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Comparative Biochemistry and Physiology Part B 122 (1999) 327–332

CBP

Inhibition of digestive proteases by vegetable meals in three fish species; seabream (*Sparus aurata*), tilapia (*Oreochromis niloticus*) and African sole (*Solea senegalensis*)

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Received 21 April 1998; received in revised form 11 January 1999; accepted 27 January 1999

Abstract

The inhibitory effect of three different vegetable foodstuffs (defatted soybean meal, corn gluten meal and wheat bran) on alkaline protease activity of seabream, tilapia and sole was evaluated. Protease inhibition on crude digestive extracts was assessed using different relative concentrations of plant meals and represented by constructing inhibition curves. SDS-PAGE zymograms were utilised to obtain further details in the characterisation of sensitivity of some fish enzymes to protease inhibitors. Tilapia showed the greatest sensitivity to protease inhibitors present in the assayed meals. A high resistance of sole digestive proteases to inhibition produced by soybean meal or corn gluten meal was detected, although they were sensitive to protease inhibitor activity in wheat bran. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Fish proteases; Inhibition; *Oreochromis niloticus*; *Solea senegalensis*; *Sparus aurata*; Vegetable meals

1. Introduction

The increasing demand of ingredients for aquaculture feeds all over the world has driven an important research effort towards the nutritive evaluation of vegetable protein sources. One of the main obstacles for a generalised inclusion of significant amounts of vegetable foodstuffs in fish feeds is the low quality of such proteins. Nevertheless, a great number of experiments has demonstrated that amino acid imbalances existing in these proteins may be overcome by a careful combination of different ingredients in the formula [24,30]. The other main limitation to the use of plant protein sources is the existence of antinutritional factors that may reduce the activity of fish digestive enzymes [12], for example, protease inhibitors present mainly in legume seeds [11,20]. For this reason, certain technolog-

ical processes largely based on thermal treatment, have been developed to eliminate such inhibitors, allowing soybean and many other vegetable foodstuffs to be included at variable levels in commercial feeds for different terrestrial and water cultured species [4,21].

Nevertheless, some authors have reported that technological treatments not always guarantee complete elimination of trypsin inhibitor in fish feeds [5]. Provided that fish proteases are highly sensitive to such inhibitor, the assessment of the nutritional value of vegetable foodstuffs (particularly through the determination of the apparent digestibility coefficient of proteins) should consider interactions between such antinutritional factors and fish digestive enzymes. Nevertheless, with the exception of the study of Krogdahl and Holm [15], differences in sensitivity to a given protease inhibitor existing in various fish species are not documented.

In the present study, the inhibitory effect of three different vegetable foodstuffs (defatted soybean meal, corn gluten meal and wheat bran) on alkaline protease

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activity of seabream, tilapia and sole is evaluated using 'in vitro' assays as well as SDS-PAGE zymograms. Comparative assessment on the suitability of using each of those ingredients in feeds for these species was the main objective of the experiments.

2. Materials and methods

Sea bream (*Sparus aurata*) were obtained from a local fish farm (FRAMAR S.L., Almería, Spain). Tilapia (*Oreochromis niloticus*) were kindly provided by Dr A.F. El-Sayed from his research laboratory at the University of Alexandria (Egypt). Senegal sole (*Solea senegalensis*) were provided by the Instituto de Ciencias Marinas (CSIC, Cádiz, Spain) where they are routinely reared after being captured on the open sea. Feeding regimes and size of fish utilised in the experiment are detailed in Table 1.

Active extracts were prepared from pooled samples of ten fish after dissection followed by manual homogenisation of pancreatic and duodenal tissues in distilled water (1:10 w/v), as detailed in Moyano et al. [25]. Supernatants obtained after centrifugation ($16\,000 \times g$ 30 min at 4°C) were stored at -20°C and further utilised for enzyme analysis. Concentration of soluble protein in extracts was determined by Bradford method using bovine serum albumin (1 mg ml^{-1}) as a standard. Alkaline proteinase activity was measured using the casein method of Kunitz [17] as modified by Walter [33], using substrate casein (0.5%) on 50 mM Tris/HCl buffer, pH 9.0. The mixture was incubated for 30 min at 25°C and the reaction was stopped by addition of trichloro acetic acid (TCA) 20%. The absorbance of the soluble TCA peptides was recorded at 280 nm. A unit of enzyme activity was defined as 1 µg of tyrosine released per minute, using the extinction coefficient for tyrosine = $0.005 \text{ ml } \mu\text{g}^{-1} \text{ cm}^{-1}$. All measurements were carried out in triplicate.

Solutions of three different plant protein sources were prepared by manual homogenisation of extracted soybean meal (ESM) or wheat bran (WB; 100 mg ml^{-1} in distilled water) and corn gluten meal (CGM; 100 mg ml^{-1} glycine-HCl buffer, pH 2.0), followed by centrifugation at 1500 g for 10 min to eliminate residues. After solubilisation of CGM in acid buffer, neutral pH was restored by addition of NaOH (0.1 M).

Inhibitory effect of the different meal solutions on fish digestive proteases was tested by measuring the reduction in protease activity, following the procedure described by García-Carreño et al. [8]. Duplicate sets of tubes were used in each assay; one of them (test set) was utilised to assess the effect of preincubating extracts (60 min, room temperature) in the presence of the plant protein solutions. A second set of tubes was used as a control of activity, being the extracts incubated in the presence of distilled water only. After this step, residual protease activity was tested by incubating the mixtures for a new time period (30 min) with the previously detailed casein solution. Each set of tubes was run with their own blank, with TCA added immediately prior to casein. This way, absorbance due to the reagents in control tubes, and also to protein hydrolysis in test tubes, could be subtracted. Protease inhibition was evidenced by a reduction in protease activity in test tubes when compared to that determined in control tubes, and it was expressed as a percentage of the control activity. In order to simulate the inhibition resulting from a variable intake of the different meals, assays were carried out using increasing ratios of plant meal/Unit of protease activity (from 12.5 to $2000 \mu\text{g U}^{-1}$). Results obtained for each protein source with the different fish species were plotted separately.

SDS-PAGE was performed in 10% polyacrylamide and $8 \times 10 \times 0.075 \text{ cm}$ gels following the protocol described by Laemmli [18]. Samples were prepared by preincubating ESM or WB solutions and tilapia digestive extracts at ratios ranging from 25 to $500 \mu\text{g}$ of meal ($\text{Unit of activity}^{-1}$) for 60 min at room temperature. Electrophoresis was performed at a constant voltage of 100 V gel^{-1} for 60 min at 5°C. Preparation of zymograms of proteinase activities was done according to García-Carreño et al. [7]; after electrophoresis, gels were washed and incubated in 0.5% Hammerstein casein, pH 9, for 30 min at 5°C, and then transferred to a freshly prepared casein solution at 25°C for 90 min without agitation. Thereafter, gels were washed and 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).

Table 1

Body weight, feeding regime and specific digestive alkaline protease activity of the fish species studied^a

Fish species	Weight (g)	Feeding regime	Protease activity (U mg protein^{-1})
<i>Sparus aurata</i>	25–50	Commercial feed (40% protein)	244 ± 35
<i>Oreochromis niloticus</i>	14–20	Experimental feed based on fish meal (30% protein)	1297 ± 86
<i>Solea senegalensis</i>	70–90	Natural food	37 ± 5

^a Means of three replicates \pm SD.

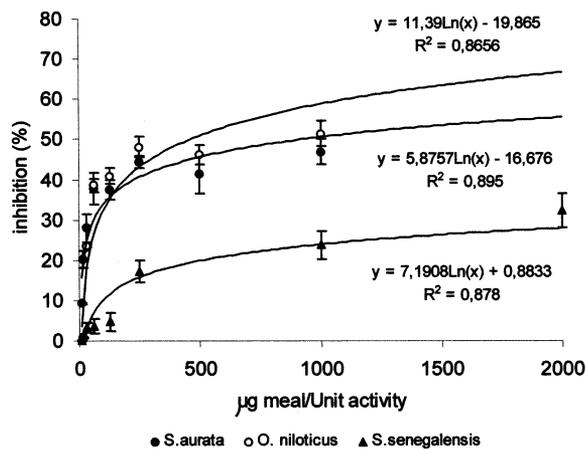


Fig. 1. Inhibition curve of alkaline protease activity obtained after 1 h incubation of digestive extracts of different fish species in the presence of increasing concentrations of soybean meal. Each point is the mean of three replicate measures \pm SD.

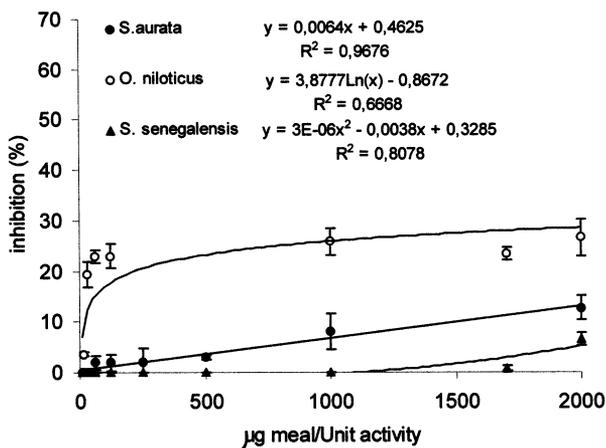


Fig. 2. Inhibition curve of alkaline protease activity obtained after 1 h incubation of digestive extracts of different fish species in the presence of increasing concentrations of gluten meal. Each point is the mean of three replicate measures \pm SD.

3. Results

Total alkaline protease activity of extracts utilised in the experiments is detailed in Table 1. Great differences in specific activity were obtained for the three species, likely a consequence of variations in feeding regime and nutritional status. Those results provided the basis for the design of the inhibition experiments, allowing to prepare incubation mixtures containing the same units of protease activity.

The inhibition curves with different relative concentrations of the protein solutions are represented in Figs. 1–3. In the case of ESM meal, clear differences in the response to inhibition were evidenced for each species (Fig. 1). Seabream and tilapia digestive proteases showed great sensitivity to protease inhibitor present in

this protein source since high inhibition rates (of nearly 40%) were reached even when mixtures contained very low amounts of the meal solution (62.5 μg Unit of activity⁻¹). In contrast, inhibition of activity measured on digestive proteases of sole was only a half of that determined sea bream and tilapia.

A similar experiment with solutions of CGM also showed differences related to fish species (Fig. 2). In this case, almost no inhibition in protease activity of sole or seabream extracts was measured, whereas a significant reduction, exceeding 20%, was determined for tilapia digestive extracts.

Finally, assays carried out with WB solutions revealed that seabream proteases were not very sensitive to low concentrations of protease inhibitors present in this vegetable feedstuff. Nevertheless, inhibitory response increased linearly up to nearly 40% of total activity when a very high (and improbable) intake of WB was simulated. Reduction in protease activity measured in tilapia digestive extracts accounted for nearly 20%, but the highest inhibition was obtained when sole digestive extracts were tested, showing inhibition rates which exceeded 40%, even when incubated in the presence of low concentrations of WB (Fig. 3).

Some of these results were confirmed by SDS-PAGE zymograms. Differences in the inhibitory effect of compounds present in WB or ESM on tilapia proteases are shown in Figs. 4 and 5, respectively. In the first case, a progressive decrease in the intensity of all bands was noted as the concentration of meal increased. In the second case, inhibition is revealed by a selective disappearance of some bands, and by the formation of protease-inhibitor compounds, still retaining some hydrolytic activity.

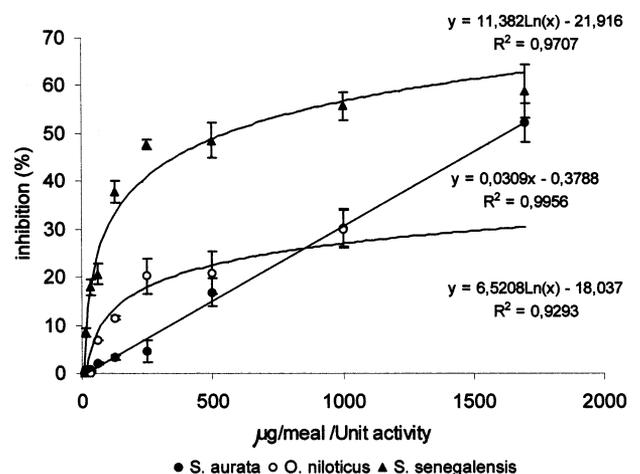


Fig. 3. Inhibition curve of alkaline protease activity obtained after 1 h incubation of digestive extracts of different fish species in the presence of increasing concentrations of wheat bran. Each point is the mean of three replicate measures \pm SD.

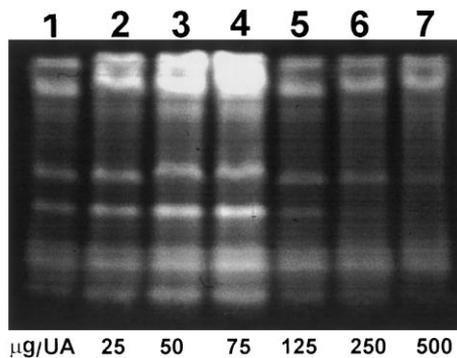


Fig. 4. Substrate-SDS-PAGE zymogram of tilapia digestive proteases obtained after 1 h incubation of extracts with increasing concentrations of wheat bran (μg of meal (U activity) $^{-1}$). Lane 1: control without inhibition.

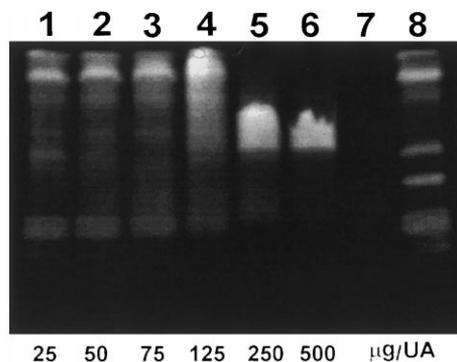


Fig. 5. Substrate-SDS-PAGE zymogram of tilapia digestive proteases obtained after 1 h incubation of extracts with increasing concentrations of soybean meal (μg of meal (U activity) $^{-1}$). Lane 8: control without inhibition.

4. Discussion

Reductions in nutritive value of plant proteins for fish, due to the presence of antinutritional compounds is well documented [13,31,32]. Taking into account their effect on fish performance, the more relevant of these substances are protease inhibitors. Protease inhibitors are protein-based substances with the ability to reduce the activity of proteolytic enzymes within the gastrointestinal tract of animals. The negative effect of using protease inhibitor-containing diets on fish growth should be related to different factors such as: (i) the type of meal; (ii) the level of such meal used in the diet; (iii) the extension of feeding period; and (iv) the sensitivity of a given fish species to the antinutritional compound.

4.1. The type of meal

It is evident that neither all protease inhibitors existing in plant foods are similar, nor are they present in the same amounts. Such compounds have been

identified in a great diversity of vegetable species, being particularly abundant in legume seeds, but also in cereal grains and by-products [19]. Although some technological processes have been designed specifically to ensure their elimination, results are not always satisfactory, and meals utilised in fish feeds still retain significant amounts of protease inhibitory activity [23]. In the case of leguminous seeds, like soybean, this can be explained by the existence of two types of inhibitors; the heat-labile Kunitz inhibitor, with a molecular mass of 20–25 kDa with relatively few disulphide bonds and selective for trypsin, and the more heat-stable Bowman–Birk inhibitor, with a molecular mass of 6–10 kDa, a high proportion of disulphide bonds and capable of partially inactivating trypsin and chymotrypsin. The reported persistence of inhibitors after processing of seeds [14,26] has been confirmed in the present work for ESM.

Protease inhibitors are also present in WB, since protease activity is reduced in the three fish species when their digestive extracts were incubated with solutions of such cereal by-product. This could explain the low digestibility reported for WB when included in tilapia feeds [3,10] and the negative effect on the growth of this species when levels of such by-product in the diet exceeded 18% [2].

Differences between the effect of inhibitors on digestive proteases of tilapia were evident in zymograms (Figs. 4 and 5). Thus, protease inhibition produced by WB could be classified as ‘general’ or ‘non-specific’, since it implied a similar reduction in activity for all the proteases present in the extract. Protease inhibition produced by soybean meal could be defined as more ‘specific’, since it affected only some proteases. As detailed previously, after thermal treatment of soybean a persistence of Bowman–Birk inhibitor may be noticed, resulting in a reduction of activity for chymotrypsin and trypsin. Digestive proteases of tilapia are particularly rich in chymotrypsin [22], these being the first bands affected by inhibition in Fig. 5, even at very low relative concentrations. As the amount of inhibitor increased, also the upper bands, mainly related to trypsin activity, were also affected. The formation of complexes enzyme-inhibitor still showing a certain protease activity was evidenced in such plate as artefacts obtained at relative concentrations of 250 and 500 μg Unit $^{-1}$. From this result one can expect additive or complementary inhibitory effects on digestive protease activity of fish, when different plant ingredients are used in the formulation of feeds. In fact, an increased adverse effect of feeds containing both wheat bran and soybean meal on feed utilisation by the tilapia has been demonstrated [6].

4.2. The amount of plant meal in feeds

Inhibition curves for protease inhibitor-containing feeds using fish digestive extracts, are an easy way to evaluate the effect that variations in the intake of different feeds could have on the protease activity [24]. Curves show that physiological responses to inhibition obtained when plant proteins are incubated at different concentrations in the presence of digestive extracts of a given species (i.e. seabream), may vary from linear (CGM or WB) to exponential (ESM). Equations defining such curves allow us to predict the expected percentage of reduction in protease activity of the fish, once the values of total protease activity and food intake for such fish are known. Following the equation in Fig. 1, for a 40-g sea bream releasing around 1300 Units of total protease activity after a meal [1] and consuming 1.5% of its weight in a meal, a reduction of nearly 35% in the activity of its proteases can be expected, if the feed contains 30% soybean meal.

4.3. Extension of the feeding period

Physiological responses of fish to the intake of feeds containing protease inhibitors are controversial. Compensation of protease inhibition by increased pancreatic enzyme secretion and resorption in distal intestine have been reported in rainbow trout and other salmonids fed on diets containing small amounts of soybean meal [9,16]. Nevertheless, although the digestive process could be satisfactorily completed under such conditions, the energetic cost for the fish could be higher, as a result of additional synthesis of proteases. In addition, a possible increased dietary requirement for sulphur-containing amino acids has been reported [16] as a consequence of an increased synthesis of proteases (which are rich in cystine). Under such conditions, a long feeding period on such feeds should result in clear reductions in fish performance and growth. On the other hand, this is supported by results obtained in experiments based on feeding tilapia using different vegetable meals, where poorer growth was obtained in fish fed on soybean meal [29]. Additionally, the elimination in faeces of undigested enzyme-inhibitor complexes should negatively affect the determination of protein digestibility, as measured by the apparent digestibility coefficients [3,27].

4.4. Sensitivity to inhibition

To date, the assessment of differences in sensitivity of fish species to protease inhibitors has been concentrated to experiments performed with soybean meals. Construction of inhibition curves allows an easy assessment of the different sensitivities to a given substance existing in digestive proteases of any fish species. Thus, it was

demonstrated that sensitivity of seabream or tilapia digestive proteases to the inhibitor present in ESM was quite high, a result also reported in salmonids [16]. Other fish, like sea bass or yellowtail, seem to be more resistant to that inhibitory effect [28], as found for the sole in the present study. In contrast, digestive proteases of sole were highly sensitive to the protease inhibitor present in WB. Those interspecific differences were much more evident when testing the inhibition produced by CGM (Fig. 2).

5. Conclusion

In summary, tilapia showed the greatest sensitivity to protease inhibitors present in vegetable feedstuffs assayed in the present work. This is surprising, considering the herbivorous feeding habits of this fish, but it should be in agreement with results obtained in previous experiments oriented to the characterisation of digestive proteases in this species, which also showed great sensitivity to artificial inhibitors [22]. The high resistance of sole digestive proteases to the inhibition produced by soybean meal could lead to use this protein source in the formulation of compound feeds for this species with less restrictions than in other fish. A similar conclusion can be drawn for CGM, but it seems that WB has a clear antinutritional effect on sole proteases. Therefore, the amount of WB should be reduced in feeds for this species.

Acknowledgements

This work was partially supported by Project MAR97-0924-C02-02 from the C.I.C.Y.T. (Spain). We are grateful to Dr A.F. El-Sayed for his kind supply of complete freeze-dried digestives of tilapia.

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