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Effect of inhibitors present in protein sources on digestive proteases of juvenile sea bream (*Sparus aurata*)

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Abstract — The presence of protease inhibitors in different ingredients used in fish feeds may negatively affect their digestive utilisation. The effect of inhibitors present in several protein sources on the activity of digestive proteases of sea bream was assessed using 'in vitro' assays. Inhibition produced by extracts of plant proteins ranged from 25 to 50 % of total activity, whereas that obtained using animal protein sources ranged from 1 to 20 %. The exception was ovoalbumin, which showed the highest measured value (62 %). A plot of the inhibition values obtained by changing the relative concentrations enzyme/inhibitor resulted in different curves for different protein sources. The use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) zymograms allowed visualisation of the aforementioned differences in inhibition. The importance of measuring protease inhibition in the preliminary evaluation of fish feed ingredients is discussed. © 1999 Ifremer/Cnrs/Inra/Ird/Cemagref/Éditions scientifiques et médicales Elsevier SAS

Protease inhibition / proteases / 'in vitro' assays / *Sparus aurata* / sea bream

Resumen — Efecto de inhibidores presentes en fuentes proteicas sobre las proteasas digestivas de dorada (*Sparus aurata*). La presencia de inhibidores de proteasas en diferentes ingredientes utilizados para elaborar alimentos para peces puede afectar negativamente su utilización digestiva. El efecto de inhibidores presentes en diferentes fuentes de proteína sobre las proteasas de la dorada se evaluó utilizando diferentes ensayos «in vitro». La inhibición producida por los extractos de proteínas vegetales osciló entre el 25 y el 50 % de la actividad total, en tanto que la determinada usando proteínas animales osciló entre el 1 y el 20 %, con la única excepción de la ovoalbúmina, la cual mostró el más alto valor encontrado (62 %). La representación de los valores de inhibición obtenidos al cambiar las concentraciones relativas enzima:inhibidor produjeron curvas diferentes en las distintas fuentes proteícas. El uso de zimogramas permitió la visualización de diferencias. Se discute la importancia de medir la inhibición de proteasa en la evaluación preliminar de ingredientes para piensos de peces. © 1999 Ifremer/Cnrs/Inra/Ird/Cemagref/Éditions scientifiques et médicales Elsevier SAS

Inhibición de proteasas / ensayos « in vitro » / *Sparus aurata* / dorada

1. INTRODUCTION

An increasing world demand for ingredients used in aquaculture feeds has resulted in increased research oriented towards the nutritive evaluation of plant protein sources. One of the main obstacles to the use of significant amounts of plant foodstuffs in fish feeds is the low quality of such proteins. Nevertheless, a great number of experiments have demonstrated that amino acid imbalances, normally occurring in these proteins, may be overcome using a careful combination of different ingredients during feed formulation [19]. Another limitation to the use of plant proteins is the presence of antinutritional factors reducing the activity of fish digestive enzymes [8, 9, 22]. Some of the best known of such compounds are protease inhibitors, which are mainly present in legume seeds [16]. Technological processes, based to a great extent on thermal treatments, have been developed to inactivate such compounds, allowing the use, at variable levels, of soybean and many other plant foodstuffs in commercial feeds for both terrestrial and aquatic species [3, 21], although some authors have reported the possibility of using untreated legume seeds in the elaboration of fish feeds [17]. However, a not complete elimination of protease inhibitors in feeds results in a reduc-

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tion in protein digestibility [18]. This effect is particularly noteworthy in fish since the digestive proteases of some of them are more sensitive to inhibitors than those of mammals [11, 12]. Taking this into account, the assessment of the nutritional value of plant proteins in fish feeds, which routinely includes evaluation of protein digestibility, should also evaluate possible interactions between antinutritional factors and fish digestive enzymes [7]. In the present work, the effect of protease inhibitors, present in animal or plant protein sources, on the efficiency of the digestive process of a marine fish, was assayed using different 'in vitro' techniques. The selected species was the sea bream (*Sparus aurata*), an omnivorous fish, the culture of which is well developed in the Mediterranean basin and for which an increasing demand for feeds is expected in a near future.

2. MATERIALS AND METHODS

2.1. Preparation of enzyme extracts

Live specimens of sea bream, ranging from 25 to 40 g were provided by a local fish farmer (FRAMAR S.L.; Almería, Spain). Fish were routinely fed on a commercial diet (45 % protein) three times a day to reach 3.5 % body weight. Following 8 h fasting, specimens were killed by submersion in cold water (4 °C). The digestive tract was removed, opened and cleaned with distilled water and dissected into stomach and pyloric caeca. Samples of stomach were mechanically homogenised (Heidolph RZR1 homogeniser, Germany) in distilled water (1:10 w/v), whereas portions containing pyloric caeca and proximal gut were homogenised (100 $mg \cdot mL^{-1}$) in cold 50 mM Tris-HCl buffer, pH 7.5. Supernatants obtained after centrifugation at 16 000 *g* for 30 min at 4 °C were stored at –20 °C, being further utilised for enzyme analysis. Concentration of soluble protein in pooled samples was determined by the Bradford method [4] using bovine serum albumin (1 $mg \cdot mL^{-1}$) as a standard.

2.2. Preparation of protein extracts

Protein solutions were prepared by manual homogenisation (using a Potter-Eveljehm) of different animal and plant foodstuffs in distilled water (100 $mg \cdot mL^{-1}$). The selected protein sources were: fish meal (F), blood meal (B), squid meal (SQ), meat and bone meal (M& B), ovoalbumin (OA), raw soybean meal (RSB), extracted soybean meal (ESB), green pea meal (GP) and corn gluten meal (CG). Compound feeds prepared either at the lab or provided by commercial firms were also used in assays. In this latter case, feeds were only identified as COM (1 or 2) in order to maintain anonymity since the objective was not to test whether a given feed had inhibitory compounds, but if some of them may present this effect. The origin and protein content of each material used in the assays are detailed in *table I*. Soybean trypsin inhibitor (SIGMA T9128) and ovomucoid (SIGMA T9253) were used as a reference in some experiments.

Table I. Crude protein content (g per 100 g d.w.) in the feedstuffs utilised in the present work.

Code	Protein source	Protein
OA	ovalbumin[1]	90.0
S	squid meal[2]	74.8
F	fish meal[3]	62.4
B	blood meal[4]	87.0
M& B	meat and bone meal[5]	64.1
ESB	extracted soybean meal[6]	49.9
LM	lupin meal[7]	42.9
CG	corn gluten meal[8]	60.0
RSB	raw soybean meal[9]	24.5
HB	horse bean meal[10]	25.5
GP	green pea meal[11]	22.0

[1] (A-5253) purchased from SIGMA; [2, 3, 6, 9, 10, 11] provided by a local supplier; [4] provided by APROCAT, S.A (Barcelona, Spain); [5, 7] provided by Grupo de Investigación en Acuicultura. Las Palmas de Gran Canaria, Spain; [8] provided by CAMPOEBRO INDUSTRIAL S.A. (Zaragoza, Spain).

2.3. Determination of protease activity in fish digestive extracts

Alkaline protease activity of extracts was measured according to Walter [23], using casein (0.5 %) substrate in 50 mM Tris/HCl buffer at pH 9.0. Acid protease activity was evaluated according to Anson [2], using 0.5 % haemoglobin in 0.1 M glycine/HCl, pH 2.0. The mixtures were incubated for 30 min at 25 °C and reaction was stopped by addition of 0.5 mL 20 % trichloroacetic acid (TCA). Absorbency was recorded at 280 nm and one unit of enzyme activity was defined as 1 µg of tyrosine released per minute. All measurements were carried out in triplicate.

2.4. Evaluation of the rate of inhibition of different proteins on sea bream digestive protease activity

The inhibitory effect of different protein sources on sea bream alkaline proteases was tested using a modification of the method described by García-Carreño [5]. The method is based on the measurement of remaining protease activity after incubation of enzyme extracts with the different solutions containing inhibitors. Two sets of enzyme extracts (20 µL) were used: one set was incubated in 500 µL HCl Tris buffer (50 mM, pH 9.0 for 60 min, 25 °C) with the protein solutions (20 µL), and the second, serving as the control, with an equivalent amount of distilled water. Then, 500 µL of a casein solution (10 $g \cdot L^{-1}$ in distilled water) were added and incubation was continued for 120 min. The reaction was stopped by addition of 500 µL TCA (200 $g \cdot L^{-1}$). Blanks were prepared for

each set using the same ingredients, but TCA was added prior to the casein. Protease inhibition was assessed as the reduction in protease activity relative to that of the controls. Dose–response curves were obtained performing different assays as previously described, but changing the ratio inhibitor/enzyme (expressed as μg of meal per unit of protease activity) from 12.5:1 to 1 000:1. All the assays were carried out in triplicate.

2.5. Effect of acid digestion on protease inhibitors

To mimic the effect of gastric HCl, solutions containing inhibitors were incubated for 60 min at 25 °C in a solution of 0.1 M HCl (pH 2.0), prior to mixing with fish extracts containing alkaline proteases. The rest of the assay was developed as previously detailed.

2.6. Identification of protease inhibitors using electrophoresis

Fish enzyme extracts were incubated at 25 °C for 60 min under continuous stirring with solutions of different protein sources (CG, RSB, ESB, GP, plus lupin seed meal (LS) and horse bean meal (HB)) prepared in 50 mM Tris-HCl buffer (pH 7.5) at a ratio of 500 μg protein per unit of activity. Then, mixtures were centrifuged at 12 000 *g* for 10 min. Clear supernatants were utilised for electrophoretic studies. The control was constructed by incubating the extract only in the presence of buffer.

SDS-PAGE of the proteins in the preparations was undertaken according to Laemmli [14], using 12 % acrylamide in 8 × 10 × 0.075 cm gels. Five microlitres of molecular weight marker (MWM) were loaded onto each plate. Zymograms revealing proteinase activity in fractions separated by electrophoresis were carried out according to García-Carreño et al. [6]. The amount of total protein in samples loaded ranged between 25 and 35 μg. Electrophoresis was performed at a constant voltage of 100 V per gel for 45 min at 5 °C. After electrophoresis, gels were washed and incubated for 30 min at 5 °C in 50 mM TRIS-HCl buffer, pH 9.0, containing 0.5 % casein Hammerstein. After this, gels were washed, fixed in 12 % TCA and stained: 0.1 % brilliant blue Coomassie (R-250) in methanol–acetic acid–water solution (50:20:50), left overnight at room temperature and distend with methanol–acetic acid–water (35:10:55). Active bands in fish digestive extracts were observed and comparisons were made between samples incubated or not in the presence of potential inhibitors.

2.7. Statistical analysis

Data were subjected to one- or two-way ANOVA when required. Differences between means at $P < 0.05$ were analysed using the Tukey test. The Statistix version 4.0 package (Analytical Software, Arizona, USA) was used. Fitting of data for plotting curves in inhibition assays was performed using the statistical module of the Excel 5.0 spreadsheet (Microsoft, USA).

Table II. Inhibition (mean ± SD) of sea bream alkaline digestive proteases after incubation of extracts with solutions containing different protein sources. Values not sharing a common superscript are significantly different with $P < 0.05$.

Code	Protein source	Percentage inhibition
OA	ovoalbumin	62.0 ± 3.5^{a}
GP	green pea meal	53.3 ± 1.9^{ab}
HB	horse bean meal	49.1 ± 2.6^{bc}
LM	lupin seed meal	42.9 ± 2.4^{c}
RSB	raw soybean meal	42.6 ± 6.7^{c}
ESB	extracted soybean meal	39.9 ± 3.0^{c}
CG	corn gluten meal	24.7 ± 0.5^{d}
S	squid meal	9.0 ± 2.1^{e}
M&B	meat and bone meal	4.4 ± 1.5^{e}
F	fish meal	3.5 ± 1.0^{e}
B	blood meal	1.0 ± 2.0^{e}

3. RESULTS

Reduction in alkaline protease activity measured in sea bream digestive extracts after incubation with different animal and plant protein sources, as well as with experimental and commercial feeds, are shown in *tables II* and *III*. Mean values of inhibition obtained with animal proteins did not exceed 10 % (with the exception of OA: 62 %), whereas those obtained with plant proteins in all cases exceeded 20 % and even reached 50 % (GP). Inhibition of protease activity by compound feeds ranged from 6 to 22 %. This last value was obtained with a commercial formula.

A significant reduction in the inhibitory effect after acid pretreatment of protein solutions prior to the inhibition assays was observed in pure ovomucoid, as well as in CG and in one of the commercial feeds (COM1) (*table IV*).

Table III. Inhibition (mean ± SD) of sea bream alkaline digestive proteases after incubation of extracts with solutions prepared using different experimental (EXP) and commercial (COM) feeds. Experimental feeds contained 70 % fish meal protein and 30 % of lupin seed, soybean, gluten or meat and bone meals, respectively. Commercial feeds belonged to three different firms. Values not sharing a common superscript are significantly different with $P < 0.05$.

Compound feeds	Percentage inhibition
EXP-Lupin	16.1 ± 1.6^{b}
EXP-Soybean	13.2 ± 1.3^{bc}
EXP-Gluten	7.7 ± 2.0^{d}
EXP-Meat and bone	6.3 ± 1.8^{d}
COM1	21.9 ± 0.9^{a}
COM2	8.4 ± 2.0^{cd}
COM3	8.2 ± 0.7^{d}

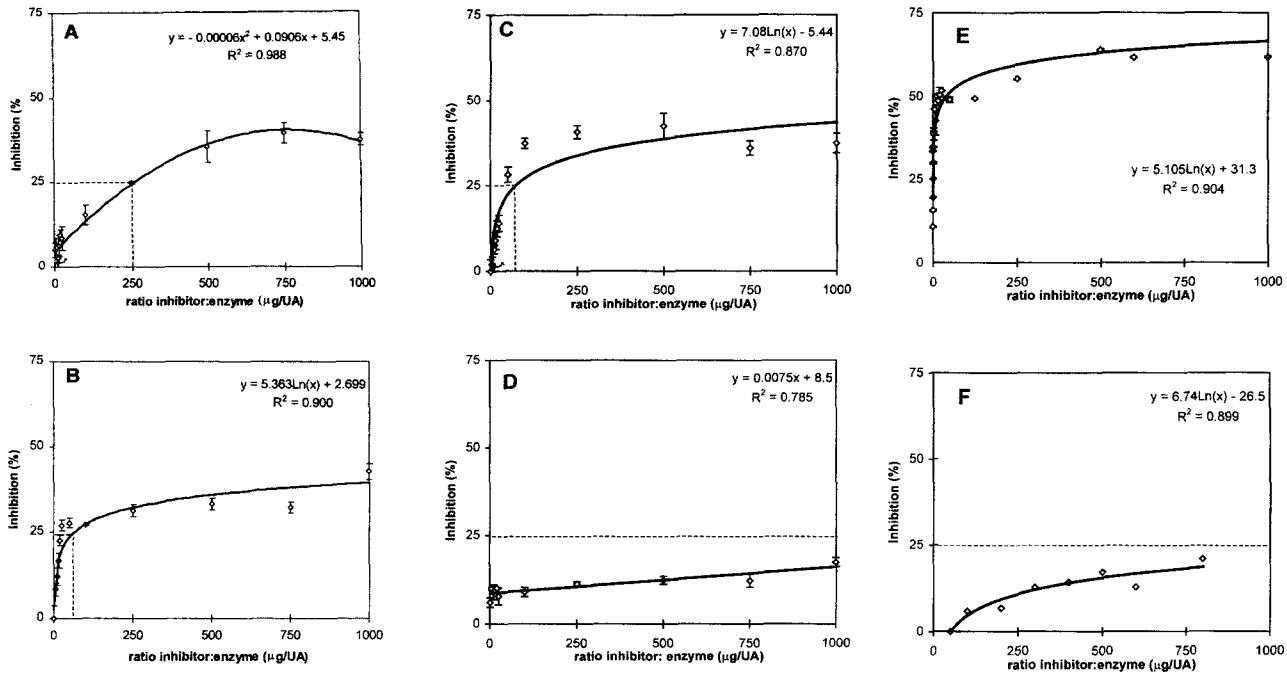

Figure 1. Inhibition of sea bream alkaline proteases obtained using different relative concentrations of protein substrate/enzyme activity in: A) extracted soybean; B) raw soybean; c) lupin seed; D) corn gluten meal; E) ovoalbumin; and F) commercial feed. The relative concentration corresponding to a 25 % inhibition is indicated by a dotted line

The inhibition curves obtained when testing different relative concentrations of some protein solutions are presented in *figure 1*. Clear differences in response to inhibition were found for the assayed proteins, resulting in different kinetics of inhibition, which mainly fitted to exponential, but also to polynomial or lineal functions. Such differences were particularly noticeable in the case of soybean meal. ESB or RSB produced the same maximum inhibition of fish enzymes at high concentrations of the inhibitor. However, the response was different at low concentrations. Under such conditions, ESB yielded less inhibition, needing a relative concentration of around 0.5 mg per unit of activity to produce a similar effect to that of RSB. Lupin meal (LM) produced a curve similar to that obtained with RSB. The inhibition produced by CG never exceeded 25 % of total activity, whereas that produced by OA was shown to be very high even at low relative concentrations (12.5 $\mu g \cdot U^{-1}$).

Table IV. Effect of an acid pretreatment on the inhibition produced by pure inhibitors (ovomucoid; Sigma, T-9253) and SBTI (Sigma T-9128), protein sources (soybean, lupin and corn gluten meals) and a commercial feed on alkaline proteases of sea bream. Values are expresssed as mean ± SD. Values not sharing a common superscript are significantly different with $P < 0.05$; the first one is related to columns, the second to rows.

	Percentage inhibition	
	Control	Acid treated
Ovomucoid	76.39 ± 0.12^{aa}	62.37 ± 1.92^{ab}
SBTI	55.38 ± 1.51^{ba}	55.27 ± 1.11^{ba}
Lupin meal	47.53 ± 0.68^{ba}	43.02 ± 2.02^{cb}
Extracted soybean meal	32.43 ± 2.53^{ca}	33.00 ± 3.53^{da}
Corn gluten meal	13.69 ± 2.53^{ea}	8.98 ± 1.18^{eb}
COM1	24.13 ± 0.68^{da}	14.31 ± 0.51^{eb}

Zymograms performed on fish intestinal proteases after incubation of enzyme extracts with several protein solutions are shown in *figure 2*. The presence of protease inhibitors was visualised by a partial or total disappearance of one or more of the bands shown in the control extracts. Inhibition could be classified into two types: one reducing the activity in specific bands and the other, less specific, which reduced the activity of all proteases. The first one was found when testing CGM, LM and RSB (*figure 2*, lanes 2, 3 and 7), the second one was detected when testing ESB, HB or GP (*figure 2*, lanes 4, 5 and 6). The maximum inhibition was detected when sea bream proteases were incubated in the presence of the solution prepared with GP. The pattern of bands in zymograms of ESB or RSB was quite different and a lower effect of the former protein source on total protease activity in extracts was found.

4. DISCUSSION

Results obtained in the present work confirm the existence of protease inhibitors in foodstuffs of both

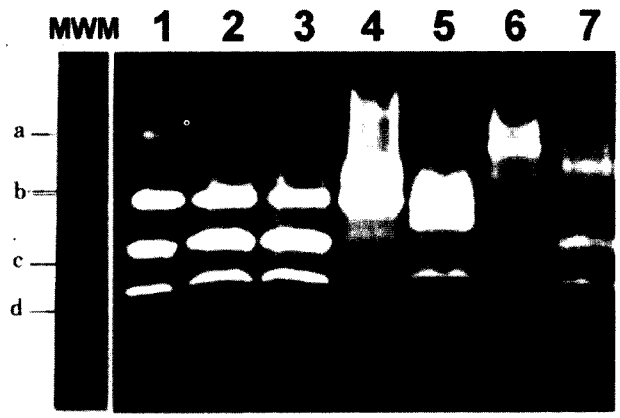

Figure 2. SDS-PAGE zymograms obtained after incubation of sea bream digestive extracts with solutions of different protein sources. Lanes 1: control, 2: corn gluten meal, 3: lupin seed, 4: extracted soybean, 5: horse bean, 6: green pea, 7: raw soybean. Molecular weight markers (MWM): a, bovine albumin (67 000 kDa); b, ovoalbumin (43 000 kDa); c, carbonic anhydrase (30 000 kDa); and d, β-lactoglobulin (18 400 kDa).

animal and plant origin. Such compounds have been identified in a great diversity of plant species, being particularly abundant in legume seeds, but also in cereal grains and by-products [15]. It was surprising to find that processing of soybean for extraction of lipids, which is carried out at a high temperature (105 °C or more) did not significantly reduce the inhibition produced by this protein source on sea bream proteases. A possible explanation may be that an inadequate thermal treatment was unable to completely eliminate the effect of inhibitors. In fact, two types of inhibitors have been described in legume seeds: the heat-labile Kunitz inhibitor, with a molecular mass of 20 000–25 000, which has relatively few disulphide bonds and binds to trypsin; and the heat-stable Bowman-Birk inhibitor, having a molecular mass of 6 000–10 000, a high proportion of disulphide bonds and capable of inactivating both trypsin and chymotrypsin [13].

A persistence of inhibition after processing of seeds has been confirmed in the present work when using ESB, and it could also explain the inhibitory effect produced by the commercial feed, in which formula a certain amount of this protein source is routinely included substituting the more expensive fish meal. Protease inhibition ranging from 10 to 86 % induced by commercial feeds has been reported in salmonids [18].

Some authors have reported an inactivation of protease inhibitors after transit into the stomach [10], but it has been demonstrated that stomach digestion is capable of destroying only Kunitz-type inhibitors, whereas the more stable Bowman-Birk-type retain their activity. A high resistance to denaturation produced by an acid incubation was also demonstrated for some of the protease inhibitors tested in the present work (*table IV*). It is deduced that very stable molecules, not affected by the acid treatment, produced most of the inhibitory effects found.

Inhibition curves are an easy way to evaluate how variations in the intake of different feeds could affect digestive protease activity in a given fish [1]. Such curves show that the expected physiological responses to inhibition may vary from lineal (CG) to exponential (RSB). Construction of inhibition curves may allow the assessment of the different sensitivities existing in digestive proteases of different fish species for a given substance [21]. Equations defining such curves may be used to predict the expected percentage of reduction in protease activity of a fish, once enzyme activity in the digestive tract and the amount of food ingested by such fish are known. For example, in the case of a 40-g sea bream, total protease activity released after a meal is around 1 300 units [1]. If that fish consumes 1.5 % of its weight in a meal, and the feed contains 30 % soybean meal, the ratio mg meal per units of activity should determine a reduction of nearly 35 % in the activity of its proteases.

Differences in the way in which inhibitors affect digestive proteases of sea bream were demonstrated in zymograms (*figure 2*). In spite of the interesting information it can offer, few works are based on the use of this technique in the assessment of protease inhibition [20]. Results obtained in the present paper support the idea that protease inhibition produced by GP could be classified as 'general' or 'unspecific', since it yielded a similar reduction in activity for most of the proteases observed in zymograms, whereas protease inhibition produced by RSB could be defined as more 'specific', since it mainly affected particular proteases. Differences found when testing either RSB or ESB confirmed that thermal processing was only partially effective in destroying protease inhibitors. When compared to that of raw soybean, zymogram of extracted soybean revealed a less marked protease inhibition, as well as differences in mobility of bands in relation to those present in the control extract. This indicates a modification in the molecular mass of active proteases present in the extract, as a result of a reaction with inhibitors present in the protein source. Such differences were in agreement with results obtained in the inhibition curves.

It can be concluded that a great number of plant protein sources contain inhibitors that affect, to a great extent, digestive proteases of fish other than salmonids. A more accurate assessment of such an inhibitory effect must consider the elaboration of dose–response experiments to construct inhibition curves. Additional information may be obtained using SDS-PAGE zymograms. Validation of the results obtained by the described 'in vitro' tests are nevertheless demanding of 'in vivo' confirmation, to enable their use in feed formulation, helping fish nutritionists in the selection of the most suitable ingredients and potential amounts of inclusion in feeds for each species.

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