fish. The dietary leucine requirement is suggested to be 0.88% (2.00% of dietary protein) for optimum growth performance of olive flounder.

Experiment 5 (Chapter 6) focuses on the optimum dietary phenylalanine requirement in juvenile olive flounder (*Paralichthys olivaceus*) by free form of phenylalanine. This study was conducted to determine the optimum dietary phenylalanine requirements for the optimum growth of juvenile (initial weight, 0.81 ± 0.02g) olive flounder. Six semi-purified diets were formulated to contain 45% crude protein with six graded levels of phenylalanine levels of 0.4, 0.7, 1.0, 1.3, 1.6 and 1.9% (designated as P-0.4, P-0.7, P-1.0, P-1.3, P-1.6 and P-1.9, respectively). Each diet was fed to triplicate groups of fish in a flow-through system for 6 weeks. At the end of the feeding trial, growth of the fish fed the P-1.0 diet was not significantly different from that of the fish fed 0.7, 1.3, 1.6 or 1.9% dietary phenylalanine, but it was significantly higher than that of the fish fed the 0.4% diet. No improved growth performance

was observed beyond 1.0% dietary phenylalanine levels (1.3-1.6%). The requirement of leucine in diets for the fish was estimated at 0.8% based on broken-line regression analysis.

The results from this stdy clearly demonstrated the hypothesis that the amino acid requirements in fish might have been over-estimated in most previous studies. The availability of amino acids could be better in fish when they are fed with dipeptides forms rather than free forms. Dipeptides can be used as promising AA source for AA requirement study in fish. Therefore, it is assumed that an accurate amino acids requirement could be estimated by a “New experimental model for amino acids requirement” from this study. It is also suggested from this study that the requirement of other essential amino acids should be re-evaluated based on this model.

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2012년 01월 05일

새로운 출발을 위해 늦은밤 사무실에서

LIST OF PUBLICATIONS

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1. **Sung-Sam Kim**., Kyeong-Jun Lee. 2012. Comparison of leucine requirements in olive flounder (*Paralichthys olivaceus*) by free or dipeptide forms of leucine. Aquaculture (Submitted, November, 2011)
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