ELSEVIER

Journal of Experimental Marine Biology and Ecology
262 (2001) 1–12

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.nl/locate/jembe

Characterization of α-amylase activity in five species of Mediterranean sparid fishes (Sparidae, Teleostei)

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Received 4 August 2000; received in revised form 26 October 2000; accepted 16 February 2001

Abstract

In the present study, amylase activity existing in the gut of different species of sparid fish was measured and characterized. The study was conducted on five species of Mediterranean sparids, (F. Sparidae), some of which coexist in the same coastal waters; *Pagrus pagrus*, *Pagellus erytrhinus*, *P. bogaraveo*, *Boops boops* and *Diplodus annularis*. Main differences were found in the optimal pH for amylase activity as well as in sensitivity to temperature, with resistance to heating very low in *B. boops* and very high in *D. annularis*. PAGE zymograms revealed the presence of two isoforms in all the species, while the analysis of extracts using IEF showed a range from one to three active bands. It is suggested that differences in amylase activity are related to variations in feeding habits of the studied fish, and could also represent an ecological advantage for those species showing a more diversified amylase equipment. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Fish; Sparids; Amylase; Digestive enzymes; SDS-PAGE

1. Introduction

The study of digestive enzymes in fish has a wide range of potential interest. Biochemical information about digestive enzyme equipment in fish can be related to their feeding habits and abilities, since whatever may be the food habit of the fish,

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PII: S0022-0981(01)00228-3

adaptations of the digestive system of different species exhibit closer correlation with their diet than on their taxonomic category, allowing a more accurate evaluation of their specific role in aquatic ecosystems. On the other hand, the assessment of the activity of digestive enzymes in cultured species may be helpful in the selection of feed ingredients (Lan and Pan, 1993), even allowing the onset of "in vitro" assays oriented to the evaluation of commercial feeds (Alarcón et al., 1999). Since protein is the major ingredient in fish feeds, a great number of biochemical studies have been oriented to the characterization of protease activity in different marine fish species (Haard, 1992; Moyano et al., 1998; Díaz et al., 1998). In contrast, other enzymes, like carbohydrases and lipases, have been much less studied, although several authors have reported the presence of a noticeable carbohydrase activity in the gut of freshwater and marine fish species (Chiu and Benitez, 1981; Ugwumba, 1993; Munilla-Moran and Saborido-Rey, 1996; Hidalgo et al., 1999).

Fish of the family Sparidae are widely distributed along the coastal waters of the Mediterranean Sea, being considered traditionally as an important resource for low-scale fishing. From an ecological perspective, the coexistence and overlapping of habitats for a certain number of Mediterranean sparids are very interesting subjects for study (Corbera et al., 1998). From an applied point of view, the increasing interest in the culture of some species of sparids, whose diet is dependent on algae to a certain extent, may justify a closer approach to their digestive abilities. Good examples are the well established industrial culture of sea bream (*Sparus aurata*) and the promising results obtained with some others species (*Pagellus, Dentex*, etc.), which are currently under different stages of pilot culture (Abellán et al., 1994). Taking these into account, it may be highly interesting to get a better knowledge about some physiological and biochemical features of Mediterranean sparids.

In the present work, several techniques, including the combination of a specific inhibitor and SDS-PAGE, were used to characterize digestive amylase activities present in some Mediterranean sparids. Results obtained are discussed from the aforementioned perspectives.

2. Materials and methods

2.1. Preparation of extracts

Wild fish (*Pagrus pagrus, Pagellus erythrinus, P. bogaraveo, Boops boops* and *Diplodus annularis*), weighing between 100–250 g, were captured by a local fisherman, stored in ice until arrival to the laboratory, and then immediately dissected. Pooled samples of pyloric caeca and about 2 cm of proximal intestine obtained from 6 to 10 fish were mechanically homogenized (200 mg ml^{-1}) in cold 10 mM phosphate buffer, pH 8.0, and after, sonicated to ensure total rupture of tissues. Extracts were centrifuged (16,000 g, 30 min at 4°C) and supernatants obtained after centrifugation were stored at −20°C until use for enzyme analysis. Concentration of soluble protein in samples was determined by the Bradford (1976) method using bovine serum albumin (1 mg ml^{-1}) as a standard.

2.2. Determination of amylase activities

Amylase activity was determined according to the Somogy–Nelson method using soluble starch (2% w/v) as substrate, as described in Robyt and Whelan (1968). Briefly, 20 μl of the enzyme preparation was 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. Activity was measured by estimating the reducing sugars released after this time. 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 per minute.

2.3. Optimal pH and temperature ranges

Optimal pH for amylase activity was determined using Universal Buffer (Stauffer, 1989), ranging from 2 to 12. The effect of pH on stability of amylase was determined by preincubation of extracts at different pH for 60 min, prior to the assay. Optimal temperature for amylase activity was determined by incubating enzyme extracts with a substrate pre-equilibrated at temperatures ranging from 25°C to 70°C. The effect of temperature on the stability of amylase activity was tested by preincubation of extracts at different temperatures (30°C, 40°C, 50°C, 60°C) for 30 min, followed by measurement of residual activity. Assays were performed in triplicate.

2.4. Sensitivity to inhibitor

Sensitivity of fish amylases to the inhibitor type I (Sigma, A1520) from triticale was tested by performing an amylase activity assay similar to those previously described, but including in the tubes a given volume of inhibitor solution. This amylase inhibitor was the only commercially available product from a plant source, and it was used as a reference for comparison with results obtained with other species. In all cases, 100 U of amylase activity was incubated in the presence of 15 U of inhibitor for 30 min, and after this time, the released amount of reducing sugars was compared to that obtained in control tubes incubated without inhibitor. Assays were performed in triplicate and the resulting activity in tests tubes expressed as a percentage of that obtained in control tubes.

2.5. PAGE-zymograms

The possibility of performing amylase SDS-PAGE zymograms (gels revealing not all protein bands, but only those showing a given enzyme activity), was previously tested by assaying the effect of SDS (Sigma, L5750) on the activity of such enzymes. An inhibition study, carried out in a similar way to that described for amylase inhibitor type I, showed a great sensitivity of fish amylases to this compound, since activities were reduced in a range from 60% to 80% when extracts were incubated with an SDS solution at 0.1% (w/v). This result oriented further assays and zymograms were performed under native conditions according to Davis (1964), using a discontinuous system of gel concentration of polyacrylamide, 4% in stacking gel and 10% in resolving

Table 1
Amylase activity found in extracts obtained from samples of digestives obtained from different sparids

Species	*n*	$U \times 10^3$/mg protein	Percent inhibition
B. boops	8	40.9 ± 1.9^a	ND
P. erithrynus	7	19.2 ± 1.5^b	65
P. bogaravεo	10	11.0 ± 0.4^c	ND
P. pagrus	10	9.5 ± 0.2^c	30
D. annularis	6	6.2 ± 0.5^d	61

Values are triplicate measurements from pooled samples of different fishes $(n) \pm$ SD. Values not sharing a common superscript are significantly different with $p < 0.05$. ND = not detected.

gel. Samples containing 6 μg of protein were placed in $8 \times 10 \times 0.075$-cm gels, and electrophoresis was first performed at 30 V for 20 min, and after, at 100 V for 45 min, per gel. Then, gels were immersed in a starch solution (1% w/v) containing phosphate–citrate buffer (0.1 + 0.05 M Na Cl^2 + 1 mM $CaCl^2$) at pH 8.0 for 60 min at 37°C. Thereafter, gels were washed and stained with an iodine/KI solution (10%) until appearance of bands. Activity is revealed as clear bands over a dark-brown background. Washing with distilled water stopped the reaction. Stained gels were photographed for recording. The relative electromobility (*Rf*) of enzymes was determined by measuring the distance in millimeter from the stacking gel to the active band, and comparing such value to the distance to the migration front. A extract obtained from tilapia (*Oreochromis niloticus*) was prepared as previously described and used as a reference in addition to a commercial porcine amylase (Sigma, A6255).

The isoelectric point (Ip) of amylases was determined by isoelectrofocusing in $125 \times 65 \times 0.4$-mm gels prepared with 5% polyacrilamyde, 5% glycerol and 20% anfolines Bio-Lyte, pH 3/10. Assays were performed at 100 V for 15 min, thereafter at 200 V for 15 min, and finally at 450 V for 60 min. Amylase activity was revealed as

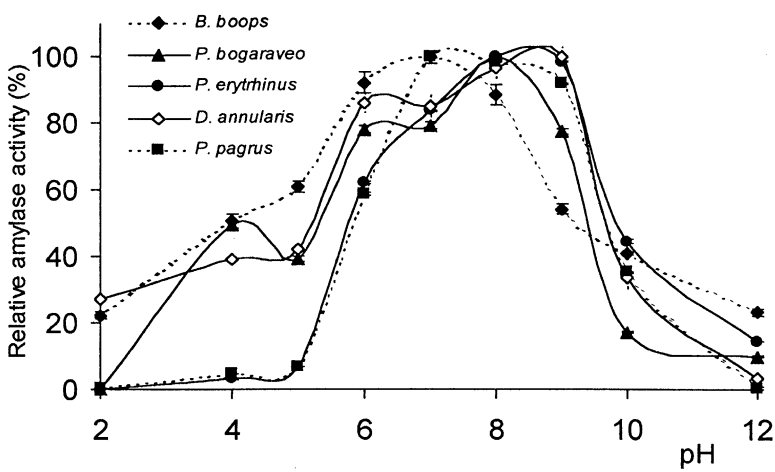

Fig. 1. Optimum pH for amylase activity in the five species of sparids used in the study.

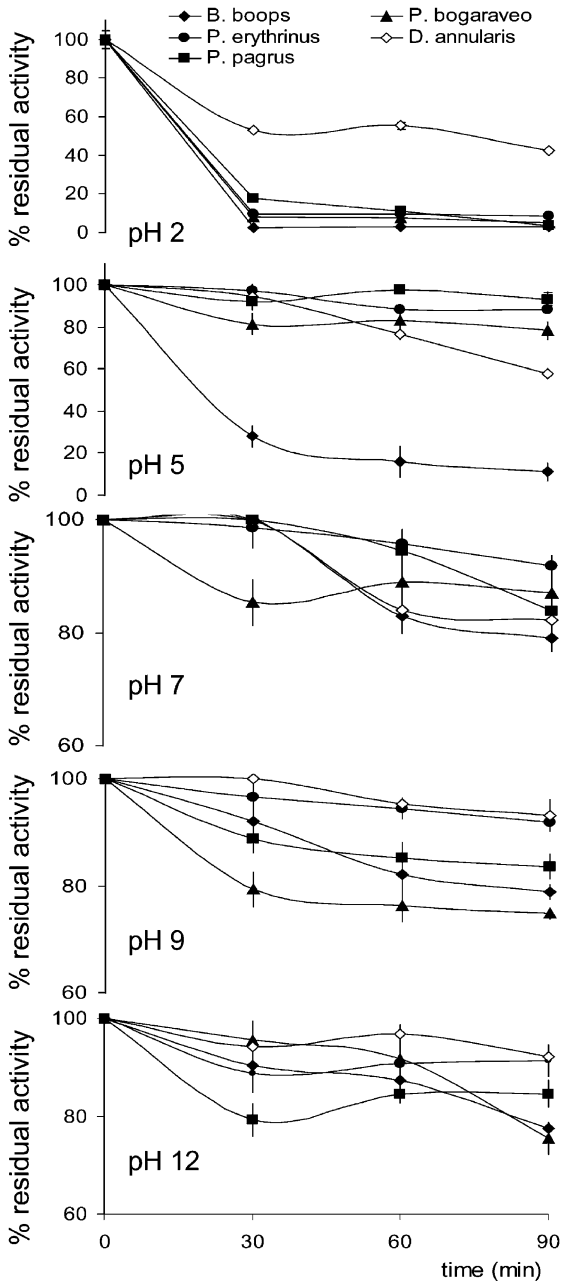

Fig. 2. Sensitivity of sparid amylases to preincubation for 30 min at different pH.

previously detailed. Commercial porcine amylase was used as a reference for calculation of Ip.

2.6. Statistical analysis

Data were subjected to one-way ANOVA when required. Differences between means at $P < 0.05$ were analyzed using the Tukey test. The Statistix version 4.0 package (Analytical Software, AZ, USA) was used.

3. Results

3.1. Specific activity and sensitivity to inhibitor

Values of specific amylase activity in pyloric caecae and sensitivity to inhibitor (amylase inhibitor Type I) were determined in the different fish species and are detailed in Table 1. Values ranged from the 40.9×10^3 units found in *B. boops*, to 6.24×10^3 units found in *D. annularis*. The reduction in activity produced by a specific inhibitor ranged from 30% in *P. pagrus* to 61% for *D. annularis* or 65% for *P. erythrinus*.

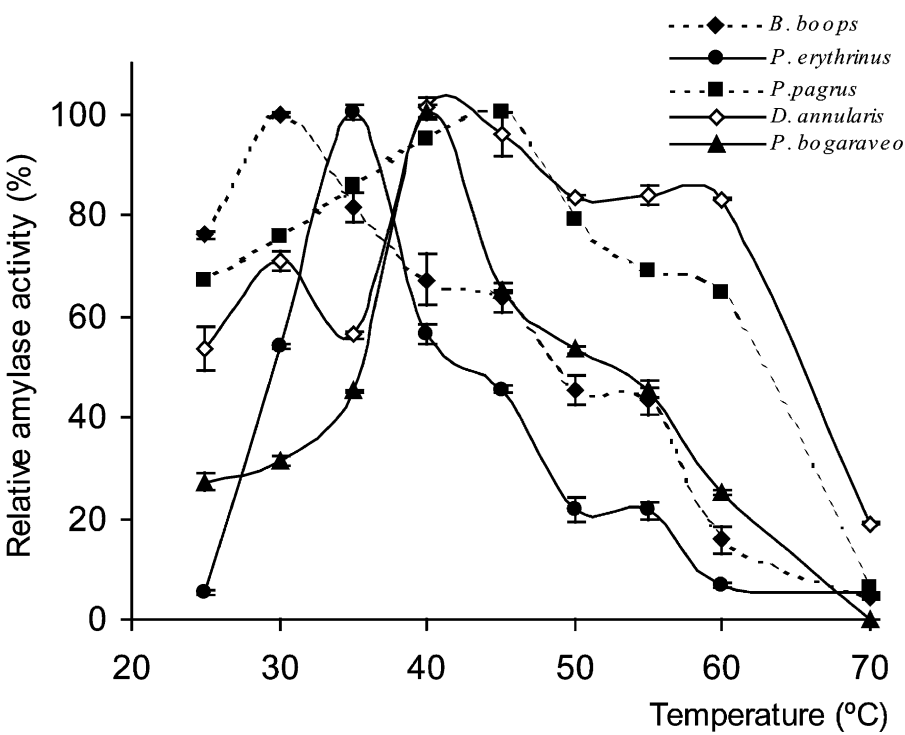

Fig. 3. Optimum temperature for amylase activity in the five species of sparids used in the study.

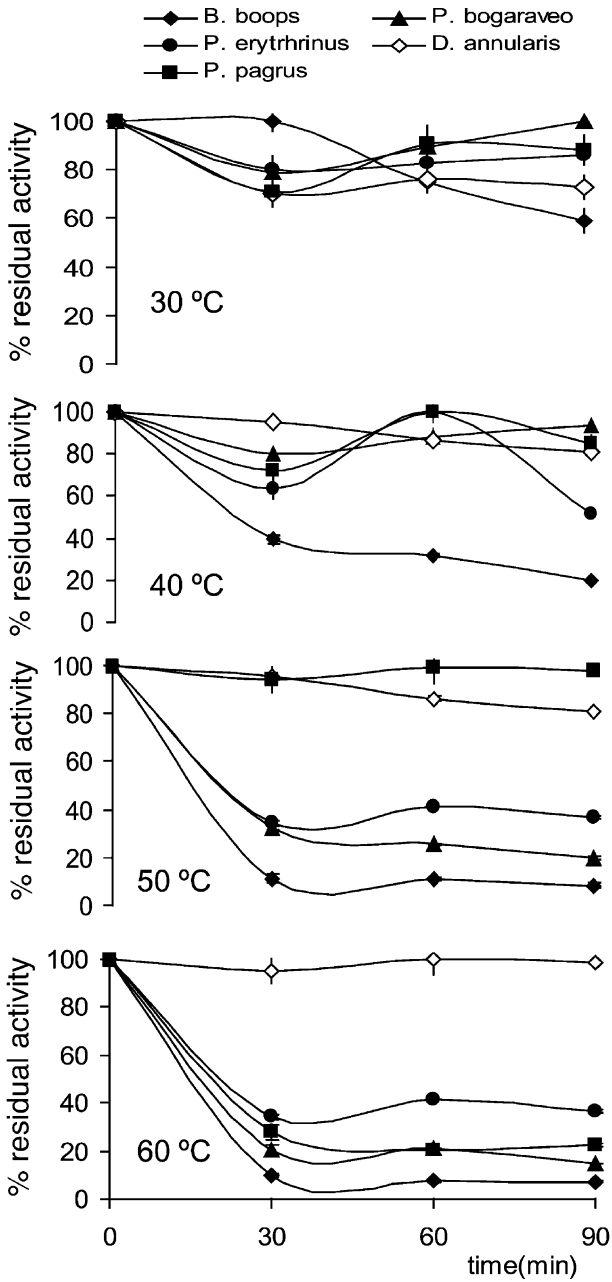

Fig. 4. Sensitivity of sparid amylases to preincubation for 30 min at different temperatures.

3.2. Optimum pH and stability

Optimum pH for amylase activity in the different species is shown in Fig. 1. Due to the great differences found in the activity for the different species, data were transformed in percentages of the maximum value found in each case, for a better comparison of profiles. A one-peak pattern was found in *P. pagrus* and *B. boops*, which showed an optimum at pH 7.0. Two-peaks pattern were found in *P. erytrhinus* (pH 7.0 and 9.0) and *D. annularis* (pH 6.0 and 9.0). Finally, *P. bogaraveo* showed a three-peak pattern at pH 4.0, 6.0 and 8.0.

Residual activity of amylase in each species after preincubation at different pH is shown in Fig. 2. Results showed a low stability of amylases at acid pH (2.0), since a short time of incubation (30 min) reduced activities to less than 30% in most of the cases, with the exception of *D. annularis*. Sensitivity to a less acid pH (5.0) was less evident, with the exception of *B. boops*. In contrast, all the amylases showed a greater stability after incubation at neutral or alkaline pH, retaining 80% activity after incubation for 30 min at pH 9.0, or even 12.0.

Optimum temperature for activity of amylases is shown in Fig. 3. A wide range of optimal temperatures was found, ranging from 30°C in *B. boops* to 45°C in *D. annularis*. Differences were found not only between optimal temperatures, but also in the relative activity measured in each species at 25°C. A very low activity was measured for *P. erythrinus* and *P. bogaraveo* (20% or less of their maximum measured activity). In contrast, enzymes of *P. pagrus*, *D. annularis* or *B. boops* showed from 50% to 70% of maximum activity at such temperature.

Sensitivity of amylases to preincubation at different temperatures is shown in Fig. 4. The greater sensitivity was found in *B. boops*, whose activity was reduced by less than half after 30-min incubation at 40°C, and was almost reduced to 0 when temperatures were increased. In contrast, amylases of *P. pagrus* and *D. annularis* showed a great resistance to heating, mainly in this latter case, since no reduction in activity was appreciated even after 90 min of preincubation at 60°C.

3.3. PAGE-zymograms

Amylase activity in fish extracts was revealed in zymograms in the form of two clear bands (Fig. 5). A similar pattern was obtained for all the assayed sparids, in contrast to

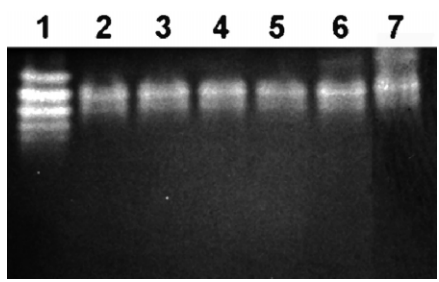

Fig. 5. PAGE zymogram showing amylase activity in digestive extracts of different sparid fishes. Lanes 2–6 show amylase bands found in *B. boops*, *D. annularis*, *P. bogaraveo*, *P. erythrinus* and *P. pagrus*. Lanes 1 (*O. niloticus*) and 7 (porcine amylase) were used as references.

the more complex pattern existing in the tilapia (*O. niloticus*) extract used as a reference. Two of the bands showed to be similar to those observed in tilapia and purified porcine amylase.

The *Rf* estimated for the two isoforms found in the different species was 0.11 and 0.16. Such values were coincident with those showed by two of the bands found in tilapia extracts (0.06, 0.11, 0.16, 0.20 and 0.23)

Determination of the Ip resulted in the detection of one more active band in *B. boops* and *P. pagrus* (Ip 4.0), two active bands in *D. annularis* (Ip 3.4 and 4.0) and three bands in *P. erytrhinus* (Ip 6.5, 7.8 and 8.7).

4. Discussion

Feeding regimes of sparids are very diversified, and for this reason, differences in their digestive enzyme equipment should be expected. *B. boops* is described as omnivorous, mainly feeding on algae and small animals living in a wide range of habitats (rocky and sandy beds and even submarine praires). *D. annularis* shares the habitat with *B. boops*, living at medium depth and mainly predating on molluscs and crustaceans. *P. bogaraveo* is found at a greater depth, mainly over sandy substrates and predates on small fish. *P. pagrus* and *P. erythrinus* are found at a medium depth over sandy substrates, as well as over those showing vegetation. Both are predators of molluscs and crustaceans and occasionally fed on algae.

Specific activity of amylases showed a clear difference between the high value found in a mainly herbivorous species (*B. boops*) and the lower ones found in fish showing a more diversified (*P. erythrinus*) or strictly carnivorous (*D. annularis*) feeding habits. Different authors have reported a close relation between herbivorous feeding habits and a high activity of amylases (Hsu and Wu, 1979; Hofer et al., 1982; Hidalgo et al., 1999). Nevertheless, amylase activity has been reported in a wide range of species that show quite different feeding habits (Kawai and Ikeda, 1971; Kuzmina, 1996).

Optimum pH for amylases close to the neutral value of 7.0 was found in the species studied, and it has been reported in other freshwater and marine fishes (Ugwumba, 1993; Munilla-Morán and Saborido-Rey, 1996). Nevertheless, the existence of more than one peak of optimal pH found in all the species (with the exception of *P. pagrus* or *B. boops*) points to the possible existence of isoenzymes. Those peaks were detected at alkaline pH (8.0 or 9.0), this being in agreement to results obtained in assays testing sensitivity of amylases to different pH. In all cases, amylases were shown to be highly sensitive to a low pH, indicating that the digestion of carbohydrates in the guts of the studied species takes place mainly in alkaline medium. A sharp reduction in amylase activity, when extracts were incubated a pH 5.0, was only found in *B. boops*, being a particular feature that could be related to the lack of a well defined stomach. The absence of acid secretion in the digestive tract of this species could be a factor influencing the evolutionary development of amylase types not adapted to an acid environment. Nevertheless, the presence of a stomach could be valuable for herbivorous fish, since it is reported that acid digestion may initiate the lysis of algal cells, this enhancing further activity of amylases (Zemke-White et al., 1999).

The determination of optimum temperature for maximum activity of an enzyme may be interesting for comparative studies, offering some information about its expected activity under physiological conditions. The range of optimal temperatures measured for the studied sparids (from 25°C to 50°C) has been also reported for amylases in other species (Ugolev et al., 1983; Munilla-Morán and Saborido Rey, 1996). Important differences were found between species, not only in relation to the optimum temperature, but also when relative activity was measured at 25°C. In addition, the specific activity of amylase was nearly nine times higher in *B. boops* than in *P. erythrinus*, when measured at such temperature, being only 0.5 times higher when assayed at 30°C. All this data support the existence of a greater activity of amylases in *B. boops* at physiological temperatures.

Important differences were also found when the remaining activity after incubation at different temperatures was tested. Such differences were particularly evident when comparing the highly sensitive amylases of *B. boops* (which reduced their activity to less than 50% after 1-h incubation at 40°C) and the highly resistant of *P. pagrus* or *D. annularis* (which retained 100% activity after 1.5-h incubation at 60°C). This feature does not seem to be an adaptive mechanism to different environmental conditions, since all the species studied coexist in the same waters. From a biological point of view, it is difficult to envisage any kind of advantage in possessing amylases showing different resistance to heating, since water temperatures are in the range of 12–28°C. Such differences should be the result of variations in the amino acid pattern of such enzymes (e.g. a greater number of cysteine or methionine residues and then a greater number of disulphide bonds) and could reflect taxonomical relations between the species.

The existence of important differences in the molecular structure of amylases in different species is also supported by differences found in sensitivity to inhibitors, for example between *P. pagrus* and *P. erythrinus*, with the latter showing twice as much inhibition as the former. Differences can also be explained by the existence of amylase isoforms, as indicated by results obtained in electrophoretic studies. Those great differences in sensitivity to inhibitors have been reported both in freshwater (Natarajan et al., 1992) and marine fishes (Fernández et al., 2001). Since wheat and wheat by-products are used as dietary ingredients for cultured fish, assessment of such inhibition also has a commercially oriented perspective.

Zymograms are useful in revealing the presence of different digestive enzymes, allowing both the identification of the number of active forms present in a species, and the differences existing in their relative molecular mass. They have been used in the assessment of protease activity in different fish species (Alarcón et al., 1998). On the other hand, PAGE performed under native conditions has been used to assess amylase activity in crustaceans (Van Wormhoudt et al., 1995), but the conditions described by those authors were not applicable to fish extracts, and up to date, no zymograms of fish amylases have been published. In the present work, different factors (substrate concentration, buffers, etc.) were assayed until the desired results are obtained, and the presence of amylases was clearly revealed. Nevertheless, results obtained using PAGE or IEF were not completely in agreement. In the first case, two active bands were detected in extracts of all species, in contrast to the five active bands found in tilapia extracts. In contrast, IEF revealed more differences between species; a single form was

found in *B. boops*, whereas the rest of species showed two or more bands. Polymorphism of amylases has been reported for crustaceans, which showed the presence of up to seven forms in some species (Van Wormhoudt et al., 1995), this being related to changes occurring during larval development or intermolt cycles (Fernández et al., 1977). In addition, the possible existence of amylases, produced not only by the pancreas but also by intestinal microflora, has been demonstrated by Sugita et al. (1997) in several freshwater fish, but this could not explain the results obtained in the present study, since only the duodenal portion of fish gut was sampled. It is difficult to relate to the existence of carnivorous feeding habits the presence of a more diversified amylase equipment. Nevertheless, some authors suggest that the production of amylase is not food-dependent, but family-specific (Hofer et al., 1982; Chakrabarti et al., 1995), in contrast to those arguing that amylase activity is mediated by the composition of the diet (Reimer, 1982). It is widely recognised that fish may change their food habits due to various environmental constraints, however, unless a fish can digest a food, such a change becomes useless. The ability of a fish to digest different kinds of food may be considered a strategy truly reflecting the possibilities of resource utilisation. This ability is closely related to the presence of different isoenzymes, showing activity in a wide range of pH and temperature, and this may represent an ecological advantage for a species like *D. annularis* in contrast to *B. boops*. In addition, considering the potential interest of sparids as aquacultured species, the existence of a well developed amylase equipment in some of them, described as carnivorous, may be an interesting feature from the perspective of feed formulation.

Acknowledgements

This work was supported by Project AGL2000-0697-C02-02 from the MCYT (Spain). *[SS]*

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