# Inhibition of Modori-Associated Proteinases by Legume Seed Extracts in Surimi Production

J.A. RAMÍREZ, F.L. GARCÍA-CARREÑO, O.G. MORALES, AND A. SÁNCHEZ



**ABSTRACT: The modori phenomenon from several fish species has been associated with endogenous proteinolytic enzymes. We evaluated the presence of modori-associated proteinases in Mexican flounder and Atlantic croaker, and the decrease of the modori effect by inhibition of the responsible enzymes with serine-proteinase inhibitors from legume seeds. The presence of modori was evaluated by changes in shear stress and shear strain at failure in surimi gels incubated for 30 min at 60 °C. Modori was associated with proteolytic degradation of myosin by SDS-PAGE analysis. Crude extracts from kidney bean, pea, chickpea, lentil, and soybean inhibited proteolytic activities of both fish to different extents.**
**Keywords: legume seed, modori, proteinase inhibitor, proteolytic activity, surimi**

## Introduction

MODORI IS A TERM USED TO DESCRIBE THE HEAT-ASSOciated gel degradation when surimi paste is incubated at temperatures close to 60 °C. Modori causes an irreversible destruction of the gel structure of surimi. The result is a decrease in the gel strength, giving brittle, nonelastic gels (Alvarez and others 1999). The most accepted explanation for modori is the presence and activity of endogenous proteolytic enzymes in the fish muscle (Niwa 1992; An and others 1996). Alternate hypotheses proposed have been that heat affects protein-protein interactions (coagulation), and the involvement of specific nonenzymatic proteins (Niwa 1992; Alvarez and others 1999).

Muscle proteinases are found in the soluble sarcoplasmic component of muscle tissue, in association with cellular organelles, connective tissues and myofibrils, and in the interfiber space. Concentration, and the physicochemical and catalytic properties of muscle proteinases are influenced by many factors, both intrinsic and extrinsic: age, diet, exercise, habitat temperature, water depth, and other developmental and environmental factors (Ashie and Simpson 1997). According to their optimum pH of activity, muscle endogenous proteinases are generally classified as acid, neutral, and alkaline proteinases. The neutral and alkaline muscle proteinases are responsible for early postmortem changes, having undesirable effects on rheological properties of fish muscle proteins intended for surimi production (Kolodziejska and Sikorski 1996). Proteinases are classified by the International Union of Biochemistry and the Enzyme Commission (EC), according to their mechanism of peptide bond hydrolysis, into serine-, cysteine-, aspartic-, and metalloproteinases. Serine proteinases (EC 3.4.21.x) have a serine residue in the active center, as well as histidine and aspartic residues (García-Carreño 1992).

Heat-stable alkaline proteinases have often been reported as responsible for the texture deterioration of surimi gels. This phenomenon is called modori. Most of the proteinases associated with modori are cathepsin-cysteine proteinases (Hase and others 1980; Wasson and others 1992; An and others 1996). However, the presence of myofibril-bound serine proteinases has been reported for anchovy *Engraulis japonica* (Ishida and others 1995), carp *Cyprinus carpio* (Osatomi and others 1997; Cao and others 1999), lizard fish *Saurida wanieso* (Cao and others 2000a), and white croaker *Argyrosmus argentatus* (Cao and others 2000b), as well as in mouse skeletal muscle (Fukusen and Aoki 1996; Sangorrin and others 2000). Sangorrin and others (2000) suggested that myofibril-bound serine proteases might be involved in the mechanism of continual degradation and resynthesis of myofibrillar proteins. Recently, it has been found that serine proteinases can also be associated with modori (Toyohara and others 1990; Ramos-Martínez and others 1999; Yongsawatdigul and others 2000). Both classes, cystein- and serin-proteases have been identified in fish skeletal muscle of white croaker (*Micropogon opercularis*; Busconi and others 1984; Folco and others 1984), and in surimi from Atlantic croaker (*Micropogon undulatus*), barred grunt (*Conodon nobilis*), sole or Mexican flounder (*Cyclopsetta chittendeni*), Northern kingfish, (*Menticirrhus saxatilis*) and striped searobin (*Prionotus evolans*; Ramos-Martínez and others 1999).

Legume seeds contain protease inhibitors that are specific for serine-proteinases; they are classified into 2 families: Kunitz, specific for trypsin-like proteinases and having a molecular weight of 21000; and Bowman-Birk, specific for chymotrypsin-like proteinases and having a molecular weight of 8300 (Macedo and Xavier-Filho 1992; Whitaker 1996). García-Carreño and others (1996) proposed the use of legume seed extracts to reduce the proteinolytic activity of serine- and cysteine-proteinases in fish flesh. Sánchez and others (1998) caused the inhibition of the activity in vitro of proteinases present in surimi from Mexican flounder (*Cyclopsetta chittendeni)* and Atlantic croaker (*Micropogon undulatus*) by using legume seed extracts. Our objective was to evaluate the decrease of modori from Mexican flounder and Atlantic croaker surimi by proteinase inhibitors from legume seed extracts. SDS-PAGE analysis was used to follow changes in the electrophoretic patterns of myofibrillar proteins.

## Material and Methods

### Frozen surimi

Atlantic croaker (*Micropogon undulatus*) and Mexican flounder (*Cyclopsetta chittendeni*) were obtained as shrimp by-catch along the shoreline of Tamaulipas, Gulf of Mexico. The fish weighed an average of 150 g and the length was about 15 cm.


© 2002 Institute of Food Technologists

**Table 1—Effect of thermal treatment on shear stress and shear strain of surimi gels from Mexican flounder and Atlantic croaker.**

| Thermal treatment | Mexican flounder | | Atlantic croaker | |
|---|---|---|---|---|
| | Shear stress (kPa) | Shear stress (Dimensionless) | Shear stress (kPa) | Shear stress (Dimensionless) |
| 90°C–15 min (control) | 57.67 (6.74)[a] | 1.498 (0.055)[a] | 76.88 (10.27)[a] | 77.05 (11.91)[a] |
| 40°C-30 min + 90°C–15 min | 80.83 (7.63)[b] | 1.642 (0.031)[b] | 77.05 (11.91)[a] | 1.028 (0.019)[a] |
| 60°C-30 min + 90°C–15 min | 28.20 (6.74)[c] | 1.098 (0.066)[c] | 56.02 (7.36)[b] | 0.899 (0.042)[b] |

*Mean values of 2 experiments with 6 replicates. Value in parenthesis indicates Standard deviation (SD).

Fish were washed immediately after catching and kept on ice until processing. Fish were processed into surimi about 12 h after being caught. Fish were beheaded, gutted, and washed. Skin and bones were removed with a Bibun deboning machine (Model NF2DX; Fujivama, Japan) with a 5 mm dia perforations drum. The mince was washed in wash tanks using water at < 10 °C and a meat:water ratio of 1:3 (w/v). Washings were followed by manual dewatering using cheesecloth as the filtering material. Surimi was mixed with 8% sucrose as cryoprotectant, using a Hobart (Model VCM; Troy, Ohio, U.S.A.) mixer. Surimi was packed into polyethylene bags (2 kg), frozen within 5 h at – 30 °C in a Crepaco plate freezer (Model B-5854-AM12; Crepaco, Inc., Chicago, Ill., U.S.A.) and stored at – 20 °C until needed.

### Surimi gel preparation

Samples of 500 g surimi were partially thawed at room temperature, cut into small pieces, and chopped in a 5-qt-capacity Hobart cutter (Model 84145; Troy, Ohio, U.S.A.) for 3 min with 2.5% NaCl. The final chopping temperature was maintained below 15 °C. The paste was stuffed into stainless steel tubes (dia = 1.87 cm; length = 17.75 cm) that have been sprayed with regular commercial vegetable oil to prevent sticking. Tubes were capped before the thermal treatments; 40 °C for 30 min, 60 °C for 30 min, and 90 °C for 15 min. After cooking, tubes were immediately removed, placed in a cold-water bath and cooled for 30 min at 4 to 5 °C. All gels were removed from the tubes and stored overnight at 4 °C in polystyrene bags prior to testing.

### Torsion test

Gels were kept at room temperature prior to the torsion test. Gels were cut into 3.0 cm lengths and milled into an hourglass shape with a minimum dia of 1 cm at the center. Each gel was placed in a modified torsion apparatus (Brookfield digital viscometer Model 5XHBTD; Brookfield Engineering Laboratories, Inc., Stoughton, Mass., U.S.A.). The texture of each gel was then measured by twisting the sample at 2.5 rpm until structural failure occurred. Shear stress and true shear strain at failure were calculated as described by Hamann and others (1990). Six replicates were obtained for each thermal treatment.

### Seed extracts

Seeds extracts were obtained from the commercial legumes kidney bean (*Phaseolus vulgaris*), pea (*Lahtyrus sativus*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), and soybean (*Glycine max*) according to García-Carreño and others (1996). The seeds were ground in an electric mill (Model CGP-134 M; Koblenz S.A., México, D.F.). The powder was treated with 3 volumes of 50 mM phosphate buffer ($NaH_2PO_4$), pH 7, by shaking for 120 min at room temperature, then for 22 h at 4 °C. The water extracts were clarified by using a 2-step centrifugation, for 10 min at 2500 x *g* and for 30 min at 5000 x *g*, both at 5 °C. The clear extracts were stored for 16 to 20 h at 4 °C. Protein concentration was evaluated using the Lowry technique (Yongsawatdigul and others 2000).

### Evaluation of proteolysis in surimi

### Statistical analysis

Statistical analysis of data was carried out by using one-way analysis of variance. Differences among mean values were established using the least significant difference (LSD) multiple range test. Differences between means were considered significant when $p < 0.05$.

## Results and Discussion

SURIMI GELS FROM ATLANTIC CROAKER INCUBATED FOR 30 MIN AT 40 °C showed an increase in shear stress, but not in shear strain (Table 1). This phenomenon is called setting and has been associated with the presence of an endogenous calcium-dependent TGase (Lee and Park 1998; Ramírez and others 2000). Mexican flounder did not show the setting phenomenon. This behavior could be associated with a low level of calcium caused by the washing-dewatering steps during surimi production. In comparison, incubation of the surimi from both fish species for 30 min at 60 °C decreased the shear stress and shear strain (Table 1). This phenomenon is called modori and has been reported in other fish species and yields a reduction in mechanical properties of the surimi gel (Saeki and others 1995; Hamann and others 1990). The loss of mechanical properties during incubation at 50 to 70 °C has been associated with the presence and activation, by tem-

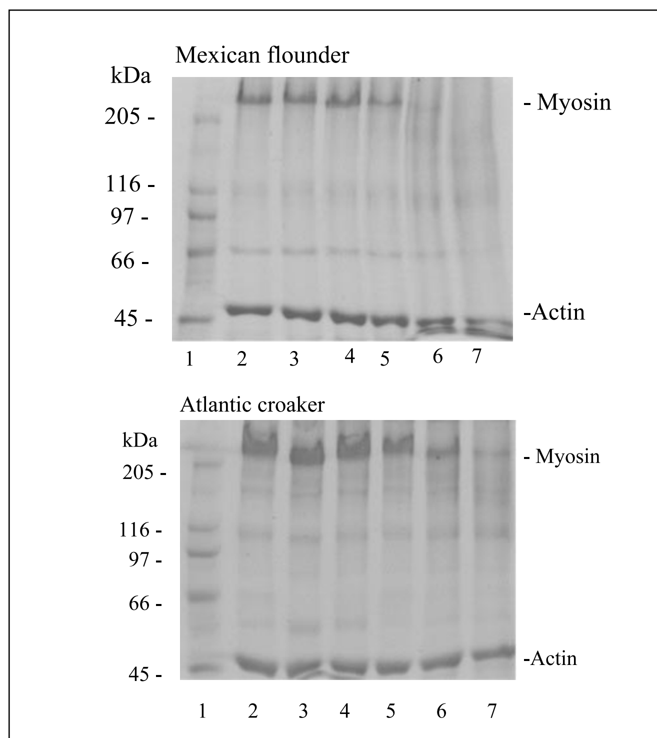


**FIGURE 1. SDS-polyacrylamide gel electrophoresis of surimi from Mexican flounder and Atlantic croaker incubated at 60 °C. Lanes: (1) molecular weight marker; (2 and 3) controls not incubated; (4) control incubated at 90 °C for 15 min; (5) 60 °C for 15 min; (6) 60 °C for 1 h; (7) 60 °C for 3 h.**

**Table 2—Protein content of legume seed crude extracts.**

| Legume | Protein (mg/mL)* |
|---|---|
| Kidney bean | 10.1 (0.23) |
| Pea | 6.0 (0.14) |
| Chickpea | 4.3 (0.07) |
| Lentil | 3.5 (0.09) |
| Soybean | 11.6 (0.34) |

*Mean values of 2 experiments with 3 replicates. Value in parenthesis indicates Standard deviation (SD).

Food Chemistry and Toxicology

perature, of muscle proteinases, mainly cathepsins (An and others 1996). In our work, the incubation of surimi gels at 60 °C caused a reduction of the molecular weight of myosin in surimi, as shown by electrophoretic patterns (Figure 1). When Mexican flounder was incubated for 3 h at 60 °C, the myosin band disappeared. In Atlantic croaker, after 3 h at 60 °C, the myosin band was reduced dramatically. In both fish species, the band corresponding to actin showed negligible changes when compared to the myosin band. Loss of protein bands in SDS-PAGE is indicative of proteolytic activity (Yongsawatdigul and others 2000). This result confirmed the presence of modori in both fish species, Mexican flounder and Atlantic croaker, and that it is associated with proteolytic degradation of myosin.

Although most of the proteinases associated with modori are cathepsin-cysteine proteinases (Hase and others 1980; Wasson and others 1992; An and others 1996), the presence of myofibril-bound serine-proteinases has been reported in several fish species (Ishida and others 1995; Kotodziejska and Sikorski 1996; Osatomi and others 1997; Cao and others 1999, 2000a, 2000b) and to be associated with modori (Yongsawatdigul and others 2000). In a previous report (Ramos-Martínez and others 1999), we found that myosin proteolysis of surimi from Atlantic croaker, Mexican flounder, and 3 other fish species from the Gulf of Mexico could be partially inhibited employing cystein-protease-specific inhibitors (PHMB, E-64, and cistatin) and serine-protease-specific inhibitors (PMSF, TLCK, and TPCK), showing that serine-protease is present in muscle tissue and remains in the surimi paste after the washing-dewatering steps, thus contributing extensively to degradation of surimi gels during thermal treatments around 50 to 70 °C. The importance of serine-proteases on surimi gel degradation during incubation at 60 °C needs to be studied more extensively.

Legume seeds contain protease inhibitors that are specific for serine-proteinases. In this work, several commercial legume seeds were studied to evaluate the presence of serine-proteinase inhibitors. The legume seed extracts assayed contained different concentrations of protein (Table 2) and their electrophoretic patterns by SDS-PAGE were similar (Figure 2, control). All legume extracts showed proteinase inhibitors for trypsin (Figure 2, trypsin) with molecular weight of about 21000, and for chymotrypsin (Figure 2, ß-chymotrypsin) with molecular weight < 18000. No cysteine-proteinase inhibitor was detected in the legume seed extracts from pea, chickpea, lentil, and soybean (Figure 2; papain). The crude extract of kidney bean contained an abundant protein with a molecular weight of 66000 (Figure 2). This protein was resistant to proteolytic degradation by the 3 proteinases studied. García-Carreño and others (1996) found, in palo blanco seed (*Lysiloma candida*), a protein with similar molecular weight and resistant to proteolytic degradation by chymotrypsin and papain.

Incubating surimi paste at 60 °C induced changes seen on the electrophoretic patterns of surimi proteins from all fish species. A decrease in intensity of the myosin band (Figure 3; Lanes 2 to 4) was observed for both fish species. The myosin band was almost totally eliminated after 3 h in both fish species. Seed extracts dif-

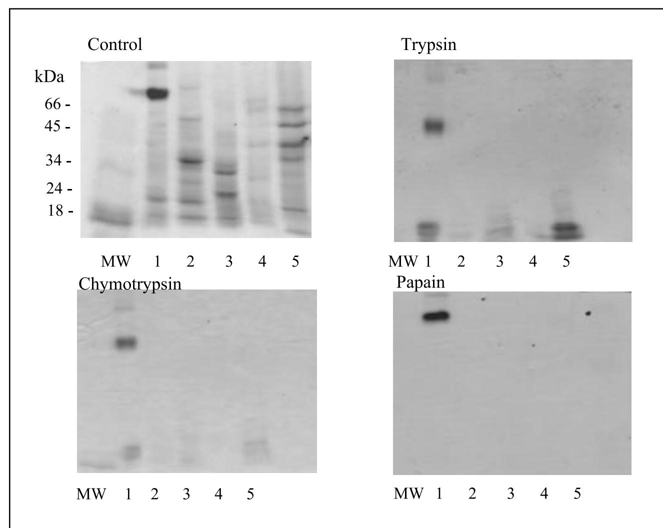


**FIGURE 2. SDS-polyacrylamide of legume seed crude extracts for protein composition (control) and identification of protease inhibitors for trypsin, alpha-chymotrypsin and papain. Lanes: (1) molecular weight marker (MW); (2) kidney bean; (3) pea; (4) chickpea; (5) lentil and (6) soybean**

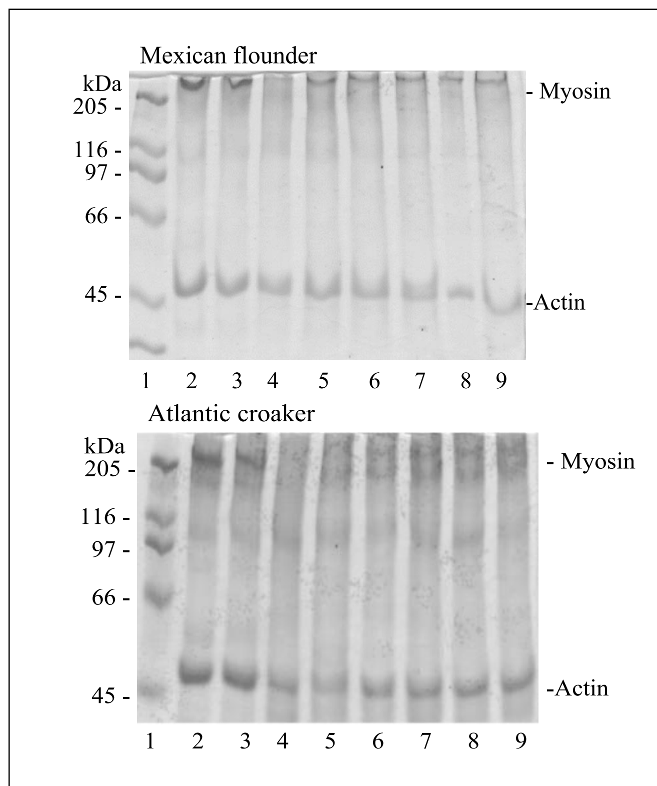


**FIGURE 3. SDS-polyacrylamide of surimi from Mexican flounder and Atlantic croaker added with legume seed crude extracts. Lanes: (1) molecular weight marker; (2) controls not incubated; (3) 60 °C for 15 min; (4) 60 °C for 3 h; (5-9) 60 °C for 3 h with kidney bean, pea, chickpea, lentil, and soybean.**

ferentially inhibited the proteolytic activity in surimi gels obtained from Mexican flounder and Atlantic croaker (Figure 3; Lanes 5 to 9) and helped reduce the hydrolysis of myosin and actin. These results showed that using crude extracts of legume seeds partially inhibited myosin degradation, suggesting that modori is associated with an endogenous serine proteinase in fish muscle.

## Conclusion

THIS STUDY SHOWS THE POTENTIAL OF USING PLANT INHIBITORS TO reduce degradation of the gel formed by the fibrous protein of fish muscle to increase the quality of surimi produced, and the potential of fish from the Gulf of Mexico to be used for surimi production. More studies about characteristics of serine proteases as well the importance of such enzymes in intrinsic muscle metabolic reactions are needed. Purification and characterization of protease inhibitors present in legume seed extracts are needed, as well as affinity, thermal stability and others biochemical and physicochemical properties of such protease inhibitors.

## References


Alvarez C, Couso I, Tejada M. 1999. Thermal gel degradation (modori) in sardine surimi gels. J Food Sci 64(4):633-637.

An H, Margo YP, Seymour TA. 1996. Roles of endogenous enzymes in surimi gelation. Trends in Food Sci and Technol 7(10):321-326.

Ashie INA, Simpson BK. 1997. Proteolysis in food myosystems-a review. J Food Biochem 21(2):91-123.

Busconi L, Folco EJ, Martone C, Trucco RE, Sánchez JJ. 1984. Identification of 2 alkaline proteinases and trypsin inhibitor from muscle of white croaker (*Micropogon opercularis*). FEBS Lett 176(1):211-214.

Cao MJ, Osatami K, Pangkey H, Hara K, Ishihara T. 1999. Cleavage specificity of a myofibril-bound serine proteinase from carp (Cyprinus carpio) muscle. Comp Biochem Physiol B Biochem Mol Biol 123(4):399-405.

Cao MJ, Osatami K, Hara K, Ishihara T. 2000a. Identification of a myofibril-bound serine proteinase (MBSP) in the skeletal muscle of lizard fish Saurida wanieso which specifically cleaves the arginine site. Comp Biochem Physiol B Biochem Mol Biol 125(2):255-264.

Cao MJ, Osatami K, Matsuda R, Ohkubo M, Hara K, Ishihara T. 2000b. Purification of a novel serine proteinase inhibitor from the skeletal muscle of white croaker (*Argyrosomus argentatus*). Biochem Biophys Res Commun 272(2):485-489.

Folco EJ, Busconi L, Martone C, Trucco RE, Sánchez JJ. 1984. Action of 2 alkaline proteinases and trypsin inhibitor from white croaker skeletal muscle (*Micropogon opercularis*) in degradation of myofibrillar proteins. FEBS Lett 176(1):215-218.

Fukusen N, Aoki Y. 1996. Purification of novel trypsin-like serine proteases from mouse spleen. J Biochem (Tokyo) 119(4):633-638.

García-Carreño FL. 1992. Protease inhibition in theory and practice. Biotechnol Edu 3(4):145-150.

García-Carreño FL, Navarrete A, Díaz-López M, Hernández-Cortéz MP, Ezquerra JM. 1996. Proteinase inhibition of fish muscle enzymes using legume seed extracts. J Food Protect 59(3):312-318.

Hamann DD, Amato PM, Wu MC, Foegeding EA. 1990. Inhibition of modori (gel weakening) in surimi by plasma hydrolysate and egg white. J Food Sci 55(3):665-669.

Hase J, Kobashi K, Nakai N, Mitsui E, Iwata K, Takedera T. 1980. The quaternary structure of carp muscle alkaline protease. Biochem Biophys Acta 611(1):205-213.

Ishida M, Sugiyama N, Sato M, Nagayama F. 1995. Two kinds of neutral serine proteinases in salted muscle of anchovy, *Engraulis japonica*. Biosci Biotechnol Biochem 59(6):1107-1112.

Kotodziejska I, Sikorski ZE. 1996. Neutral and alkaline muscle proteinases of marine fish and invertebrates a review. J Food Biochem 20(5):349-363.

Laemmli U. 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 277(259):680-685.

Lee N, Park JW. 1998. Calcium compounds to improve gel functionality of Pacific whiting and Alaska pollock surimi. J Food Sci 63(6):969-974.

Macedo ML, Xavier-Filho, J. 1992. Purification and partial characterization of trypsin inhibitor from seeds of *Clitoria ternatea*. J Sci Food Agric 58(1):55-58.

Niwa E. 1992. Chemistry of surimi gelation. In: Lanier TC, Lee CM, editors. Surimi technology. New York: Marcel Dekker, Inc. P 389-427.

Osatomi K., Sasai H, Cao M, Hara K, Ishihara T. 1997. Purification and characterization of myofibril-bound serine proteinase from carp *Cyprinus carpio* ordinary muscle. Comp Biochem Physiol B Biochem Mol Biol 116(2):183-190.

Ramírez JA, Rodríguez-Sosa R, Morales OG and Vázquez M. 2000. Surimi gels from striped mullet (*Mugyl cephalus*) employing microbial transglutaminase. Food Chem 70(2000):443-449.

Ramos-Martínez E, Morales OG, Ramírez JA, García-Carreño FL, Montejano-Gaitán JG. 1999. Determination of the modori phenomenon and its origin in surimi of 5 fish species from the Gulf of Mexico. Int J Food Sci and Tech 5(5):397-406.

Saeki H, Isega Z, Sugiura S, Seki N. 1995. Gel forming characteristics of frozen surimi from chum salmon in the presence of protease inhibitors. J Food Sci 60(5):917-921, 928.

Sánchez A, Ramírez JA, Morales OG, Montejano JG. 1998. Detección de inhibidores de proteasas en extractos de leguminosas y su efecto sobre proteasas endógenas del músculo de pescado. Cienc Tecnol Aliment 2(1):12-19.

Sangorrin MP, Martone CB, Sanchez JJ. 2000. Identification of myofibril-bound serine protease and its endogenous inhibitor in mouse skeletal muscle. Int J Biochem Cell Biol. 32 (11-12):1213-1222.

Toyohara H, Sakata T, Yamashita K, Kinoshita M, Shimizu Y. 1990. Degradation of oval-filefish meat gel caused by miofibrillar proteinase(s). J Food Sci 55(2):364-366.

Wasson DH, Babbitt JK, French JS. 1992. Characterization of a heat stable protease from arrowtooth flounder (*Arteresthes stomias*). J Aquatic Food Product Technol 1(3/4):167-182.

Whitaker JR. 1996. Protease inhibitor. In: Fennema OR, editor. Food Chemistry. New York: Marcel Dekker, Inc. P 486-487.

Yongsawatdigul J, Park JW, Virulhakul P, Viratchakul S. 2000. Proteolytic degradation of tropical tilapia surimi. J Food Sci 65(1):129-133.


MS 20010034 Submitted 1/22/01, Revised 4/24/01, Accepted 4/26/01, Received 4/26/01

The authors thank Maria de los Angeles Navarrete del Toro (CIBNOR) for her technical assistance. The authors are grateful to CONACYT-SIREYES for the financial support of this work (Proj. 9704001017). Thanks to Ellis Glazier for editing the English-language text.

*Author García-Carreño is with the Centro de Investigaciones Biológicas del Noroeste. La Paz, B.C.S., México. Author Morales is with the Instituto de Ecología y Alimentos (UAT), Victoria, Tamaulipas, México. Authors Ramírez and Sánchez are with the Unidad Académica Multidisciplinaria Reynosa Aztlán. Universidad Autónoma de Tamaulipas. A.P. 1015 Reynosa, Tam. 88700 México. Direct inquiries to author Ramírez (E-mail: ramirez@quirey.uat.mx).*