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Interactive Effects of Microbial Transglutaminase and Recombinant Cystatin on the Mackerel and Hairtail Muscle Protein

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Interactive effects of microbial transglutaminase (MTGase) and recombinant cystatin on the mackerel and hairtail water soluble protein (WSP), salt soluble protein (SSP), and muscle protein (MP) were investigated. According to sodium dodecyl sulfate−polyacrylamide gel electrophoresis and enzymic activity analyses, cross-linking of mackerel and hairtail myosin heavy chain and low molecular mass compounds and formation of ϵ-(γ-glutamyl)lysine cross-links were observed on samples with MTGase, while the recombinant cystatin could effectively inhibit the cathepsins and subsequently prevent degradation of proteins during setting. The cathepsins and MTGase activities in WSP, SSP, and MP solutions decreased, but the recombinant cystatin activity increased during setting at 45 °C.

KEYWORDS: MTGase; recombinant cystatin; cathepsin; fish protein; ϵ-(γ-glutamyl)lysine

INTRODUCTION

Mackerel (*Scomber australasicus*) and hairtail (*Trichiurus lepturus*) have been used to produce surimi. However, because of the poor gel-forming ability, it is still not commercialized. According to our previous study (*1*), the combined use of microbial transglutaminase (MTGase) and recombinant cystatin revealed a synergistic effectiveness on improving the gel-forming ability of mackerel and hairtail surimi. MTGase (TGase; EC 2. 3. 2. 13) can catalyze the acyl transfer reaction between a glutaminyl residue and a primary amino group of various substrates (*2*) and form a ϵ-(γ-glutamyl)lysine cross-link, which is very important in enhancing the gel properties (*3*). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis also indicated the formation of a cross-linked myosin heavy chain (MHC) in MTGase-containing pollack, mackerel, and hairtail surimi during the setting process (*1*, *4*, *5*).

The cystatin superfamily comprises a group of proteinase inhibitors that are widely distributed in animal tissues and that could form tight complexes with cysteine proteases such as cathepsins B, H, L, and S (*6*). In fish, high levels of cystatin are found in developing eggs, which are considered to be involved in embryogenesis, regulation of pathological processes, and protection against microorganisms (*7*). Recently, cystatins have been cloned and studied at the molecular level and include human cystatin C (*8*), chicken cystatin (*9*), and rat cystatin (*10*). A high level of recombinant chicken cystatin was expressed in *Pichia pastoris* X-33 (*11*). It substantially inhibited the proteolysis of myosin and gel softening, which consequently improved the gel properties of mackerel and hairtail surimi. The objective of this study was to investigate the interactive effects of the combined use of MTGase and recombinant cystatin on the mackerel and hairtail muscle protein (MP).

MATERIALS AND METHODS

Materials. Tris(hydroxymethyl)aminomethane, carbobenzoxyl-L-glutaminyl-glycine (CBZ-L-Gln-Gly), SDS, papain (type III), and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO). Coomassie Blue G-250, β-mercaptoethanol (β-Me), glycerol, and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). Yeast extract and soluble starch were obtained from Difco (Detroit, MI), while the protein marker was from Promega Inc. (Madison, WI). Z-Phe-Arg-MCA was purchased from Wako (Peptide Institute, Inc., Osaka, Japan), while the *Streptoverticillium ladakanum* ATCC 27441 was obtained from the Taiwan Culture Collection and Research Center (Hsinchu, Taiwan). All chemicals were reagent grade.

Preparation of MTGase. MTGase was obtained according to Ho et al. (*12*). One loop of spore suspensions of *S. ladakanum* stock culture was activated in a 125 mL Erlenmeyer flask containing 50 mL of medium (1% glycerol, 1.5% yeast extract, 0.2% K_2HPO_4, and 0.1% $MgSO_4$, pH 7.0) and incubated in a 28 °C shaking incubator (150 rpm) for 2 days. One milliliter of culture suspension was then transferred to 150 mL of fresh medium (pH 7.0). After 4 days of incubation at 28 °C with shaking, the culture fluid was filtered first through filter paper (Whatman No. 1) and subsequently through a 0.22 μm filter membrane. The filtrate was then chromatographed on CM

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Sepharose CL-6B and Blue Sepharose Fast Flow column. Fractions with MTGase activity were collected. The purified MTGase was then stored at −70 °C until use.

Preparation of Recombinant Cystatin. Recombinant cystatin was prepared according to Chen et al. (*11*), and a cDNA encoding chicken cystatin was cloned into the pGAPZαC expression vector and then transformed into the *P. pastoris* X-33 expression host. The *P. pastoris* X-33 strain integrated with recombinant pGAPZαC-cystatin DNA was cultivated in 5 mL of YPDS broth (2% tryptone, 1% yeast extract, 2% dextrose, and 18.2% sorbitol) containing 100 μg/mL Zeocin in a 50 mL flask in a 30 °C shaking incubator (300 rpm) overnight. One milliliter of the resulting culture was inoculated into 50 mL of fresh YPD broth (2% tryptone, 1% yeast extract, and 2% dextrose) in a 250 mL flask and cultivated in a 30 °C shaking incubator (300 rpm) for 3 days. After 10 min of centrifugation at 3000*g* to remove the *Pichia* cells, the supernatant was then chromatographed on Sephacryl S-100 HR and Superdex 75 column. Fractions with cystatin activity were collected. The purified cystatin was then stored at 4 °C until use.

Determination of MTGase Activity. The MTGase activity was measured according to Folk (*13*). The reaction mixture, containing 50 μL of enzyme, 350 μL of 0.1 M Tris-acetate buffer (pH 6.0), 25 μL of 2.0 M hydroxylamine, and 75 μL of 0.1 M CBZ-L-Gln-Gly, was incubated at 37 °C for 10 min and then stopped by adding an equal volume (500 μL) of 15% TCA containing 5% $FeCl_3$. After 15 min of 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 the standard. One unit of MTGase activity was defined as the amount of enzyme that can catalyze the formation of 1 μmol of hydroxamic acid within 1 min of reaction at 37 °C.

Determination of Recombinant Cystatin Activity. The recombinant cystatin activity was measured according to Chen et al. (*11*). The enzyme mixture was comprised 50 μL of papain, 250 μL of 0.4 M sodium phosphate buffer (pH 6.0) containing 8 mM cysteine and 4 mM EDTA, 400 μL of distilled water, and 50 μL of cystatin. The reaction was started by adding 250 μL of 40 μM Z-Phe-Arg-MCA solution and stopped by adding 1.0 mL of sodium acetate buffer containing 0.1 M sodium monochloroacetate (pH 4.3). The amount of liberated aminomethylcoumarin was determined by a spectrofluorometer at an excitation of 350 nm and an emission of 460 nm. One unit of inhibitory activity was defined as the amount of cystatin that can inhibit one unit of the proteolytic activity of papain, while one unit of proteolytic activity was the amount of proteinase that can hydrolyze Z-Phe-Arg-MCA and release 1 nmol of aminomethylcoumarin within 1 min of reaction at 37 °C.

Determination of Cathepsin Activity. Cathepsin activity was measured according to Lee et al. (*14*). The enzyme mixture was comprised of 50 μL of cathepsin, 250 μL of 0.4 M sodium phosphate buffer (pH 6.0) containing 8 mM cysteine and 4 mM EDTA, and 450 μL of distilled water. The reaction was started by adding 250 μL of 40 μM Z-Phe-Arg-MCA solution and stopped by adding 1.0 mL of sodium acetate buffer containing 0.1 M sodium monochloroacetate (pH 4.3). The amount of liberated aminomethylcoumarin was determined by a spectrofluorometer at an excitation of 350 nm and an emission of 460 nm. One unit of cathepsin activity was defined as the amount of cathepsin that can hydrolyze Z-Phe-Arg-MCA and release 1 nmol of aminomethylcoumarin within 1 min of reaction at 37 °C.

Role of the MTGase in the Gelation of Surimi Gel. Mackerel and hairtail surimi were prepared according to our previous study (*1*). The water soluble proteins (WSPs) of the mackerel and hairtail surimi were extracted by using sodium phosphate buffer (SPB, 50 mM, pH 7.0), while the salt-soluble protein (SSP) was isolated according to the method of Noguchi and Matsumoto (*15*). The protein concentrations of WSP, SSP, and MP solutions were measured by the micro-Biuret method (*16*). The effects of protein concentration on the MTGase activity were then investigated. The amounts of added MTGase for mackerel and hairtail samples in this study were 0.5 and 1.0 unit/mL, respectively. MTGase was added into various protein concentrations of WSP, SSP, and MP solutions (0−3.0 mg/mL). After 20 min of setting at 45 °C, the residual MTGase activity was determined. The setting conditions for samples with/without MTGase were at 30 and 45 °C for 2 h as described in our previous study (*1*). Then, MTGase was added into SPB, WSP, SSP, and MP solutions (1 mg/mL). Samples were then set at 30 and 45 °C for 2 h prior to the determination of residual MTGase activity.

Role of Recombinant Cystatinin Preventing the Gel Softening. The effects of recombinant cystatin on the inhibition of cathepsins were then investigated. The setting conditions for samples with/without recombinant cystatin were at 30 and 45 °C for 2 h as described in our previous study (*1*). The amounts of added recombinant cystatin for mackerel and hairtail samples in this study were 0.002 unit/mL. Then, recombinant cystatin was added into SPB, WSP, SSP, and MP solutions (1 mg/mL). Samples were then set at 30 and 45 °C for 2 h prior to the determination of residual cathepsins and recombinant cystatin activity.

Analysis of ε-(γ-Glutamyl)lysine Bonds Content. The effects of protein concentration on the formation of ε-(γ-glutamyl)lysine bonds by MTGase were then investigated. Varying concentrations of mackerel WSP, SSP, and MP solutions (0, 1.0, 2.0, and 3.0 mg/mL) with/without MTGase (0.5 unit/mL) were incubated at 45 °C for 20 min. After inactivation of MTGase by heating at 100 °C for 15 min, the proteolytic digestion of resulting samples was carried out by sequential addition of proteolytic enzymes (*17*). The digested samples were then lyophilized and subjected to a fractionation of ε-(γ-glutamyl)lysine with high-performance liquid chromatography (HPLC). The elution time and ε-(γ-glutamyl)lysine content were estimated by synthetic ε-(γ-glutamyl)lysine as a reference (*18*).

SDS−PAGE Analysis. WSP and MP solutions with/without MTGase and/or recombinant cystatin were incubated at 30 or 45 °C for 2 h. Samples (0.1 mL) were incubated with 0.2 mL of dissociation buffer (2% SDS, 5% β-Me, and 62.5 mM Tris-HCl, pH 6.8) in a water bath (95 °C) for 3 min. The resulting samples were analyzed by SDS−PAGE according to Hames (*19*) using 7.5% polyacrylamide. After electrophoretical running, gels were stained with Coomassie Brilliant Blue G-250 (*20*).

RESULTS AND DISCUSSION

Effects of MTGase on Mackerel and Hairtail WSP and MP. According to Jiang et al. (*21*), MTGase participated in the setting process of mackerel and hairtail surimi gels and mainly accelerated the cross-linking of MHC. The MHC of MTGase-contained mackerel and hairtail MP decreased greatly, while the cross-linked MHC became predominant during the early 20 min of incubation (**Figure 1A**,**E**). No cross-linked MHC was observed on samples without MTGase (**Figure 1B**,**F**). Similar trends were also observed in the changes of low molecular mass compounds of mackerel and hairtail WSP without MTGase

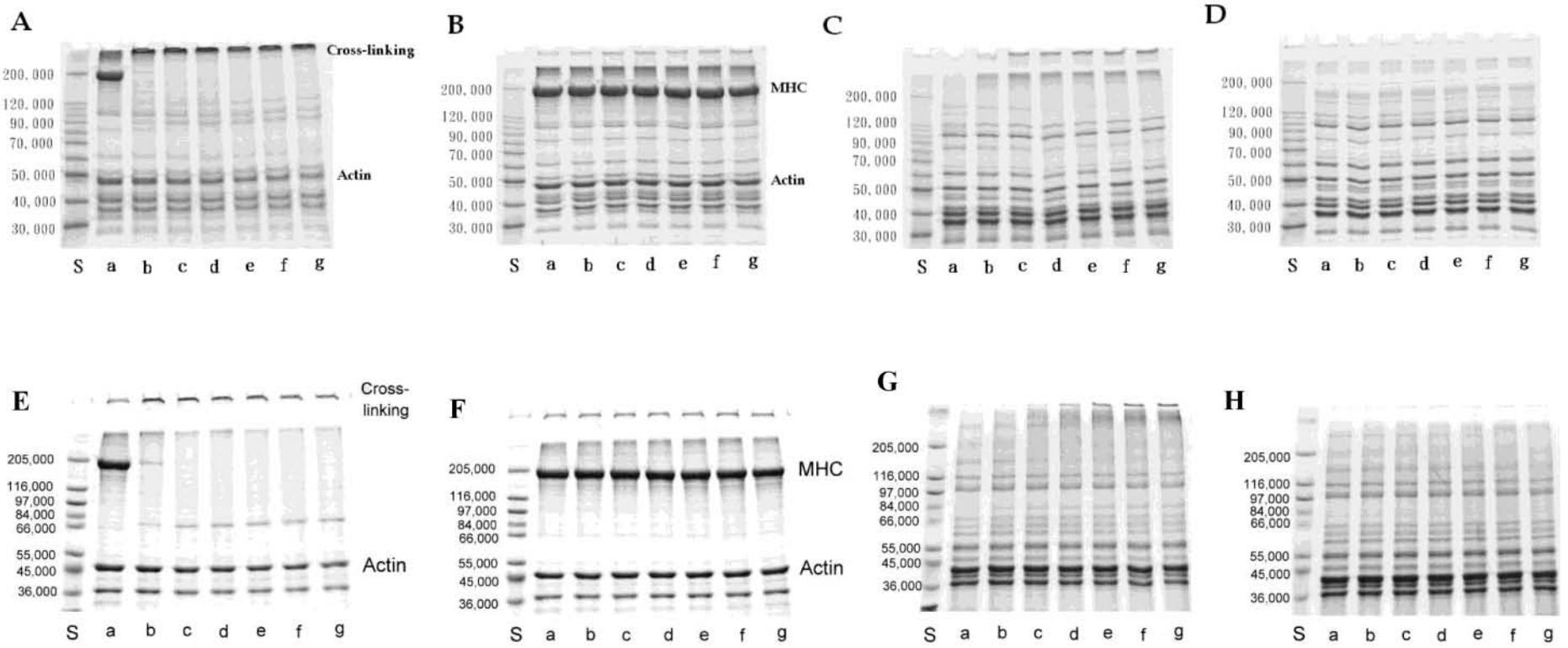

Figure 1. Change in SDS–PAGE profiles of mackerel and hairtail WSP and MP with/without MTGase incubated at 30 °C for various time periods. (**A**) Mackerel MP with MTGase; (**B**) mackerel MP without MTGase; (**C**) mackerel WSP with MTGase; (**D**) mackerel WSP without MTGase; (**E**) hairtail MP with MTGase; (**F**) hairtail MP without MTGase; (**G**) hairtail WSP with MTGase; and (**H**) hairtail WSP without MTGase. S, protein marker; a, 0 min; b, 20 min; c, 40 min; d, 60 min; e, 80 min; f, 100 min; and g, 120 min.

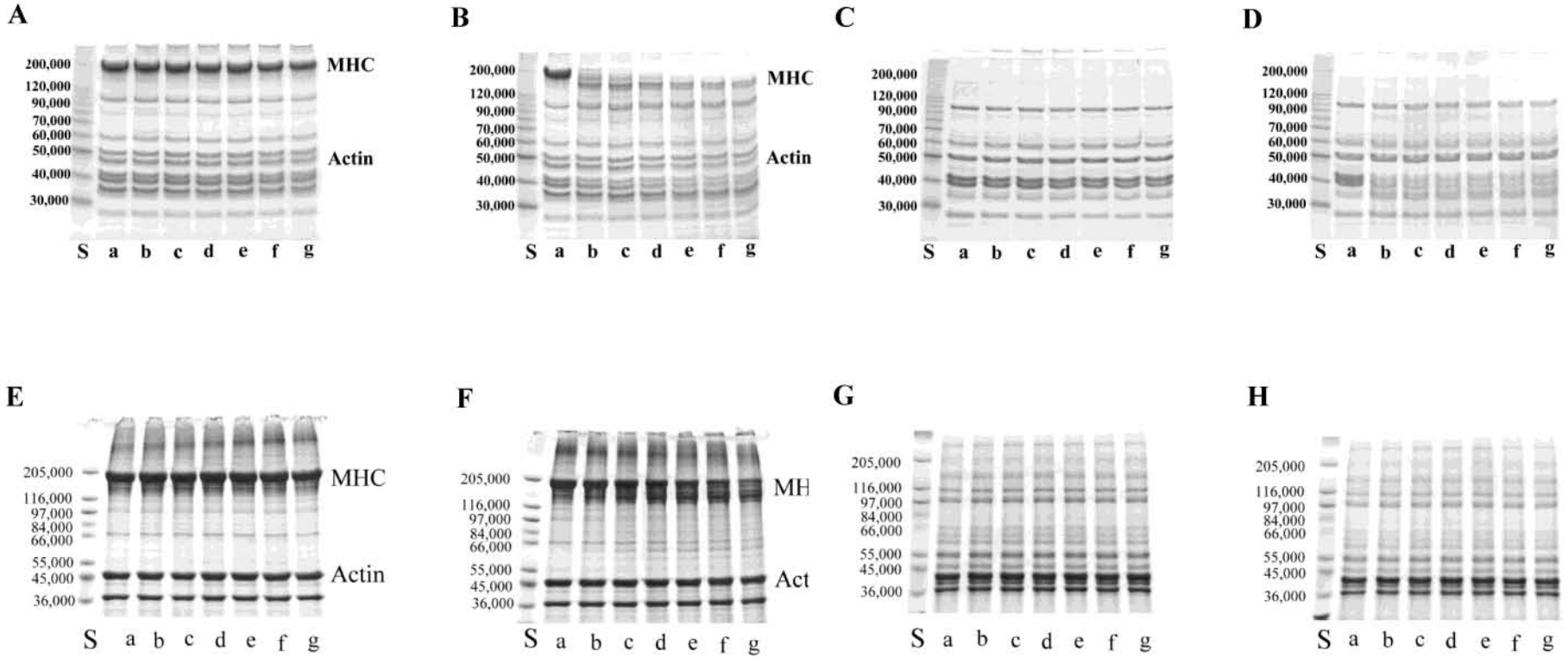

Figure 2. Change in SDS–PAGE profiles of mackerel and hairtail WSP and MP with/without recombinant cystatin incubated at 45 °C for various time periods. (**A**) Mackerel MP with recombinant cystatin; (**B**) mackerel MP without recombinant cystatin; (**C**) mackerel WSP with recombinant cystatin; (**D**) mackerel WSP without recombinant cystatin; (**E**) hairtail MP with recombinant cystatin; (**F**) hairtail MP without recombinant cystatin; (**G**) hairtail WSP with recombinant cystatin; and (**H**) hairtail WSP without recombinant cystatin. S, protein marker; a, 0 min; b, 20 min; c, 40 min; d, 60 min; e, 80 min; f, 100 min; and g, 120 min.)

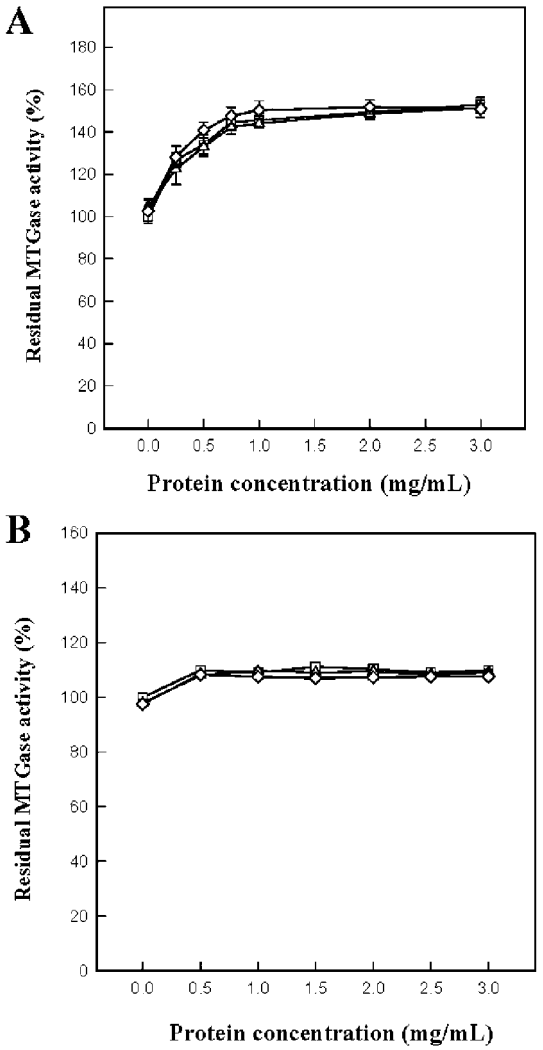

Figure 3. Effects of protein concentration on the MTGase activity. Samples incubated at 45 °C for 20 min. (**A**) Mackerel samples and (**B**) hairtail samples. WSP solution, □; SSP solution, △; and MP solution, ◇. Samples with MTGase, solid symbols; samples without MTGase, open symbols. Vertical bars represent the standard deviation.

(**Figure 1D**,**H**). However, the formation of cross-linked high molecular mass polymers of the MTGase-contained WSP appeared on the top of the gels after 40 min of incubation (**Figure 1C**,**G**). These results suggested that the MHC was a good substrate for MTGase.

Effects of Recombinant Cystatin on Mackerel and Hairtail WSP and MP. Chen et al. (*11*) reported that the MHC degradation could be inhibited by recombinant cystatin. No degradation of MHC of recombinant cystatin-contained MP was observed (**Figure 2A**,**E**), while the MHC of MP without recombinant cystatin disappeared after 120 min of incubation (**Figure 2B**,**F**). The significant degradation of MHC was evidenced by the addition of endogenous cysteine proteases such as cathepsins B, L, and L-like (*22*). Similar trends were also observed in the changes of low molecular mass compounds (40 and 42 kDa) of WSP with/without recombinant cystatin (**Figure 2C**,**D**,**G**,**H**). These results indicated that the recombinant cystatin could prevent the degradation of MP during the setting process.

Effects of Protein Concentration and Incubation Condition on MTGase. After 20 min of incubation at 45 °C, the residual MTGase activity in mackerel and hairtail WSP, SSP, and MP solutions increased with the increase of protein concentration (**Figure 3A**,**B**). However, no further increase was

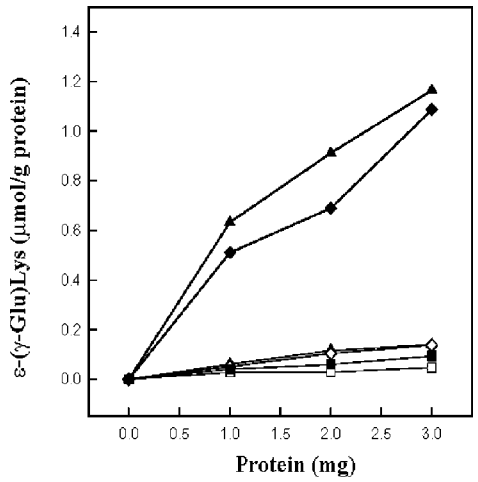

Figure 4. Effects of protein concentration on the ϵ-(γ-glutamyl)lysine bonds content. Mackerel samples incubated at 45 °C for 20 min. WSP solution, □; SSP solution, △; and MP solution, ◇. Samples with MTGase, solid symbols; samples without MTGase, open symbols. Vertical bars represent the standard deviation.

observed on mackerel and hairtail samples with higher than 1.0 and 0.5 mg/mL, respectively. These results suggest that the components of mackerel and hairtail could enhance the MTGase activity. Stefano et al. (*23*) reported the chemical, physical, and biological factors, which can affect the activity of transglutaminase. Furthermore, after 20 min of incubation at 45 °C, the ϵ-(γ-glutamyl)lysine bonds in mackerel WSP, SSP, and MP solutions increased with the increase of protein concentration (**Figure 4**). The ϵ-(γ-glutamyl)lysine bonds in mackerel SSP and MP with MTGase were 0.63 and 0.51 μmol/g protein, respectively, which were higher than that of the control (without MTGase). Sakamoto et al. (*24*) reported that the ϵ-(γ-glutamyl)lysine in foods ranged from 0.2 to 135 μmol/100 g protein, while high levels were found in fish paste products. It was noted that the ϵ-(γ-glutamyl)lysine bonds in mackerel SSP and MP were higher than in mackerel WSP. These results also evidenced that the WSPs were not good substrates for MTGase.

The residual MTGase activity in mackerel and hairtail WSP, SSP, and MP solutions (1 mg/mL) was substantially increased after 20 min of incubation at 30 and 45 °C (**Figure 5A**−**D**). All of the residual MTGase activities of the samples were higher than that of the sample without MP (control, SPB, 0 mg/mL). However, those incubated at 45 °C, despite WSP, SSP, and MP solutions, decreased significantly during prolonged incubation (**Figure 5B**,**D**). According to Duran et al. (*25*), the reaction of MTGase with proteins increased with the increase in incubation temperature up to 50 °C; however, the thermal denaturation occurred and consequently declined the reaction rate when the incubation temperature was >50 °C. Ho et al. (*12*) also reported that the rate constants (K_D) for thermal inactivation of the purified MTGase at 50 and 55 °C were 2.3×10^{-4} and 1.1×10^{-3} min^{-1}, respectively. These results suggested that the MTGase is very stable at <30 °C.

Effects of Incubation Condition on Cathepsins and Recombinant Cystatin. No significant changes in residual cathepsins activity in mackerel and hairtail SSP, WSP, and MP solutions at 30 °C were observed, but the catheptic activities markedly decreased on samples incubated at 45 °C (**Figure 6A**−**D**). These results suggest that the denaturation of cathepsins occurred during incubation at 45 °C. Lee et al. (*14*) reported that the inactivation rate constants (K_D) of mackerel cathepsins L and L-like at 50 °C were 5.1×10^{-5} and 6.9×10^{-4} s^{-1},

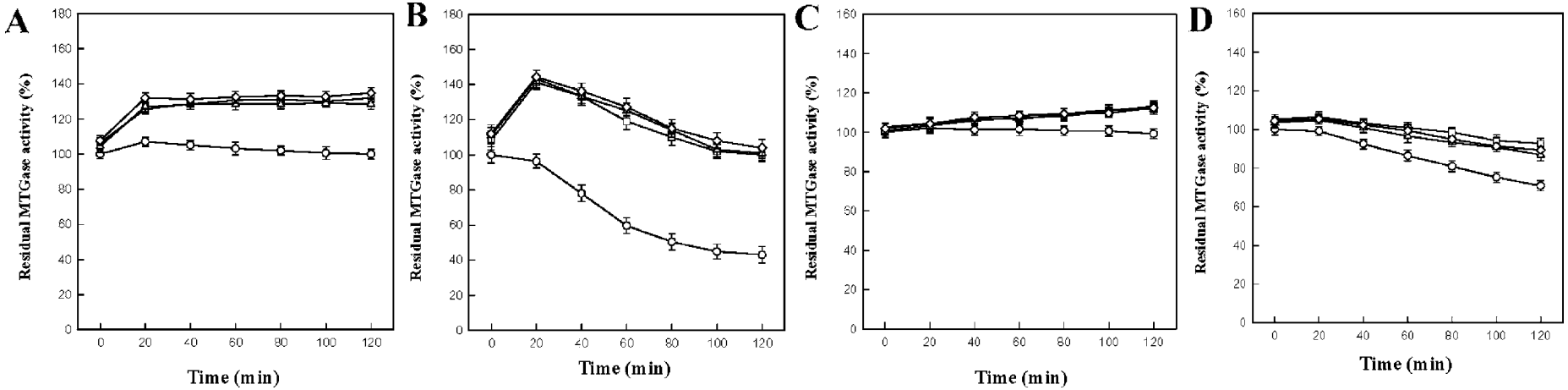

Figure 5. Effects of setting condition on the MTGase activity. (**A**) Mackerel samples incubated at 30 °C; (**B**) mackerel samples incubated at 45 °C; (**C**) hairtail samples incubated at 30 °C; and (**D**) hairtail samples incubated at 45 °C. Sodium phosphate buffer (SPB), ○; WSP solution, □; SSP solution, △; and MP solution, ◇. Vertical bars represent the standard deviation.

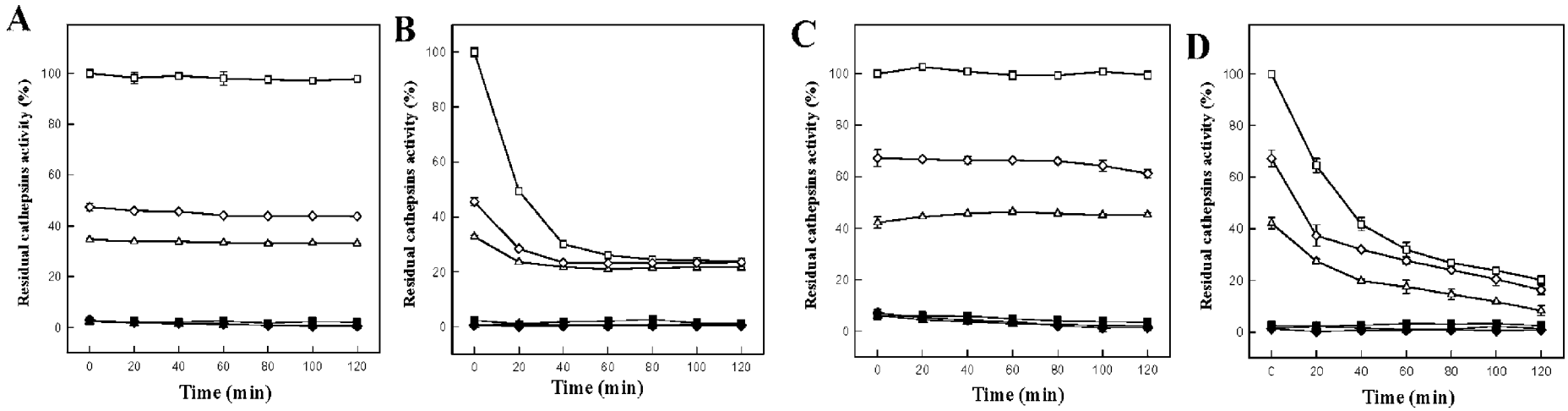

Figure 6. Effects of setting condition on the cathepsins activity. (**A**) Mackerel samples incubated at 30 °C; (**B**) mackerel samples incubated at 45 °C; (**C**) hairtail samples incubated at 30 °C; and (**D**) hairtail samples incubated at 45 °C. SPB, ○; WSP solution, □; SSP solution, △; and MP solution, ◇. Solid symbol, samples with recombinant cystatin; open symbol, samples without recombinant cystatin. Vertical bars represent the standard deviation.

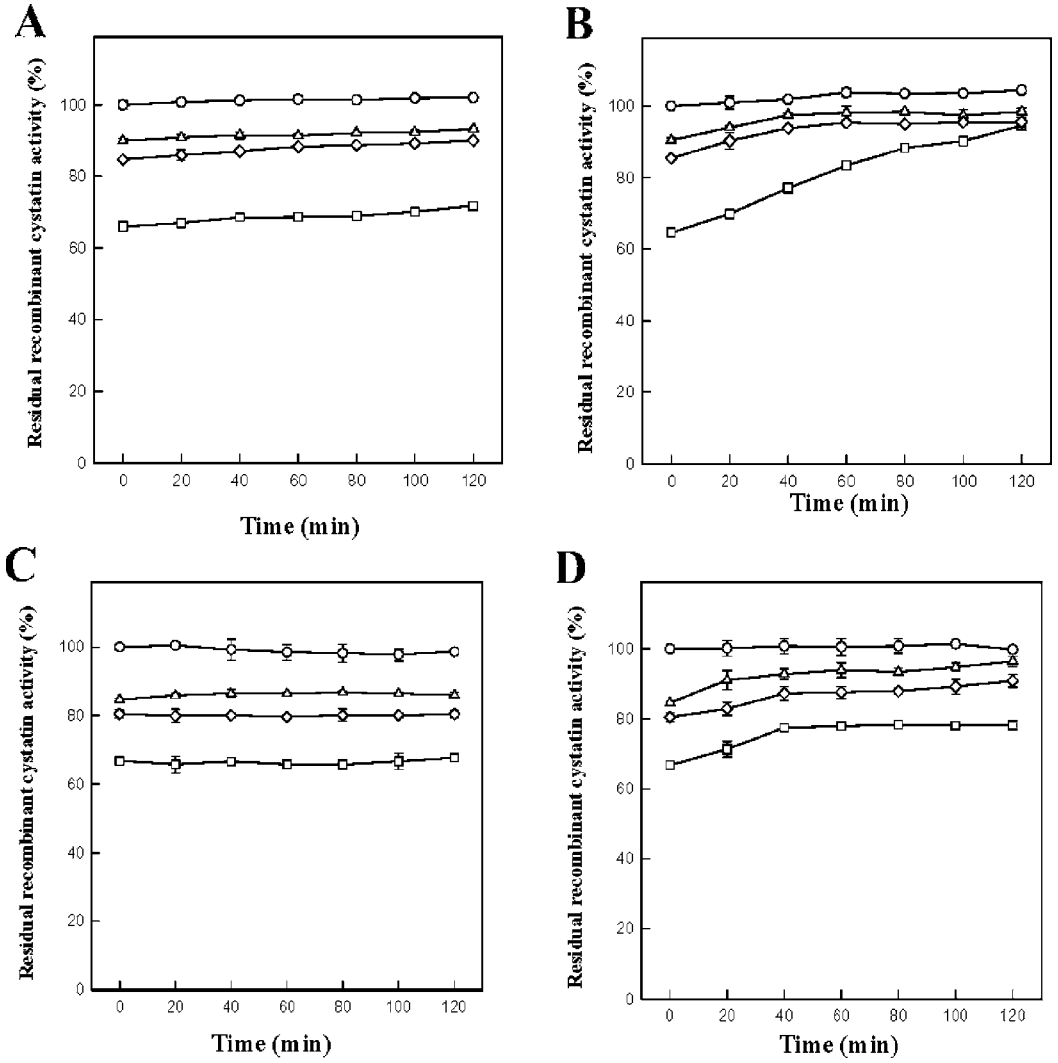

Figure 7. Effects of setting condition on the recombinant cystatin activity. (**A**) Mackerel samples incubated at 30 °C; (**B**) mackerel samples incubated at 45 °C; (**C**) hairtail samples incubated at 30 °C; and (**D**) hairtail samples incubated at 45 °C. SPB, ○; WSP solution, □; SSP solution, △; and MP solution, ◇. Vertical bars represent the standard deviation.

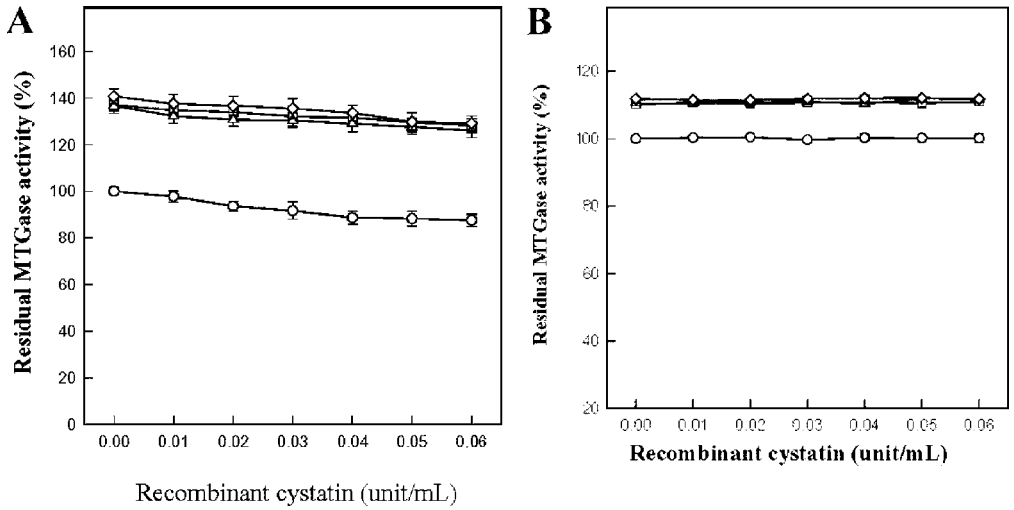

Figure 8. Effects of recombinant cystatin on the MTGase activity. (**A**) Mackerel samples and (**B**) hairtail samples. Samples incubated at 45 °C for 20 min. SPB, ○; WSP solution, □; SSP solution, △; and MP solution, ◇. Vertical bars represent the standard deviation.

respectively. Furthermore, when the recombinant cystatin was added into the mackerel and hairtail SSP, WSP, and MP solutions, the catheptic activities were completely inhibited by recombinant cystatin.

As compared with the cystatin activity of mackerel and hairtail WSP samples incubated at 45 °C with those at 30 °C, it significantly increased during the 120 min incubation and was higher (**Figure 7A−D**). This phenomenon suggested that the

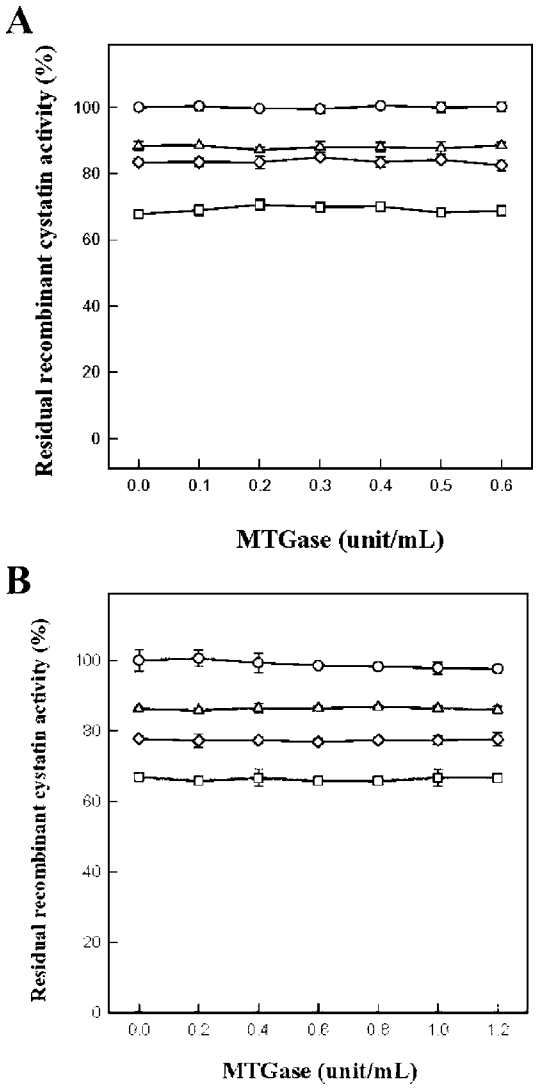

Figure 9. Effects of MTGase on the recombinant cystatin activity. (**A**) Mackerel samples and (**B**) hairtail samples. SPB, ○; WSP solution, □; SSP solution, △; and MP solution, ◇. Vertical bars represent the standard deviation.

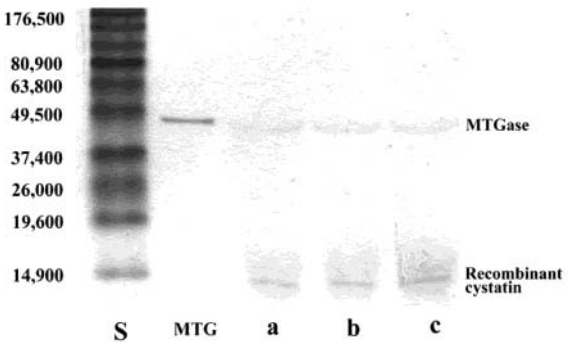

Figure 10. Change in SDS–PAGE profiles of MTGase with recombinant cystatin incubated at 30 °C for various time periods. S, protein marker; MTG, purified MTGase; a, 0 min; b, 30 min; and c, 60 min.

denaturation of cathepsins occurred at >45 °C, which consequently increased the residual recombinant cystatin activity in WSP solutions.

Effects of Recombinant Cystatin on MTGase. Effects of recombinant cystatin on the MTGase activity were also investigated. As indicated in **Figure 8A**,**B**, there were no significant changes in residual MTGase activity, when varying concentrations of recombinant cystatin were added into SPB, WSP, SSP, and MP solutions. These results suggest that the MTGase was not the substrate for the recombinant cystatin. As mentioned previously, the MTGase activity markedly increased with the increase of protein concentrations. Therefore, it was also observed that the residual MTGase activity in mackerel and hairtail WSP, SSP, and MP solutions (1 mg/mL) was higher than in SPB.

Effects of MTGase on Recombinant Cystatin. As shown in **Figure 9A**,**B**, when varying concentrations of MTGase were added into SPB, WSP, SSP, and MP solutions, there were no significant changes in residual recombinant cystatin activity. The SDS−PAGE analysis also indicated that no cross-linked recombinant cystatin was observed on samples with MTGase after 60 min of incubation (**Figure 10**). Several studies indicated that MTGase catalyzes the acyl transfer reaction between a γ-carboxamine group of a peptide-bound glutaminyl residue and a primary amino group of various substrates (*26*, *27*). In this study, the results suggest that the recombinant cystatin was not the substrate for the MTGase.

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