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Inhibition of protease activities in discus *Symphysodon* spp. by three plant meals

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Abstract. Based on biochemical assays and electrophoretical methods, the inhibitory effects of three plant meals (soybean meal, wheat meal, winged bean meal) on digestive alkaline proteases of discus were investigated. Casein assays revealed that increasing levels of soybean meal caused a linear inhibitory effect on activity of protease. SDS-PAGE images revealed that trypsin and chymotrypsin were the inhibited enzymes. Soybean showed the lowest inhibition rate followed by wheat meal and raw winged bean. There was a quadratic relationship between wheat meal levels and its inhibition of protease acitivity. The highest inhibitory effect was obtained with the winged bean meal with inhibition of caseinolytic activities ranging from 3.6–98.6%. Results from this study showed the potential of both soybean meal and wheat meal as ingredients for practical diet for discus, while demonstrating the need for further improvement in processing method for winged bean meal.

Introduction

Farming of tropical fish for the aquarium trade is an important souce of income for several Asian countries (Andrews 1990). Ng and Tan (1997) estimated that South East Asian countries produced ornamental fishes valued at USD 80–150 million annually. Discus, *Symphysodon* spp., originally consisting of 2 species and several subspecies, is widely cultured in South East Asian countries for the ornamental trade (Chapman et al. 1997; Koh et al. 1999).

Discus farmers currently rely on freshly–prepared moist feed consisting of various feedstuffs such as beef-heart, cockles, shrimps and blended plant ingredients. These feeds may result in good growth but have a detrimental effect on water quality as they disintegrate easily during feeding causing high-nutrient content effluents. In addition, nutrient levels of these feeds could be unnecessarily above or below the optimum level required by the fish, hence the need to develop nutritionally optimized cost-effective dry diets for farming activities. However, until now, knowledge of nutritional requirements was limited to a protein requirement that had been estimated in an earlier study (Chong et al. 2000).

Fish meal is traditionally used as a protein-source in aquaculture due to its high protein content, balanced essential amino acid profile and good digestibility. The global shortage in fish meal production together with the increasing demand from

poultry and livestock feed industry have caused an increase in fish meal prices. The need for more readily available and cheaper dietary protein sources has led to evaluation studies on the use of various plant proteins as fish meal replacers in aquaculture diets. The use of plant proteins however, is often hampered by various factors such as inbalanced amino acid profile, low digestibility, palatibility and the presence of anti-nutritional factors (Francis et al. 2001). Anti-trypsin inhibitors present in soybean for instance, have been proven to reduce the capability to digest proteins in rainbow trout, coho salmon and Atlantic salmon (Krogdahl et al. 1994; Olli et al. 1994; Haard et al. 1996). Inadequately-heated soybean meal reduces both protein and energy digestibility, and growth rate in common carp and channel catfish (Viola et al. 1983; Wilson and Poe 1985). Krogdahl and Holm (1983) also reported that trout proteases were more sensitive to soybean trypsin inhibitors as compared to other animals. In this study we investigated the inhibitory effect of three plant proteins (soybean, winged bean and wheat meals) on the alkaline protease activity of discus juveniles. This was based on casein assays and the use of substrate SDS-PAGE electrophoresis as an early indication on the suitability of these materials as feedstuffs in diets for discus.

Materials and methods

Fish crude enzyme extract

Juvenile discus were obtained from stock maintained at the Aquaculture Research Complex, Universiti Sains Malaysia. Ten week old fish (5.2 ± 0.8 g) were selected for digestive protease sampling. The fish were previously maintained on *Artemia* for the first three weeks after hatching followed by dry pellets (Tetrabits®) at 5% body weight/day. Fish were starved for approximately 12 hours prior to sampling and subsequently frozen at −20 °C for 30 minutes. Then, whole intestine was dissected followed by removal of its contents and rinsing with cold distilled water. Intestinal tissues were homogenised in cold Tris-HCl 50 mM buffer (pH 7.5) at 1 g tissue/ml buffer using a hand-held glass homogenizer. Homogenate was then centrifuged at 4 °C at 10,000 g for 15 min. Enzyme extract was then stored at −70 °C before analysis. The soluble protein content of enzyme extract was measured according to Lowry et al. (1951).

Plant material substrate

Winged bean seeds (crude protein 47.0%), were crushed and oven heated for 15 min at 80 °C. For soybean (crude protein 46.8%), a standard treatment of the whole seeds with hexane followed by heating (15 min at 80 °C) was used. Crushed wheat grain meal (crude protein 13.4%) was also used. Solutions were prepared from the different plant material by homogenization of the meals in distilled water (2 mg/ml)

followed by centrifugation at 10,000 g for 1 hour. Supernatants were then collected and stored at 4 °C prior to analysis.

Casein assay

Protease activity was assayed using the casein hydrolysis method of Kunitz (1947) as modified by Walter (1984). For the control, an enzyme-substrate mixture consisting of 0.3 ml 1% (w/v) casein in water, 0.5 ml buffer (Tris-HCl 0.1 M, pH 9.0) and 0.3 ml of crude enzyme extract, was incubated in a water bath for 1 hour at 37 °C. A total of 0.5 ml trichloroacetic acid (TCA, 12% w/v) was added to the reaction mixture to stop the reaction. This mixture was allowed to stand for 1 hour at 4 °C before centrifuging at 8,000 g for 15 min. Absorbance of the supernatant was recorded at 280 nm, to measure the amount of tyrosine produced. The blank used for this assay was prepared by incubating a mixture of the crude enzyme extract, buffer and water for 1 hour at 37 °C, followed by the addition of TCA and casein. One unit of specific discus enzyme activity was defined as the amount of enzyme needed to produce 1 μg tyrosine/minute/mg soluble protein of enzyme extract (U per mg protein).

The inhibitory effect of the test plant meals was measured by incubating enzyme extracts with selected concentration of extracted plant supernatant for 60 minutes at 28 °C prior to casein assay. In order to evaluate the effect of different levels of the test plant materials, different concentration of the test plant meals per unit of protease activity U (mg meal/U) were studied. Inhibitor level was determined as percentage of reduction in U per mg protein between control and treatment with the test plant materials.

SDS-PAGE electrophoresis

Zymograms for substrate SDS-PAGE electrophoresis were also used to observe the inhibitory effects of plant meals on the alkaline proteases present in the crude enzyme extract (Bollag and Edelstein 1991; Garcia-Carreno and Haard 1993). Crude enzyme extract was mixed in sample buffer (Tris-HCl 1 M pH 6.8, glycerol, SDS, bromophenol blue) at a ratio of 2:1(v/v). A total of 5 μl of this mixture was loaded into SDS-PAGE gels (6.0 × 8.0 cm) with 0.5 mm thickness consisting of 5% of stacking gel and a 12% separating gel (Bollag and Edelstein 1991). Electrophoresis was conducted at 120V using the Mini Protean III® electrophoresis system (BIO-RAD Laboratories, California) for approximately 90 minutes at 4–6 °C with electrophoresis buffer of Tris-glycine-SDS. The gel was then immersed in casein solution (3% in 50 mM Tris-HCl at pH of 7.5) at 4 °C for 30 minutes to allow absorption of casein into gel. This zymogram was then removed and placed in a water bath at 27 °C for an additional 90 minutes to allow proteases in the gel to digest the casein. This was followed by staining with Coomassie Brilliant Blue R-250 (BIORAD® Laboratories, California) dissolved in a solution containing acetic acid, methanol and distilled water (1 g Brilliant Blue per 450 ml methanol, 450 ml distilled water and 100 ml of acetic acid) for 30 minutes. This process will stain

the whole gel blue due to the presence of adsorbed casein except in areas containing proteases which had digested the casein. Destaining the gel in a methanol-acetic-acid-distilled water solution for additional 60 minutes will enhance these areas, indicating the presence of alkaline digestive protease. Classification of proteases was based on study by Chong et al. (2002). For inhibitory studies, crude enzyme extracts were incubated under similar conditions with a selected concentration of plant material for 60 minutes at room temperature prior to electrophoresis.

Results

Figure 1 shows the effect of incubating discus alkaline proteases with different concentrations of soybean, winged bean and wheat meals. All three plants meals showed different rates of inhibition towards fish proteases. Processed soybean showed the lowest rate followed by wheat meal and raw winged bean meal. A linear relationship ($y = 7.76x - 0.23$, $r^2 = 0.95$) was obtained between concentrations of soybean per unit activity (mg/U) of fish protease and percentage of inhibition, with inhibition levels ranging from 0.0–13.5% within levels tested. As for wheat meal, it showed a quadratic relationship between wheat meal levels and its inhibitory effects ($y = -8.13x^2 + 44.98x - 4.84$, $r^2 = 0.98$). In comparison, the highest inhibitory effect was obtained with the incubation of discus proteases with winged bean meal where an exponential curve was obtained ($y = 32.86\ln x + 74.82$) with inhibition ranging from 3.6–98.6%.

Figures 2, 3 and 4 show the effect of different levels of the three meals on different classes of alkaline proteases. SDS-PAGE electrophoresis revealed further details of the inhibitory effect of these meals in terms of the classes of proteases affected. High levels (0.93–1.87 mg meal/U activity) of soybean meals inhibited both the trypsin and chymotrypsin proteases (Figure 2). In wheat meal, the serine proteases and chymotrypsin groups were the first to be affected while trypsin bands were only affected at high wheat meal concentrations. Gel image for winged bean inhibition also confirmed results obtained with casein assay where most of the proteases are inhibited by the presence of this plant ingredient. Trypsin, serine protease and chymotrypsin were completely inhibited at the 0.56 mg meal/U activity level while metalloprotease was completely inhibited at 0.93 mg meal /U activity. The complete disappearance of all bands at high concentrations of winged bean corresponds with the complete inhibition of casein digestion (Figure 1).

Discussions

Both the casein assay and substrate SDS-PAGE electrophoresis methods provided useful information on the effect of the test plant ingredients on the digestive capabilities of discus alkaline proteases. Different curves (linear, quadratic and exponential) to relate substrate concentration and inhibition effect were obtained for all

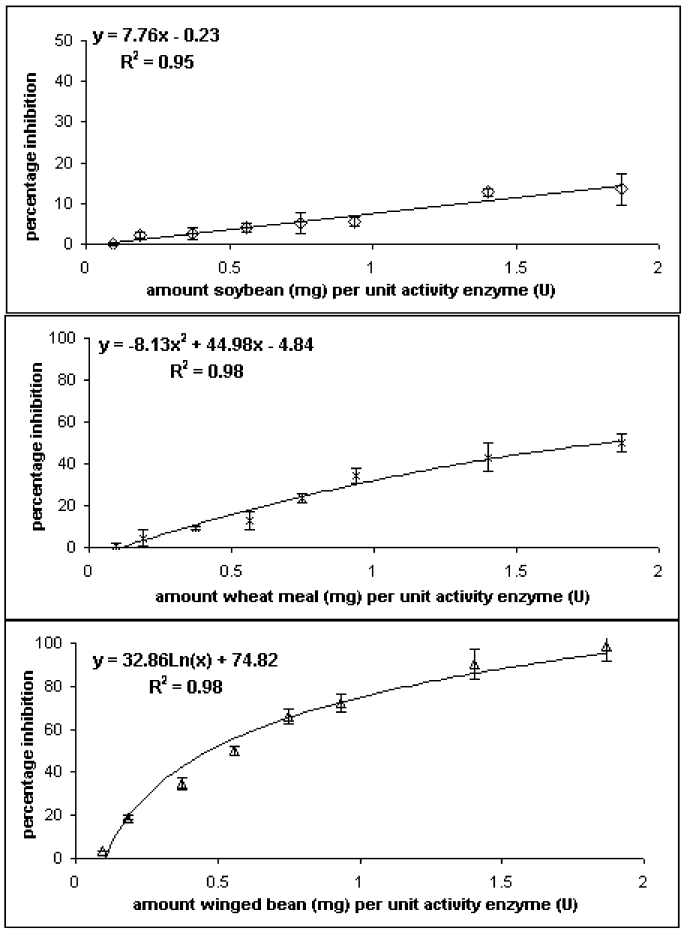

Figure 1. Percentage of inhibition of discus alkaline proteases by three different plant meals at different concentrations based on casein hydrolysis assays. Percentages are mean value of three separate assays.

three meals with the casein assay while electrophoretical method gave further information of the effect of each meal on the different protease classes. Lopez et al. (1999) deduced that plant inhibitory rate towards fish proteases is affected by type of plants, its level in feed, feeding duration and sensitivity of particular fish species towards the inhibitor present. Differences in inhibitory percentage versus meal concentrations curves among different meals or fish species also demonstrated the existence of such variations (Viola et al. 1983; Wilson and Poe 1985; Alarcon et al. 1999; Lopez et al. 1999; Refstie et al. 2000).

Results of this study indicate that soybean meal is preferred as a protein source for discus feed since it exhibited low inhibitory effects towards trypsin and chymotrypsin activities, except at very high concentration levels (> 0.93 μg meal/U activity). Elsewhere, Dabrowski et al. (1989) and Lopez et al. (1999) reported that

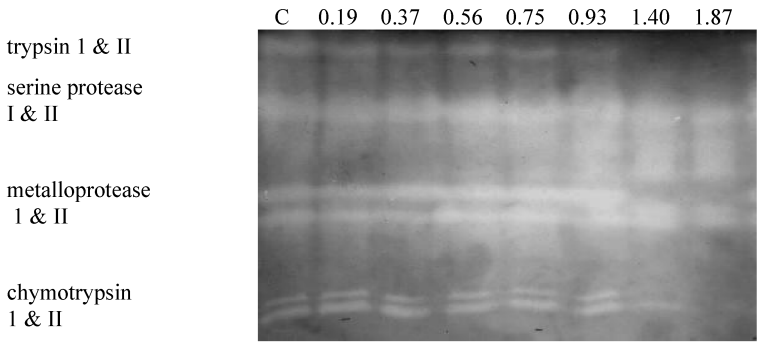

Figure 2. Substrate-SDS PAGE gel of discus alkaline proteases after 1 hour incubation with increasing concentration of soybean meal (mg meal/U activity). Lane C: extract without plant meal as control. Identification of different protease classes are according to Chong et al. (2002).

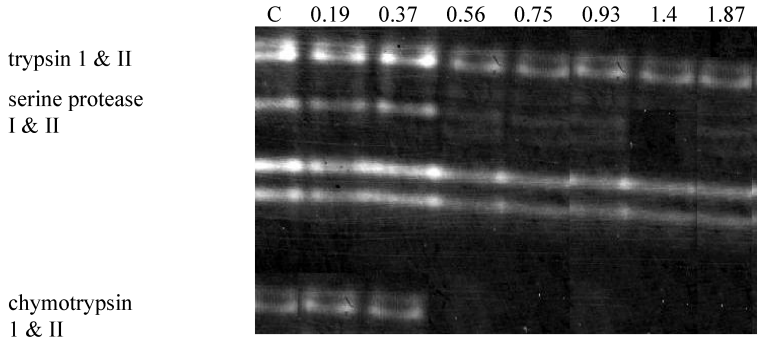

Figure 3. Substrate-SDS PAGE gel of discus alkaline proteases after 1 hour incubation with increasing concentration of wheat meal (mg meal/U activity). Lane C: extract without plant meal as control. Identification of different protease classes are according to Chong et al. (2002).

soybean showed inhibitory effects at high inclusion levels toward both protease groups in rainbow trout and Nile tilapia, respectively. It has been suggested that at low inclusion levels, the fish digestive system is able to overcome the inhibitory effects of soybean antinutritional factors through higher secretion of trypsin or by increasing the adsorption of protein throughout the whole gut (Hofer 1982; Krogdahl and Berg-Lea 1992; Krogdahl et al. 1994; Olli et al. 1994).

Heating is the most common method utilised for soybean processing but it does not completely destroy all natural-occurring inhibitors, especially trypsin inhibitor. The Bowman-Birk chymotrypsin and trypsin inhibitor in soybean, which possess a higher inhibitory rate towards both these proteases as compared to the Kunitz trypsin inhibitor, is also reported to have higher resistance to heat treatment (Obara and Watanabe 1971; Olli et al. 1994).

The present study also demonstrated that discus alkaline proteases showed high degree of sensitivity towards the presence of winged bean. At the 0.56 μg meal/U activity level for instance, 50% inhibition was obtained with winged bean as com-

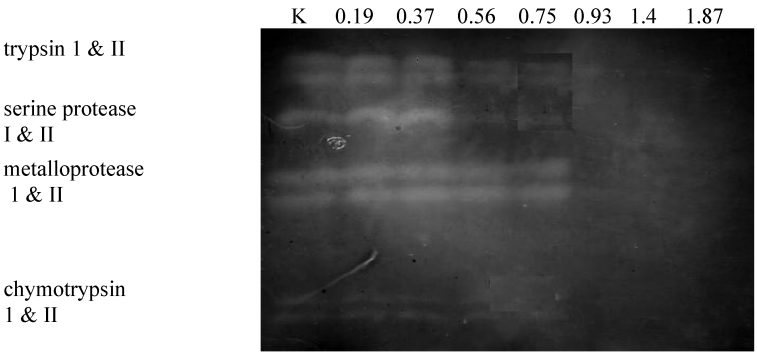

Figure 4. Substrate-SDS PAGE gel of discus alkaline proteases after 1 hour incubation with increasing concentration of winged bean (mg meal/U activity). Lane C: extract without plant meal as control. Identification of different protease classes are according to Chong et al. (2002).

pared to 4.70% for soybean and 14.29% for wheat meal. Elsewhere, a similarly processed winged bean meal has been reported to successfully replace 33% of fishmeal for red tilapia (Hashim et al. 1994). However this replacement level is lower than the 80% replacement of menhaden fishmeal reported in African catfish with a similar meal autoclaved or roasted at 125 °C and 110 °C for 30 minutes, respectively (Fagbenro 1999). This indicates that a higher processing temperature at longer duration is needed to further reduce protease inhibitors present in this meal. As for wheat meal, our finding here also showed that it can be included at low dietary levels without affecting protein digestion in discus. This concurs with other reports on reduced protease activities and overall digestibility of vegetable meals in tilapia (Lopez et al. 1999).

Results obtained here also enable us to predict the expected reduction of protease activity in discus when fed with diets with different inclusion levels of any of three tested plant meals. Following the equation for winged bean ($y = 32.86\ln X + 74.82$), a meal containing 30% winged bean fed to a 5 g discus consuming 2% of its weight will result in 35.2% reduction in protease activity if a total of 100 U activity of protease is produced by fish after the meal. A correlation of these values with growth and feed utilization parameters of fish will provide further useful information on effect of plant meal inclusion levels in this species.

Conclusions

This present study showed that the methods applied were able to detect the degrading action of anti-protease inhibitors in different test plant meals more rapidly than the usual trials which involve feeding the animals diets with different inclusion levels of ingredients and monitoring subsequent growth and digestibility. Soybean meal and wheat meal remain potential choices as practical ingredients for discus diet formulation when inclusion levels are kept low since significant inhibition was

only evident at high concentrations. As for winged bean, an improved processing method compared to the one used in this study is needed to further remove the anti-proteases inhibitors present.

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