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Substrate-SDS-PAGE determination of protease activity through larval development in sea bream

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Identification of alkaline proteases produced during larval stages of gilthead sea bream, *Sparus aurata* was achieved using SDS-PAGE and specific inhibitors. Such techniques were also applied to determine proteases existing in rotifers, *Brachionus plicatilis*, and *Artemia* nauplii, which are used as live food for these larvae, as well as proteases of adult fish. The results show a great prominence of trypsin-like proteases during the 4 weeks after hatching, but the number of enzyme species was reduced in adult fish. Alkaline proteases present in the rotifers and *Artemia* showed clear differences when compared with those of the larvae and were not detected in extracts obtained from fed larvae. The results obtained provide information about the role of exogenous enzymes in larval feeding of sea bream.

KEYWORDS: Alkaline proteases, *Artemia*, Chymotrypsin, Larval development, Proteinase classes, Rotifers (*Brachionus plicatilis*), Sea bream (*Sparus aurata*), Trypsin, Zymogram

INTRODUCTION

A detailed knowledge of the digestive physiology of marine fish larvae is necessary when designing artificial diets for cultured species. In this context, protease enzymes are important because they are involved in the processes of protein degradation. Studies which characterized protease activity in larvae of different marine fish species concluded that due to a poor development of the digestive tract during the first 2 or 3 weeks after hatching, marine fish are strongly dependent on the exogenous enzymes provided by live food. This was confirmed by results of several experiments showing better growth of larvae fed on live organisms (Dabrowski, 1979; Lauff and Hofer, 1984; Munilla-Moran *et al*., 1990; Kolkovski *et al*., 1993; Walford and Lam, 1993). In contrast, some authors have suggested that, at least in some species, at the moment of first feeding, levels of the main enzymes may be enough to allow digestion of either a prey or an artificial diet (Baragi and Lowell,

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1986; Cahu and Zambonino, 1994). Clearly protease activity has become a decisive point to establish the ability of larvae to utilize artificial diets. However, many studies have been carried out by measuring enzymatic hydrolysis on specific substrates or by acrylamide-gel-electrophoresis (Alliot *et al.*, 1980; Lauff and Hofer, 1984), but none of them has utilized the combination of substrate-SDS-PAGE and specific inhibitors to obtain a more complete characterization of both larval and live food proteases. This paper summarizes results obtained with the application of such techniques to study digestive proteases of gilthead sea bream larvae (*Sparus auratus*), as well as rotifers, *Brachionus plicatilis*, and *Artemia aurata nauplii*, providing useful information to determine the relative significance of exogenous proteases in the digestive equipment of fish larvae.

MATERIALS AND METHODS

Fish

Sea bream eggs were obtained by natural spawning from fish held at 19.5 °C at the facilities at Instituto Ciencias Marinas Andalucía (ICMA), Cádiz, Spain. Incubation of eggs was at the same temperature. After hatching, larvae were transferred to 300 l cylindro-conical tanks provided with a continuous flow of seawater at 19 °C and 33‰ salinity. Larval density was maintained at 60–100 larvae l^{-1} being fed following a standard schedule using rotifers and *Artemia* nauplii (Polo *et al.*, 1992). At different intervals from 0 to 30 days, the larvae were sampled, washed, freeze-dried and stored until analysis. Samples of rotifers and *Artemia* nauplii used as live food were obtained by filtration, and were processed in the same manner. Pyloric caeca of adults ranging from 300 to 350 g, sampled at a cage farm (FRAMAR S.L.; Almería, Spain), were obtained by dissection and stored at −20 °C prior to analysis.

Analytical procedure

Reagents

Enzymes, substrate, inhibitors and general reagents were obtained from the following sources: trypsins (TRY; Type IX from porcine pancreas and TRY-G; Type XX-S from Atlantic cod, *Gadus morhua*), chymotrypsin (CHY; Type II from bovine pancreas), phenylmethylsulphonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), N-tosyl-L-Phe chloromethyl ketone (TPCK), N-CBZ-L-Phe chloromethyl ketone (ZPCK), Nα-p-tosyl-L-Lys chloromethyl ketone (TLCK), and Tris(hydroxymethyl)-aminomethane base (Tris) were obtained from Sigma Chem. Co. (St Louis, MO). Hammerstein grade casein was purchased from ICN Biomedicals, Inc. (Aurora, OH). SDS-PAGE reagents were from Bio-Rad (Richmond, CA). Sephadex G-25M and molecular mass protein standard (MWM) for electrophoresis were obtained from Pharmacia Biotech (Uppsala, Sweden).

Preparation of samples

Samples of fish larvae, rotifers, *Artemia* nauplii (35 mg ml^{-1}) and pyloric caeca (100 mg ml^{-1}) were homogenized in cold 50 mM Tris-HCl buffer, pH 7.5 and centrifuged (16 000 rpm; 30 min. at 4 °C). The supernatants were filtered through a

Sephadex G-25M column (1 × 10 cm) by centrifugation (2000 rpm, 2 min.). The elutant was then stored at −20 °C until used for enzyme analysis. The concentration of soluble protein in pooled samples was determined using bovine serum albumin (1 mg ml^{-1}) as a standard (Bradford, 1976).

Enzyme inhibition

The protease classes were determined using standard inhibitors, following the methods described by Dunn (1989) and García-Carreño (1992). The enzyme extract (20 μl) was mixed with 0.5 ml of 100 mM Tris-HCl, 10 mM $CaCl_2$ buffer, pH 9 and 10 μl of different inhibitors and incubated for 60 min at 25 °C. The mixture was assayed for protease activity using 0.5 ml 1% casein as substrate (pH 9) and incubated at 25 °C (adults) or 37 °C (larvae). The reaction was stopped with addition of 0.5 ml 20% trichloroacetic acid (TCA), and after holding for 1 h at 4 °C, the reaction mixture was centrifuged for 5 min at 12 000 **g**. Absorbance at 280 nm was recorded in supernatants against a blank of buffer-TCA 20% (50:50). The assay included internal controls for inhibition of solvents and enzyme. Control enzymes were assayed using 50 mM Tris-HCl, 20 mM $CaCl_2$ buffer, pH 7.5. Inhibitors, class of peptidases, stock concentration (final concentration was 1:50), solvents and enzyme controls utilized in the assays are indicated in Table 1. The percentage of inhibition was established taking the activity without inhibitor as 100%. All assays were performed in triplicate. Standard deviaton (SD) was never higher than 2%.

Substrate-SDS-polyacrylamide gel electrophoresis

SDS-PAGE of the proteins in the enzyme preparations were according to Laemmli (1970), using 12% acrylamide and 8 × 10 × 0.075 cm gels. MWM (5 μl) was loaded on each plate. Preparation of samples and zymograms of proteinase activities of fractions separated by electrophoresis were according to García-Carreño *et al.* (1993). Samples were diluted (1:1) in sample buffer without ß-mercaptoethanol and not boiled. Each cell of sample contained 40–45 μg of total protein. Electrophoresis was performed at a constant current of 15 mA per gel for 60 min at 5 °C. After electrophoresis, gels were washed and incubated in 50 mM TRIS-HCl buffer, pH 9, containing 0.75% casein Hammerstein for 30 min at 5 °C, and then the temperature was raised to 25 °C for 90 min without agitation. Thereafter, gels were washed, fixed in 12% TCA prior to staining with 0.1% Coomassie Brilliant Blue (BBC R-250) in a methanol–acetic acid solution (50:20:50). Destaining was carried out in a methanol–acetic acid–water solution (35:10:55).

Initial concentration of SDS in gels was 1%. This concentration was progressively diluted along three successive steps: 1:50 after washing with distilled water, 1:25 during incubation at 5 °C and lastly, 1:25 during incubation at 25 °C. This processing of samples rendered a final concentration of 3.2×10^{-5}% of SDS in gels. The elimination of SDS results in a renaturation of the alkaline proteases and the possibility of detecting their activity *in situ*, as has been reported (Lacks and Springhorn, 1980; García-Carreño *et al.*, 1993).

Characterization of protease type in SDS-PAGE zymograms using specific inhibitors was done according to García-Carreño and Haard (1993). Extracts (40 μl) were mixed with 10 μl of inhibitor stock solutions, and after incubation for 1 h at

TABLE 1. Characterization of alkaline proteases in larvae, adults and live food extracts using specific inhibitors and casein as substrate. Each assay was performed in triplicate. Enzymatic inhibition by solvents was never higher than 5%

Inhibitor	Class characterized	Stock concentration	Solvent	Enzyme control	Percentage of inhibition											
					Samples											Enzyme control
					L3	L4	L5	L6	L8	L15	L21	L30	A	R	N	
PMSF	Serine proteases	100 mM	Ethanol	TRY	0	33	22	22	21	**	23	22	27	10	100	98
				TRY-G												52
SBTI	Serine proteases	250 μM	Water	TRY	0	83	76	62	78	63	64	74	61	39	100	99
				TRY-G												53
TLCK	Trypsin	10 mM	HCl 1 mM	TRY	0	25	2	6	12	4	8	10	14	0	56	100
				TRY-G												3
TPCK	Chymotrypsin	5 mM	Ethanol	CHY	0	0	0	35	49	24	11	39	23	0	73	99
ZPCK	Chymotrypsin	10 mM	DMSO	CHY	0	0	8	39	53	7	12	47	31	0	8	98

L, sea bream larvae aged 3 to 30 days; A, pyloric caeca of adults; R, rotifer, *Brachionus plicatilis*; N, *Artemia* nauplii, **, not done.

25 °C, mixed in a 1:1 ratio with sample buffer, and 25 μl loaded on SDS-PAGE plates. Extracts incubated without inhibitor were utilized as controls, as well as commercial pure trypsins (porcine, cod) and chymotrypsin (bovine). Electrophoresis and zymograms were done as previously described. After electrophoresis, excess of inhibitors was washed using 50 mM Tris-HCl buffer, pH 9, for 15 min at room temperature before incubation with substrate.

RESULTS

The zymogram of total alkaline protease activity during larval stage of sea bream, from 3 to 30 days old is shown in Fig. 1A. The number of bands with caseinolytic activity present in zymograms, as well as their molecular weight (relative molecular mass) are detailed in Table 2. Protease activity was evident at the onset of exogenous feeding (day 4) in the form of a clear band (molecular mass, 34 kDa) and also as a diffuse band (57 kDa). Both activities were classified as trypsin-like based in the specific inhibition with TLCK (Fig. 1B; Table 2).

A more complex set of bands was observed from day 6 on (Table 2). All proteases produced during larval development ranged from 20.5 to 57 kDa, giving three main caseinolytic bands (32, 34 and 41.5 kDa) as well as another one (30 kDa) which became clearer as age of larvae progressed (Fig. 1A). A zymogram performed on extracts of the pyloric caeca in adult fish showed differences in number and molecular mass of bands, which ranged from 24 to 69.5 kDa. The major protease activities were found in bands of 30 and 41.5 kDa. The coefficient of similarity between a 30 day-old larva and an adult fish was 50%, because only three bands existing in the larva were also present in the completely developed fish, but another three bands were present only in extracts of pyloric caeca (50, 61.5 and 69.5 kDa).

Table 2 also shows results of protease characterization obtained when incubating extracts of sea bream larvae and adults, as well as live food organisms, with different inhibitors. The main proteolytic activity in larval extracts was classified as serine protease type, because SBTI inhibits more than 60% of total activity, but a lower inhibition was obtained when using PMSF. Trypsin-like activity was detected in a high amount at the moment of mouth opening (day 4), being progressively reduced (in relative proportion to total bands) as the larvae get older and in adult fish. Chymotrypsin-like activity appeared later in the development, being detected from day 6 on.

The proteinase class characterization performed on SDS-PAGE zymograms closely agreed with results obtained using *in vitro* incubation (Table 1). Most of the activity was shown to be that of serine-proteases, evidenced by specific inhibition using SBTI and PMSF (Fig. 1B and 1C). The major class was classified as trypsin-like (50%), but chymotrypsin-like activity was partially mixed with other serine activities because reduction of caseinolytic activity when using TPCK or ZPCK was not complete and a 25% activity remained unidentified (Table 2). Bands existing in larvae and in adult fish (Mr = 30 and 41.5 kDa) were identified as chymotrypsin-like proteases.

Results obtained with rotifers or *Artemia* extracts were quite different. Of the protease activity detected in rotifers, 50% can be classified as the serine protease

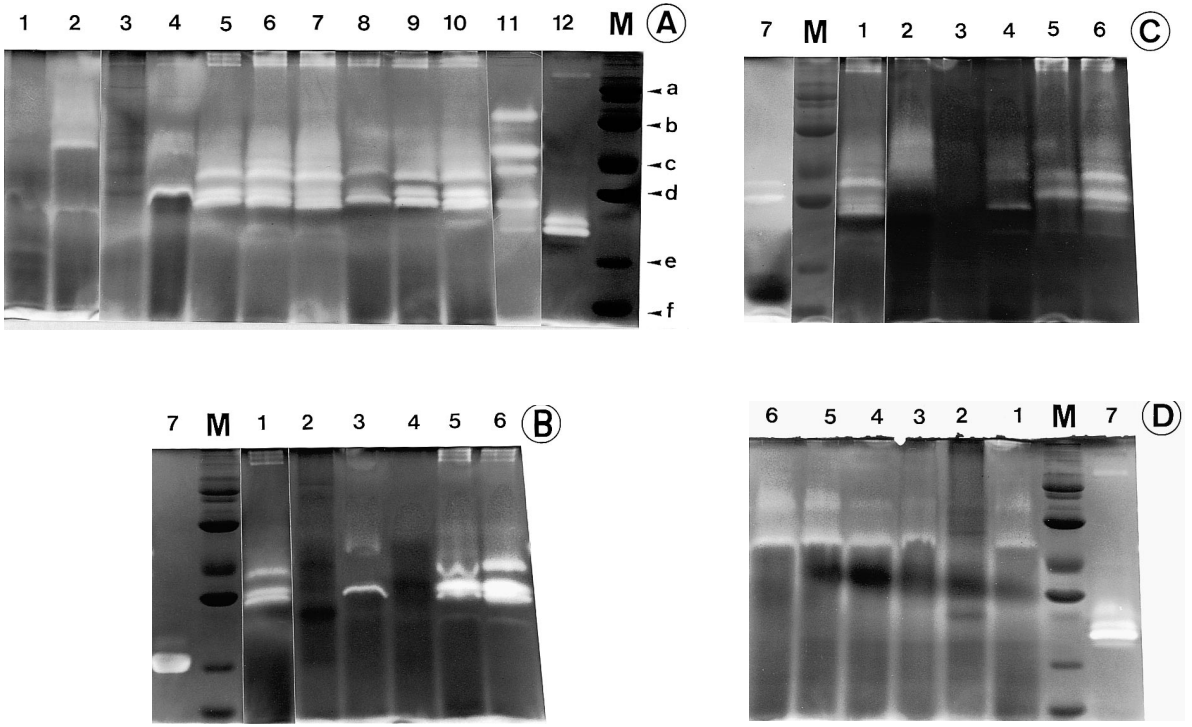

FIG. 1. (A) Substrate-SDS-PAGE of extracts obtained from sea bream larvae at different days after hatching, pyloric caeca from adults, *Artemia* nauplii and rotifers, *Brachionus plicatilis*. M, MWM (5 μg): (a) phosphorylase b (94 000); (b) bovine serum albumin (67 000); (c) ovalbumin (43 000); (d) carbonic anhydrase (30 000); (e) SBTI (20 100) and (f) α-lactalbumin (14 400). Columns: 1, *Artemia* nauplii; 2, rotifer, *Brachionus plicatilis*; 3–10, extracts of larvae at 3, 4, 5, 6, 8, 15, 21 and 30 days post hatching; 11, extract of pyloric caeca in adult fish; 12, trypsin from Atlantic cod (10 μg). (B) Five-day-old larvae. Extracts were mixed with the inhibitors indicated in each column. M, MWM; 1, extract without inhibitors; 2, SBTI; 3, PMSF; 4, TLCK; 5, ZPCK; 6, TPCK and 7, porcine trypsin (2 μg). (C) Eight-day-old larvae. Extracts were mixed with the inhibitors indicated in each column. M, MWM; 1, extract without inhibitors; 2, SBTI; 3, PMSF; 4, TLCK; 5, ZPCK; 6, TPCK and 7, CHY (10 μg). (D) Rotifer, *Brachionus plicatilis*. Extracts were mixed with the inhibitors indicated in each column. M, MWM; 1, extract without inhibitors; 2, SBTI; 3, PMSF; 4, TLCK; 5, ZPCK; 6, TPCK and 7, trypsin from cod (10 μg).

type, and chymotrypsin activity was not detected. In *Artemia* nauplii, 100% of protease activity belongs to the serine type, with trypsin and chymotrypsin each accounting for half of total activity (Table 1). Zymograms of proteinases found in extracts of rotifers and *Artemia* are shown in Fig. 1A. The rotifers presented two bands with caseinolytic activity (Mr = 53 and 71 kDa) and *Artemia* nauplii presented a very attenuated protease activity with bands of 16 and 19.5 kDa molecular mass. No bands of an equivalent mass to those present in larval or adult fish were found (Table 2). Characterization of proteinases existing in either rotifers or *Artemia* showed differences. Fractions present in rotifer extracts were inhibited by SBTI but not by PMSF, whereas proteases found in *Artemia* extracts were inhibited by SBTI, PMSF and TLCK, being classified as trypsin-like (Fig. 1D; Table 2).

Zymograms of the enzymes (trypsins) utilized as controls also showed great differences between those from mammal or fish origin (Fig. 1A, B, D). Trypsin from porcine pancreas (TRY) resulted in two bands with an Mr of 20.5 and 24 kDa, respectively (Fig. 1B,7). Trypsin from Atlantic cod (TRY-G; Fig. 1A,12; 1D,7) presented four bands with caseinolytic activity: main bands corresponded to an Mr of 24.5 and 27 kDa, and minor bands to 23 and 30 kDa. Control enzymes also showed a very different response to inhibition. TRY followed a 'standard' inhibition pattern when incubated with specific inhibitors, whereas TRY-G showed a 50% less response to PMSF and SBTI, and a 97% less response to TLCK than TRY (Table 1). Inhibition

TABLE 2. *Protein fractions with caseinolytic activity in SDS-PAGE zymogram of sea bream and the presumptive identification of class, and variation of these fractions and proteolitic activity during larval development*[1]

			Larval development (days)										
Mr	*Inhibition*[2,3]	*Class*[4]	*3*	*4*	*5*	*6*	*8*	*15*	*21*	*30*	*A*	*R*	*N*
71	*SBTI*	*Ser*										+	
69.5	**	**	−	−	−	−	−	−	−	−	+		
61.5	**	**	−	−	−	−	−	−	−	−	+		
57	*TLCK*	*Try*	−	+	+	+	+	+	+	+	−		
53	*SBTI*	*Ser*										+	
50	**	**	−	−	−	−	−	−	−	−	+		
46	*PMSF*	*Ser*	−	−	−	+	+	+	+	+	−		
41.5	*SBTI,ZPCK*	*Chy*	−	−	+	+	+	+	+	+	+		
34	*TLCK*	*Try*	−	+	+	+	+	+	+	+	−		
32	*TLCK*	*Try*	−	−	+	+	+	+	+	+	−		
30	*PMSF,ZPCK*	*Chy*	−	−	−	+	+	+	+	+	+		
24	*PMSF,TLCK*	*Ser,Try*	−	−	−	+	+	+	+	+	+		
20.5	*TLCK*	*Try*	−	−	−	−	−	−	−	+	−		
19.5	*SBTI,TLCK*	*Ser,Try*											+
16	*SBTI,TLCK*	*Ser,Try*											+

[1] *Abbreviations: Mr, relative molecular mass (molecular weight) (kDa); A, adult fish; R, rotifer; N, Artemia* nauplii.
[2] Reagent codes as explained in methods.
[3] ** indicates that inhibition was not done for adults.
[4] Chy, chymotrypsin-like; Ser, serine proteinase; Try, trypsin-like.

pattern obtained for trypsin-like proteases of sea bream closely resembled that of cod.

DISCUSSION

Proteolytic activity is detected early in marine fish larvae (Alliot *et al*., 1980; Baragi and Lowell, 1986; Cousin *et al*., 1987; Boulhic and Gabaudan, 1992). In the case of sea bream, results obtained using SDS-PAGE are in close agreement with those using *in vitro* assays (Moyano *et al*., 1996) or histochemistry (Sarasquete *et al*., 1995). Nevertheless, the use of electrophoresis allows a greater accuracy in determining and classifying protease activities, because the use of substrate SDS-PAGE makes it possible to determine exactly when a concrete activity appears during ontogeny (Alliot *et al*., 1980; Lauff and Hofer, 1984). With this technique the enzymes are isolated, with no interferences due to other substances present in the extracts that should mask activities. This fact is especially relevant when assaying extracts with a low activity, as is the case for fish larvae. In addition, data obtained using specific inhibitors of caseinolytic activity in different zymograms can be compared because a common substrate is utilized in all analyses.

Results show that a complete alkaline protease set was fully developed in 6 day-old larvae and that no differences could be observed until an age of 30 days, except in the appearance of Mr 20 500 class trypsin. Trypsin activity seems to be important, as it was detected earlier than chymotrypsin and accounted for nearly 55% of serine type activity. In this sense, inhibition of TLCK proved to be more specific, whereas PMSF acts as a general inhibitor of mammalian serine proteases, not very suitable for its use in characterization of fish proteases. In addition to the bands showing a caseinolytic activity, molecular mass and inhibition closely resembling that of trypsin or chymotrypsin, other bands with a similar activity but a higher mass were identified. This result has also been reported in decapods (García-Carreño *et al*., 1994), adult fish (Dimes *et al*., 1994) and in larval stages of other species (Alliot *et al*., 1980) and it is suggested to be due to the presence of isozymes (Lauff and Hofer,1984). The high proportion of trypsin-like proteases has also been observed, suggesting that it could be a compensation for the lack of acid proteases until development of a functional stomach. In fact, when comparing zymograms of larvae and adult sea bream it is possible to observe a net disappearance of four bands, corresponding to trypsin-like proteases. Lauff and Hofer (1987) also hypothesized that acid proteases begin to play an important role in digestion and this fact induces a decrease in alkaline proteases, either by the joint action of pepsin and hydrochloric acid or due to a decrease in the stimuli generating production of those latter enzymes. Recent studies in sea bass larvae are in agreement with this hypothesis (Walford and Lam, 1993).

A first characterization of alkaline proteases present in the rotifer *Brachionus plicatilis* was performed by Hara *et al*. (1984a). These authors found two fractions having alkaline proteinase activities, classified as a serine protease and a trypsin-like protease. By polyacrylamide disc gel electrophoresis in native conditions, the molecular masses of these proteases were estimated to be about 800 000 and 900 000 (Hara *et al*., 1984b). Wethmar and Kleinow (1993a) characterized the proteolytic activity of rotifer extracts by SDS-PAGE 'substrate-containing gel', finding

two major proteases placed in an Mr-region ranging from 45 kDa to 90 kDa. In the same conditions, we also found two caseinolytic bands of 53 kDa and 71 kDa, respectively, that were not present in zymograms of the normally fed larvae utilized in our study. If alkaline proteases existing in rotifers were quantitatively important in relation to total digestive proteases of sea bream larvae, probably they have been revealed in zymograms of larval extracts. Nevertheless, rotifers are susceptible to autolysis (Kleinow, 1993) and their acid proteases show a five times higher activity than alkaline ones (Wethmar and Kleinow, 1993b). Autolysis of rotifers may take place inside the larval gut while loriga is still intact, but in the course of digestion, the alkaline medium existing in the gut would determine the inactivation of rotifer acid proteases and the onset of the alkaline larval enzymes. A similar effect could be suggested for *Artemia* because, as in the case of rotifers, alkaline proteases are quite different from those of larvae and they did not appear in significant amounts in the extracts, but autolysis of nauplii is possible due to the presence of high amounts of cathepsins (Osuna *et al*., 1977; Warner and Shridhar, 1980; Pan *et al*., 1991).

Considering the above-mentioned data, as well as the existence of a well-developed set of alkaline proteases, evidenced by the diversity and good contrast of bands obtained in electrophoresis, it seems that exogenous enzymes provided with food only initialize protein digestion in sea bream larvae, but larval proteases play the main role in the process. This is in agreement to results obtained in other marine fish (Zambonino and Cahu, 1994) and is supported by the existence of a greater diversity in proteases of sea bream larvae when compared with adult fish, resulting in a digestion process closely resembling that of a stomachless fish, such as the carp, *Cyprinus carpio*.

CONCLUSIONS

1. The set of alkaline proteases of larval sea bream seems to be complex and well developed 3 days after hatching.
2. Most such proteases belong to the serine type.
3. The alkaline proteases differ substantially in larvae and adults; some proteases remain during development but others, mainly identified as trypsin-like, disappear with time.
4. Characterization of alkaline proteases existing in organisms commonly utilized as live food showed great differences in molecular mass and mechanism of action when compared with those of larvae. Indeed, zymograms of extracts obtained from fed larvae did not reveal the existence of such proteases in the digestive tract.
5. The role of exogenous enzymes in the digestion of larval sea bream seems limited to an autolytic process occurring in rotifers and *Artemia* due to the action of their own acid proteases. This process may be neutralized when such proteases contact the alkaline contents of larval gut.
6. Considering this fact, and the diversity of alkaline proteases identified, the possibility of developing artificial diets for larval stages of this species seems not to be limited by a theoretical inability to digest food protein. Easily digestible proteins will make acid digestion unnecessary.

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REFERENCES

Alliot, E., Pastoureaud, A. and Trellu, J. (1980) Evolution des activites enzymatiques dans le tractus digestif au cour de la vie larvaire de la sole. Variations des proteinogrammes et des zymogrammes. *Biochemical Systematics and Ecology* **8,** 441–445.

Baragi, V. and Lowell, R.T. (1986) Digestive enzyme activities in striped bass from first feeding through larval development. *Transactions of the American Fisheries Society* **115,** 478–484.

Boulhic, M. and Gabaudan, J. (1992) Histological study of the organogenesis of the digestive system and swim bladder of the Dover sole, *Solea solea* (Linneaus 1758). *Aquaculture* **102,** 373–396.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248–254.

Cahu, C.L. and Zambonino Infante, J.L. (1994) Early weaning of sea bass (*Dicentrarchus labrax*) larvae with a compound diet: effect on digestive enzymes. *Comparative Biochemistry and Physiology* **109A,** 213–222.

Cousin, J.C.B., Baudin-Laurencin, F. and Gabaudan, J. (1987) Ontogeny of enzymatic activities in fed and fasting turbot, *Scophthalmus maximus* L. *Journal of Fish Biology* **30,** 15–33.

Dabrowski, K. (1979) The role of proteolytic enzymes in fish digestion. In: *Cultivation of Fish Fry and its Live Food* (eds E. Styczynska-Jurewicz, T. Backiel, E. Jaspers and J. Persoone), Special Publications 4. European Mariculture Society: Bredene, Belgium, pp. 107–126.

Dimes, L.E., García-Carreño, F.L. and Haard, N.F. (1994) Estimation of protein digestibility, 3. Studies on the digestive enzymes from the pyloric ceca of rainbow trout and salmon. *Comparative Biochemistry and Physiology* **109A,** 349–360.

Dunn, B.M. (1989) Determination of protease mechanism. In: *Proteolytic Enzymes: A Practical Approach* (eds R.J. Beynon and J.S. Bond) IRL Press: Oxford, England, pp. 57–81.

García-Carreño, F.L. (1992) Protease inhibition in theory and practice. *Biotechnology Education* **3**(4), 145–150.

García-Carreño, F.L. and Haard, N.F. (1993) Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. *Journal of Food Biochemistry* **17,** 97–113.

García-Carreño, F.L., Dimes, L.E. and Haard, N.F. (1993) Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceus proteinase inhibitors. *Analytical Biochemistry* **214,** 65–69.

García-Carreño, F.L., Hernández-Cortés, M.P. and Haard, N.F. (1994) Enzymes with peptidase and proteinase activity from the digestive systems of a freshwater and a marine decapod. *Journal of Agricultural and Food Chemistry* **42,** 1456–1461.

Hara, K., Arano, H. and Ishihara, T. (1984a) Some enzymatic properties of alkaline proteases of the rotifer *Brachionus plicatilis. Bulletin of the Japanese Society of Scientific Fisheries* **50,** 1611–1616.

Hara, K., Arano, H. and Ishihara, T. (1984b) Purification of alkaline protease of the rotifer *Brachionus plicatilis*. *Bulletin of the Japanese Society of Scientific Fisheries* **50,** 1605–1609.

Kleinow, W. (1993) Biochemical studies on *Brachionus plicatilis*: hydrolytic enzymes, integument proteins and composition of trophi. *Hydrobiologia* **255/256,** 1–12.

Kolkovski, S., Tandler, A., Kissil, G.Wm. and Gertler, A. (1993) The effect of dietary exogenous enzymes on ingestion, assimilation, growth and survival of gilthead seabream (*Sparus aurata*, Sparidae, Linnaeus) larvae. *Fish Physiology and Biochemistry* **12,** 203–209.

Lacks, S.A. and Springhorn, S.S. (1980) Renaturation of enzymes after polyacrylamide gel eletrophoresis in the presence of sodium dodecyl sulphate. *The Journal of Biological Chemistry* **225,** 7467–7473.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lauff, M. and Hofer, R. (1984) Proteolitic enzymes in fish development and the importance of dietary enzymes. *Aquaculture* **37,** 335–346.

Moyano, F.J., Díaz, M., Alarcón, F.J. and Sarasquete, M.C. (1996) Characterization of digestive enzyme activity during larval developement of gilthead seabream (*Sparus aurata*). *Fish Physiology and Biochemistry* (in press).

Munilla-Morán, R., Stark, J.R. and Barbour, A. (1990) The role of exogenous enzymes in digestion in culture of turbot larvae (*Scophthalmus maximus* L.). *Aquaculture* **88,** 337–350.

Osuna, C., Olalla, A., Sillero, A., Günther Sillero, M.A. and Sebastián, J. (1977) Induction of multiple proteases during the early larval development of *Artemia salina*. *Developmental Biology* **61,** 94–103.

Pan, B.S., Lan, C.C. and Hung, T.Y. (1991) Changes in composition and proteolytic enzyme activities of *Artemia* during early development. *Comparative Biochemistry and Physiology* **100A,** 725–730.

Polo, A., Yúfera, M. and Pascual, E. (1992) Feeding and growth of gilthead seabream, *Sparus aurata* L. larvae in relation to the size of the rotifer strain used as food. *Aquaculture* **103,** 45–54.

Sarasquete, M.C., Polo, A. and Yúfera, M. (1995) Histology and histochemistry of the development of the digestive system of larval gilthead seabream, *Sparus aurata* L. *Aquaculture* **130**, 79–92.

Walford, J. and Lam, T.J. (1993) Development of digestive tract and proteolytic enzyme activity in seabass (*Lates calcarifer*) larvae and juveniles. *Aquaculture* **109,** 187–205.

Warner, A.H. and Shridhar, V. (1980) Characterization of an acid protease from encysted embryos of *Artemia*. In: *The Brine Shrimp. Vol. 2. Physiology, Biochemistry, Molecular Biology* (eds G. Persoone, P. Sorgeloos, O. Roels and E. Jaspers) Universa Press: Wetteren, Belgium, pp. 355–364.

Wethmar, Chr. and Kleinow, W. (1993a) Characterization of proteolytic activities stimulated by SDS or urea and two-dimensional gel electrophoresis of proteins from *Brachionus plicatilis* (Rotifera). *Comparative Biochemistry and Physiology* **106B,** 349–358.

Wethmar, Chr. and Kleinow, W. (1993b) Characterization of a 24 kDa endopeptidase and detection of a proteinase-inhibitor in homogenates of *Brachionus plicatilis* (Rotifera). *Comparative Biochemistry and Physiology* **106B,** 359–368.

Zambonino Infante, J.L. and Cahu, C.L. (1994) Development and response to a diet change of some digestive enzymes in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiology and Biochemistry* **12,** 399–408.