Proteolytic Degradation of Tropical Tilapia Surimi

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ABSTRACT: Proteolytic degradation of tropical tilapia surimi was biochemically and rheologically characterized to identify a group of proteinase(s) responsible for its textural degradation. Proteolysis of tilapia surimi occurred as the temperature increased and attained the highest activity at 65 °C. Smaller proteins with molecular weight of 116-97 kDa were noted as a result of myosin heavy chain (MHC) degradation. MHC completely disappeared when incubated at 65 °C for 4 h. Proteolysis was significantly inhibited by soybean trypsin inhibitor (SB) and leupeptin (LE). Storage modulus (G′) of surimi gels mixed with either SB or LE was higher than other inhibitors indicating that serine type proteinase(s) were involved in proteolysis of tropical tilapia surimi.

Key Words: tilapia, surimi, enzymatic degradation, serine proteinase

Introduction

TILAPIA IS A FISH WITH MILD FLAVOR AND WHITE FLESH. IT IS ONE of the most frequently aquacultured freshwater fish in the world. Park and others (1990) reported that blue tilapia (*Areochromis aureus*) surimi produced excellent gels. According to our recent comparative study with Alaska pollock and Pacific whiting (Klesk and others 2000), the gel quality of tropical tilapia (*Tilapia niloticus*) surimi was comparable to gel strength of Alaska pollock when heated at 90 °C for 15 min and was better than Pacific whiting surimi without enzyme inhibitors. Setting effects, accompanied by a decreased content of myosin heavy chain (MHC) and an increased shear stress value, were also noticed when pre-incubated for 1 h at 40 °C. These results indicate that tilapia can produce high quality surimi. When incubated at 60 °C for 30 min, a gel-weakening phenomenon was observed. This could limit its use, particularly in surimi-based products manufactured at slow heating.

It has been shown that endogenous proteinases are responsible for the gel weakening phenomenon; these proteases vary among fish species. Serine proteinases were found to be responsible for textural breakdown of threadfin bream (*Nemipterus bathybius*) (Toyohara and Shimizu 1988), oval-filefish (*Navodon modestus*) (Toyohara and others 1992), and lizardfish (*Saurda sp*.) (Suwansakornkul and others 1993). The enzyme was heat stable and effectively hydrolyzed MHC at pH 7.0 and 60 °C. Cathepsin L, a cysteine proteinase, hydrolyzed muscle proteins of Pacific whiting and caused severe textural degradation in whiting surimi (An and others 1994b; Morrissey and others 1993). The enzyme exhibited optimum activity at 55 °C and pH 5.5 and its molecular weight was found to be 28,800 daltons (Seymour and others 1994). Arrowtooth flounder is another species exhibiting high cystein proteinase activity, which was presumably related to an infection of the parasite, *Kudoa thyrsitis* (Greene and Babbitt 1990). In chum salmon surimi, gel quality was improved when L-trans-epoxysuccinylleucylamino (4-guanidino) butane (E-64) was added, indicating involvement of heat stable cysteine proteinase(s) in textural degradation (Saeki and others 1995). Heat stable alkaline proteinase also involves textural degradation of surimi gels. A distinct characteristic of alkaline proteinase is its high activity in alkaline condition (pH 8.5 to 9.1) at 60 to 65 °C (Iwata and others 1974; Boye and Lanier 1988; Makinodan and others 1985). The enzyme shows maximum activity toward MHC, actin, and tropomyosin at 60 °C.

Proteolytic degradation of fish muscle proteins can be overcome using various strategies including food grade enzyme inhibitors (Morrissey and others 1993), a rapid heating method (Yongsawatdigul and Park 1996), or high hydrostatic pressure techniques (Chung and others 1993). The optimal processing parameters can be determined based on the knowledge of proteolytic degradation patterns of fish species. Our objectives were: (1) to investigate degradation patterns of tilapia surimi at various incubation times, temperatures and salt concentration, and (2) to identify the group of proteinase(s) involved in proteolysis of tilapia surimi.

Results and Discussion

Optimum time and temperature of proteolysis

Proteolytic activity linearly increased with incubation time at 65 °C for up to 240 min (Fig. 1). Proteolysis of tilapia surimi did not take place until the temperature > 40 °C, then it gradually increased to 50 °C and rapidly reached a maximum at 65 °C (Fig. 2). Proteolytic activity was not detected at 70 °C (Fig. 2), which was likely due to thermal denaturation of proteinase(s).

Protein patterns of tilapia surimi for varied incubation times at 65 °C are shown in Fig. 3. Degradation of MHC was noted at 30

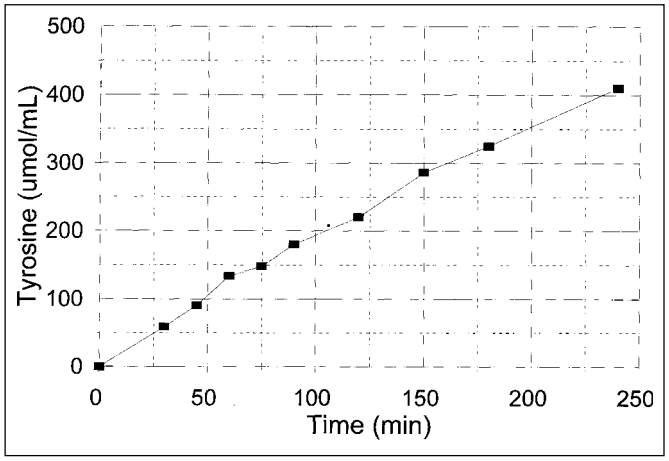

Fig. 1—Autolytic activity of tilapia surimi incubated at 65 °C

min. With prolonged incubation time, a decrease in the intensity of MHC bands resulted in smaller molecular weight proteins (97 < Mr < 205kDa). Disappearance of MHC was evident when incubated at 65 °C up to 3 h. Proteins with molecular weights between 116 and 205 kDa also disappeared with extended incubation time, resulting in protein bands at 116, 110, and 97 kDa. Furthermore, intensity of the protein band at 37 kDa increased with prolonged incubation time. This appeared to be hydrolyzed protein since the band was not seen in the control. It should be noted that proteolysis of actin was not observed throughout the 4 h incubation period. This suggested that actin was not a preferred substrate of the endogenous proteinase(s).

Optimum temperature and extent of proteolysis varied with fish species. In Pacific whiting surimi, MHC and actin were extensively hydrolyzed when slowly heated from 10 to 90 °C at 1 °C/min (Yongsawatdigul and Park 1996). The optimum temperature of proteolysis of whiting surimi, as measured using autolytic assay, was at 55 °C, which corresponded to that of purified cysteine proteinase from the whiting flesh (An and others 1994a; Seymour and others 1994). Cysteine proteinase from arrowtooth flounder completely degraded MHC in 20 min at 55 °C, but actin was unaffected during 60 min incubation (Wasson 1992). Textural degradation of oval-filefish was caused by a proteinase in a sarcoplasmic fluid fraction that associated with myofibrils that have a maximum activity at 50 and 65 °C, respectively (Toyohara and others 1990). Textural degradation of chum salmon surimi was also found at 30 and 60 °C, which was accompanied by a significant loss of MHC and an increased amount of degraded proteins with molecular weights of 90 and 150 kDa (Saeki and others 1995). However, degradation of actin was not noted even up to 6 and 10 h at 30 and 60 °C, respectively. The degradation pattern of tilapia muscle proteins in this study was different from that described above.

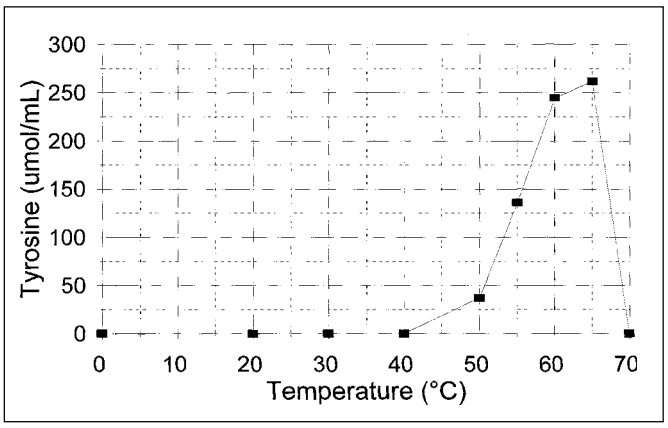

Fig. 2—Effect of temperature on autolytic activity of tilapia surimi incubated at 65 °C.

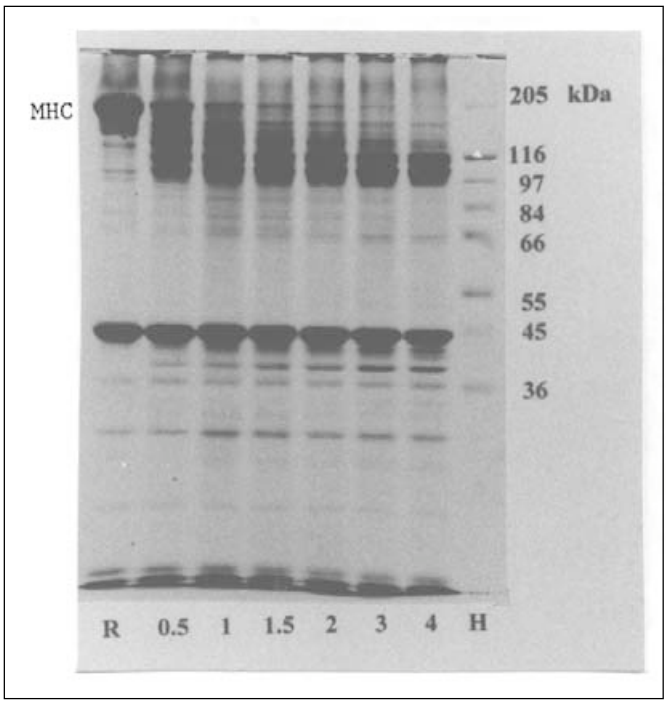

Fig. 3—SDS-PAGE pattern of tilapia surimi incubated at 65 °C for various times: 0.5 to 4 indicates incubation time in hr; (R) raw sample; (H) high molecular weight standard. Applied protein was 60 mg/lane.

Effect of proteinase inhibitors

Pepstatin (PE) had no inhibitory effect on proteolysis of tilapia surimi at a concentration up to 100 μg/g surimi; whereas E-64 at a concentration of 33.3 μg/g surimi inhibited about 36% (Fig. 4). In contrast, residual proteolytic activity decreased and became null when LE increased from 5 to 500 μg/g surimi. Proteoly-

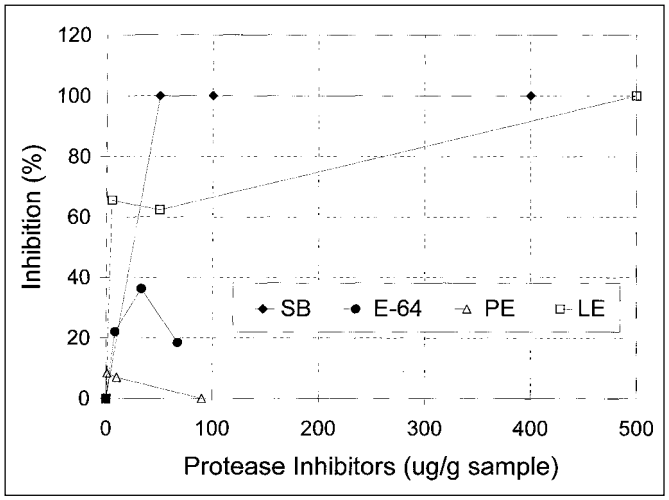

Fig. 4—Effect of various proteinase inhibitors on autolytic activity of tilapia surimi at 65 °C

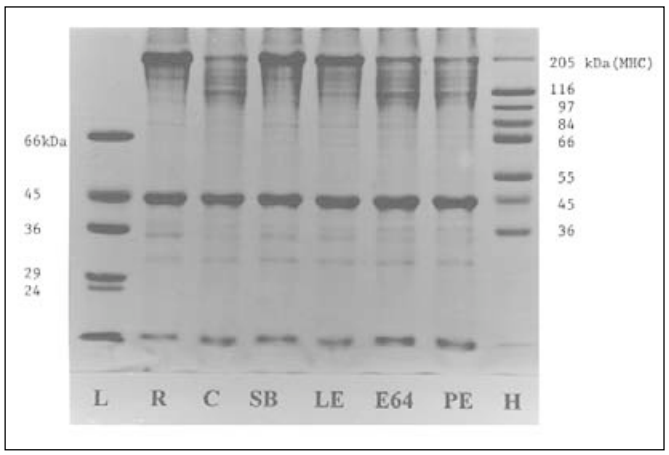

Fig. 5—SDS-PAGE pattern of tilapia surimi mixed with various proteinase inhibitors and incubated at 65 °C for 90 min. (R) raw sample; (C) sample without inhibitor; (SB) soybean trypsin inhibitor; (LE) leupeptin; (E64) E-64; (PE) pepstatin; (L) low molecular weight standard, 66.0, 45.0, 36.0, 29.0, 24.0 kDa from the top; (H) high molecular weight standard, same as Fig. 3. Applied protein was 20 mg/lane.

sis of tilapia surimi was also completely inhibited by SB at concentrations as low as 50 μg/g surimi (Fig. 4).

Results from autolytic assay agreed with protein patterns on SDS-PAGE (Fig. 5). MHC of samples incubated with the highest concentration of PE and E-64 was degraded in a similar fashion to that of the control. Smaller molecular weight proteins ($97 < Mr < 205$) were noticed in these three samples. Because PE is a specific inhibitor for metalloproteinases (Asghar and Bhatti 1987), metalloproteinases were not likely involved in proteolysis of tilapia at 65 °C. On the other hand, degradation of MHC was not observed in samples incubated with SB and LE at a concentration of 50 and 500 μg/g surimi, respectively. Inhibition of SB implied that serine proteinase(s) predominantly contributed to proteolysis of tilapia surimi. Degradation of MHC was minimized in a sample mixed with LE, which is an inhibitor for both cysteine and serine proteinases. The inhibitory effect of E-64, a cysteine proteinase inhibitor, was observed to a lesser extent. These results indicated that serine proteinase(s) was likely responsible for MHC degradation of tilapia; whereas, cysteine proteinase could play a minor role at 65 °C. Serine proteinases were also involved in textural degradation of various fish species. Toyohara and Shimizu (1988) reported that the gel weakening phenomenon of threadfin bream surimi was caused by serine proteinase exhibiting the maximum activity at 65 °C and proteolysis was inhibited by SB at a concentration of 100 to 200 μg/g surimi. This concentration was about 2 to 4 times as much as that found in this study. Suwansakornkul and others (1993) reported that SB showed a high inhibitory effect on proteolysis of washed muscle proteins from lizardfish at 60 °C. In contrast, LE and E-64 inhibited proteolysis to a lesser extent; leading to the conclusion that degradation of MHC was largely from serine proteinase(s). In addition, heat stable serine proteinase was found in myofibrils of crucian carp muscle with an optimum temperature of 50 and 60 °C at pH 6.6 to 8.0 (Kinoshita and others 1990). Calpain, a Ca^{++}-dependent cysteine proteinase, was purified from tilapia (hybrid of *Tilapia nilotica* and *Tilapia aurea*) and its optimal pH was 7.5 (Wang and others 1992). It is involved in postmortem tenderization of fish muscle. Although pH of the surimi was in the optimum range for calpain, it is unlikely that this was the enzyme responsible for MHC proteolysis observed because calpain is heat-labile and has a half-life of 0.3 min at 65 °C. Jiang and others (1990) reported that cathepsin D, a pepstatin-sensitive proteinase, hydrolyzed myofibrils of tilapia (hybrid of *Tilapia nilotica* and *Tilapia aurea*) at pH 5.5 and 6.0 and did not contribute to the degradation of myofibrils at pH 6.5. This supported the results of this study that degradation of MHC at 65 °C was not caused by pepstatin-sensitive proteinase(s).

Dynamic rheology

Storage moduli (G′) of tilapia gels at varied shear frequencies are shown in Fig. 6. G′ of surimi gels mixed with different inhibitors was dependent on shear frequency, indicating the viscoelastic nature of the sample. Although an increasing trend of G′ with shear frequency was observed in all treatments, the magnitude of G′ was different among the various added inhibitors ($p < 0.05$). G′ values of surimi mixed with SB were not different from those of LE ($p > 0.05$) and were greater than other samples over the experimental frequency range (0.06 and 63 rad/s). Gel of tilapia surimi mixed with E-64 exhibited the lowest storage modulus ($p < 0.05$). G′ of the control was similar to that of surimi treated with PE ($p > 0.05$). Dynamic rheological parameters agreed with the protein patterns on the SDS-PAGE (Fig. 5). This suggested that

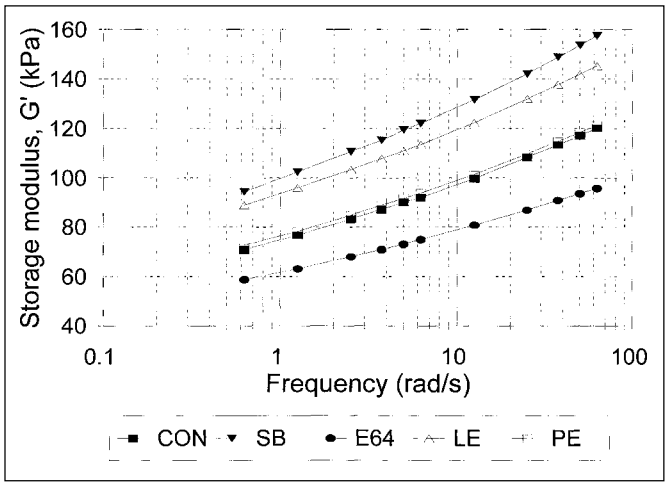

Fig. 6—Effect of various proteinase inhibitors on storage modulus (G′) of tilapia surimi gels

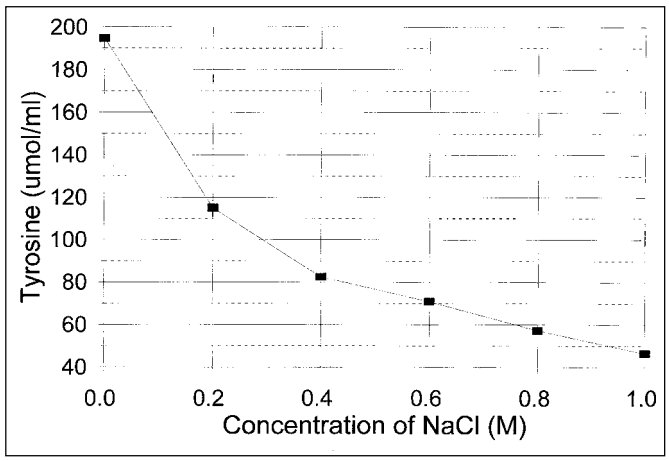

Fig. 7—Effect of NaCl concentration on autolytic activity of tilapia surimi at 65 °C

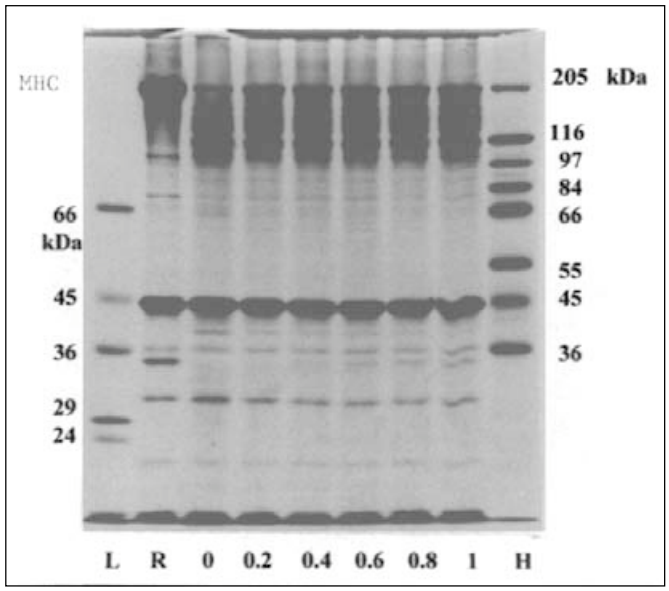

Fig. 8—SDS-PAGE pattern of tilapia surimi mixed with various NaCl concentration and incubated at 65 °C for 90 min. (0) distilled water; (0.2) 0.2M NaCl; (0.4) 0.4M NaCl; (0.6) 0.6M NaCl; (0,8) 0.8 M NaCl; (1) 1 M NaCl; (L) and (H) low and high molecular weight standard same as Fig. 5, respectively. Applied protein was 60 mg/lane.

G′ of the surimi gel reasonably reflected the gel network structure as affected by proteolysis. Since myosin was responsible for the development of a 3-dimensional network, degradation of myosin in the control and in the samples with added E-64 and PE limited the ordered aggregation of myosin. This resulted in a poor gel network and inferior rheological properties (Arntfield and others 1989).

Besides proteolysis of MHC, gel network formation could be mediated by the reaction of chemical inhibitors, particularly E-64. Since E-64 is an epoxysuccinyl peptide, it could react with the myofibrillar proteins (Rich 1986), which in turn affect the gel network formation and rheological properties. Therefore, interpretation of the rheological properties of proteolysis should be made in conjunction with the protein pattern on SDS-PAGE. G′ appeared to be a better index of proteolysis than other rheological terms, such as loss modulus (G") or loss tangent. Loss tangent of samples treated by various inhibitors was not different ($p > 0.05$) (data not shown). The extent of proteolysis of tilapia in a dynamic rheology study might be less than in SDS-PAGE because samples were heated linearly on the rheometer from 10 to 80 °C at 1°C/min. Therefore, the reaction time at the optimum temperature (65 °C) in dynamic study was less than the experiment in which the samples were incubated in a 65 °C water bath.

Effect of NaCl concentration

Proteolysis of tilapia surimi as affected by NaCl concentration is shown in Fig. 7. Oligopeptides resulting from proteolysis decreased with increased NaCl concentration. Oligopeptides decreased by about 76% compared to the control (0% NaCl) and surimi incubated in 1M NaCl at 65 °C for 1.5 h. This indicated inhibitory effects of NaCl on the degradation of muscle proteins. Results from the autolysis assay corresponded to the degradation pattern of proteins observed on SDS-PAGE (Fig. 8). Retention of MHC increased as NaCl concentration increased. Moreover, degraded proteins with 116 < Mr < 205 were hydrolyzed further in the control than others. This resulted in a higher intensity band at 110 kDa. NaCl simply reduced the extent of proteolysis of the muscle proteins. A substantial loss of MHC, as compared to the raw sample, was still noticed in the sample incubated with 1M NaCl (2.9% w/w). Degradation of myosin was not detected in the absence of NaCl. This was explained by an increased unfolding of myosin at a relatively high salt content. Consequently, myosin was more susceptible to hydrolysis by proteinase(s). Reduced proteolytic activity in the presence of NaCl could be due to its effect on proteinase(s) rather than that on a substrate. Further study is needed to purify the enzyme and study the effect of ionic strength on enzyme activity.

Conclusion

SURIMI MADE FROM TROPICAL TILAPIA EXHIBITED HIGHEST AUtolytic activity at 65 °C. Proteolysis was well inhibited by SB and LE, implying that the proteinase involved was serine proteinase. In addition, proteolysis of tilapia myosin decreased with increased salt content. Gel weakening phenomenon of tilapia surimi could be minimized when appropriate food grade serine inhibitors, salt content, and a rapid heating regime are assigned.

Materials and Methods

Sample preparation

Surimi was prepared using farm raised tropical tilapia (*Tilapia niloticus*) at the Fishery Technology Development Institute, Dept of Fisheries (Bangkok, Thailand). Frozen surimi containing 4% sucrose, 4% sorbitol, and 0.3% sodium tripolyphosphate as cryoprotectants was air-freighted with dry ice to the OSU Seafood Laboratory and stored at −30 °C. At the time of experiment, it was about three months old.

Autolysis as affected by time, temperature, inhibitors, and salt content

Preliminary studies indicated that proteolytic activity of surimi was highest at 65 °C. A time-course study at this temperature was investigated. Three grams of tilapia surimi were incubated at 65 °C for 0, 30, 45, 60, 75, 90, 120, 150, 180, and 240 min. Autolysis was stopped by adding 27 mL 5% cold trichloroacetic acid (TCA) solution. The mixture was incubated at 4 °C for 15 min and centrifuged at 6100 ∞ *g* for 15 min. The TCA soluble proteins were analyzed for the oligopeptide content using Lowry's assay and expressed as μmol/mL of tyrosine released (An and others 1994b). Sample blanks were kept on ice and analyzed to correct for oligopeptide present in fish muscle. Optimum temperature for proteolysis was re-determined using incubation times obtained from the time-course study described above. Autolysis was carried out at 0, 20, 30, 40, 50, 55, 60, 65, and 70 °C for 90 min.

Tilapia surimi was mixed with specific inhibitors at various concentrations; soybean trypsin inhibitor (SB) (50, 100, 400 μg/g surimi), leupeptin (LE) (5, 50, 500 μg/g surimi), pepstatin (PE) (1, 10, 100 mg/g surimi), trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) (8.33, 33.3, 66.6 μg/g surimi). All chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Three grams of mixed sample were incubated in a 65 °C water bath for 1.5 h and oligopeptide content was determined. The sample without any inhibitors was used as a control. Blanks for both the control and sample were mixed with inhibitors and kept on ice. TCA was immediately added. Percent inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{(TC - TC_b) - (TI - TI_b) \infty 100}{(TC - TC_b)}$$

where TC = tyrosine of control (no inhibitor) incubated at 65 °C, TC_b = tyrosine of control (no inhibitor) incubated at 0 °C, TI = tyrosine of sample (with inhibitor) incubated at 65 °C, TI_b = tyrosine of sample (with inhibitor) incubated at 0 °C.

The effect of salt concentration on proteolysis was carried out by mixing 3 g tilapia surimi with 3 mL of NaCl solutions (0, 0.2, 0.4, 0.6, 0.8, and 1.0 M). The mixture was incubated at 65 °C for 1.5 h. The degree of autolysis was determined by measuring the concentration of released tyrosine according to the Lowry assay at 750 nm (An and others 1994a).

Dynamic test

Frozen tilapia surimi was mixed homogeneously in 0.6 M NaCl solution at a 1:1 ratio (w/w) with an effective concentration of four inhibitors, which was determined from the autolytic study. The paste was placed between the parallel plate (20 mm dia.) and the plate attached to the Bohlin CS-50 (Bohlin Instrument, N.J.). Surimi paste was heated from 10 to 80 °C at a heating rate of 1 °C/min and immediately cooled to 25 °C. The moisture trap was applied to prevent evaporation during heating. A frequency sweep was performed from 0.63 to 63 rad/s using an applied dynamic shear stress of 1 kPa, which was determined to be within the linear viscoelastic range based on preliminary tests.

Sodium Dodecylsulfate Polyacrylamide Electrophoresis (SDS-PAGE)

Twenty- seven mL of heated (95 °C) 5% sodium dodecylsulfate solution was added to a 3-g sample. The mixture was homogenized for 1 min at a speed setting of 3 to 4 by a Polytron (Brinkmann Instruments, Westbury, N.Y., U.S.A.). The homogenates were incubated in an 80 °C water bath for 1 h and centrifuged at 7,000 ∞ *g* (Sorvall, DuPont Co., Newton, Conn., U.S.A.) for 10 min at room temperature. The protein concentration of supernatants was measured by the Lowry method (Lowry and others 1951), using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) as a standard.

Electrophoresis was carried out according to the procedure of Laemmli (1970). Stacking gels and separating gels were 4% (w/v) and 10% (w/v) polyacrylamide, respectively. Separated proteins were stained with 0.125% Coomassie brilliant blue R-250 (Bio-Rad, Richmond, Calif., U.S.A.), and destained in a solution containing 25% ethanol and 10% acetic acid.

Statistical analysis

Analysis of variance for frequency sweep was performed on the data at 0.63, 6.3, and 63 rad/s. Least significant differences were used to determine differences between mean at $P < 0.05$. Analysis was done using SAS statistical software (SAS, 1996).

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MS 1999-0309 received 3/2/99; revised 8/16/99; accepted 8/26/99.

Authors would like to acknowledge the partial financial supports ofthe National Center for Genetic Engineering and Biotechnology (BIOTEC) and National Science and Technology Development Agency (Bangkok, Thailand).

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