INFLUENCE OF DIET PEPTIDE CONTENT ON SURVIVAL, GROWTH AND DIGESTIVE ENZYMES ACTIVITIES OF JUVENILE CUTTLEFISH SEPIA OFFICINALIS

E. LE BIHAN*, A. PERRIN, N. KOUETA

Laboratoire de Biologie et Biotechnologies Marines, Université de Caen, 14032 Caen cedex, France

Corresponding author: estlebihan@yahoo.fr

ABSTRACT. – Juvenile cuttlefish hatched in the laboratory were reared for 40 days with different enriched diets (LBBMA4 or LBBMA25, which are viscera silages). Survival was 100% in every group. Cuttlefish fed with enriched diet had better growth and conversion rate. These results indicate that autolysed proteins from enrichment can be used in juvenile cephalopod nutrition. Differences observed in juvenile cuttlefish fed with non-enriched food and cuttlefish receiving enriched food are due for a large part to the protein and peptides contained in the enrichments. In fact, LBBMA4 contains less peptides than LBBMA25, whereas the content in proteins (> 20kDa) was similar. Low molecular weight peptides present in enriched shrimps are also responsible for the remaining specific activity of proteolytic enzymes in the juvenile cuttlefish fed with enriched shrimp. Moreover, high concentration of carbohydrates in enriched shrimps improved a decrease of amylase specific activity. Specific activity of lipases was not modified by diet.

INTRODUCTION

Marine animals are mainly commercialized in eviscerated frozen form, which improves the development of transformation factories. In consequence, many scraps are produced, principally viscera. Traditionally, viscera have been considered as waste and utilized only to a minor extent (Gildberg & Almas 1986). Therefore, given their biochemical composition, viscera could be the basis of marine autolysates to be used in aquaculture diet supplementation. Silage is described as a liquid product made only from animals. Liquefaction is caused by the action of enzymes already present in the animal, and is accelerated by the acid, which in addition to creating the right conditions for the enzymes to work, helps to break down bone and limits the growth of spoilage bacteria (Tatterson 1976). Temperature at which silage is processed may affect the nutritional value of the resulting meal, mainly due to differences in protein digestibility (e.g. Pike et al. 1990). In fact, the higher the temperature of fabrication of silage the higher the quantity of peptides compared with protein composition of silage.

Since there is no formulated diet suitable for marine larvae and juvenile, they are fed live prey. Therefore, rearing marine larvae and juveniles represents high cost that limits the development of marine aquaculture. In fact, cuttlefish eat mainly live prey such as crustaceans and fish only subadults and adults can be reared easily with inert food (Castro et al., 1993). During post-hatch development, they exclusively hunt small crustaceans such as young shrimp (Koueta et al. 2002). This is the main problem to succeed in rearing juvenile cuttlefish for aquaculture. Many attempts have been made to rear juvenile cuttlefish with alternative diets, but young animals in these trials were fragile and their growth rate was low (Castro 1991). Nevertheless, Perrin et al. (2004) obtained encouraging results in rearing juvenile cuttlefish with enriched frozen shrimps from 10-days-old. Over the last two decades, several studies have been conducted to determine the nutritional requirements of marine larvae and juveniles (Zambonino-Infante et al. 1997, Perrin et al. 2004). As demonstrated by Zambonino-Infante et al. (1997) in sea bass larvae, the molecular size of the dietary protein fraction could play a major role in larval and juvenile development. Protein hydrolysates are potential ingredients that are used in aquaculture mainly as protein supplements, attractants and palatability enhancers (Hardy 1991). The high concentration of free amino acids in silage permitted to use it as a food additive in aquaculture (Viana et al. 1993).

MATERIAL AND METHODS

Enrichment characteristic and prey enrichment: In this paper, our viscera silages made at two different temperatures were investigated in enrichment of juvenile cuttlefish diet. Table I top describes our two silages.
The Crangon crangon shrimp were captured at Luc-sur-Mer (France) and placed in a tank (90 l). Shrimp were fed with mashed shrimp. In this way, fresh shrimp surimi was soaked for 24 h at 4°C for control diet and for 24 h at 4°C in silage (500 mg powder / 2 g surimi) for test diet. Surimi was then distributed to young C. crangon at a ratio of 5 g of surimi for 10 g of live prey (Koueta 2002). After feeding, shrimp were weighted and frozen.

**Rearing:** The rearing took place at the Marine Station in Luc-sur-Mer (France) in the facility described by Koueta & Boucaud-Camou (1999). At hatching, juvenile cuttlefish were placed individually in small tanks, which were separated in 4 compartments by a transparent surface. A total of 60 juvenile cuttlefish, hatched the same day, were divided in 3 groups of 20 animals that were housed and fed separately and reared in the same conditions. Each tank contained 4 animals separated by a thick partition; the animals were housed and fed separately with the same quantity of food for each treatment and reared in the same conditions.

The 60 cuttlefish were fed ad libitum with simple live shrimps (Crangon crangon) during the first 5 days. After 5 days, the first group received simple frozen shrimps, the second group received frozen shrimps enriched with LBBMA4 and the third group received frozen shrimps enriched with LBBMA25 (ad libitum). At 40-days-old, 8 h after receiving the diet, cuttlefish were frozen in liquid nitrogen and stored at –80°C until enzymatic analysis.

**Growth parameters:** The amount of food ingested by the animals in each container was measured by weighting the food remaining in the individual tanks each day. Weight (mg) was estimated each 5 days. Food conversion efficiency (%) was calculated as (growth weight / weight of food ingested)*100.

**Enzymatic assays:**

**Total carbohydrates contained in freeze-dried shrimps (100 g) were freeze-dried and transformed to powder using a ball grinder. Total carbohydrates contained in freeze-dried shrimps (10 mg). After vortexing, the solution was stored for 12 h at room temperature. A solution of NaHC0₃ 2% in NaOH 0.1M (100 ml) was added to solutions of CuSO₄ 0.5% (1ml) and tartaric sodium potassium 1% (1 ml). Later on, 0.5 ml of sample was added to 0.5 ml of H₂SO₄ 0.5M and 5 ml of NaCl₂ solution. The incubation took place at room temperature during 10 min. After that, 0.5 ml of Folin-Ciocalteu solution 0.5M was added to the solution. After vortexing, the incubation took place at room temperature during 1h30. The intensity of coloration was estimated at 750 nm.**

Total carbohydrates contained in freeze-dried shrimps were extracted using the Staats et al. method (1999) and assayed according to Dubois et al. (1956). Dry tissue (500 mg) was added to distilled water (5 ml). Assays were placed at 35°C during 1 h, after that, assays were
centrifuged 10 min at 3000 g. Two ml of supernatant were taken and added to 8 ml of absolute ethanol. Assays were placed during 16 h at –20°C and centrifuged 30 min at 4000 g and 10°C. The supernatant contains low molecular weight carbohydrates and pellets contain high molecular weight carbohydrates. Ethanol rests on pellets and supernatant were evaporated at 60°C. We added 1 ml of distilled water on dry pellets thus obtained. Assays of carbohydrates were made adding 1 ml of phenol solution at 5% and 5 ml of sulfuric acid 96%. The incubation took place at room temperature during 30 min using glucose as standard. Intensity of coloration was estimated at 485 nm.

Total lipids contained in freeze-dried shrimps were extracted using the Bligh & Dyer method (1959) and assayed according to Marsh & Weinstein (1959). Dry tissue (10 mg) was added to 1 ml of chloroform and 2 ml of methanol. Assays were centrifuged 10 min at 4000 g. Supernatant was taken and 1 ml of chloroform and 2 ml of methanol were added to pellets. Assays were centrifuged 10 min at 4000 g. All the supernatant was taken and 4 ml of distilled water were added. Assays were centrifuged 10 min at 4000 g. Lipids were contained in the lower phase. Chloroform remains were evaporated at 60°C. Then we added 10 ml of H$_2$SO$_4$ on dry pellets thus obtained. The incubation took place at 200°C during 20 min using tripalmitate as standard. Intensity of coloration was estimated at 360 nm.

Statistical analysis: Results are given as mean ± standard deviation (n=10). Data were compared with an ANOVA followed by a Tukey’s test when significant differences (p<0.05) were found (Sokal & Rohlf 1981).

RESULTS

Molecular weight of proteins in silage

The peak, which contains proteins of a molecular weight higher than 20 kDa represents 19 or 11%, respectively in the LBBMA4 and LBBMA25, whereas the second peak (proteins >6.5 KDa) represents 81 or 89%, respectively (Fig. 1).

Growth parameter

The survival was 100% in all three groups during the experimental rearing. The cuttlefish weight significantly increased (p<0.05) from 0 to 40 days in all the groups (Fig. 2). The cuttlefish which received enriched food have significantly higher weights (p<0.05) at 10, 35, 40-days of age. The conversion rate was significantly higher (p<0.05) in the groups fed with enriched diet at 10 and 35-days-old than in the control (Fig. 2).

Enzymatic activity

Specific total proteolytic acid activity increased significantly (p<0.05) in cuttlefish fed with shrimp enriched with LBBMA25 (Table I top). Specific total proteolytic alkaline activity increased significantly (p<0.05) in cuttlefish fed with shrimp enriched with LBBMA25. Specific lipase activity increased significantly (p<0.05) in cuttlefish fed with shrimp enriched with LBBMA4 (Table I middle). Specific amylase activity increased significantly (p<0.05) in cuttlefish fed with enriched shrimp (Table I top).

Prey composition

The amount of total proteins in g/ 100g of dry weight was significantly (p<0.05) lower in enriched shrimp in comparison to non enriched shrimp (Table I bottom). The amount of total carbohydrates in g/ 100g of dry weight was significantly (p<0.05) higher in enriched shrimp in comparison to non enriched shrimp (Table I bottom).

![Fig. 1. – a, Calibration graph of molecular weight for Pharmacia G25M PD 10 Sephadex. b, Shrimp proteins molecular weight distribution using gel filtration. – Simple frozen shrimps, — — — Frozen shrimps enriched with LBBMA4, - - : Frozen shrimps enriched with silage LBBMA25, “clear grey bar”: LBBMA4, “dark grey bar”: LBBMA25.](image-url)
HMW (high molecular weight) carbohydrates in µg/g of dry weight were significantly (p<0.05) higher in shrimp enriched with LBBMA4 in comparison to non enriched shrimp. Moreover, LMW (low molecular weight) carbohydrates in µg/g of dry weight was significantly (p<0.05) lower in shrimp enriched with LBBMA4 and higher in shrimp enriched with LBBMA25 in comparison to non enriched shrimp. The amount of total lipids in g/100g of dry weight was significantly (p<0.05) lower in enriched shrimp in comparison to non enriched shrimp (Table I bottom). The quantity of proteins, which have a molecular weight higher than 20 kDa contained in shrimps was not significantly influenced by enrichment. On the contrary, the content of peptides (> 6.5 kDa) in shrimps was directly influenced by the amounts of peptides of enrichment. In this way, shrimps received the LBBMA25 contains significantly (p<0.05) more peptides than shrimps receiving the LBBMA4, which contain significantly (p<0.05) more peptides than control shrimps.

DISCUSSION

From a metabolic point of view, one of the most apparent differences found in comparison to cephalopods and other marine organisms is a high-protein content (75-85% of dry weight) due to the predominance of their amino acid metabolism (Villanueva et al. 2002). Artificial diets based on less expensive protein sources are becoming increasingly important as an alternative to live feeds in the aquaculture industry (Coutteau & Sorgeloss 1992). In fact, silage has proved to be a good protein source for sea-farmed fish (Raa & Gildberg 1982). In our silages, there are more than 81% of proteins in LBBMA4, which have a molecular weight lower than 6.5 KDa, whereas there are 89% in LBBMA25. Silages obtained contain mainly peptides, which can possess active properties and amino acids, which can be ingested rapidly by juvenile animals in rearing.

Nobody has reared juvenile cuttlefish with alternate diet before this experiment, from 5-days-old with such good survival (100%). The weight highly increased to 40-days old. In this way, cuttlefish, which received simple frozen prey, have a weight of 2.11g, cuttlefish receiving frozen enriched shrimp with LBBMA have a weight of 2.35g (+11.37% compared to control cuttlefish) and cuttlefish which received frozen enriched shrimp with LBBMA25 have a weight of 2.38g (+ 12.8% compared to control cuttlefish) after 40 days of rearing. Moreover, enrichments permit juvenile cuttlefish to have better growth and higher weight. The conversion rate was better for the cuttlefish fed with enriched frozen prey at 10, 20, 25 and 35-days-old. This higher food conversion rate shows that the methodology of enrichment of the prey with silages was effective. The animal fed with enriched diet refused excess food as previously observed by Richard (1975) and Koueta et al. 2002 in experimental rearing. Other studies showed the same phenomena in Haliotis fulgens where the addition of viscera silage to the diet permitted to induce higher growth rates compared with natural food (Viana et al. 1993). Moreover, Oliva-Telez et al. (1999) showed that the effect of silage processing temperature on fish performance is more or less pronounced, depending on the species considered. In Atlantic salmon Salmo salar (Pike et al. 1990)
diets including silage processed at low temperatures supported higher growth rates, while in rainbow trout *Oncorhynchus mykiss* (Pike *et al*. 1990) and in cuttlefish differences were not as pronounced as in salmon. Nevertheless, given the high difficulty to feed juvenile cuttlefish with artificial diet, we used enriched frozen shrimps as alternative diet. Therefore, the impact of silages supplementation on juvenile cuttlefish was made by using indirect enrichment. Prey composition shows that enrichment implied an increase of total carbohydrates and peptides. In contrast, total proteins and lipids decreased with the enrichment of shrimps. Our results show, despite the decrease of total proteins content on enriched shrimps, that the quantity of peptides increased according to the peptide content of silages. Even in indirect enrichment, we have success in obtaining alternative diet enriched on peptide and free amino acids. Nevertheless, shrimp composition was not adjustable indefinitely. Thus, since shrimp metabolism is based on carbohydrates, protein enrichment induces an increase of carbohydrate content after metabolization. We had only success to obtain alternative diet enriched on peptides and free amino acids. This limitation can explain the fact that no differences are observed in comparison to cuttlefish fed with shrimp enriched with LBBMA4 or LBBMA25. We can imagine that the utilization of indirect enrichment limits the effect of silage. In future experiments, we have to test the impact of silages using direct enrichment. At 40-days of age, the digestive system of cuttlefish was similar to the adult system (Boucaud-Camou 1973, Perrin *et al*. 2004). Therefore, all differences observed in specific digestive enzyme activities are correlated to cuttlefish diet. Our results show that total acid proteolytic activity was stimu-
lated by the contents of shrimps fed with LBBMA25. Moreover, total alkaline proteolytic activity stimulation by the contents of shrimps fed with enrichment was correlated with shrimp quantity of peptides. So, higher peptide composition of enriched shrimps improved higher proteolytic enzyme stimulation. Furthermore, the contents of shrimp carbohydrate quantity inhibited amylase activity.

The utilization of silage as enrichment of juvenile cuttlefish shows very interesting results. Thus, it permits to reduce food ration, to increase weight and to increase the food conversion rate. Several factors can be at the origin of this phenomenon. Enrichment improves higher quantity of peptides and carbohydrates in shrimps as observed by Perrin (2004). Thus, autolysate from viscera can be used as enrichment in cuttlefish rearing.

CONCLUSION

The use of dried fish protein hydrolysate in diets has been shown to improve growth and food utilization of salmonids such as Salmo salar (Berge & Storebakken 1996) and carp larvae Cyprinus carpio (Carvalho et al. 1997). This positive effect can be attributed to increased digestibility of the diet due to the enzymatic treatment; free amino acids released in the process might also act as attractants, increasing food intake and growth (Berge & Storebakken 1996, Oliva-Telez et al. 1999). In this way, incorporation of a protein hydrolysate in the diet has a beneficial effect on larval development as in Carassius auratus or Dicentrarchus labrax (Szlaminska et al. 1991, Carvalho et al. 1997, Zambinino-Infante et al. 1997). The digestive system of juvenile cuttlefish only becomes mature 30 days after hatching (Boucaud-Camou 1973, Perrin 2004). During this maturation period, important changes occur. Therefore supplementing the diet contributed to healthy and rapid growth and higher survival rate.

Autolysate is used as a food for farmed fish (Haaland & Njaa 1989). The nutritional quality of the silage may depend on the degree of autolysis and the products formed. Haaland & Njaa (1989) showed that the temperature has an influence both on the degree of autolysis reached after storage and on the degree of hydrolysis of the amide groups. Moreover, the undissolved fraction, which always remains in an autolysate, was smaller in the autolysate stored at high temperatures than in the autolysate stored at 2°C (Haaland & Njaa 1989). The liquefaction of silages is markedly favoured at acid pH values and above room temperature (Raa & Gildberg 1976). The process of ensilage is very simple and the capital cost of equipment can be low. The basic equipment could consist of a grinder, a means of adding the acid, an acid-resistant storage tank, a means of stirring the silage and containers to distribute the product (Tatterson & Windsor 1974). Thus, our experimentally silage fabrication can be easily applied industrially to produce a protein concentrate from viscera.

ACKNOWLEDGMENTS. – This work was supported by the IFOP. This study was conducted in CREC (Centre de Recherches en Environnement Côtière) at Luc-sur-Mer, Normandie, France. We are grateful to the cooperative Granvilmer for providing the biological material used in this study and to A Coulon, A Moncuit, A Meslon, H Viala, G Saﬁ and L A Gabault for their help on cuttlefish rearing. We want to thank Dr B Roel for his help in English editing.

REFERENCES


Received September 26, 2005
Accepted October 27, 2005