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Characterization of fish acid proteases by substrate–gel electrophoresis

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Abstract

Several analytical techniques based upon the use of substrate–polyacrylamide gel electrophoresis were evaluated to achieve characterization of aspartate proteases in fish stomach. Since aspartate proteases of fish are more stable at high pH than mammalian pepsins, the most accurate technique for activity assessment is electrophoresis at neutral pH and revealing of such activity at low pH with hemoglobin as substrate. The technique is suitable for characterization of proteases and in comparative assessment of acid protease activity in different sparids. © 1998 Elsevier Science Inc. All rights reserved.

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1. Introduction

Most analytical methods in digestive enzymology have been developed to study mammals or micro-organisms, mainly because samples from these two types of organisms are very easy to obtain. However, there is an increasing interest in knowing more about digestive enzymes present in different animals living in aquatic environments (both marine and freshwater), especially in the case of aquacultured species. Problems arise when the available methods are not suitable for the analysis of enzyme activities in these organisms. For example, it is well known that fish proteases have particular properties: pepsins from cold water fish exhibit lower Arrhenius activation energy, temperature optima, and thermal stability and a higher apparent Michaelis constant and pH optima, when compared to pepsins from ectotherm animals living in temperate or warm habitat or those from endotherms [14,12]. In addition, fish pepsins have high isoelectric points (IPs) (ranging from 3 to 5) when compared to mammalian pepsins, which not exceed 2 [14]. Such different properties demand the use of appropriate analytical methods for characterization.

Since substrate electrophoresis is a major technique for enzyme studies, it has been utilized in the assessment of the composition, molecular mass and classes of proteases present in crude extracts from fish digestive tracts [10,11]. Since only intestinal proteases of fish have been preferentially studied with electrophoresis, the techniques are fitted to the evaluation of proteases with an alkaline pH optimum. Nevertheless, due to the absence of a specific methodology for the evaluation of fish stomach proteases, a lack of information is evident. This could be influenced by different problems related to sensitivity of acid proteases to the composition of electrophoresis buffers, and migration to the cathode when analyzed under a low pH discontinuous system [23]. In the present work, several techniques for native electrophoresis of fish acid proteases are evaluated. We propose the use of native polyacrylamide gel elec-

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trophoresis (PAGE) at neutral pH to overcome the disadvantages of previously reported techniques. The validity and usefulness of our methodology is tested by analyzing stomach proteases of several sparid fishes with differing feeding habits.

2. Materials and methods

2.1. Source of enzymes

Most of the experiments were carried out with medium sized (50–75 g) gilthead sea bream (*Sparus aurata*), reared and supplied by a local fish farm (FRAMAR S.L. Carboneras, Almería, Spain). Additionally, we used other sparids in some of the assays: common dentex (*Dentex dentex*, 40–50 g), reared and supplied by the Instituto Español de Oceanografía (Mazarrón, Murcia, Spain), couch's sea bream (*Pagrus pagrus*), pandora (*Pagellus erythrinus*), bogue (*Boops boops*) and saupe (*Salpa salpa*), ranging from 80 to 150 g, obtained from the Mediterranean Sea by a local fisherman. Fish stomachs were separated from the esophagus and pyloric ceca, and the gastric mucosa was mechanically homogenized in distilled water (pH 6.0) (1:10 w/v). Active extracts, obtained by centrifugation for 30 min at 16900 × *g* and 4°C, showed a pH from 4.0 to 5.0 and were maintained overnight at 4°C in order to activate pepsinogen. After that, the aqueous extracts were stored at −20°C until use. The amount of soluble protein in the extracts was measured using the Bradford method [3].

2.2. Reagents

Porcine pepsin (pepsin A, EC 3.4.23.1), hemoglobin (Hb), phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), Tris, glycine, alanine, sodium dodecyl sulfate (SDS), and EDTA were purchased from Sigma Química (Madrid, Spain). Pepstatin A was obtained from ICN Biomedicals (Costa Mesa, CA), electrophoresis chambers and reagents from Bio-Rad (Richmond, CA), and Sephadex G-25S® and molecular weight markers from Pharmacia Biotech (Uppsala, Sweden).

2.3. Enzyme assay

Acid protease activity was evaluated using hemoglobin as the substrate as follows: 1 ml of 0.5% Hb in 0.1 M Gly·HCl, pH 2.0, was mixed with 20 μl of enzyme extract. The reaction mixture was incubated for 30 min at 25°C. The reaction was stopped by adding 0.5 ml of 20% trichloroacetic acid (TCA). The TCA precipitate was chilled for 30 min at 4°C. The absorbance of the soluble TCA peptides was recorded at 280 nm in a UV–Vis spectrophotometer. One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μg of tyrosine from hemoglobin per minute at the conditions of the assay. Commercially obtained porcine pepsin was used as control.

The evaluation of the protease class was based on the method of García-Carreño and Haard [9,10]. Enzyme extracts were incubated with different specific protease inhibitors such as PMSF and SBTI for serine proteases, pepstatin A for aspartate proteases, and inactivators such as chelators (EDTA) for metalloproteases. Five microliters of a solution containing the inhibitor was mixed with 20 μl of the enzyme extract and incubated for 60 min at 25°C. Then, 1 ml of a solution of substrate containing 0.5% Hb in 0.1 M Gly·HCl, pH 2.0, was added. Assays including the solvents of the inhibitors were used as controls. Activity in inhibition assays is reported as a percentage, considering the activity measured in the absence of inhibitor or chelator as 100%. Assays were done in triplicate.

2.4. Electrophoresis

Assessment of proteins present in active extracts was done by PAGE, according to Laemmli [18], using 8 × 10 × 0.075 cm gels. Electrophoresis of fish pepsins was performed using three different nondissociating discontinuous methods (Table 1): (1) *neutral electrophoresis*, where the gel and electrophoresis buffers were prepared by modifying the method of Williams and Reisfeld [28]; (2) *alkaline electrophoresis*, done according to Davies [7]; and (3) *acid electrophoresis*, done according to Reisfeld et al. [23].

The time for gel polymerization ranged from 30 min at alkaline pH to several hours for neutral to acid pH. The use of riboflavin instead of ammonium persulfate-TEMED is recommended to accelerate polymerization of acrylamide [15]; however, good results were obtained in the present work when the ammonium persulfate-TEMED *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine concentration was increased (Table 1).

Samples of stomach extracts were prepared for electrophoresis as follows: (a) the analysis of protein composition SDS-PAGE was done according to Laemmli [18]; and (b) the analysis of protease composition (substrate–SDS-PAGE) was done by mixing one volume of sample with one volume of the sample buffer (Table 1). The diluted samples were not boiled. Samples for substrate–SDS-PAGE contained three units of protease activity in less than 35 μg of soluble protein.

In methods (1) and (2), the cathode (−) was attached to the upper buffer cell and the anode (+) to the lower buffer cell. In method (3), when analyzing porcine pepsin, the cathode and anode were connected in the same position as in methods (1) and (2), but when analyzing the fish protease, the position of elec-

Table 1
Nondissociating discontinuous system electrophoresis: components and development

	Alkaline	Neutral	Acid
Stacking gel			
Final % PAA[a]	3.5	3.5	3.5
Stacking buffer	0.125 M Tris·HCl, pH 6.8	0.1 M Tris·H_3PO_4, pH 5.5	0.127 M acetic acid, 0.12 M KOH, pH 6.8
10% ammonium persulphate (μl)	30	40	50
TEMED (μl)[b]	6	15	8
Resolving gel			
Final % PAA[a]	12	11	11
Resolving buffer	0.375 M Tris·HCl, pH 8.8	0.07 M Tris·HCl, pH 7.5	0.376 M acetic acid, 0.06 M KOH, pH 4.3
10% ammonium persulphate (μl)[c]	50	75	75
TEMED (μl)[c]	6	8	50
Electrode buffer	0.025 M Tris, 0.192 M Gly, pH 8.3	5 mM Tris, 0.62 M Gly, pH 7.0[d]	0.14 M acetic acid 0.35 M β-Ala, pH 4.5
Sample buffer (diluted 1:1 with samples)	0.125 M Tris·HCl, pH 6.8, 20% glycerol, 0.04% bromophenol blue	0.1 M Tris·H_3PO_4, pH 5.5, 20% glycerol, 0.02% methylene blue	0.127 M acetic acid, 0.12 M KOH, pH 6.8, 20% glycerol, 0.02% methylene blue
Electrophoresis time/tracking dye electromobility[e]	1 h 30 min/4 cm	1 h/6 cm	4–5 h/2 cm

All the molarities of solutions are the final concentrations during the electrophoresis and were obtained by diluting the corresponding stock solutions, except for the sample buffer [2].
[a]PAA (polyacrylamide), acrylamide–bisacrylamide, 30:0.8 (fresh solution or less than 1 month old).
[b]Final volume = 5 ml.
[c]Final volume = 10 ml.
[d]This buffer system was used instead of the oroginal barbital buffer [28].
[e]Constant voltage, 100 V per gel. Length in resolving gels.

trodes was inverted. All assays were performed at a constant voltage of 100 V per gel and variable amperage (mA). The electrophoretic separation was closely related to the method used (Table 1). Further information about the electrophoresis techniques can be obtained from the comprehensive chapter by Hames [15].

2.5. Development of enzyme activity (zymograms)

After electrophoresis, gels were removed from the cell and soaked in 0.1 M HCl to reduce the pH to 2.0, for the enzymes to become active. After 15 min, the gel was soaked for 30 min in a solution containing 0.25% Hb in 0.1 M Gly·HCl, pH 2.0 and 4°C, then for 90 min in a fresh Hb solution at 37°C. Gels were washed in distilled water and fixed for 15 min in 12% TCA solution.

2.6. Gel staining

After development of the enzyme activity, gels were stained by using a filtered 0.1% solution of Coomassie brilliant blue R-250 in methanol–glacial acetic acid–water (5:2:5). Destaining was carried out using an aqueous solution of 30% methanol and 10% acetic acid. Clear zones revealing activity of acid proteases could be seen in a few minutes, although well-defined zones were obtained only after 2–4 h staining. Stained gels were photographed for recording and dried.

2.7. Characterization of protease type in zymogram PAGE

Characterization of proteases using specific inhibitors was done according to García-Carreño and Haard [10]. Active extracts (40 μl) were mixed with 10 μl of inhibitor or inactivator stock solution. After incubation for 1 h at 25°C, the mixture was diluted with sample buffer (1:1) and 25 μl of the resultant solution were loaded on SDS-PAGE plates. Extracts incubated without inhibitor were used as controls. Electrophoresis, development of enzyme activity, and staining of zymograms were done as previously described. After electrophoresis, excess of inhibitors was removed by washing of gels for 15 min at room temperature with 0.1 M HCl, pH 2.0, (solution:gel, 100:1), before incubation with the substrate.

2.8. Determination of IPs

Prior to the determination of their IP, proteases were purified by electroelution following Harrington [16]. The IP was estimated by isoelectric focusing (IEF)

Table 2
Activity in hemoglobin of seabream and dentex stomach, and porcine pepsin after incubation with inhibitors

Target class	Inhibitors[a]			Inhibitor solvents		
enzyme[b]	SBTI serine protease	PMSF serine protease	Pepstatin A aspartate protease	EDTA metalloprotease	Ethanol	DMSO
Seabream	88 (0.2)	93 (0.3)	1 (0.2)	124 (0.2)	90 (0.3)	85 (0.1)
Dentex	93 (0.5)	91 (0.5)	1 (0.1)	113 (0.2)	92 (0.3)	90 (0.3)
Pepsin[c]	90 (0.3)	95 (0.2)	2 (0.1)	111 (0.1)	96 (0.2)	94 (1.0)

[a] Stock solution: SBTI, 250 M in water; PMSF, 100 mM in ethanol; Pepstatin A, 1 mM in DMSO; EDTA, 0.5 M in water.
[b] Residual activity in % (± S.D.). Data are the mean of triplicate determinations.
[c] Porcine pepsin was prepared as a 1 mg ml^{-1} solution.

using 125 × 65 × 0.4 mm gels in a chamber model 111 mini-IEF cell (Bio-Rad). Five microliters of enzyme preparation were applied, using the paper sample applicators, onto IEF gel containing 5% monomer acrylamide, 5% glycerol, and 1:20 dilution of Bio-Lyte®, pH 3–10. The focusing condition was 100 V for 15 min, 200 V for 15 min, and 450 V for an additional 60 min [2]. After this time, the gel was treated as before for development of enzyme activity.

3. Results

Stomach proteases detected in extracts of seabream and dentex were classified as aspartate proteases (E.C. 3.4.24.x.): inhibitors for serine proteases did not affect their activity, but pepstatin A, a specific inhibitor for aspartate proteases, reduced it to almost zero, in a similar way to that observed for porcine pepsin (Table 2). Additionally, fish enzymes showed no dependence on divalent ions, since addition of EDTA to the reaction mixture did not reduce, but enhanced their activity.

Seabream acid protease was measured in the presence of either buffers or SDS utilized in electrophoresis. The enzyme was highly sensitive to incubation at pH values greater than 7.0, retaining only 25% of its activity after 30 min of preincubation (Fig. 1A). In contrast, porcine pepsin, used as reference, was completely inactivated under the same assay conditions (Fig. 1B). The fish enzyme activity was recovered after incubation in 0.1 M HCl for 15 min at room temperature. Incubation of extracts in SDS at the concentration utilized in the electrophoresis systems (0.1%) reduced activity of seabream protease by 50% after 20 min incubation (Fig. 2). This denaturation was not reversible.

Electrophoresis performed at neutral or alkaline pH (which included SDS) yielded no activities of either seabream or porcine pepsins, even when an acid buffer was utilized for development. To avoid this problem, different non-dissociating discontinuous electrophoresis systems were tested.

3.1. PAGE at alkaline pH

In electrophoresis performed under alkaline pH, activity of seabream acid protease was clearly revealed, although resolution of bands was poor. Activity of porcine pepsin was not detectable using this system (Fig. 3).

3.2. PAGE at acid pH

The nondissociating substrate–PAGE system at acid pH revealed the activity of both porcine pepsin and fish (seabream and dentex) acid proteases (Fig. 4). However, all activities could not be seen simultaneously in

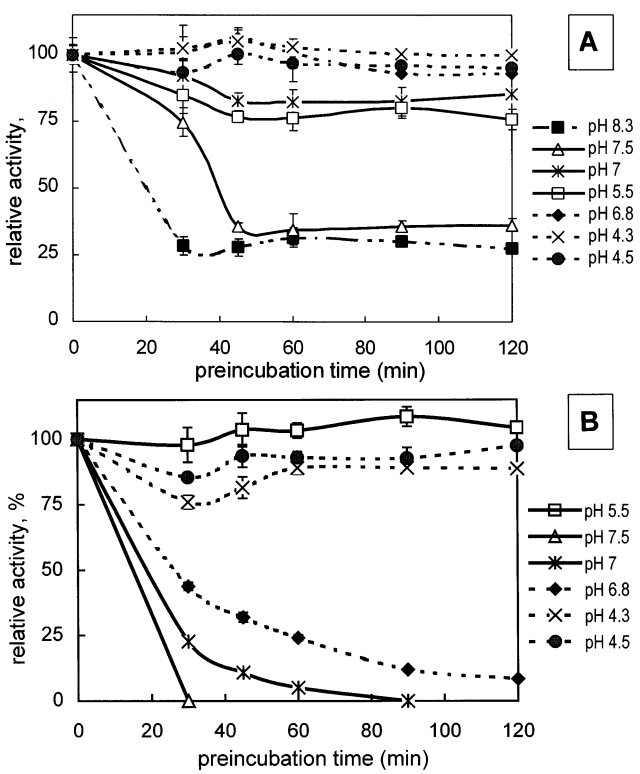

Fig. 1. Effect of pH and composition of PAGE system buffers on enzyme activity of stomach extracts of seabream (A) and porcine pepsin (B). For buffer systems, see Table 1.

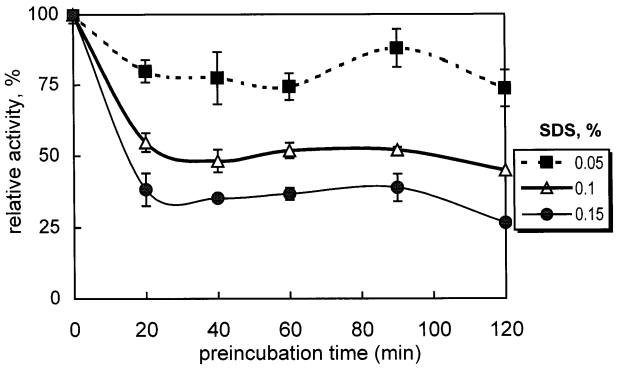

Fig. 2. Effect of SDS on enzyme activity of stomach extracts of seabream.

the vertical electrophoresis system. When electrodes were connected as usual, only porcine pepsin migrated because of its negative charge, while fish proteases did not migrate into the electrophoresis gel. It was necessary to reverse the polarity of the current by placing the cathode in the lower buffer cell to show activity of fish extracts (Fig. 4, lane 1 and 2). Under these conditions, only fish acid proteases migrated, since they were positively charged under the pH of this electrophoresis system (Fig. 4, lanes 3 and 4). Zymograms obtained for acid proteases of both seabream and dentex were almost identical, showing a single band (Fig. 4, lanes 5 and 6) with a slow migration, in spite of the electrophoresis lasting about 5 h.

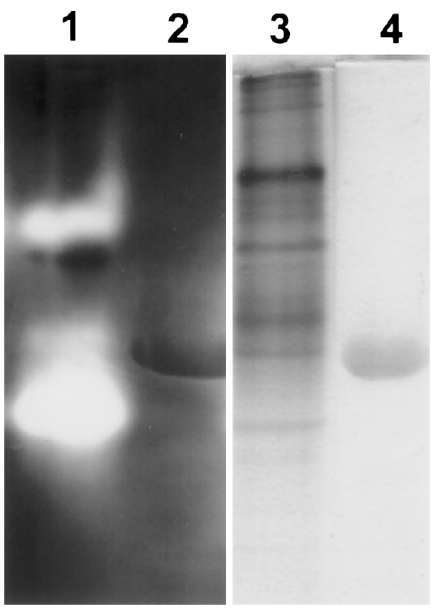

Fig. 3. Native PAGE system at alkaline pH of acid proteases in stomach extracts of seabream. Lanes 1 and 2, zymograms of protease activity (clear zones) using hemoglobin as the substrate; lanes 3 and 4, protein electropherogram stained with Coomassie Brilliant Blue R-250, as detailed in Section 2. Lanes 1 and 3, *Sparus aurata*; lanes 2 and 4, porcine pepsin.

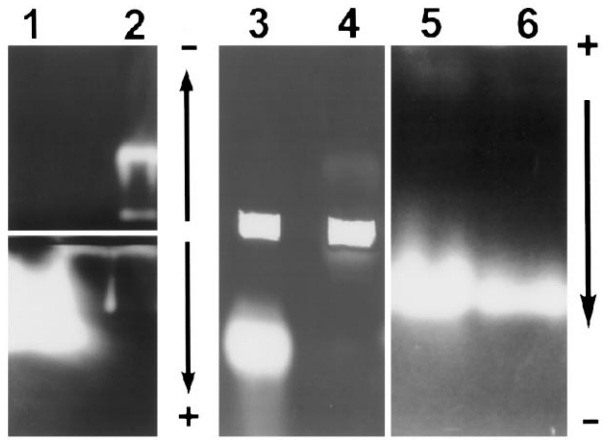

Fig. 4. Substrate–PAGE system at acid pH. Lanes 1 and 2 are two different and complementary zymograms prepared in vertical electrophoresis systems (lane 1, porcine pepsin; lane 2, seabream stomach extract). Acid protease of seabream is visualised in the upper gel (lane 2), the lower gel shows porcine pepsin (lane 1). Lanes 3 and 4 are the same but in a horizontal electrophoresis system. Lane 5 (*Sparus aurata*) and lane 6 (*Dentex dentex*) are from a vertical electrophoresis system.

In order to explain such anomalous behaviour of fish acid proteases, some additional assays were performed: (1) identification of seabream pepsin in zymograms, using a specific inhibitor (pepstatin A, Fig. 5); (2) purification through electroelution, starting from electrophoresis at neutral pH and resulting in a protein with a molecular mass of 67 kDa (Fig. 6); (3) calculation of the IPs of seabream pepsin by IEF. Two bands with IPs of 4.9 and 4.1 (Fig. 7, lane 1) were identified; only the band with an IP of 4.1 was inhibited by pepstatin A (lanes 3 and 4).

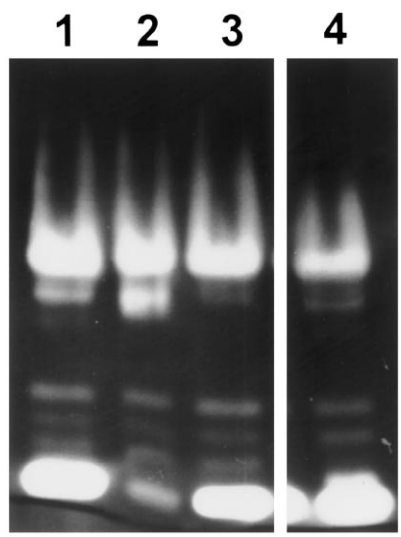

Fig. 5. Substrate–PAGE at neutral pH of stomach extracts in seabream treated with several protease inhibitors. Lane 1, EDTA; lane 2, Pepstatin A; lane 3, SBTI; lane 4, control without inhibitor.

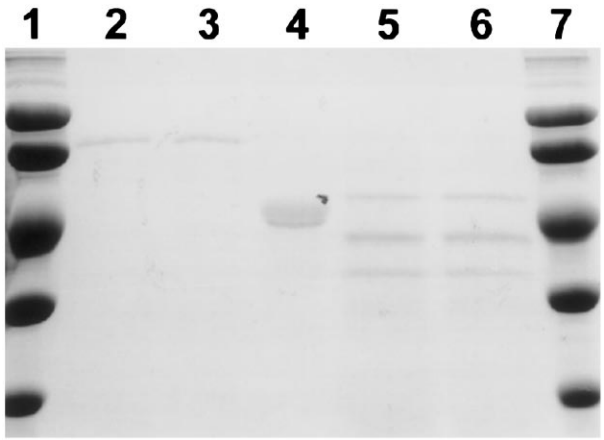

Fig. 6. SDS-PAGE of stomach extracts in seabream. Lane 1 and 7, molecular weight markers (phosphorylase *b* (94000), bovine albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), and soybean trypsin inhibitor (20100)); lanes 2 and 3, purified extracts of seabream; lane 4, porcine pepsin; lanes 5 and 6, crude extracts of stomach.

3.3. PAGE at neutral pH

Assays carried out at neutral pH gave clear zones as a result of hydrolysis of the hemoglobin substrate, indicating activity of seabream acid protease, but not of porcine pepsin (Fig. 8). Zymograms of acid proteases present in stomach extracts of several fish species obtained using the neutral electrophoresis system are shown in Fig. 9. Seabream, *Pagrus* and *Pagellus* had two or three active bands (lanes 1–4) while *Sarpa* and *Boops* only showed one low activity band each (lanes 5 and 6).

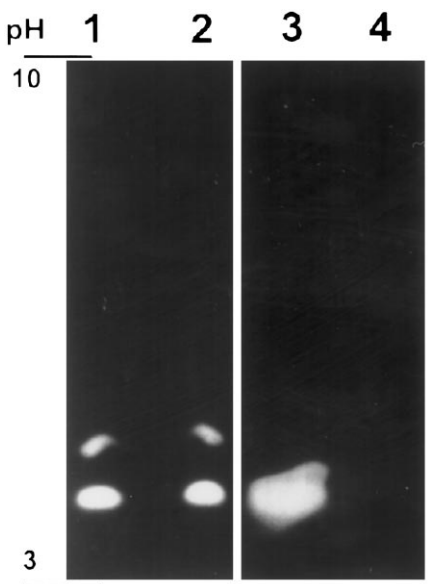

Fig. 7. Substrate IEF of acid proteases in stomach. Lane 1, crude extracts of seabream; lane 2, crude extracts of common dentex; lane 3, purified extract of seabream stomach; lane 4, purified extracts treated with Pepstatin A.

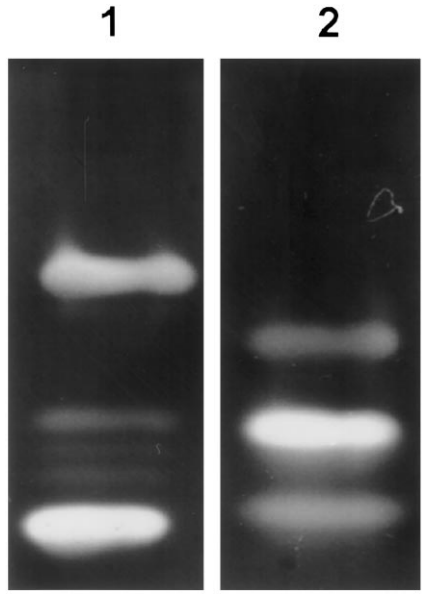

Fig. 8. Substrate–PAGE system at neutral pH of acid proteases present in stomach homogenates of seabream (lane 1) and common dentex (lane 2).

A summary of differences in resolution power and ability of the three methods for identification of seabream acid proteases is shown in Fig. 10.

4. Discussion

A number of studies have demonstrated that, according to its inhibition pattern, at optimum pH for activity, stability and origin, the major protease in fish stomach is pepsin [6]. Several important differences between mammal and fish enzymes have been reported

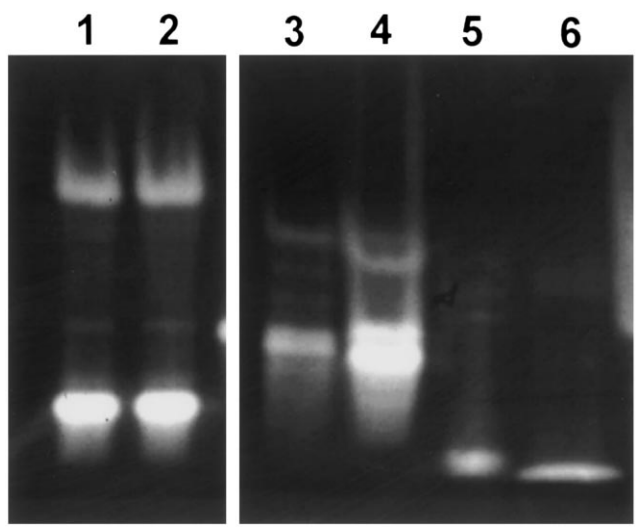

Fig. 9. Substrate–PAGE at neutral pH of acid proteases present in stomach extracts of seabream (lanes 1 and 2), couch's sea bream (lane 3), pandora (lane 4), bogue (lane 5) and saupe (lane 6).

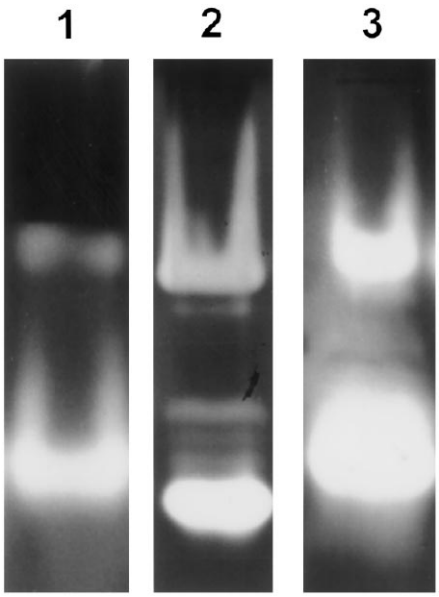

Fig. 10. Substrate–PAGE of stomach homogenates in seabream obtained with different nondissociating electrophoresis systems as detailed in Table 1. Lane 1, acid system; lane 2, neutral system; lane 3, alkaline system.

in species such as salmon [21], tuna [22], bonito [17], hake [24], Greenland cod [25], Atlantic cod [4], tilapia [30], and sardine [20]. Some other studies demonstrated a greater stability of fish acid proteases in alkaline environments, since they show the ability of renaturation after incubation in high pH buffers [1,19]. In addition, they have a higher IP and a different sensitivity to substances like SDS. This unusual stability of fish acid proteases to alkaline buffers may be related to their amino acid composition, characterized by a high proportion of basic residues when compared with those of mammals [14].

All these properties determine that the electrophoretic techniques commonly used for identification of activity in digestive extracts of mammals do not offer satisfactory results for fish pepsins. Therefore, we developed a suitable technique to allow comparative studies between pepsins of different fish species. In the preliminary assays, activity of fish pepsins could not be observed using this electrophoresis system, since enzymes of both seabream and dentex were sensitive to the SDS concentration on the PAGE gels, and washing of gels before incubation with the hemoglobin substrate did not result in a better resolution. Therefore, seabream and common dentex pepsins were studied using nondissociating substrate–PAGE. Three PAGE systems (alkaline, neutral, and acid) were assayed. By analyzing in the test tube and by electrophoresis, it was demonstrated that buffer salt composition of the three nondissociating systems did not affect fish pepsin activity.

Surprisingly, the acid system utilized for identification of mammalian pepsins offered the worst results. The low resolution obtained with this technique did not allow comparisons between species. In addition, the time needed for completing the assay was very long (4–6 h). In order to explain that abnormal behaviour found in seabream pepsin, different experiments aimed at the determination of its IP were performed. High IPs found in both seabream and dentex acid proteases should explain the particular behaviour of their enzymes when assayed under acid conditions. The IP is a key factor in electrophoretic study of these enzymes. The IP is related to the electric charge of the protein and probably to the stability of the enzyme. Net electric charge of the enzyme and the pH of the electrophoresis system affect its mobility and, hence, the resolution of the zymogram. Fish gastric proteases have a higher IP [13], than porcine pepsin [29]. In opposition to porcine pepsin, seabream and dentex pepsins were positively charged at the pH of the acid electrophoresis system. Thus, electrode polarity should be reversed to allow electrophoretic mobility of such enzymes within the gels.

The alkaline electrophoretic system made it possible to obtain a better identification of the proteases than the acid PAGE, but the resolution power remained low. It was also surprising that enzymes retained activity after being treated for 90 min at pH 8.8. This could be explained by the ability of fish pepsins to renaturalize when incubated in acid pH. From a physiological point of view, considering both the increases in stomach pH resulting from food and water ingestion determined in different fish species (pH 4.3 in seabass [8], pH 5.2 in eel [26], or around pH 4.0 in yellowtail [5]), and the need for a suitable pH for the activity of acid proteases, it may be concluded that acid proteolysis will not occur until secretion of acid is sufficient to decrease the stomach pH. The stability of seabream or dentex acid proteases until secretion of acid establishes suitable conditions for their activity, seems to be a good adaptation towards complete protein digestion. This feature is especially interesting considering that fish are ectotherms, their physiological responses being much slower than those of mammals. This particular feature should also explain the artifacts found by several authors, who have identified acid proteases in fish intestines that logically could not be produced in such tissues, but are contributed by the stomach [19,27].

Clear differences in sensitivity to alkaline pH were noted when comparing fish and mammalian pepsins; porcine pepsin could only be studied using the acid substrate–PAGE system, whereas zymograms of fish enzymes were similar, regardless of the pH of the PAGE system. This is because fish enzymes are only

slightly sensitive to pH values greater than 8.0 and denaturation by alkaline pH can be reversed when extracts are incubated in HCl.

Because of their stability at alkaline pH, fish pepsins are best analyzed by the neutral substrate–PAGE system. In the neutral electrophoresis system, a mobility close to 6 cm h^{-1} is possible and good resolution is obtained, showing differences between the two species studied. Using this technique, both the composition and class of proteases present in fish stomach extracts can be assessed. A preliminary screening performed on several sparids showed the existence of a more developed enzymatic equipment in mainly carnivorous species such as seabream, common dentex and *Pagrus*, which showed up to three proteolytic fractions. Omnivorous and herbivorous species, such as *Sarpa* or *Boops*, showed a single proteolytic fraction with low activity. In the latter species, stomach proteolysis does not seem to be a major determinant in the overall digestive process. In summary, the methodology developed in the present work may be useful in the evaluation of digestive proteases present in any fish species. Up to now, most studies only focused on intestinal proteases, and overlooked proteolytic stomach enzymes, in spite of their importance in digestive processes.

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