Combination Effects of Microbial Transglutaminase, Reducing Agent, and Protease Inhibitor on the Quality of Hairtail Surimi

S.-T. JIANG, J.-F. HSIEH, M.-L. HO AND Y.-C. CHUNG

ABSTRACT: A combination of microbial transglutaminase (MTGase), reducing agent and protease inhibitor was employed to improve the quality of underutilized fish surimi. SDS-PAGE indicated that cross-linking of myosin heavy chain (MHC) occurred in MTGase-contained samples, while MHC of samples without MTGase disappeared after 120 min incubation at 45 °C. Although the gel-forming ability increased with MTGase added up to 0.6 unit/g, it was still too low to be commercially acceptable. However, the combined use of 0.1% $NaHSO_3$, 0.01 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and 0.35 units MTGase/g substantially improved the quality of hairtail surimi. Based on this result, the combined use of E-64, MTGase and $NaHSO_3$ seemed to be a better way to improve gel-forming ability of hairtail surimi.

Key Words: surimi, MTGase, reducing agent, protease inhibitor, gelation, and hairtail

Introduction

SURIMI-BASED PRODUCTS, SUCH AS KAMABOKO, TEMPURA, AND chikuwa are very popular in Asia markets because of their unique texture properties (Lee 1984). As a result of overfishing Alaska pollock for surimi production, some underutilized species have been used to produce surimi. However, some fish species, such as mackerel, are basically not suitable for surimi production because of their poor gelation ability. Mackerel also has poor resistance to frozen storage due to protein denaturation and residual cathepsins B, L, and L-like (Jiang and others 1986, 1996), while Pacific whiting has soft-texture problem caused by protease if no enzyme inhibitors are used. (An and others 1994; Kudo and others 1973). The freeze-denaturation of muscle proteins was considered to be due to the formation of hydrophobic interactions, hydrophilic interactions, and disulfide bonds among protein molecules (Goldblith 1969; Sikorski 1978, 1980). According to previous studies (Jiang and others 1986, 1987a, b, c, 1998a), the addition of reducing agents or sulfite nicotinamide adenine dinucleotide phosphate (NADPH) reductase significantly increased the 8 M urea-unfolded total sulfhydryls (SHs) of fish actomyosin before and after freezing or freeze-drying processes due to the reduction of disulfide bonds. This improved the gelation of the reductant- or reductase-added surimi-based products. However, the surimi-based products from some fish species have gel-softening ability or their muscles have softening problems due to the existence of endogenous proteases. Whey protein concentrate has been used as a protease inhibitor in Pacific whiting surimi, while protease inhibitors, such as E-64 and cystatin, are also employed to inhibit the cysteine proteases of frozen fish minces (Jiang and others 1997; Weerasinghe and others 1996).

Except for the reductants (for example, sodium bisulfite) or reductase which can recover the native muscle proteins and inhibitors which can inhibit the proteinase actions, TGase was found to be able to catalyze the formation of ε-(γ-glutamyl) lysine crosslinks. This is a covalent isopeptide bond and affects gel properties significantly (Seki and others 1990; Sakamoto and others 1995; Seguro and others 1995). Kimura and others (1991) found that the ε-(γ-glutamyl) lysine isopeptides occurred in kamaboko gels and considered TGase to be a suwari-promoting enzyme. Furthermore, SDS-PAGE analysis revealed the increase in cross-linked MHC when TGase-contained pollock and mackerel surimi were set (Jiang and others 1994, 1996; Nowsad and others 1994). The purpose of this study was to investigate the combined effects of MTGase (*Streptoverticillium ladakanum*), reducing agents, and protease inhibitors on improving the gel forming ability of hairtail surimi.

Results and Discussion

Effects of MTGase on the gel-forming ability of hairtail surimi

The breaking force and deformation of hairtail surimi gels both increased with addition of MTGase ($p < 0.05$, Fig. 1). Several

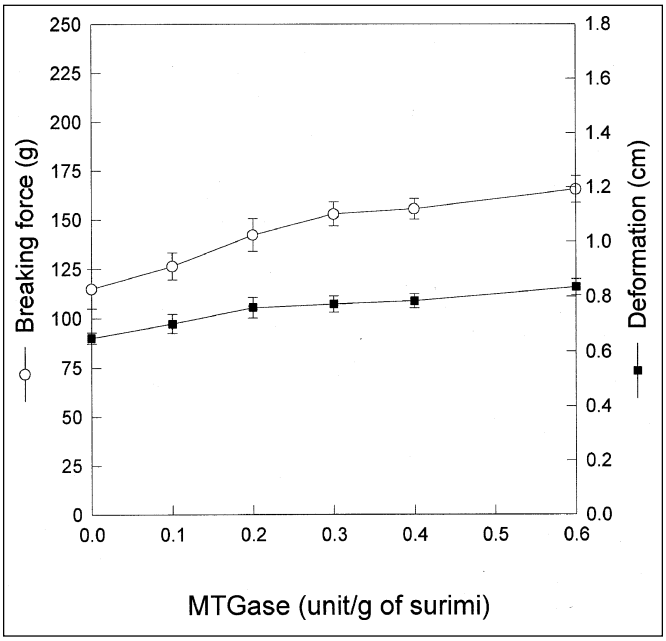

Fig. 1—Effect of MTGase on the gel properties of hairtail surimi. Vertical bars represent standard deviation.

previous studies indicated that excess of MTGase caused a decrease in gel-forming ability (Asagami and others 1995; Tsai and others 1996; Jiang and others 1998b). This phenomenon was not observed in this study. This might be because the concentrations of MTGase were not high enough to affect the texture. However, the gel-forming ability of hairtail surimi was much lower than other surimi even though 0.6 unit MTGase/g was added. It was obvious that MTGase alone was not sufficient to produce a high quality of hairtail surimi.

Effects of setting condition on gel-forming ability of MTGase-contained hairtail surimi

Boye and Lanier (1988) reported that gelation of myosin occurred at 2 stages: low temperature setting (0 about 45 ±Ψ≥ and high temperature (90 ±Ψ≥ heating. The low temperature setting was responsible for the polymerization of myosin heavy chain (MHC) catalyzed by TGase (Nowsad and others 1994). In this study, the breaking force and deformation of MTGase-contained hairtail gels increased with duration up to 6 h during setting at 20 °C (Fig. 2). However, the deformation reached the highest level after 3 h setting. The breaking force and deformation were higher than that of the control (gels without MTGase), separately. The breaking force and deformation of MTGase-contained gels increased to a maximum value after 40 min setting at 30 °C or after 20 min setting at 45 °C (Fig. 3). However, the breaking force of samples with MTGase reached a peak value after 40 min setting at both temperatures. The breaking force and deformation of MTGase-containing hairtail gels were higher than that of the control ($p < 0.05$). It was noted that the breaking force and deformation of control samples set at 30 °C or 45 °C decreased

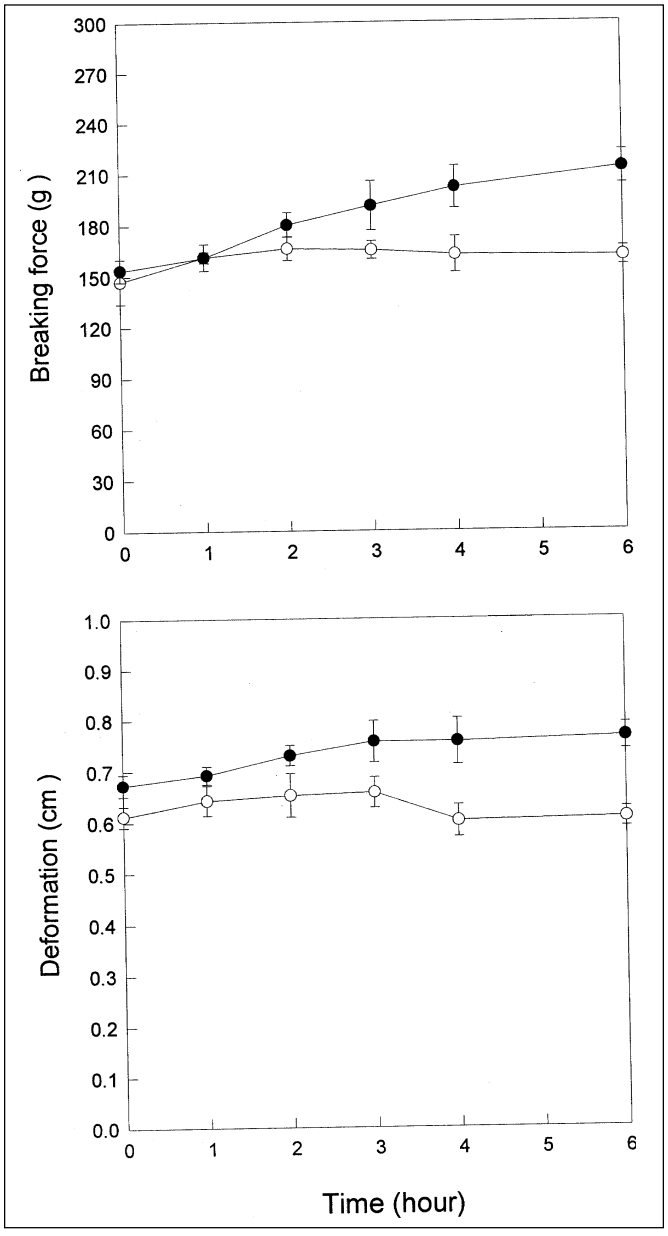

Fig. 2—Effect of setting time on the gel properties of hairtail surimi with/without MTGase at 20 °C. Gels with 0.35 units of MTGase/g: solid symbols; Gels without MTGase: open symbols. Vertical bars represent standard deviation.

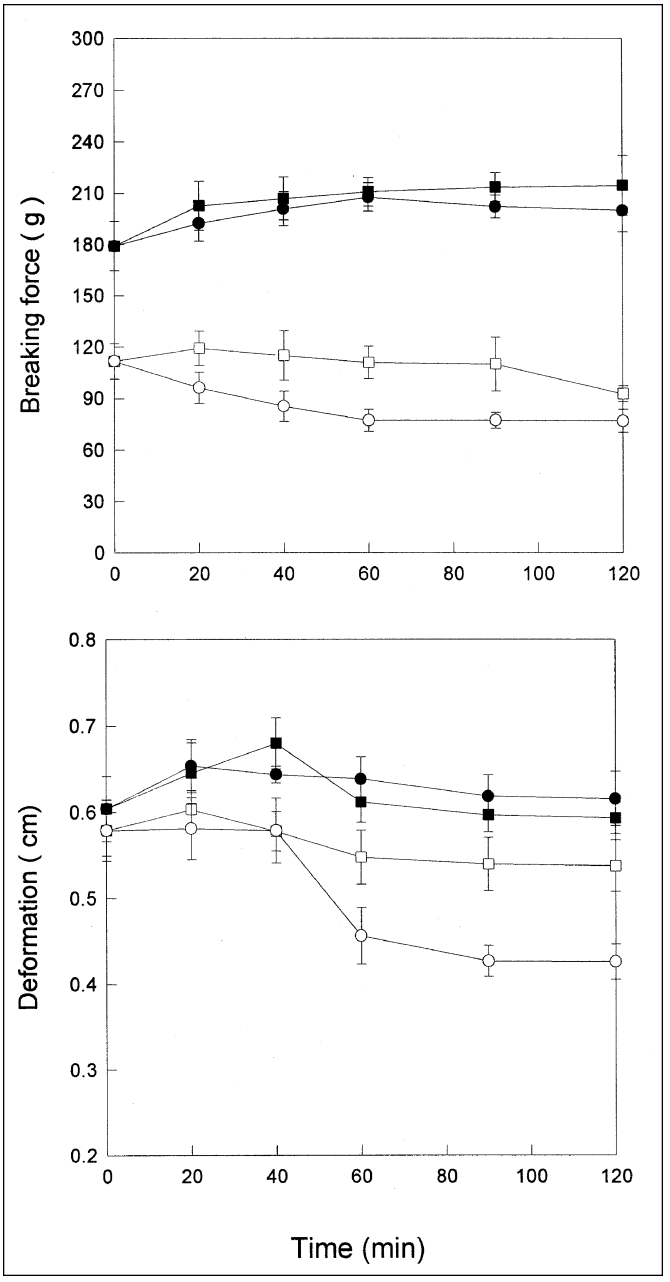

Fig. 3—Effects of setting temperature and time on the gel properties of hairtail surimi with/without MTGase. Gels set at 30 °C, □ ■ ; 45 °C, ○ ●. Gels with 0.35 units of MTGase/g: solid symbols; Gels without MTGase: open symbols. Vertical bars represent standard deviation.

right after the setting proceeded (Fig. 3). This might be due to presence of endogenous protease. The further assay indicated high protease activity in hairtail surimi (data not shown).

Effects of MTGase on cross-linking of hairtail actomyosin

According to SDS-PAGE analysis, MHC intensity decreased as the concentration of MTGase increased (Fig. 4). High molecular weight cross-links (>200 kDa) were clearly observed on the top of acrylamide gels. These components were considered resulting from cross-linking of MHC (Nowsad and others 1995; Seguro and others 1995; Jiang and others 1998b). Disappearance of MHC evidenced the occurrence of cross-linking of MHC, since the MHC cross inked by MTGase was difficult to dissociate by SDS during SDS-PAGE analysis (Jiang and others 1998a).

When MTGase-containing hairtail AM was incubated at 30 °C or 45 ±Ψ≥ the MHC decreased greatly at the first 20 or 5 min, respectively, and after that the degradation of MHC was not noticeable. On the other hand, cross inking of MHC also became prominent within a short period (Figs. 5 and 6). Disappearance of MHC was more rapid at 45 °C than at 30 °C. However, no cross-linked MHC was observed on samples without MTGase during 120 min incubation at 30 °C or 45 °C. After 120 min incubation at 45 ±Ψ≥ MHC of hairtail AM without MTGase disappeared. This phenomenon further confirmed the existence of endogenous proteases as explained later.

Effects of sodium bisulfite and E-64 on the gel-forming ability

As mentioned previously, MTGase-containing hairtail surimi was not equivalent to most of other surimi even though MTGase did improve the gel-forming ability. The existence of protease in frozen hairtail surimi suggested that protease inhibitors might be able to improve the quality and consequently produce high quality surimi analogues. Barrett and others (1982) reported that E-64 could be used as an inhibitor of cysteine proteases including cathepsins B, H and L and other proteases. Jiang and others (1986) also reported that native proteins could be recovered by addition of sodium bisulfite to the denatured proteins. Therefore, combined use of sodium bisulfite, E-64, and MTGase was thought to improve the gel forming ability of hairtail surimi. The effects of sodium bisulfite and/or E-64 on the gel-forming ability of the hairtail surimi were more pronounced when the MTGase was additionally added. Breaking force and deformation increased as setting time was extended (Fig. 7). Combined use of 0.1% sodium bisulfite, 0.01mM E-64 and 0.35 unit/g MTGase had the highest breaking force and deformation. These data suggested that the addition of sodium bisulfite could recover the sulfhydryl groups. E-64 substantially prevented the texture degradation caused by endogenous proteases, and MTGase further catalyzed the cross-linking of MHC of the sodium bisulfite, E-64 and MTGase-added hairtail surimi.

Conclusion

MTGASE COULD CATALYZE THE MHC CROSS-LINKING AND INcrease the gel-forming ability of hairtail surimi. The texture degradation caused by the endogenous proteases could be inhibited by the addition of inhibitor. The best solution to improve

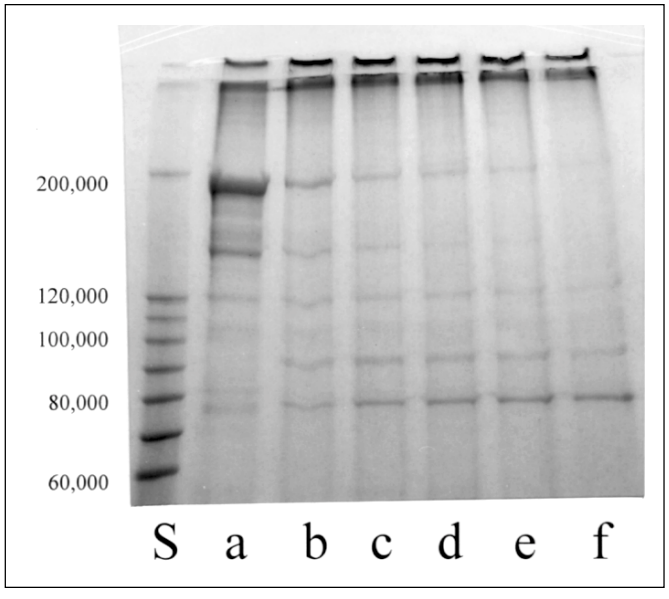

Fig. 4—Change in SDS-PAGE profiles of hairtail AM with various amounts of MTGase incubated at 30 °C. for 30 min. (S: protein marker; a: 0 unit; b: 0.1 units; c: 0.2 units; d: 0.3 units; e: 0.4 units; f: 0.6 units of MTGase/g AM)

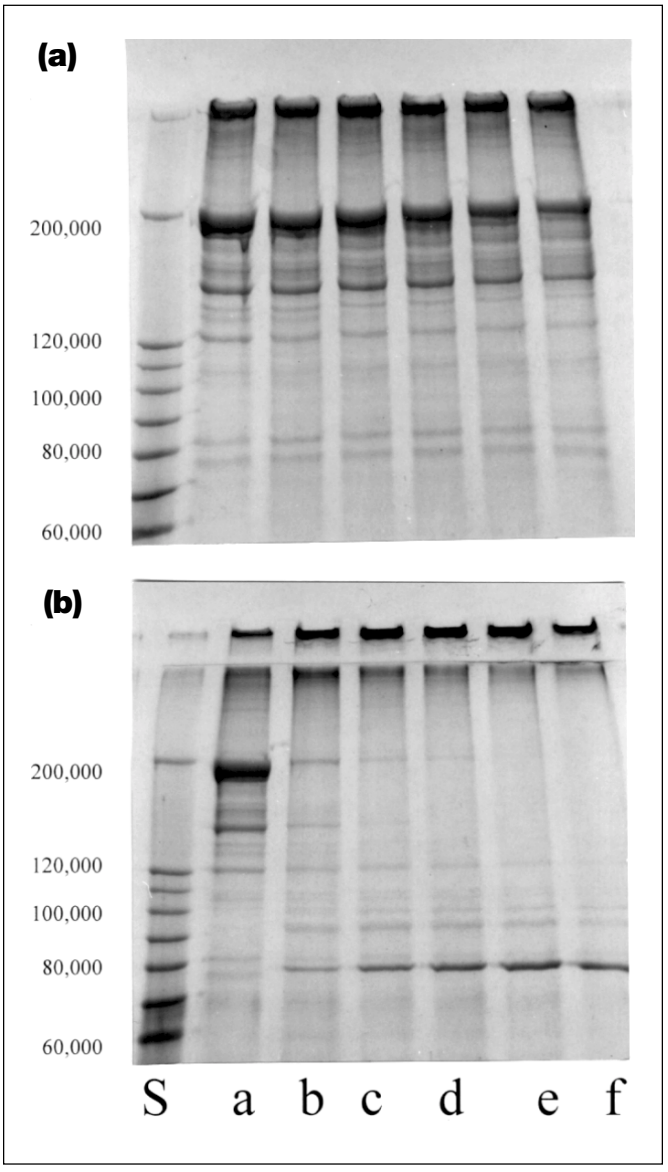

Fig. 5—Change in SDS-PAGE profiles of hairtail AM with/without MTGase incubated at 30 °C. for various time periods. A: without MTGase; B: with 0.35 units of MTGase/g. (S: protein marker; a: 0 min; b: 20 min; c: 40 min; d: 60 min; e: 90 min; f: 120 min.)

the gel forming ability of frozen hairtail surimi was the combined use of MTGase, sodium bisulfite, and E-64. However, economic justification for the use of these chemicals in frozen surimi needs to be investigated.

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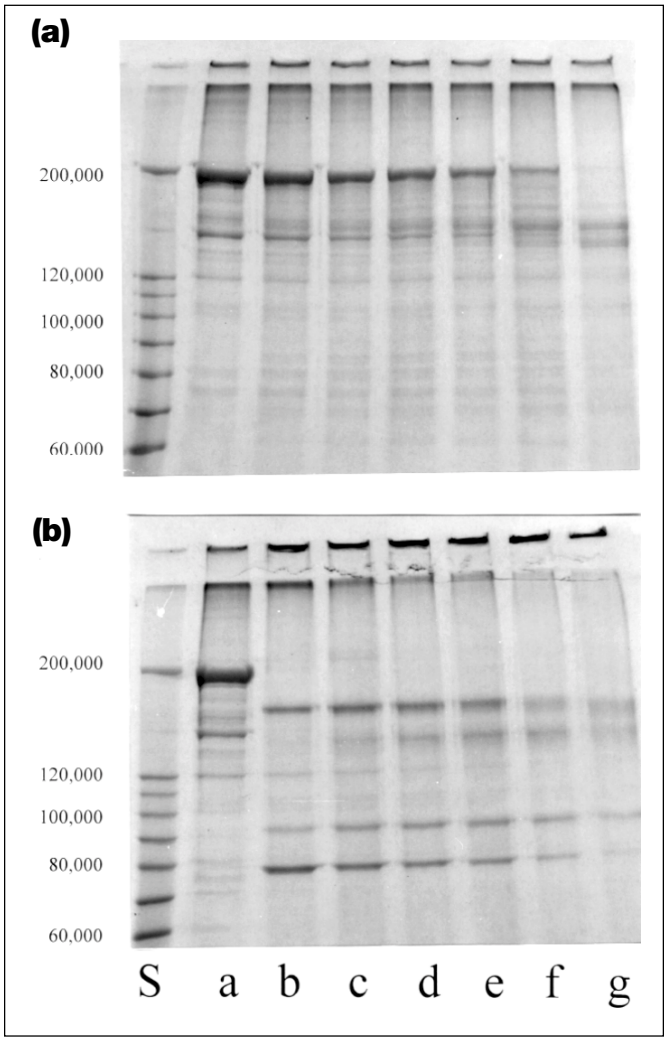

Fig. 6—Change in SDS-PAGE profiles of hairtail AM with/without MTGase incubated at 45°C for various time periods. A: without MTGase; B: with 0.35 units of MTGase/g. (S: protein marker; a: 0 min; b: 5 min; c: 10 min; d: 15 min; e: 20 min; f: 25 min g: 120 min.)

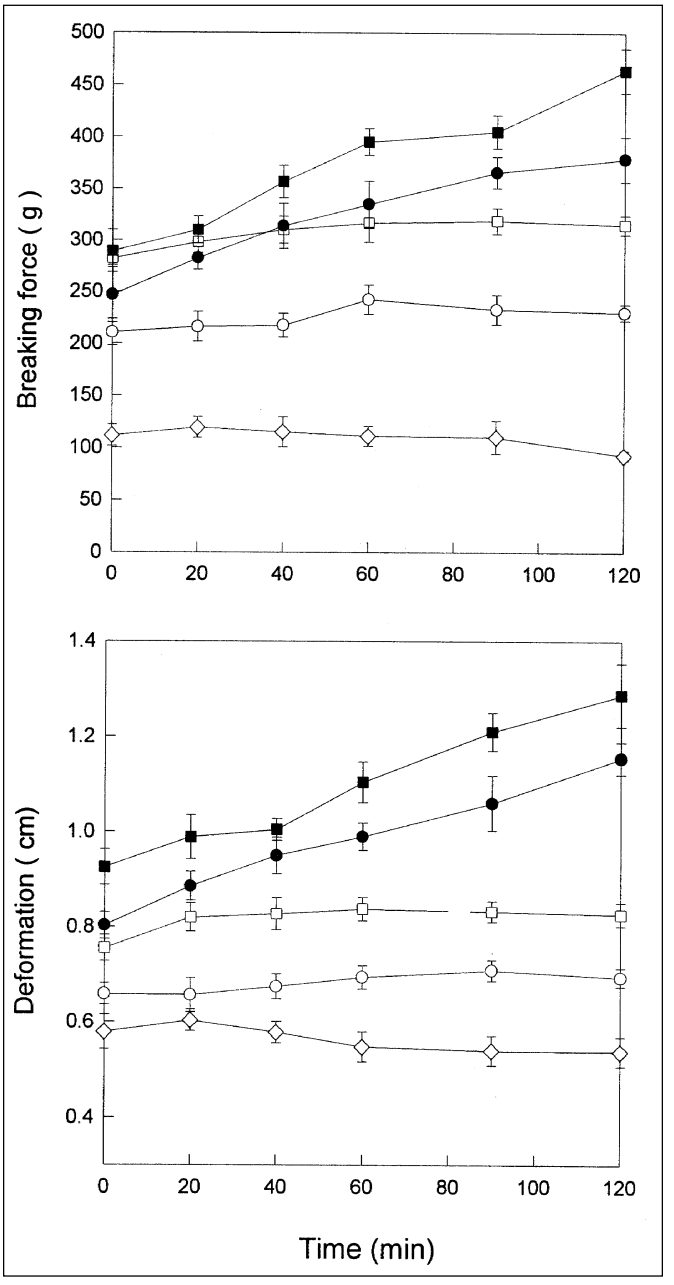

Fig. 7—Effect of MTGase, sodium bisulfite and E-64 on the gel properties of hairtail surimi with/without MTGase set at 30 °C. Gels without additives, ◇; Gels with 0.1% sodium bisulfite, ○ ● ; Gels with 0.1% sodium bisulfite and 0.01 mM E-64, □ ■ . Gels with 0.35 units of MTGase/g: solid symbols; Gels without MTGase: open symbols. Vertical bars represent standard deviation.

Materials and Methods

Chemicals

Tris(hydroxymethyl)aminomethane, γ-globulin, carbobenzoxyl-L-glutaminyl-glycine (CBZ-L-Gln-Gly), sodium dodecyl sulfate (SDS), Tween 20, L-glutamic acid-γ-monohydroxamic acid, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), sucrose and β-casein (dephosphorylated, from bovine milk) were the products of Sigma (St. Louis, MO). β-Mercaptoethanol (β-Me), Coomassie blue G-250, ammonium sulfate, glycerol and trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany). Yeast extract, Bacto-agar and soluble starch were obtained from Difco (Detroit, Mich., U.S.A.). *Streptoverticillium ladakanum* ATCC 27441 was obtained from the Taiwan Culture Collection and Research Center, Hsinchu, Taiwan. All other chemicals were reagent grade.

Production of MTGase

One loop of spore suspensions of *S. ladakanum* frozen stock culture was activated in a 125 mL Erlenmeyer flask containing 50 mL medium (1 % glycerol, 1.5 % yeast extract, 0.2 % K_2HPO_4, 0.1 % $MgSO_4$, pH 7) and cultured with shaking (150 rpm) at 28 °C for 48 h. One mL of culture suspension was then transferred to 150 mL of fresh medium (pH 7.0). After 4-d incubation at 28 °C with shaking, the culture fluid was filtered firstly through filter paper (Whatmam No. 1) and then through a 0.22 μm filter membrane. The filtrate was used as crude enzyme preparation of MTGase and stored at −30 °C until use.

Determination of MTGase activity

MTGase activity was measured by the method described previously by Folk (1970). Reaction mixture, containing 50 μL enzyme, 350 μL 0.1 M Tris-acetate buffer (pH 6.0), 25 μL 2.0 M hydroxylamine, and 75 μL 0.1 M carbobenzoxyl-L-glutaminyl-glycine, was incubated at 37 °C for 10 min and then stopped by adding equal volume (500 μL) of 15% TCA containing 5% $FeCl_3$. After 15 min centrifugation at 4000 × g, the supernatant was collected and the absorbance at 525 nm was measured. The calibration was performed using L-glutamic acid-γ-monohydroxamic acid as standard. One unit of MTGase activity was defined as the amount of enzyme that can catalyze the formation of 1 mole of hydroxamic acid within 1 min reaction at 37 °C.

Preparation of surimi gels

Frozen hairtail surimi was purchased from Hungmin frozen Foods Co. (Keelung, Taiwan) and it was 1 month old when purchased. According to the manufacturer, hairtail (*Trichiurus lepturus*) surimi was processed on vessel and stored at −20 °C for 3 months during experiments. After being thawed in running tap water to around 0 ±Ψ≥ hairtail fish was headed, eviscerated, deboned and processed into frozen surimi.

For gel preparation, frozen surimi was thawed at 4 °C until the core temperature reached 0 °C. Surimi was sliced before grinding using a mortar and pestle for 5 min. After adding 2.5% NaCl, grinding was resumed for another 20 min. Various amounts of MTGase (0, 0.1, 0.2, 0.3, 0.4 and 0.6 unit /g surimi) were added and ground for another 5 min. The amounts of salt, water and MTGase were calculated based on the initial weight of surimi. The temperature of surimi was maintained below 2 °C during grinding. After grinding, the surimi solutions were stuffed into polyvinylchloride tubes (1.5 cm diameter) and held at 30 °C for 30 min. The resulting samples were then heated at 90 °C for 30 min to fix the protein gel. For investigating the effect of setting temperature on gel-forming ability, MTGase-containing hairtail surimi paste (0.35 unit/g surimi) were prepared and set at three different conditions (20 °C for 6 h, 30 °C for 2 h or 45 °C for 2 h) before final cooking at 90 °C for 30 min. After heating, surimi gels were chilled in ice water for 30 min and kept at 4 °C overnight prior to gel analysis. Four formulas were used in this study including (1), sodium bisulfite alone; (2), sodium bisulfite with MTGase; (3), sodium bisulfite with E-64; 4), sodium bisulfite, E-64 with MTGase. The effects of sodium bisulfite and E-64 on gel-forming ability of MTGase-containing surimi gels were also investigated. Surimi gels were prepared as described previously except that 0.1% $NaHSO_3$, 0.01 mM E-64, 0.35 unit MTGase/g surimi or their combination was added into surimi paste during grinding.

Assessment of gel quality

The gel strength was determined according to Seki and others. (1990). Surimi gels were set at room temperature for 30 min to equilibrate the gel temperature. After cutting into pieces with a thickness of 3 cm, samples were subjected to the puncture test. The breaking force (g) and deformation (cm) of samples were measured using a Rheometer (Model CR-200D, Sun Scientific Co., Ltd.) equipped with a ball plunger (5 mm in diameter) at a compression speed of 60 mm/min. For each treatment, 12 determinations were performed and the mean values were calculated.

SDS-polyacrylamide gel electrophoresis analysis (SDS-PAGE)

Actomyosin (AM) was extracted from hairtail surimi according to the method described by Noguchi (1974). AM with or without MTGase was incubated at 30 °C or 45 °C. After 120 min incubation, 0.1 mL of sample was mixed with 0.4 mL of dissociation buffer (2% SDS, 5% β-Me, 62.5 mM Tris-HCl, pH 6.8), and incubated in a water bath (95 ±Ψ≥ for 3 min to terminate the reactions. The resulting samples were analyzed by SDS-PAGE according to Hames (1990) using 7.5% polyacrylamide. The gels were stained with Coomassie brilliant blue G-250 (Neuhoff and others 1988). The changes in electrophoretic profiles were used to evaluate the cross-linking caused by MTGase.

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