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Intestinal absorption of amino acids in the Pacific bluefin tuna (*Thunnus orientalis*): *In vitro* uptake of amino acids using hydrolyzed sardine muscle at three different concentrations

Emmanuel Martínez-Montaño [a], Emyr Peña [a], Ulfert Focken [b], María Teresa Viana [c,*]

[a] Programa de Maestría y Doctorado en Oceanografía Costera, Facultad de Ciencias Marinas, Universidad Autónoma de Baja California (UABC), Ensenada BC, Mexico
[b] Department of Aquaculture Systems and Animal Nutrition in the Tropics and Subtropics, University of Hohenheim, 70593 Stuttgart, Germany
[c] Instituto de Investigaciones Oceanológicas, UABC, PO Box 453, Ensenada BC 22860, Mexico

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ABSTRACT

The absorption rates of different amino acids (AAs) were compared using the everted intestine technique with fresh proximal intestinal sections from the Pacific bluefin tuna (*Thunnus orientalis*). Sardine muscle was hydrolyzed in two steps; first with porcine pepsin, followed by an alkaline pancreatic bluefin tuna extract. The hydrolyzed extract was tested in triplicate samples at three concentrations (0.14, 0.09, and 0.06 mg of soluble protein mL^{-1}). In general, the non-essential amino acids (NEAAs; mainly Ala, Glu, and Gly) were assimilated in preference to the essential amino acids (EAAs). It was observed that certain AAs, such as Arg and Lys, had more affinity with the cationic transporters than did His. The glucogenic AA Glu always displayed double the absorption rate observed for Asp, probably to satisfy an energy requirement of the cells. The neutral AAs, Ala and Gly, showed the highest absorption rates at all concentrations. Other AA relations are also discussed. This is the first study that evaluates the use of this gastrointestinal system to estimate AA absorption and kinetics from a natural pool of AAs obtained from sardine muscle hydrolyzed with bluefin tuna digestive enzymes.

1. Introduction

Pacific bluefin tuna (*Thunnus orientalis*) mariculture is one of the fastest-growing aquatic industries (FAO, 2007) and has become quite important in México over the last 5 years (Zertuche-González et al., 2008) with exports of up to 4350 metric tons (mt) per year at an average price of US$20,000/mt. The production method involves the capture of juveniles into net cages near the coastline, followed by a fattening period of 4–9 months. Pacific bluefin tuna are usually fed with fresh or frozen Pacific sardine (*Sardinops sagax*) or mackerel, and occasionally whole squid. According to the statistics for bluefin tuna production, an estimated 20,000–30,000 mt of fresh feed needs to be consumed in order to obtain the commercial product required for the international market. Thus, the feed conversion ratio (FCR) when using fresh or frozen sardine is calculated to be 17 kg of sardines per kilogram of bluefin tuna (Sylvia, 2007), which is more than four times higher than that reported by Naylor et al. (2000) as the average for carnivorous marine fish in aquaculture. In a parallel study, Zertuche-González et al. (2008) demonstrated that the sardine fishery has not been similarly affected by this new commercial activity since the maximum sustainable yield of 250,000 mt per year has not yet been attained. However, since there is great concern about using fresh high-quality fish such as sardine to feed farmed tuna, resulting in a negative overall production due to the FCR mentioned above, the development of formulated diets undoubtedly needs to be investigated.

Protein is the most important component in feeds, not only because of its high economic cost and high requirements in carnivorous fish diets but also because growth promoters are associated with some protein sources (Wilson, 2002). Protein quality is determined by many factors, such as amino acid profile and digestibility, which are both important for improving protein turnover (protein ingestion *vs.* protein accretion) and decreasing the organic content excreted in feces. The study of digestibility involves examining the digestion and absorption of nutrients for a given species, which is essential knowledge in aquaculture in order to determine the nutritional requirements of the species. However, this evaluation traditionally requires the collection of fecal material, which is a very difficult or impossible task in many aquatic species (Bureau et al., 1999). Moreover, even if these studies could be carried out, it would still be necessary to confine the animals in tanks, which for some species is not an option, either due to their pelagic behavior or their physical size. In consequence, the development of an *in vitro* methodology to represent the physiological digestion and absorption processes will undoubtedly be an important step toward the development of compound feeds.

* Corresponding author. UABC, PO Box 453, Ensenada BC 22860, Mexico. Tel.: +52 646 1745462x190; fax: +52 646 1745303.
E-mail address: viana@uabc.mx (M.T. Viana).

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All cells require a continuous supply of amino acids (AA) to meet their metabolic needs. These AAs can be obtained by endocytosis by means of transport proteins or paracellular movement by carrier-independent passive diffusion. Amino acids can also be transported by ion gradient-independent carriers and by energy-dependent carriers coupled with ionic gradients (Ferraris and Ahearn, 1984). Transport systems are generally defined as proteins which recognize and transfer a selected group of substrates across the cellular membrane, either acting singly or in combination with other proteins (Matthews, 2000). Transport systems for AAs have been grouped into families according either to their required energy sources, kinetics of absorption, and/or substrate specificities, such as carriers for anionic (basic), cationic (acids), neutral (zwitterionic), and imino amino acids (Matthews, 2000).

Several *in vitro* and *in vivo* methods have been reported for measuring AA absorption and absorption, including the incubation of gut samples, everted intestine or plasma studies in radiolabeled medium, and nutrient absorption by cellular culture tissues (Ferraris and Ahearn, 1984; Buddington et al., 1987; Hidalgo and Li, 1996; Berge et al., 2004). Recently, in our laboratory, Rosas et al. (2008) used the everted intestine system to compare the absorption rates of AAs in various carnivores from freshwater and marine habitats. The innovation in this approach was the evaluation of AA intestinal absorption in a dynamic manner using the whole complex mixture of AAs from a commercial tryptone (Bacto™ Tryptone; Difco Cat. No. 211705), a pancreatic digest of casein consisting of a mixture of single AAs, filtered with an Amicon ultrafiltration cell (10 kDa cut-off). However, it is still necessary to develop more complex *in vitro* systems, which simulate not only absorption but also digestive processes, in order to generate more knowledge on the bioavailability of protein or AA supplements under the environmental conditions normally found, and then to use this information to formulate novel aquafeeds.

Thus the aim of this work was to evaluate AA *in vitro* uptake in the proximal intestinal region of the Pacific bluefin tuna (*T. orientalis*) using an AA pool obtained from fresh sardine hydrolyzed with a pancreatic enzyme extract from the bluefin tuna.

2. Materials and methods

2.1. Fish samples

Pacific bluefin tuna (15–18 kg bodyweight) were obtained from a commercial sea farm (Maricultura del Norte S de RL de CV, Ensenada BC, México). The pancreas tissue and/or intestinal section (12 fish) were removed and transported in iced and oxygenated Ringer solution (at 4 °C) for further investigation at the laboratory (1 h away) as recommended by Rosas et al. (2008) to avoid a decrease in cell viability.

The pancreas tissue was sliced and homogenized to obtain a pool sample from 12 fish, using one volume of pancreatic tissue per two volumes of iced saline solution (10% NaCl according to Viana and Raa, 1991). The homogenate was then centrifuged for 30 min at 3000 *g* at 4 °C and the supernatant was decanted. Alkaline protease activity was determined using azocasein prior to storing the supernatant at −80 °C until it was ready to be used for the hydrolysis of feed proteins.

The intestinal section just after the pyloric ceca (referred to as the proximal intestine) was sliced, placed in iced marine Ringer solution (117 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM $KH_2PO_4 \cdot 7\ H_2O$, 2.5 mM $CaCl_2$; pH 7.4; Buddington et al., 1987), with an additional 150 mM NaCl to simulate the ion strength in marine organisms according to Berge et al. (2004). The intestine slices were utilized immediately upon arrival at the laboratory.

2.2. Experimental procedures

To simulate the digestion process in tuna, a protein hydrolyzate solution from sardine muscle was prepared. For this purpose, acid digestion followed by an alkaline enzyme treatment was carried out using porcine pepsin followed by a bluefin tuna pancreatic homogenized enzyme pool as described above and outlined in Fig. 1.

The first stage of hydrolysis (acid step) was performed according to Alarcón et al. (2002). Here, an appropriate amount of muscle from sardine (*Sardinops sagax caerulea*) to yield 1.6 g of protein dry weight was homogenized, sieved through a 200-μm mesh, and dissolved with 180 mL of distilled water. The pH was adjusted to 2.0 with 1 M HCl solution and stirred for 30 min at 37 °C. To start the hydrolysis reaction, 20,000 U of porcine pepsin (Sigma P-7012) was added, at 12.5 U per mg protein, which is equivalent to the pepsin enzyme activity reported for the bluefin tuna (Matus-de-la-Parra et al., 2007). The solution was maintained at 37 °C and the activity was stopped after 1 h by adjusting the pH to 7.8 with 1 M NaOH. The alkaline step of hydrolysis was performed using the pH STAT system, as recommended by Navarrete-del-Toro and García-Carreño (2002). In summary, to start the reaction, a 10-mL enzymatic extract from the bluefin tuna pancreas was added (63.8 ± 0.5 U of alkaline protease activity). The reaction mixture was maintained for 2 h at 37 °C and the degree of hydrolysis (DH) was calculated according to Navarrete-del-Toro and García-Carreño (2002) as follows:

$$DH = B \times N_B \times 1 / \alpha \times 1 / M_P \times 1 / h_{tot} \times 100\%$$

where B is the volume (in mL) of 0.1 mol L^{-1} NaOH solution required to maintain the pH of the reaction mixture at 8.0; N_B corresponds to the normality of the titrant; α is the average dissociation degree of the α-amino groups; M_P is the quantity of protein (in g) present in the reaction mixture; and h_{tot} is the sum of individual AAs (mmol per gram protein) associated with the used protein source. The hydrolyzed solution was then centrifuged for 15 min at 3000 *g* at 4 °C and the supernatant was decanted and stored in an ice bath for immediate use.

2.3. Amino acid absorption analysis in proximal intestine

The technique using everted intestine is a modification of a system previously reported by Rosas et al. (2008). Briefly, a fragment of everted proximal intestine previously rinsed with iced marine Ringer solution was attached to a glass tube (9.5 mm i.d.) with an elastic bandage. The serous layer of the intestine (serosa) was then facing toward the inside of the tube, and the intestinal mucosa was facing outward so that it would come into direct contact with the hydrolyzed protein. The exposed area for absorption corresponded to 0.785 cm^2. The tube with the attached intestinal fragment was then placed in a specially designed flask with 25 mL volume capacity (Fig. 2). Oxygen was bubbled through a thin glass tube located to one side of the glass tube.

All the intestinal fragments were pre-incubated for 5 min at room temperature (22 °C) in marine Ringer solution constantly gassed with a mixture consisting of 95% O_2 and 5% CO_2. After being bubbled, the marine Ringer solution was exchanged for the experimental hydrolyzed solutions consisting of hydrolyzed sardine muscle made with the digestive enzymes from Pacific bluefin tuna, as previously described, at three different concentrations: 0.14 ± 0.01, 0.09 ± 0.01, and 0.06 ± 0.003 mg mL^{-1}. A control group was incubated in marine Ringer solution on its own, in order to correct for any amino acids released from the endogenous protein (Awati et al., 2009).

Each of the glass tubes with intestinal fragments attached was filled with 300 μL of marine Ringer solution and the tubes were submerged in flasks containing the hydrolyzed solutions. A 95/5 (%) O_2/CO_2 mixture was gassed through the flasks to keep the solution oxygenized. Individual samples of 30 μL were taken from each sampling tube at 0, 15, and 30 min and stored at −80 °C until analyzed. Samples taken at time 0 were considered to be the specific blank for each replicate. Individual samples were evaluated for AA content