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Effect of plant protease inhibitors on digestive proteases in two fish species, *Lutjanus argentiventris* and *L. novemfasciatus*

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Abstract

This work provides a comparative study of the inhibitory effect of several plant protein sources on digestive proteases of two snappers: yellow snapper (*Lutjanus argentiventris*) and dog snapper (*Lutjanus novemfasciatus*). Seed extracts did not affect gastric proteases whereas they significantly inhibit intestinal proteases. Inhibition of alkaline proteases showed that pancreatic proteases of *L. argentiventris* were more sensitive to seed protease inhibitors than those of *L. novemfasciatus*. Legume seeds showed the highest inhibitory capacity on alkaline proteases causing inhibition higher than 50% in total proteolytic activity. Protease inhibition on digestive extracts was assessed using different relative concentration of seed extracts and represented by constructing dose response curves. In order to reduce the inhibitory effect, seed extracts were acid-treated before the inhibition assay. Results showed that acid treatment did not affect the inhibitory capacity of seeds on alkaline proteases in both species. However, when the action of gastric enzymes was simulated on seed extracts, the inhibitory capacity was reduced significantly, mainly in the case of *L. novemfasciatus*. The responses of fish enzymes to heat-treated seed extracts were also tested. Only higher temperatures were capable of reducing the inhibitory capacity of seed, with the specific response to the snapper species. The use of biochemical assays allows us to quantify the action of inhibitors on total proteolytic activity. In addition, zymograms obtained by substrate-SDS-PAGE provided qualitative information about the number and type of proteases affected by each inhibitor. Each seed extract produces a characteristic profile of inhibition on alkaline protease. The results obtained are important for future formulation of feeds for these snapper species.

Introduction

Mexican aquafarming is a developing industry and economic activity. The most important cultivated species is shrimp, mainly white shrimp *Penaeus vannamei*. Some efforts have recently been made to diversify the number of species, with emphasis in commercially important fish. Species along the Pacific Mexican coast, fish of the genus Lutjanus, are the focus of research. At CIBNOR (BCS, México), some studies with two snapper species are being done. Yellow snapper *Lutjanus argentiventris* (Peters) and dog snapper *Lutjanus novemfasciatus* (Gill) are currently under study for culture (Serrano-Pinto and Caraveo-Patiño 1999). However, the species are not yet domesticated. One important condition for domestication is to determine the biology of the organisms (Diamond 1997), including their nutrition. Nutrition is a complex phenomenon involving characteristics of both the food and the organism. Protein digestion is one of the most important activities in nutrition, for several reasons. Reasons include the cost of the proteinaceous ingredients, the digestion and assimilation of digestion products, and the release of undigested nitrogenous materials into the farm ponds.

Plant proteins, mainly from legume seeds, are readily available sources of protein with potential for use as 'fishmeal replacers' in aquafeeds. However, the use of legume seeds is usually limited because of the presence of antinutritive factors like enzyme inhibitors. An enzyme inhibitor is any substance that reduces the measured rate of an enzyme-catalyzed reaction (Whitaker 1994). Protease inhibitors are proteins that act in biological phenomena, like regulation of blood coagulation, fibrinolysis, complement activation, and inflammatory response in mammals (Potempa et al. 1994). In fish nutrition, the presence of antinutritional compounds in feeds affects protein digestibility, causing adverse physiological effects and reducing growth (Olli et al. 1994). Proteinaceous protease inhibitors from plants have been extensively studied (Ryan 1979). According to their specificity, protease inhibitors can be divided into four types; those inhibiting serine-, cysteine-, metallo-, or aspartic proteases (García-Olmedo 1987; Strukelj 1992). Serine and cysteine protease inhibitors are abundant in seed and storage tissues of plant (Reeck et al. 1997; García-Carreño et al. 1996, 1997). Protease inhibitors accumulate during seed or tuber maturation, which suggest that they facilitate protein accumulation (5–15% of total protein in staple crops, cereals, beans, and potatoes) by attenuating the activities of proteases. Some authors support the general hypothesis that protease inhibitors have the potential to protect plants against a broad range of herbivore digestive proteases (Broadway 1995).

Digestive proteases are classified into four major groups; serine proteases, e.g. trypsin and chymotrypsin; cysteine proteases, e.g. catepsin; metalloproteases, e.g. some aminopeptidases; and acid proteases, e.g. pepsin, gastricin. Differences between specificities into each subgroup were established according to the molecular mechanism of hydrolysis (Whitaker 1994). Digestion of protein in the stomach of fish is done by the action of pepsin, helped by the acid environment. When the chyme arrives at the intestine, several proteases secreted by the pancreas continue the hydrolysis. The most important proteases acting within the intestine belong to the group of serine proteases (Munilla-Morán and Saborido-Rey 1996; Alarcón et al. 1999).

Fish species differ considerably in their sensitivity and response to protease inhibitors (Kroghahl and Holm 1983; Tacon 1997; Krogdahl et al. 1994; Moyano et al. 1999; Alarcón et al. 1999). With some exceptions, no comparative studies have been made to ascertain the precise sensitivity and tolerance of individual fish species to protease inhibitors within feeds.

This study was made to compare the sensitivity and effect of protease inhibitors in plant seeds on digestive proteases of *L. argentiventris* and *L. novemfasciatus*. The differences may have important implications in the future formulations for aquafeeds by understanding how much an inhibitor affects digestion and the threshold amount of inhibitor permissible in a feed.

Materials and methods

Reagents

All reagents were supplied by Sigma Chemical Co. (St. Louis, Missouri, USA).

Animals

Live specimens of *L. argentiventris* and *L. novemfasciatus*, 25 to 50 g, were collected in Ensenada de la Paz, BCS, in the mangroves of the Barra el Mogote from April to June 1999. Organisms were maintained in 200-l fiberglass tanks (150% water exchange daily) and constant aeration, at the CIBNOR facilities (La Paz, BCS, México). Fish were fed on a commercial feed (40% protein), twice a day at 0900 and 1700 h. Organisms that were at the same stage of development and were fed the same feed were used to ensure the accuracy of results.

Preparation of enzyme extracts

Fish were fasted for 12 h before submersion in 2 °C water for anesthesia. The digestive tract was dissected into stomach and pyloric caecum at 4 °C. Crude enzyme extracts of the stomach and pyloric ceca were standardized by homogenizing with two volumes of distilled water in a Waring blender. To eliminate feed residues, lipids, and cell debris, samples were centrifuged for 20 min at 12,000 *g* at 4 °C in an Eppendorf centrifuge (Model 5403). Aqueous supernatants were stored at −20 °C until assayed. Concentration of soluble protein in pooled samples was evaluated by the Bradford method (1976).

Preparation of seed extracts

Most of the seeds used in this study are from legume plants of the Baja California Peninsula. The reasons

for using these seeds are legume seeds are widely used in aquafeeds, so endemic legume seeds are good candidates as protein ingredients, and protease inhibitors have been found in aqueous extracts of some of them (García-Carreño et al. 1996). Legume seeds are pigeon pea (*Cajanus cajan*, L. Huth), guamuchil (*Pithecellobium dulce*, Robx. Benth), ironwood (*Prosopis palmeri*, S. Wats), palo blanco (*Lysiloma candida*, Brandegee), mezquite (*Prosopis articulata*, S. Wats), soybean (*Glicine max*, L. Merr), chick-pea (*Cicer arietinum*, L.), and green pea (*Pisum sativum*, L.). The cereal seed is sorghum (*Sorghum bicolor*, L. Moench).

Inhibitor extracts were obtained according to García-Carreño et al. (1997). In brief, the seeds were ground and the powder extracted with three volumes of distilled water by shaking for 120 min at room temperature and for 22 h at 4 °C. The mixture was centrifuged for 20 min at 12,000 g and 4 °C. The supernatant was stored at 4 °C until use.

Protease activity assay

Acid protease activity of stomach and alkaline protease activity of pyloric ceca were measured as detailed previously in Alarcón et al. (1998). Briefly, acid protease activity was measured by using 0.5% haemoglobin in 0.1 M glycine.HCl, pH 2.0. Alkaline protease was measured using 0.5% azocasein as the substrate, in 50 mM TRIS·HCl buffer, pH 9.0. The mixtures were incubated for 60 min at 37 °C and the reaction was stopped by addition of 0.5 ml of 20% trichloroacetic acid (TCA). Absorbance was recorded at 280 nm (acid proteases) and 366 nm (alkaline proteases). The amount of extracts in all enzyme assays was adjusted to provide a lineal increase of absorbance (ΔOD) of 0.5 in 60 min at 37 °C. The presence of protease activity in seed-meal extracts was also tested in the same experimental conditions detailed above. All assays were done each in triplicate and repeated three times. Units of protease activity (U) were defined as $\Delta\text{OD}\,\text{h}^{-1}$.

Inhibition of protease activity by seed meal extracts

Inhibition was evaluated according to Alarcón et al. (1999). In brief, 10 μl of seed extracts, 10 μl of enzyme extract, and 500 μl of buffer were mixed. For acid proteases, 0.1 M Glycine.HCl, pH 2.0, and for alkaline proteases 50 mM TRIS·HCl, pH 9.0 were used. Then the mixture was incubated for 60 min at 25 °C. Residual protease activity was evaluated by using 500 μl of 0.5% haemoglobin or 500 μl 0.5% azocasein. Enzyme extracts were adjusted to provide 0.5 U. Blanks were prepared by adding TCA before adding the substrate. Protease inhibition was assessed as the percentage of reduction in protease activity relative to that of the controls. Controls for each set were made by using distilled water instead of the seed extracts.

Dose response curves were obtained for soybean and sorghum meals performing different assays as previously described, but changing the relation seed extract/enzyme. All the assays were made with three different pools and in triplicate.

Effect of acid treatment on protease inhibitors

Ten μl of seed extract were incubated for 30 min at 25 °C in 20 μl of 0.1 M HCl, pH 2.0. Then, pH was neutralized with 500 μL of 50 mM TRIS·HCl, pH 9.0 buffer before mixing with pyloric ceca extracts. The rest of the assay was developed as previously detailed.

Effect of gastric digestion on protease inhibitors

Physiologically, it is interesting to evaluate the sensitivity of protease inhibitors to gastric enzymes. To evaluate it, seed extract is incubated with gastric proteases, then the remaining inhibitory ability on intestine proteases is measured. To measure the effect of gastric proteases on the protease inhibitors, 10 μl of seed extract were incubated for 30 min at 25 °C in 20 μl of 0.1 M HCl, pH 2.0 and 10 μl of stomach extract (containing 1.5 U). The inhibitory capacity of the treated seed extract on alkaline proteases was evaluated, using as a control the untreated seed extract. The inhibition assay was done as above.

Effect of temperature on protease inhibitors

Seed extracts were incubated in a water bath for 20 min at 40, 60, 80, and 100 °C, then centrifuged for 2 min at 12,500 *g* to eliminate any precipitate and the supernatant used for the inhibition assay as detailed above.

Detection of the effect of inhibitors on particular enzymes by substrate-SDS-PAGE

10 μl of pyloric ceca extracts (containing 0.5 U) were mixed with 10 μl of seed extract. The solution was incubated at room temperature for 60 min under continuous stirring. Then, mixtures were centrifuged for 3 min at 12,500 *g* and clear supernatants were mixed

with sample buffer and used for electrophoretic studies. Controls were made by using 10 μl of distilled water instead of seed meal extracts. Electrophoresis was done according to Laemmli (1970). Zymograms revealing protease activity bands were made according to García-Carreño et al. (1993). After electrophoresis, gels were washed and incubated for 30 min at 4 °C in 0.5% casein, pH 9.0. Subsequent casein digestion by bands containing proteases was done at 37 °C for 90 min in a fresh 0.5% casein solution. After electrophoresis, gels were stained in 0.1% Coomassie brilliant blue, in methanol-acetic acid-water (50:20:30). Gels were washed with methanol-acetic acid-water (40-10-50) for 2 h. Proteases were visualized as clear bands on a blue background after staining the gel with Coomassie brilliant blue. Bands were compared between samples incubated or not in the presence of seed extracts.

Statistical analysis

Results are given as mean $\pm s$ ($n = 3$). For comparison, the percentage of inhibition was arcsin ($x^{1/2}$) transformed. Values were subjected to one- or two-way ANOVA when required. Differences between means at $p < 0.05$ were analyzed by discriminate analysis, followed by Tukey's HSD test. The data were analyzed by using the Statistica software for PCs (Statsoft Inc., Tulsa, Oklahoma, USA).

Results

Total proteolytic activities and soluble protein measured in stomach and pyloric ceca extracts of both snappers are shown in Table 1. Activities measured in yellow snapper extracts were higher than the dog snapper, mainly alkaline proteases. Values of protein concentration are similar in both species. No proteolytic activity in the seed extracts was detected under the experimental conditions used in this work (pH 2.0 or 9.0, 60 min and 37 °C).

None of the seed extracts studied inhibit proteolytic activity of stomach extracts. For this reason, subsequent inhibitory assays were carried out only on pyloric ceca extracts. Inhibition on activity of pyloric ceca extracts after incubation with seed extracts are shown in Figure 1. Before the inhibition assay, seed extracts were subjected to different treatments: T-1, none, T-2, acid treatment and T-3, acid digestion, according to Materials and methods. Mean values of protease inhibition were significantly higher for yellow snapper enzymes than for dog snapper ($p < 0.05$). Guamuchil, palo blanco, chick-pea, and soybean extracts without previous treatment had the highest inhibitory activity for either yellow or dog snapper proteases, from 70 to 80%, whereas sorghum extract did not exceed 40% for both fish enzyme extracts (T-1). A previous treatment of seed extracts with 0.1 M HCl did not significantly reduce their inhibitory activity, but the sorghum extract was decreased by 30% for both species (T-2). However, the simulated action of gastric enzymes from dog snapper reduced the inhibitory capacity of seed extracts greater than those of yellow snapper (T-3). For example, green pea extract inhibitory activity was reduced to 42% when incubated with the dog snapper gastric extract, whereas the inhibitory activity of the same extract was reduced to 63% when incubated with the yellow snapper gastric extract. Data of inhibition are influenced significantly by the type of meal, species, or treatment ($p < 0.05$). Under the same experimental conditions, statistical analysis showed that *L. novemfasciatus* is less sensitive to protease inhibitors present in seed extracts than *L. argentiventris*.

Dose response curves with different relative proportions of seed extracts were constructed for soybean and sorghum meal (Figure 2). In all cases, inhibition curves have a similar trend, however some differences were evidenced for each snapper. For the same assay conditions, effect of seed extracts on pyloric ceca proteases were higher for yellow snapper than for dog snapper. Soybean extract yielded a higher inhibition than sorghum extract even when they were incubated at low relative concentrations.

Seed extracts were heated at different temperatures before inhibition assays. When heat-treated seed extracts were used to evaluate their inhibitory activity on enzymes from the snappers, a complex picture arose (Figure 3). Soybean extract was the less affected by temperature, whereas sorghum and green pea extracts were the most affected for both fish enzymes. Inhibitors in soybean kept 100% of the inhibitory activity after 20 min at 100 °C. Sorghum was the most sensitive, losing most of the inhibitory activity at 80 °C. The alkaline proteases of the yellow snapper were more sensitive to the remaining inhibitory activity of heat-treated seed extracts. The dog snapper alkaline protease activity was less affected by the same heat-treated extracts, progressively keeping more activity than that of the yellow snapper when temperature of inhibitor treatment was increased.

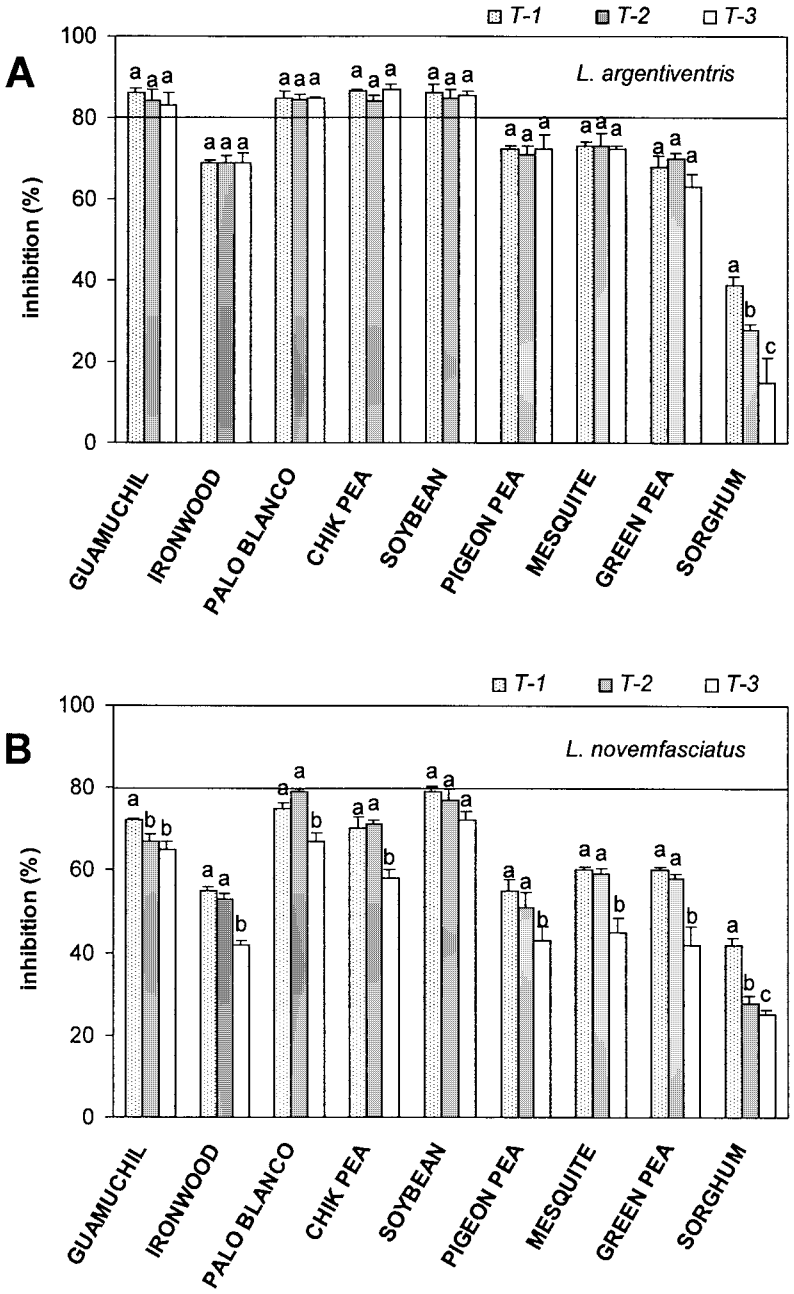

Figure 1. Effect of seed extracts on activity of alkaline proteases of *L. argentiventris* (A) and *L. novemfasciatus* (B). T-1: Untreated seed extracts; T-2: acid treated seed extracts and T-3: simulated acid digestion on seed extracts. Enzyme extracts were adjusted in order to produce an increase in ΔOD 366 nm in TCA-soluble fractions of 0.5 in 60 min at 37 °C. The volume of seed extract employed in the inhibitory assay was equivalent to 3 mg of meal. 80% of inhibition was indicated by a line. Results are given as means $\pm s$ ($n = 3$) with different superscript letters being significantly different ($p < 0.05$).

Table 1. Soluble protein and protease activity in stomach and pyloric ceca extracts from yellow and dog snapper. Data are the mean of three determinations $\pm s$.

Source	Stomach		Pyloric ceca	
	Soluble protein[a]	Protease activity[b]	Soluble protein	Protease activity
Yellow snapper	0.5 ± 0.1	737.0 ± 33.8	2.9 ± 0.2	52.3 ± 3.9
Dog snapper	0.4 ± 0.1	691.4 ± 33.7	3.1 ± 0.2	17.2 ± 1.1

[a]amount of protein in extracts (mg ml^{-1}).
[b]specific activity of the enzymatic extracts (U mg $protein^{-1}$).

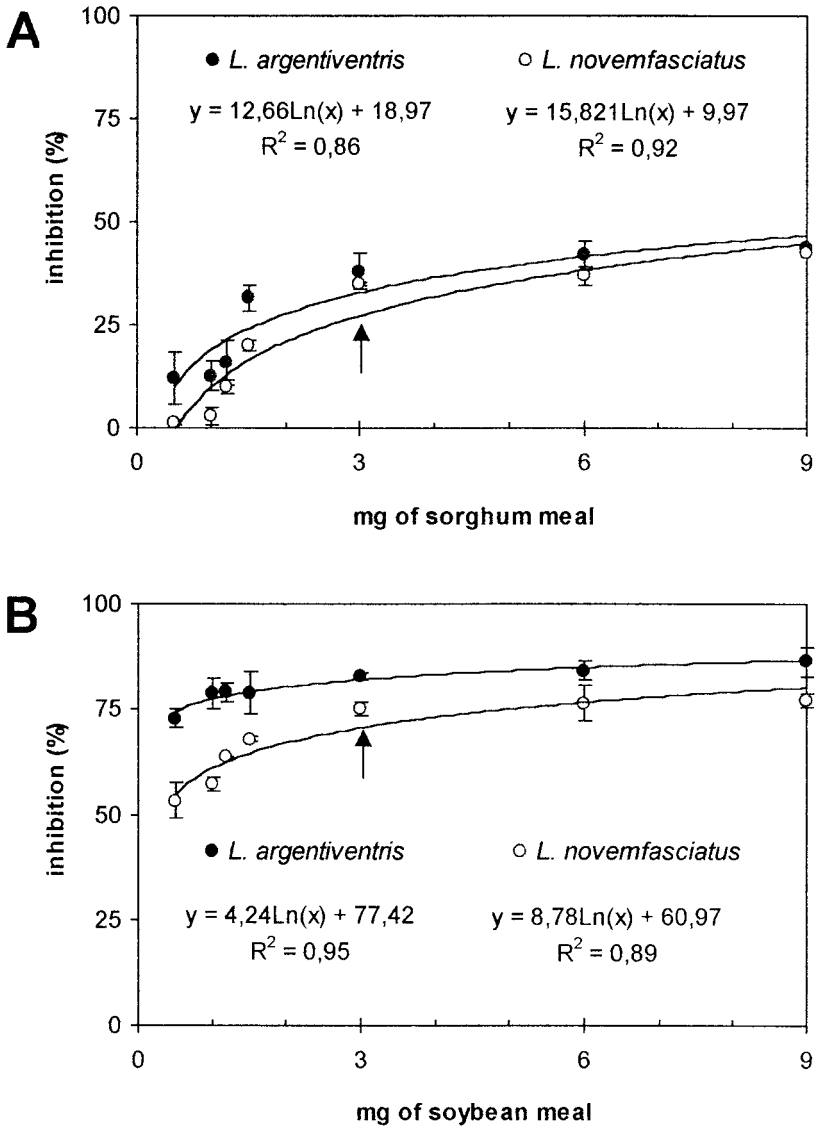

Figure 2. Inhibition curves of alkaline protease activity obtained after 1 h incubation of digestive extracts of snappers in the presence of increasing concentration of sorghum (A) and soybean (B) extracts. Each point is the mean of three replicate $\pm s$. Volume of enzymatic extract (0.5 U) was kept constant in each point. Arrows indicate the relative proportion used in the rest of the experimental assays.

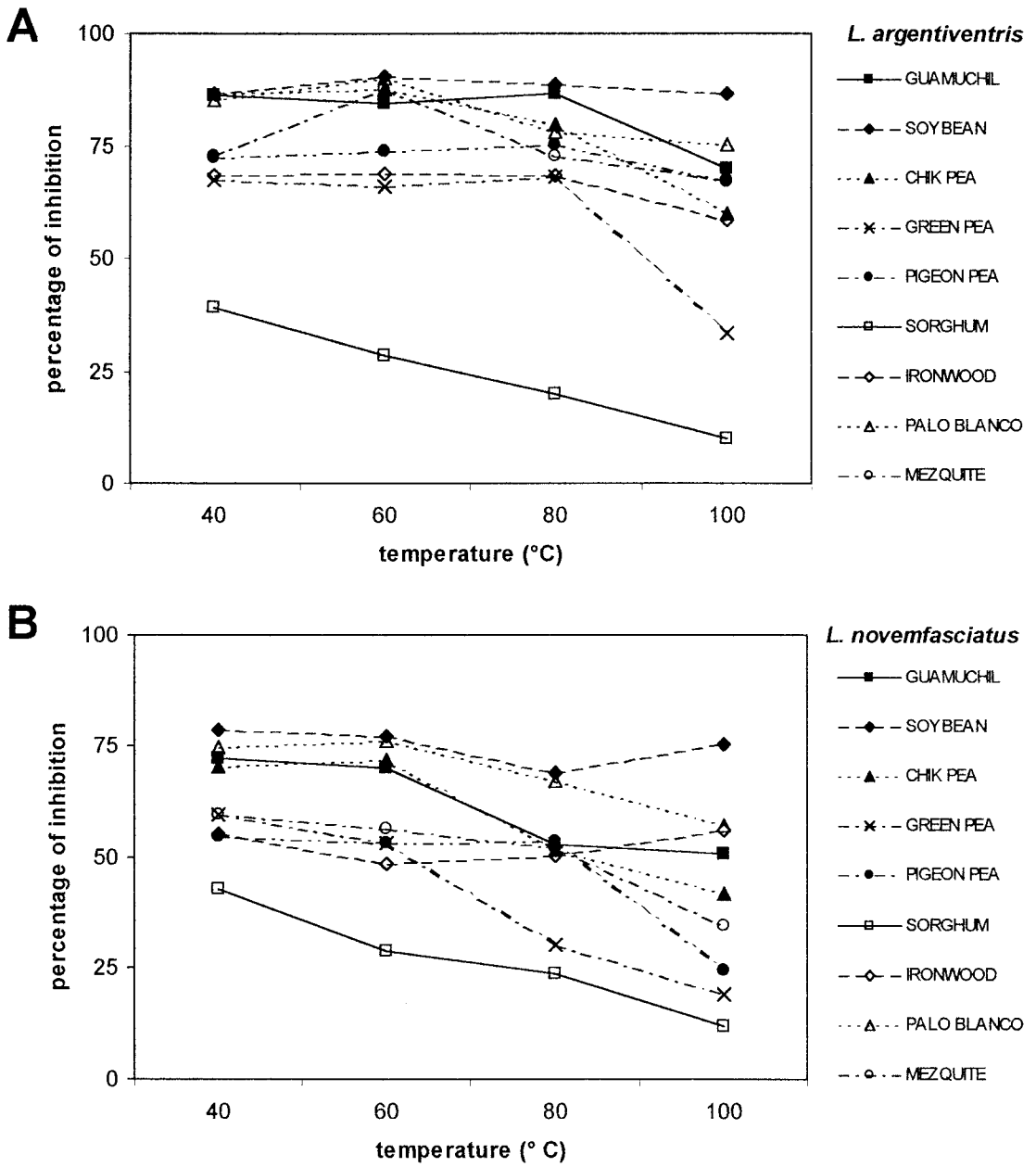

Figure 3. Inhibition of yellow snapper (A) and dog snapper (B) alkaline proteases by seed extracts heated at different temperatures. Seed extracts were heated for 20 min at different temperatures before measuring their inhibitory effect on fish digestive proteases.

Zymograms made for pyloric ceca extracts incubated with seed extracts revealed the effect of inhibitors on specific proteases (Figure 4). Effect of protease inhibitors on digestive proteases was visualized by a decrease in the intensity of active bands when compared with the control (column 1). Zymograms of yellow snapper showed a noticeable reduction in the intensity of several active bands when mixed with the seed extracts (columns 2 to 9). Each seed extract caused a characteristic pattern of inhibition. Palo blanco inhibitors dramatically reduced the bands of activity of both fish enzymes, mostly those bands between 20 and 30 kDa. These bands were also inhibited by the soybean inhibitors. The same bands were inhibited to a lesser degree by most of the plant inhibitors, except for sorghum and mesquite.

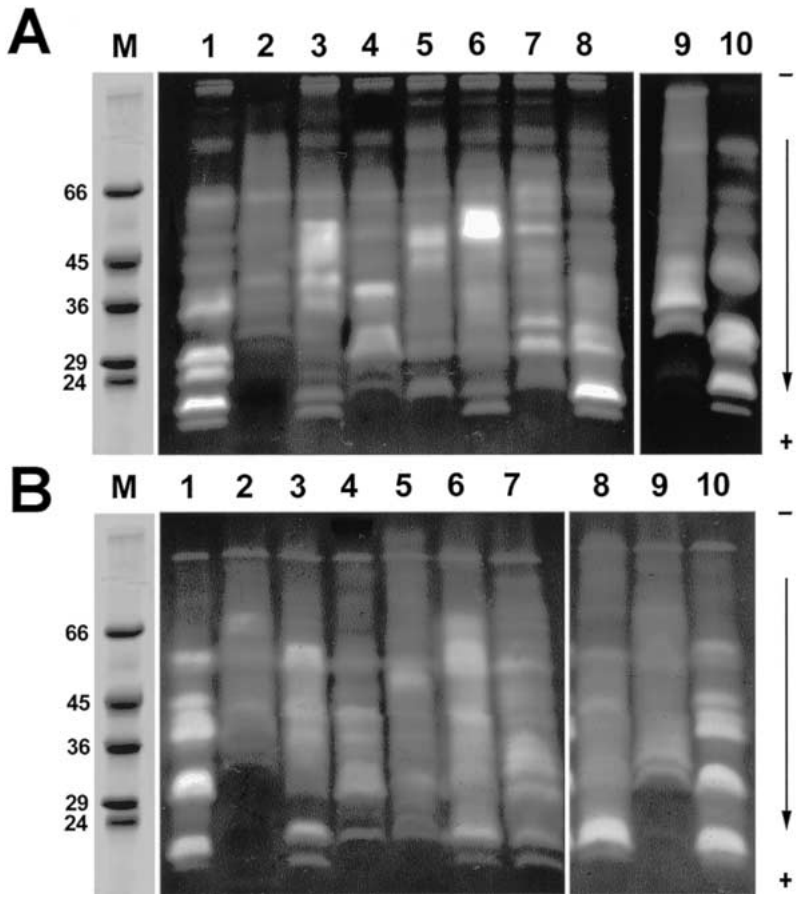

Figure 4. Substrate-SDS-PAGE obtained after incubation of yellow snapper (A) and dog snapper (B) digestive extracts (0.5 U) with 10 μl of aqueous solution of seed meals. M = molecular weight markers, Column 1 = control without inhibitor (10 μl of distilled water were used instead of seed extract), column 2 = palo blanco (aqueous extracts equivalent to 3 mg of meal), column 3 = pigeon pea, column 4 = guamuchil, lane 5 = chick-pea, column 6 = ironwood, column 7 = green pea, column 8 = mesquite, column 9 = soybean, and column 10 = sorghum. Molecular weight markers are: albumin bovine (66 kDa), albumin, egg (45), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36), carbonic anhydrase from bovine erythrocytes (29) and trypsinogen from bovine pancreas (24).

Discussion

Protein is the most expensive ingredient in aquafeeds, accounting for >50% of the cost of feed production. The most common proteinaceous ingredient is fish meal, which often is scarce and expensive. Because aquafeeds are formulated to have a high protein concentration, there is a growing demand for less expensive, alternative protein sources substituting for fish meal, especially those from plants. Some plant seeds, like soybean, have been used to partially replace fish meal in aquaculture feeds (Lim and Akiyama 1992). Usually, plant protein is less expensive than animal protein. However, the presence of antinutrient factors may limit their use (for general reviews see Liener 1980; Tacon 1997). Among antinutrients, protease inhibitors have the ability to reduce the activity of proteolytic enzymes secreted into the intestine lumen. The degree of inhibition may vary considerably among individual species (Shimeno et al. 1994; García-Carreño et al. 1997; Moyano et al. 1999). Recently, García-Carreño et al. (1996, 1997) reported the presence of inhibitors in the legume seeds used in this study. These inhibitors were effective for several proteases, both commercial and experimental; porcine trypsin, bovine chymotrypsin, and shrimp digestive proteases. In the present work, activity of digestive alkaline proteases of yellow and dog snapper were also severely reduced by the seed inhibitors. Palo blanco, guamuchil, chick-pea, and soybean seed extracts inhibited >50% of the caseinolytic activity of both snapper enzymes. Though

yellow snapper has the highest proteolytic activity, it was the most affected by seed inhibitors.

Gastric proteases were not inhibited to any extent by seed inhibitors. Plant inhibitors affecting fish gastric proteases have not been reported, whereas inhibitors for pancreatic digestive proteases are widely distributed among seed plants. Results agree with data reported for other fish species (Krogdhal et al. 1994; Shimeno et al. 1994; Alarcón et al. 1998, 1999). The inhibitors contained in seed extracts were resistant to acid denaturation (Figure 1). When seed inhibitors were treated with gastric enzymes, they lost some of their ability to inhibit intestinal proteases, mainly in dog snapper (Figure 1). Krogdhal and Holm (1981) have reported an inactivation of protease inhibitors after transit into the stomach, but some inhibitors, like the Bowman-Birk-type, are more stable and keep their ability to affect intestinal proteases. Dog snapper gastric digestion of the inhibitors was more extensive than yellow snapper gastric digestion.

The mechanism of inhibition of protease inhibitors has been extensively studied in mammals (Gallarher and Schneeman 1984). However, it has been reported in mammals (Kroghahl and Holm 1979, 1983; Holm and Krogdahl 1982), fish (Moyano et al. 1999) and shrimp (García-Carreño et al. 1997) that the effect of a given inhibitor is species specific. In this way, the use of standardized enzymes (porcine and bovine trypsins) for evaluation of inhibitors content in plant protein sources give results irrelevant in the nutrition of a given species. To understand the particularities of any aquatic species we need to make detailed studies on each one. Various works have demonstrated that the ingestion of soybean trypsin inhibitor, as part of a feed, results in significant reduction of protein digestibility and weight gain (Krogdahl et al. 1994; Olli et al. 1994). These symptoms are caused by pancreatic hypertrophy and hyperplasia, resulting from stress on the pancreatic tissue to continually produce proteases to compensate for the presence of protease inhibitors (Flavin 1982). The binding of protease inhibitors to the proteases causes the pancreas to secrete larger amounts of digestive enzymes to overcome the inhibitor and to digest the feed protein (Haard et al. 1996). Results obtained in the present paper suggest that protease inhibitors contained in some seeds could persist within the gastrointestinal tract of fish (resistant to acid and alkaline hydrolysis), and could cause adverse effects when ingested.

According to Tacon (1999), carnivorous fish will accept '*green revolution*' aquafeeds if they are fabricated by improved feed processing techniques. Heat-treatment is a conventional procedure that improves product quality and makes the best nutritional use of the raw protein sources (De Silva and Anderson 1995; El-Dahhar 1999; Fagbenro 1999). However, each legume seed should be studied individually because the sensitivity of inhibitors to heat is species specific. For example, sorghum inhibitors were inactivated progressively when temperature was increased, whereas soybean meal and palo blanco resisted high temperatures up to 20 min (Figure 3). Usually, fish nutritionists assume that processing methods like heating destroy the protease inhibitors in raw materials. However, our results indicate that this is not true for all the seed assayed. In this sense, the presence of protease inhibitors was found in commercial aquafeeds, indicating that thermal treatments are not enough to eliminate the inhibitors (Alarcón et al. 1999; Mitchell et al. 1993). Thus, the response of the same heat-treated seed extract is different in each studied species (Figure 3). Intestinal proteases of yellow snapper were more affected by the same heat-treated extract than those of dog snapper.

The effect of inhibitors on intestinal proteases of yellow and dog snapper was analyzed by susbstrate-SDS-PAGE (Figure 4). The information obtained with this technique gives information about the number of proteases. Seed inhibitors yield a characteristic pattern of inhibition on digestive proteases. It was similar for both snappers. According to biochemical data, palo blanco and soybean meals inhibit to a great extent the different caseinolytic fractions detected in zymograms. In some cases, the interaction between proteases and inhibitors is reversible, producing a new protein that could maintain the caseinolytic activity (In Figure 4A, columns 3 and 6; some active fractions are not present in control column 1). Such observations are in agreement with results obtained in others marine species, like sea bream (Alarcón et al. 1999), Senegal sole, and tilapia (Moyano et al. 1999).

Data obtained in the present work suggest that the presence and effect of protease inhibitors must be studied when a new ingredient or species is intended for aquafeeds. A study *in vivo* is currently in progress to evaluate the effects of inhibitors on histological, histochemical, biochemical, and physiological variables in both fish species, using soybean, green pea, and chick-pea.

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