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Characterization of digestive carbohydrase activity in the gilthead seabream (*Sparus aurata*)

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Abstract

The presence of digestive carbohydrases was determined in seabream intestines, and the main activity identified was amylase. Optimum activity for this enzyme was found at pH 8.0 at 40 °C. Amylase activity was highly sensitive to extreme pH, and temperatures exceeding 50 °C. The use of SDS–PAGE zymograms allowed identification of amylase in the form of a high molecular mass fraction exceeding 100 kDa. Results confirm the existence of a well developed amylase equipment in this species which supports the possibility of increasing the amount of carbohydrates in the formulation of its commercial feeds.

Introduction

Marine aquaculture has been intensively developed during the last 30 years. The increasing production of intensively cultured marine fish relies to a great extent on an improved knowledge about feeding habits, nutritional requirements and digestive capabilities in different species. In this sense, a great number of studies on digestive enzymes of marine fish have been carried out (Glass et al., 1989; Eshel et al., 1993; Kuzmina, 1996; Munilla-Morán & Saborido-Rey, 1996a; Alarcón et al., 1998). Since protein is the major ingredient in fish feeds, most of these studies have been focused on the characterization and evaluation of digestive proteases. In contrast, other enzymes, like carbohydrases and lipases have not been studied as much, although several authors have reported the presence of noticeable carbohydrase activity in the gut of freshwater and marine fish species (Chiu & Benítez, 1981; Ugwumba, 1993; Hidalgo et al., 1999). Recent advances in fish nutrition have revealed the possibility of increasing the level of energy in feeds for some carnivorous fish using highly digestible carbohydrates (Cardenete et al., 1997). This point, together with the development of new technologies in feed processing (extruded pellets), has lead to an increasing interest in obtaining a better knowledge of the digestive carbohydrases present in different fish species. In the present paper, several biochemical and electrophoretical techniques are utilized to obtain more complete information about the digestive carbohydrases present in gilthead seabream (*Sparus aurata*), one of the most important fish species cultured in the Mediterranean basin. Although the activity of the main digestive enzymes in this species has been recently assessed (Munilla-Morán & Saborido-Rey, 1996a; Alarcón et al., 1998), little attention has been paid to the activity of its carbohydrases.

Materials and methods

Fish samples

Twenty live seabream (*Sparus aurata)* with average weight of 50 g were obtained from a fish farm (FRAMAR S.L.; Almería, Spain). Fish were fed on a commercial diet (45% protein) four times a day at regular intervals, but starved for a 12 h period prior to sampling. After sacrificing the specimens by

submersion in cold water (0 °C), the digestive tract was ligated at specific points to separate six distinct regions, namely: stomach, pyloric caeca, anterior, medium and posterior intestine and rectum. Samples of each region were pooled and after manually homogenized with a potter Eveljehm (100 mg ml^{-1}) in cold 50 mm Tris–HCl buffer at pH 7.5. Supernatants obtained after centrifugation (16 000 ***g***; 30 min. at 4 °C) were stored at −20°C and further utilized for enzyme analysis. Concentration of soluble protein in samples was determined by the Bradford method (Bradford, 1976).

Enzyme activity

Activity of carbohydrases present in extracts was measured using different substrates prepared as solutions in distilled water (w/v): 2% starch (polymeric substrate), 2% glycogen (polymeric substrate), 10% raffinose (α-galactosidic linkages), 1% lactose (β-galactosidic linkage), 1% sucrose (α-glucosidic linkages). Twenty μl of the enzyme preparation were mixed with 0.125 ml of 0.1 m phosphate-citrate pH 7.5 at 25 °C. Reaction was initiated by the addition of 0.125 ml of substrate and stopped 30 min later. The reducing sugars released after this time were estimated using the Somogyi-Nelson procedure described by Robyt & Whelan (1968). A blank without substrate and a control containing no enzyme extract were run simultaneously with the reaction mixture. All the assays were carried out in triplicate. One unit of activity was defined as the amount of enzyme able to produce 1 μg of maltose min^{-1}.

Effect of pH and temperature on amylase activity and stability.

Optimal pH for amylase activity was determined using Universal Buffer (Stauffer, 1989) ranging from 2 to 11. The effect of pH on stability of total protease activity was determined by preincubation of extracts at different pHs for 30–180 min, prior to assaying for amylase activity using soluble starch as a substrate. Optimal temperature for amylase activity was determined by incubating enzyme extracts with soluble starch pre-equilibrated at temperatures ranging from 20–70 °C. The influence of temperature on the stability of amylase activity was assayed by preincubating extracts at different temperatures for various time intervals. After cooling in a water bath, residual activity was measured at 25°C. Assays were performed in triplicate.

Table 1. Distribution of carbohydrase activity in the gut of seabream. Data are mean of three replicate measurements ± SD. Values in a column not sharing a common superscript are statistically different at $p < 0.05$

	Substrate	
	Starch	Glycogen
stomach	22.6 ± 14.4^{a}	14.6 ± 8.9^{a}
pyloric caeca	1555 ± 206^{c}	942 ± 69^{b}
anterior intestine	1454 ± 215^{c}	1683 ± 144^{c}
medium intestine	6625 ± 356^{d}	5002 ± 258^{d}
posterior intestine	1846 ± 266^{c}	1018 ± 89^{b}
rectum	112.2 ± 42.5^{b}	25.8 ± 19.3^{a}

Electrophoresis

SDS–PAGE of the proteins in the enzyme preparations was done according to Laemmli (1970), using 12% polyacrylamide and 8 × 10 × 0.075 cm gels. Preparation of samples and zymograms of amylase activity were done according to Lacks & Springhorn (1980). The amount of protein sample applied is indicated in the legends of the figures. Electrophoresis was performed at a constant voltage of 100 V per gel for 45 min at 5 °C. Following electrophoresis, gels were subjected to different treatments in order to reveal enzyme activity or to identify the main proteins present in extracts. Enzyme activity was revealed in gels by gently washing in distilled water (15 min) to eliminate the excess detergent. After, gels were incubated in a solution of 2% soluble starch buffered with 0.1 m citrate-phosphate, 500 mm NaCl, pH 7.5, for 30 min at 5°C, and then transferred to the same solution at 37 °C for 60 min without agitation. Thereafter, gels were washed and stained with an iodine/KI solution. Clear zones revealing activity of amylases could be seen in a few seconds. Stained gels were photographed for recording. Identification of main proteins was carried out in gels after fixation in 12% TCA and further staining with 0.1% Coomassie Brillant Blue (BBC R-250) in a methanol-acetic acid solution (50:20:50). Destaining was carried out with the same solution without BBC. A Molecular Weight Marker from Pharmacia Biotech (Uppsala, Sweden) MWM was used as standard.

Results

Table 1 shows the distribution pattern and degree of hydrolysis of starch and glycogen produced by crude

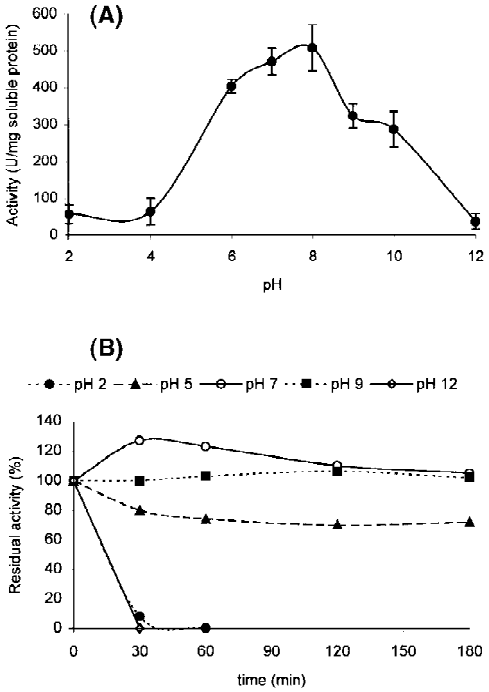

Figure 1. Effect of pH on activity (A) and stability (B) of intestinal amylases in seabream.

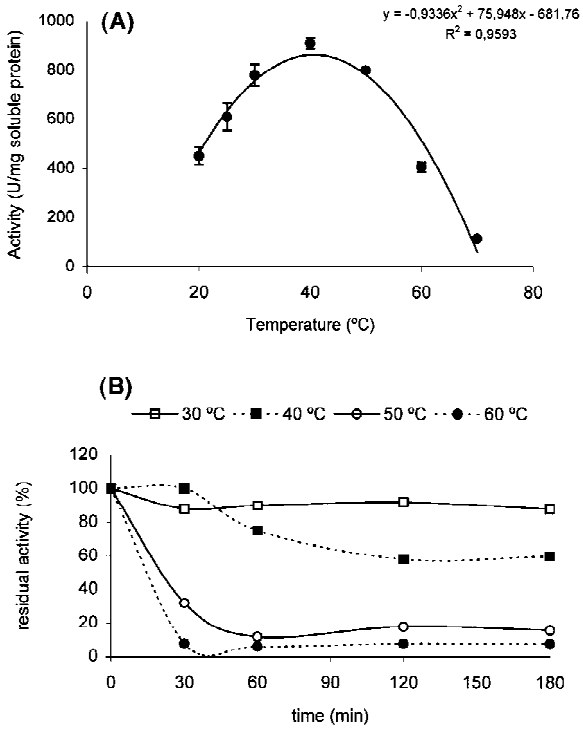

Figure 2. Effect of temperature on activity (A) and stability (B) of intestinal amylases in seabream.

extracts obtained from various regions of seabream digestive tract. No hydrolysis was measured with the rest of the assayed substrates. Amylase activity was the main activity identified along the digestive tract, being mainly observed in the pyloric caeca and the intestine extracts. Once this activity was identified, the rest of analysis was performed using soluble starch as a substrate, assuming that the main carbohydrase activity in the gut of seabream was that of amylase.

The activity profiles of amylase in relation to pH and temperature were determined using crude extracts obtained from pyloric caeca. Amylase activity of seabream was low at a pH below 4.0, showed a well-defined optimum at pH 8.0 and quickly decreased as pH increased (Fig. 1A). Determination of residual activity of amylase after incubation at different pHs was utilized as a measure of its stability (Fig 1B). Results indicated a great resistance of seabream intestinal amylase to alkalinization since it retained 100% activity after 2 h of incubation at pH 9.0. In contrast, an acid environment (pH 5.0) significantly decreased its hydrolyzing ability.

Optimum temperature for amylase activity was 40 °C (Fig. 2A). Thermostability of amylase is shown in Figure 2B. Nevertheless, a great sensitivity to temperature was evidenced, since less than 50% of maximum activity was retained after a 120-min incubation at such temperature.

SDS–PAGE confirmed the existence of amylase activity in crude enzymatic extracts obtained from pyloric caeca and the intestine (Fig. 3). It was revealed as a single active band corresponding to a protein with a molecular mass exceeding 100 kDa.

Discussion

Important carbohydrase activity has been reported in different species of cultured fish like tilapia, *Oreochromis niloticus* (Ugwumba, 1990); couch's sea bream, *Pagrus major* (Kawai & Ikeda, 1971); salmon, *Oncorhynchus keta* (Ushiyama et al., 1965) and Dover sole, *Solea solea* (Clark et al., 1984). Results obtained in this paper confirm the presence of carbohydrase activity in the gut of seabream, which was detected in different gut sections, from the stomach to the rectum, but mainly present in the pyloric caeca and the intestine. A similar distribution pattern has been reported in white salmon, *Chanos chanos* (Chiu & Benítez, 1981) and mullet, *Mugil* sp. (Establier et al., 1985). The

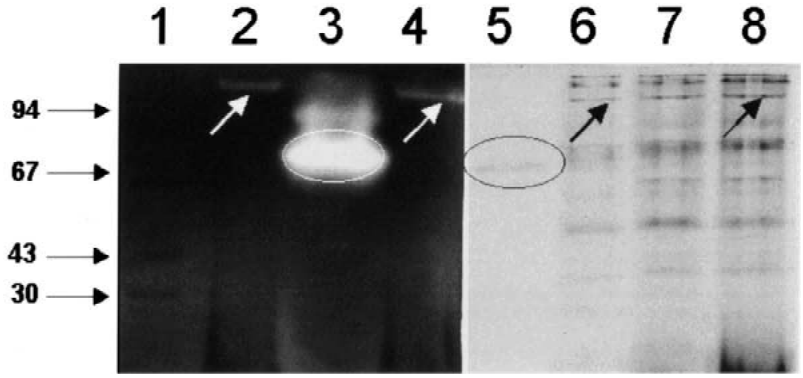

Figure 3. Substrate-SDS–PAGE (12% PAA) of amylase activity revealed on digestive extracts of *Sparus aurata.* Lanes 2–4 is a zymogram of amylase activity using starch as substrate; lanes 5–8 is a gel stained directly with BBC as detailed in 'Materials and Methods'. Lane 1: Molecular Weight Marker (MWM en kDa): phosphorylase *b* (94), bovine albumin (67), egg albumin (43), carbonic anhydrase (30). Lanes 2 and 6: pyloric caeca extract (35 μg protein cell^{-1}). Lanes 4, 7 and 8: intestinal extract (35 μg protein cell^{-1}). Lanes 3 and 5: α-amylase from *Bacillus subtilis* (1.5 μg cell^{-1}). Seabream amylase was indicated by arrows. Amylase from *Bacillus subtilis* was included into ellipses.

activity seems to be mainly due to the existence of an α-amylase and not to α-galactosidase, β-galactosidase or α-glucosidase, since extracts hydrolyzed glycogen and starch, but not raffinose, lactose or sucrose. This is in agreement to the results reported in couch's sea bream (Kawai & Ikeda, 1971) and tilapia (Ugwumba, 1990) and clearly contrasts to the results obtained in other species, like rainbow trout (*Oncorhynchus mikiss),* carp (*Cyprinus carpio*) or Dover sole, where the presence of such carbohydrases has been described (Kitamikado & Tachino, 1960; Kawai & Ikeda, 1971; Clark et al., 1984).

Several authors have reported the presence of amylase activity in stomach extracts of different fish. Ugwumba (1993) described the presence of amylase in the stomach of the African bony-tongue (*Heterotis niloticus*) and a similar result has been reported in mullets, redfish, seabream and turbot (Establier et al., 1985; Munilla-Morán & Saborido-Rey, 1996b). Activity of amylase in the stomach of sea bream has also been detected in the present work, but it was considered an artifact resulting from contamination of stomach extracts with pancreatic tissue during manipulation for dissection. In fact, it should be difficult to explain the presence and activity of these enzymes, which have an optimum pH ranging from neutral to alkaline, considering the acid environment existing in the stomach.

The pH profile of amylase activity present in pyloric caeca extracts, which showed an optimum activity at pH 7–8.5, closely resembled those previously reported in other freshwater or marine fish (Hofer & Sturmbauer, 1985; Ugwumba, 1993; Munilla-Morán & Saborido-Rey, 1996b). The presence of a single peak of optimum pH (8.0) suggested the existence of only one active form of amylase in the extracts. The optimum temperature for the intestinal amylase activity of seabream was about 40 °C, being in agreement to previous data obtained in other species, like the redfish (*Sebastes mentella*) or the turbot (*Psetta maxima*) by Munilla-Morán & Saborido-Rey (1996b). The determination of optimum temperature for an enzyme may be interesting for comparative studies, but offers little information about its activity under physiological conditions. In this sense, assays determining enzyme resistance to denaturation after incubation at different temperatures seem to be more useful. Amylase activity in seabream extracts was reduced almost to zero after 2 h of incubation at 60 °C, which evidenced a great sensitiveness to the increase of temperature. From a biological point of view, it is difficult to find any kind of advantage in possessing amylases showing different resistance to heating, since water temperature rarely exceed 28 °C, but from a biotechnological perspective it may be interesting to have information about active and easily denaturalizable amylases potentially useful in biotechnological processes (Haard, 1992).

Results obtained by substrate SDS-PAGE confirm the presence of a single α-amylase in enzyme preparations. This contrasts to the existence of several α-amylase isoenzymes reported in other species like the rat (two isoenzymes; Sander & Rutter, 1972), humans (four isoenzymes in saliva; Malacinski & Rutter, 1966) and shrimp (one to six isoenzymes; Wormhoudt et al., 1995). Surprisingly, the molecular mass of

seabream amylase (> 100 kDa) is much higher than that reported for most animal, vegetable and bacterial amylases (50 kDa) (Hagenimana et al., 1994; Wormhoudt et al., 1995). Due to the lack of similar studies it is not possible to compare the electrophoretical pattern obtained in the present paper with other fish species.

According to Nikolsky (1963), the pattern of carbohydrases could be species specific and related to the natural feeding habits. In general, the activity of amylase is greater in herbivorous or omnivorous than in carnivorous fishes (Hidalgo et al., 1999). Wild seabream primarily feeds on molluscs and crustaceans, though occasionally it consumes algae (Arias, 1980). It is known that the plant carbohydrates may be well digested if the cellular membrane is partially broken, exposing the content of cells to the digestive enzymes. In seabream, this initial process of hydrolysis may be ensured by the acid environment of the stomach (Bowen, 1976). Further hydrolysis of starch is completed in the intestine by the action of amylase. Currently commercial feeds utilised for rearing of seabream do not include carbohydrates as a significant source of energy. Results obtained in the present paper point to the existence of a well developed carbohydrase activity in the gut of this species, possibly allowing the use of an increased amount of such nutrients in the feed formulations.

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