J Mar Biotechnol (1997) 5:36–40

Journal of Marine Biotechnology
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# Digestive shrimp proteases for evaluation of protein digestibility in vitro. I: Effect of protease inhibitors in protein ingredients

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Received: 8 May 1996/Accepted 14 June 1996

**Abstract.** The digestive protease system from shrimp hepatopancreas is currently used to evaluate the in vitro digestibility of protein in ingredients for shrimp feeds, such as legume seed meals. The effect of antiphysiological inhibitors in legume seeds on digestive proteases of white and brown shrimp was analyzed. All the tested seed meals showed inhibitory activity for shrimp and commercial proteases. An increase in the degree of hydrolysis (DH) of the seed meal protein by the shrimp enzyme system was shown after heating the meal. Based on their protein content, three of six seed meals were found to be suitable as ingredients for shrimp feed production, provided the digestive enzyme inhibitors of the meal are expeditiously reduced by a physical treatment. The evaluation of DH is a useful tool to predict the protein digestibility of farmed shrimp.

Biotechnology is the use of biological processing methods in productive activities. In this study, the protease digestive system of shrimp hepatopancreas was used to evaluate the *in vitro* protein digestibility of ingredients for shrimp feeds. It is generally accepted that about 50% of the cost of shrimp farming goes to feed. World shrimp production is about 2.2 million metric tons, including 700,000 tons by farming. With an average feed conversion ratio of 2.5 kg of feed for 1 kg of shrimp, the world production of shrimp feed is about 1,750,000 tons. Since more than 30% of the protein included in the shrimp feed comes from seed meals, there is increasing interest in alternative sources of protein. One choice is seed from legumes. The peninsula of Baja California has many endemic and introduced legumes that produce significant amounts of seeds. These plants germinate, sprout, flower, and release seeds under severe arid conditions. Legume seeds are known to have elevated concentrations of high-quality protein and to produce protease inhibitors (Laskowski and Laskowski 1954; García-Carreño *et al.* 1996). Recently, we reported on inhibitors for several proteases derived from a dozen legume seeds (García-Carreño *et al.* 1996); these included inhibitors for procine trypsin, bovine chymotrypsin and papain, and cathepsins from fish muscle. The presence of inhibitors in the seed meals can be a limitation to their use as aquaculture feed. The effect of inhibitors in the shrimp digestive system needs to be studied for possible side effects produced by the seed meals. Likewise, a detection procedure for protease inhibitors should also be included in the quality control of shrimp feed.

Food protein is hydrolyzed by digestive enzymes. The crustacean hepatopancreas, also called midgut gland, produces and releases into the digestive tract several enzymes, including proteases and peptidases (García-Carreño and Haard 1993, 1994; García-Carreño *et al.* 1994). Proteinases split the protein into medium-size polypeptides, while peptidases are responsible for further hydrolysis, producing small peptides and free amino acids. These enzymes are sensitive to inhibitors. Proteinase inhibitors are ubiquitous active molecules (García-Carreño 1996). The aim of this work is to evaluate: (1) the protein content in legume seed meals, the presence of protease inhibitors in the meals, and the effect of the meal inhibitors in the protein digestion, and (2) to develop a technique to predict the digestibility *in vitro* using an enzyme extract of the shrimp hepatopancreas, based on the experience with salmonids that had the highest correlation coefficient ever (Dimes and Haard, 1994; Dimes *et al.* 1994a,b).

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## Materials and methods

Reagents including the enzymes porcine trypsin (catalog number: T0134), bovine chymotrypsin (C4129), porcine peptidase (P7500), bacterial protease (P0652), papain (P3125), substrate azocasein (A2765), and general reagents were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Seeds were obtained from legume plants that are either endemic or introduced to the Baja California Peninsula: guamuchil *(Pithecellobium dulce),* palo fierro *(Olneya tesota),* palo blanco *(Lysiloma candida), soybean (Glycine max),* pigeon pea *(Cajanus cajan),* and chick pea *(Cicer arietinum).* In the summer

of 1994 and 1995, seeds were collected and processed as described by García-Carreño *et al.* (1996). Seeds were stored dry at 4°C for several months. Dry seeds were ground in a coffee mill to produce the seed meals. Total nitrogen content of the seed meals was evaluated using the Kjeldahl method (AOAC 1990). Soluble protein content was evaluated in an aqueous extract of the seed meals by the Bradford method (1976).

Seed extracts for inhibition assays were obtained according to García-Carreño *et al.* (1996) as follows: each seed meal was extracted with three volumes of 50 mM Tris HC1, pH 7.5, by shaking for 120 min at room temperature, then for 22 h at 4°C. The extracts were obtained by centrifugation for 30 min at 5000*g*, 5°C. The clear extracts were stored at 4°C.

White shrimp, *Penaeus vannamei,* were purchased from a local farm, Acuacultores de la Península, and transported alive to the laboratory in an ice chest containing seawater at 10°C. Brown shrimp, *Penaeus californiensis,* were provided by the CIBNOR aquaculture facilities. Animals were adults, averaging 15.56 ± 1.5 g and 12.6 ± 0.8 cm for the white shrimp and 8.62 ± 2.5 g and 11.24 ± 0.82 cm for the brown shrimp. The hepatopancreas were removed from the decapitated shrimp at 10°C. The tissue was standardized by homogenizing with one volume of water in a Waring blender at the same temperature, centrifuged for 30 min at 10,000*g*, 4°C. The aqueous supernatant was frozen and stored at −20°C.

Enzyme activity and inhibition were evaluated according to García-Carreño (1992). In brief, 500 μl of Tris HC1, pH 7.5 buffer, 5 μl of enzyme preparation, and 500 μl of the substrate 1% azocasein in Tris buffer were mixed at zero time. Addition of the substrate azocasein marked the beginning of the reaction. The mixture was incubated at 25°C for different periods. The reaction was stopped by adding 500 μl of 20% TCA. TCA-insoluble protein was separated by incubating the reaction mixture for 10 min at 4°C and centrifuging for 5 min at 12,500*g*, 20°C. The absorbance for soluble TCA peptides was recorded at 366 nm with no further treatment. For the inhibitory assay, 5 μl of inhibitor preparation, 5 μl of enzyme preparation, and 500 μl of Tris buffer were mixed. The mixture was incubated for 60 min at 25°C. The inhibitor–enzyme mixture was analyzed for enzyme activity as above. Blanks were prepared by adding 500 μl of 20% TCA before addition of the substrate.

### *Inhibitor thermostability*

The seed extracts containing the inhibitor(s) were incubated for 20 min at 40, 60, 80, and 100°C in a water bath, then centrifuged for 3 min at 12,500 *g*. The precipitate was discarded and the supernatant was used for the inhibition assay. One unit of enzyme activity was defined as the absorbance at 366nm per minute. The specific activity was units per milligram of protein content in the enzyme preparation.

The protein digestibility was determined by evaluating the degree of hydrolysis (DH) following Adler-Nissen (1986), Dimes and Haard (1994), Dimes *et al.* (1994a), and García-Carreño and Haard (1994). The evaluation of DH was done in a pHstat 718 Stat Titrino (Metrohm Ion Analysis, Switzerland), interfaced to a PC with Metrohmdata software. The apparatus is the lab scale version of a full-scale pH controller. It was set on the following parameters: selected mode, TIP; End point 1, 8.0; dynamics, 1; maximum rate, 10 ml/min; minimum rate, 25.00 μl/min; Stop type, drift; drift, 2 μl/min; and report, assign full; using an electrode, 6.0234.100 combined micro pH glass electrode with 6.1236.040 SGJ sleeve, at measuring input 1.6.110.100 T sensor. The determination was carried out at a constant temperature of 25°C. A cocktail made of the commerical enzymes: trypsin (1.6 mg/ml), bovine chymotrypsin (3.1 mg/ml), porcine peptidase (1.3 mg/ml), and bacterial protease (7.95 mg/ml) or a shrimp hepatopancreas extract in water (20 mg/ml) was used as hydrolyzing enzyme preparation. The seed meals were homgenized in water by using a Waring blender. The amount of crude protein (the substrate) from the seed meals was adjusted to 8 mg/ml. Ten grams of the substrate suspension were poured into the hydrolysis vessel and the pH was adjusted to 8.0 by adding drops of 2 M NaOH. Then, 1 ml of shrimp extract or 0.3 ml of the commercial enzyme cocktail was added and consumption of 0.05 M NaOH was recorded. The DH was calculated by using the algorithm described by Alder-Nissen (1986), as follows:

$$DH\% = \{[(EP1*CO1*CO2)/CO3]/CO4\}*CO5$$

where EP1 is ml of 0.05 M base (NaOH) consumed; CO1 of NaOH molarity; CO2 is $\alpha^{-1}$ (calibration factor: $\alpha = 10^{pH-pK}/1 + 10^{pH-pK}$); CO3 is grams of protein substrate; CO4 is h tot (meq/g (N*fN)), N is nitrogen content and fN is convertion factor of nitrogen; and CO5 is 100.

To evaluate the effect of temperature on the seed protease inhibitors the DH of seed meal protein was evaluated after heating the seed meal for 60 min at 85°C in a water bath. The DH for casein was used as internal control. All the assays, in triplicate, were repeated at least three times.

### *Statistical analysis*

Except where indicated, results are presented as means ± SD, SD will be shown only when higher than 2. Differences among treatments were analyzed by one-way analysis of variance, followed by Tukey's multiple range test. The data were analyzed by using the Statistica software for PCs (Statsoft Inc., Tulsa, OK, USA).

## Results

The seed meal chemical composition is shown in Table 1. The palo fierro meal had the highest fat content, measuring 42% of its dry weight, while the pigeon pea meal was the lowest with 4%. The ash content of the seed meals was between 3 and 5%. The protein content of the studied seeds was over 30% for the soybean, palo blanco, and palo fierro meals. Pigeon pea, chick pea, and guamuchil meals had a protein content lower than 25%. In the present study, no correlation was found between the protein content of the meals detemined by Kjeldahl method and the water-soluble protein. However, we used the water-soluble protein as an indicator of the protein that is available, in the first instance, for enzyme digestion.

As a preliminary evaluation of protease activity, the period in which the azocaseinolytic reaction was linear was determined to guarantee that any reduction of the enzyme activity by the enzyme inhibitor was due to the effect of the inhibitor and not related to any shortage of the substrate or instability of the enzyme. The period in which the reaction was linear was 5 min for both white and brown shrimp under the conditions of our assay. The protein concentration for both white and brown shrimp standardized hepatopancreas extract was 7 and 5.4 mg/ml, respectively.

To determine the protease activity of the shrimp hepatopancreas, midgut gland extracts were evaluated using azocasein as the substrate. Table 2 shows the protease activity of the white and brown shrimp enzyme preparations. The enzyme activity of the white shrimp was higher than the brown shrimp. Specific activity and protein concentration were also higher for the white shrimp hepatopancreas. Both assays were done after grinding the organ in one volume of water. The protease composition and class, evaluated by substrate-SDS-PAGE, is reported elsewhere.

Table 3 shows the percentage of protease activity for azocasein after incubating the shrimp proteases with the seed extract. The protease activity from the white shrimp hepatopancreas was reduced 62% (38% of remaining activity) by the soybean extract and 36% by the palo fierro extract. Protease activity from the brown shrimp hepatopancreas was lower than in white shrimp, and it was thus less affected by the meal inhibitors and reduced 47% by the soybean extract and 39% by the palo fierro extract. The soybean extract showed the highest inhibitor activity for either white or brown shrimp proteinolytic activity, while the palo fierro extract showed the least for both shrimp enzyme preparations.

Protease activity in shrimp hepatopancreas was evaluated after incubating the preparations with seed extracts that were previously exposed to different temperatures. Figures

**Table 1.** Chemical composition of legume seed meals.[a]

| Seed meal | Water | Fat | Ash | Protein | NON[b] | Soluble Protein (mg/ml) |
|---|---|---|---|---|---|---|
| Chick pea | 8.55 | 11.03 | 3.64 | 18.86 | 57.92 | 16.1 |
| Palo blanco | 6.98 | 17.99 | 4.15 | 32.10 | 38.78 | 17.8 |
| Soybean | 7.78 | 21.28 | 4.99 | 32.82 | 33.13 | 26.70 |
| Palo fierro | 7.78 | 42.06 | 3.55 | 32.71 | 13.9 | 8.89 |
| Pigeon pea | 9.0 | 4.28 | 4.67 | 16.82 | 65.23 | 13.10 |
| Guamuchil | 7.08 | 14.53 | 2.98 | 22.22 | 53.19 | 31.0 |

[a] Units as percentage; 100% will include carbohydrates; SDs were lower than 2 ($N = 3$)
[b] Nonprotein nitrogenous compounds.

**Table 2.** Protease activity and protein concentration of shrimp hepatopancreas extracts.

| | White shrimp | Brown shrimp |
|---|---|---|
| Protease activity | 0.055 ± 0.004[a] | 0.03 ± 0.005 |
| Protein conc. (mg/ml) | 7.0[b] | 5.4 |
| Specific activity | 1.57[c] | 1.1 |

[a] Units of activity = abs/min or 100% of activity for inhibition assays.
[b] Amount of protein in the hepatopancreas exract.
[c] Specific activity of the hepatopancreas extract using azocasein as the substrate (units/mg protein).

**Table 3.** Effect of seed extract inhibitors on protease activity of shrimp hepatopancreas.[a]

| Seed extracts | White shrimp | Brown shrimp |
|---|---|---|
| Chick pea | 54 ± 2.05* | 54 ± 0.81 |
| Palo blanco | 42 ± 0.81 | 59 ± 3.29 |
| Soybean | 38 ± 1.41 | 53 ± 4.32 |
| Palo fierro | 64 ± 4.18 | 61 ± 3.29 |
| Pigeon pea | 45 ± 1.69 | 56 ± 3.29 |
| Guamuchil | 49 ± 4.32 | 58 ± 5.43 |

[a] Values are the percentages of remaining protease activity of shrimp extracts after incubation for 60 min at 25°C with the seed meal extracts containing the inhibitors, then assayed for azocasein in test tube.

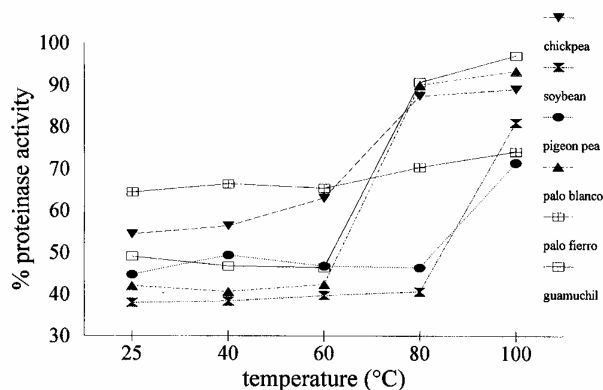


**Fig. 1.** The effect of temperature on the seed meal protease inhibitors for the white shrimp proteases.

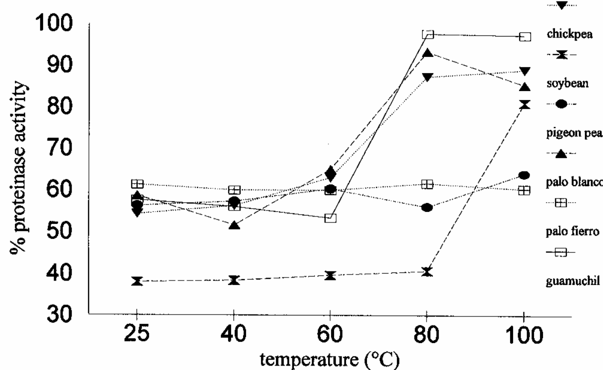


**Fig. 2.** The effect of temperature on the seed meal protease inhibitors for the brown shrimp proteases.

1 and 2 show the effect of temperature on the inhibition ability of seed extracts on proteases from shrimp. The temperature affected the seed extracts by reducing their inhibitory capacity. Guamuchil extract was the most affected by high temperatures (100°C), reducing inhibition ability to 3% for both white and brown shrimp. On the other hand, pigeon pea and palo fierro inhibitors were the least sensitive to temperature. They kept more than 25% of their inhibitory activity for shrimp protease preparations when heated for 20 min at 100°C. Moreover, palo fierro extract showed no significant difference in the ability to reduce the shrimp protease activity when treated for 20 min at 20–100°C. The other seed extracts showed no change in their inhibition ability when heated up to 60°C, but this was reduced by higher temperatures.

The degree of hydrolysis of the seed protein was evaluated to determine the effect of the seed inhibitors on the *in vitro* digestibility of protein in seed meals. Table 4 shows the DH of the seed meals after treatments at 25 and 85°C for 60 min. The DH of the seed meal protein at 25°C was not always higher when using the commercial enzyme cocktail than it was when we used the white or brown shrimp hepatopancreas. This was despite the fact that the cocktail was made from highly purified enzymes, containing endopeptidases as well as exopeptidases. This is evidence of the powerful nature of protein-hydrolyzing enzymes from the shrimp hepatopancreas and illustrates why shrimp are capa-

**Table 4.** Effect of temperature treatment of seed meals on degree of hydrolysis of seed meal protein by shrimp hepatopancreas extracts and commercial enzyme cocktail.

| | Degree of hydrolysis[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | White shrimp | | | Brown shrimp | | | Enzyme cocktail | | |
| Seed meal | 25°C | 85°C | f | 25°C | 85°C | f | 25°C | 85°C | f |
| Chick pea | 14 | 17 | 1 | 16 | 17 | 1 | 13 | 17 | 1 |
| Palo blanco | 33 | 37 | 1 | 11 | 26 | 2 | 27 | 33 | 1 |
| Soybean | 10 | 22 | 2 | 12 | 13 | 1 | 12 | 15 | 1 |
| Palo fierro | 6 | 21 | 3 | 9 | 15 | 2 | 7 | 25 | 3 |
| Pigeon pea | 10 | 38 | 4 | 12 | 27 | 2 | 13 | 39 | 3 |
| Guamuchil | 21 | 30 | 1 | 19 | 40 | 2 | 23 | 38 | 1 |

[a] f is the ratio between the DH of the seed meal treated at 85°C and 25°C; DH values are the average of three replicates. The coefficient of variation was less than 10%.

ble of obtaining amino acids from food protein, even though the food remains in the digestive tract for a short period of time.

The treatment of the seed meals at 85°C resulted in a higher DH regardless of the origin of the seed and the enzyme preparation. The DH of palo fierro and pigeon pea seed meals was increased two- and threefold by the temperature treatment, while soybean, chick pea, guamuchil, and palo blanco seed meals increased DH to a lesser degree. The results show that proteins contained in the seed meals are hydrolyzed more effectively after heat treatment. A reasonable explanation of the results is the inactivation of the protease inhibitors by heat denaturation, which agrees with the results of inhibition reduction in the test tubes (Table 3) and the results reported by García-Carreño et al. (1996) when using the commercial enzymes trypsin, chymotrypsin, and papain. An alternative explanation is that heat treatment alters the seed meal protein's three-dimensional structure, allowing the proteases better access to the target-splitting peptide bonds. This possibility remains to be explored.

## Discussion

As confirmed by their protein content, three of the six seed meals studied are promising protein sources for aquaculture feed production; they are soybean, palo blanco, and palo fierro. The amino acid composition of these legume seed meals is currently under evaluation. Palo fierro and palo blanco, the most promising seed-producing plants because of their seed meal protein content and seed production, are wild organisms. A study of seed yield and agronomic potential is currently in progress. The fat content of palo fierro is high, and its composition is also under investigation.

The fat content in the palo fierro seed meal was extraordinarily high. Although the *in vitro* results of this research showed no effect on the DH of the protein, the influence of the chemical composition on shrimp nutrition has to be addressed.

White shrimp extract showed a higher activity and specific activity than brown shrimp extract. The relationship between the higher proteolytic activity and the higher growth rate by the white shrimp remains to be assessed by *in vivo* studies.

The azocaseinolytic activity of the white and brown shrimp hepatopancreas was severely reduced by the seed meal inhibitors in test tube assays. The soybean meal extract was the best inhibitor for proteinolytic activity in both shrimps. Brown shrimp proteinolytic activity was reduced to about 55% by all seed extracts. Except for palo fierro, all the inhibitors were affected by temperature, provided they lost ability to reduce the white and brown shrimp enzyme activity (Figures 1 and 2).

White and brown shrimp hepatopancreas preparations were able to hydrolyze seed meal protein to different DH, even when the seed meal extracts reduced the shrimp azocaseinolytic activity in the test tube assays at 25°C. A differential increase in the DH was shown after the seed meals were treated for 60 min at 85°C. The DH was increased more than twice when assaying the hydrolysis of soybean, palo fierro, and pigeon pea seed protein by the white shrimp extract. The same happened when assaying the hydrolysis of palo blanco, pigeon pea, and guamuchil seed protein by the brown shrimp extract.

The presence of digestive protease inhibitors must be investigated when a new ingredient is intended for feed production. The hydrolysis and absorption of protein are possible targets for the inhibitors, as reported by Yamamoto et al. (1994). High levels of mortality can be caused by protease inhibitors at concentrations as low as 0.1% in diets with low or moderate protein concentrations (Burgess et al. 1994). However, some organisms are able to compensate for digestive proteases when challenged with inhibitors equivalent to 5 mg/g feed (Olli et al. 1994).

In the present work, several seeds were found to be suitable for shrimp feed production, provided the protein content is similar to soybean and the possible antiphysiological activity of the seed protease inhibitors can be reduced by heat treatment. Nutrition is a complex phenomena involving palatability, digestibility, and absorption, when considered in terms of the organism. When considering the properties of the food, amino acid balance, digestibility, and bioavailability are important, as well as the presence of antinutritional factors. The *in vitro* method to evaluate protein digestibility is promising as a predictive technique, which will reduce the time and cost of protein digestibility evaluation by avoiding the feeding trials that are laborious and costly. A study of the correlation between *in vivo* and *in vitro* digestibility is currently in progress.

*Acknowledgments.* The authors thank B. Francisco Magallón for launching this project, Dr. Michael T. Morrissey at OSU for his suggestions to the early draft of the manuscript, ant CONACyT for the grant (3589-N) given

to Dr. García-Carreño, which partially supported this research. The chemical evaluation of the meals by Sonia Rocha is appreciated.

## References

Adler-Nissen J (1986) Enzymic hydrolysis of foodprotein. Elsevier Applied Science, London

AOAC International (1990) *Official methods of analysis,* 15th ed. Association of Official Analytical Chemists, Washington, DC

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal Biochem 72:248–254

Burgess E, Main C, Stevens P, Christeller J, Gatehouse A, Laing W (1994) Effect of protease inhibitor concentration and combinations on the survival, growth and gut enzyme activities of the black field cricket, *Teleogryllus commodus.* J Insect Physiol 40:803–811

Dimes L, Haard N (1994) Estimation of protein digestibility. I. Development of an *in vitro* method for estimating protein digestibility in salmonids. Comp Biochem Physiol 108A:349–362

Dimes L, Haard N, Domg F, Rasco B, Forster I, Fairgrieve W, Arndt R, Hardy R, Barrows F, Higgs D (1994a) Estimation of protein digestibility. II. *In vitro* assay of protein in salmonid feeds. Comp Biochem Physiol 108A:363–370.

Dimes L, García-Carreño FL, Haard N (1994b) Estimation of protein digestibility. III. Studies on the digestive enzymes from the pyloric ceca of rainbow trout and salmon. Comp Biochem Physiol 109A:349–360

García-Carreño FL (1992) Protease inhibition in theory and practice. Biotechnol Educ 3:145–150

García-Carreño FL (1996) Proteinase inhibitors. Trends Food Sci Technol 7:197–204

García-Carreño FL, Haard N (1993) Characterization of protease classes in langostilla *(Pleuroncodes planipes)* and crayfish *(Pacific astacus)* extracts. J Food Biochem 17:97–113

García-Carreño FL, Haard N (1994) Preparation of an exopeptidase enriched fraction from the hepatopancreas of decapods. Process Biochem 29:663–670

García-Carreño FL, Hernández-Cortés MP, Haard N (1994) Enzymes with peptidase and protease activity from digestive system of fresh water and marine decapods. J Agric Food Chem 42:1442–1456

García-Carreño FL, Navarrete del Toro M.A., Díaz-López M, Hernández-Cortés MP, Ezquerra JM (1996) Protease inhibition of fish muscle enzymes using legume seed extracts. J Food Protection. 59:312–318

Laskowski M, Laskowski M Jr (1954) Naturally occurring trypsin inhibitors. Adv Protein Chem 9:203–242

Olli J, Hjelmeland K, Krogdahl A (1994) Soybean trypsin inhibitor in diets for Atlantic salmon (*Salmo salar,* L): Effects on nutrient digestibilities and in pyloric ceaca homogenate and intestinal content. Comp Biochem Physiol 109A:923–928

Yamamoto A, Taniguchi T, Rikyuu K, Tsuji T, Fujita T, Murakami M, Muranishi S (1994) Effects of various protease inhibitors on the intestinal absorption and degradation of insulin in rats. Pharm Res 11:1496–1499