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Suwari gel properties as affected by transglutaminase activator and inhibitors

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Abstract

Effect of transglutaminase activator and inhibitors on textural properties and cross-linking of myofibrillar proteins in suwari gel of surimi from four fish species, including bigeye snapper, threadfin bream, barracuda and bigeye croaker was investigated. Breaking force and deformation of suwari increased as calcium chloride added increased ($P<0.05$); however, a slight decrease was observed with an excessive amount of addded calcium chloride. With addition of calcium chloride, the concomitant decrease in solubility of suwari gel, in a mixture of sodium dodecyl sulfate, urea and β-mercaptoethanol, suggested increased non-disulfide covalent bond formation. Conversely, the addition of transglutaminase inhibitors, including *N*-methylmaleimide (NEM), ammonium chloride and EDTA, resulted in a marked decrease in breaking force and deformation, especially with increasing concentration. The decrease in gel-forming ability was associated with the decrease in non-disulfide covalent cross-linking, as indicate by an increase in solubility and more myosin heavy chain (MHC) retained. The results indicate that endogenous transglutaminase played an essential role in setting, at high temperature (40 °C), of surimi from tropical fish.

Keywords: Setting; Suwari; Surimi; Transglutaminase; Tropical fish

1. Introduction

Surimi gel strength can be enhanced by subjecting sols to setting at the temperatures ranging from 0 to 40 °C prior to heating (Alvarez, & Tejada, 1997; An, Peters, & Seymour, 1996; Kamath, Lanier, Foegeding, & Hamann, 1992). The gel obtained after setting is generally referred to as 'suwari gel'. During setting, myosin heavy chain (MHC) undergoes polymerization via formation of non-sulfide covalent cross-links, catalyzed by an endogenous transglutaminase (TGase) (Kumazawa, Numazawa, Seguro, & Motoki, 1995; Kimura et al., 1991). TGase has been known to catalyse acyl transfer reactions between the γ-carboxamide groups of glutamine residues within protein and suitable acceptors, usually primary amines (Folk & Chung, 1973; Kishi, Nozawa, & Seki, 1991). Formation of an ε-(γ-glutamyl) lysine isopeptide was reported to serve for gel strengthening (Kumazawa et al., 1995). Complete suppression of myosin cross-linking of walleye pollack surimi gel was associated with the inhibition of endogenous TGase (Takeda & Seki, 1996). Additionally, Kumazawa et al. (1995) reported that the addition of EDTA and ammonium chloride resulted in the suppression of ε-(γ-glutamyl) lysine formation.

Setting response can be varied, depending upon fish species (Benjakul & Visessangaun, 2003; Morales, Ramirez, Vivanco, & Vazuez, 2001; Shimizu, Machida, & Takenemi, 1981). Different species have different optimum setting temperatures. Pollock and croaker had optimum setting temperatures of 25 and 40 °C, respectively (Kamath et al., 1992). The rate of TGase-mediated cross-linking of MHC may be dependent, primarily, on the conformation of the substrate myosin at a given temperature rather than on the temperature optimum of TGase (Kamath et al., 1992). The reactivity of TGase, toward various fish actomyosins, varied, depending on the conformation of actomyosin (Araki & Seki, 1993). In essence, a sufficient amount of Ca^{2+}-ion is required for TGase activity and varied among fish species (Nozawa, Mamogoshi, & Seki, 1997). Stronger gel was obtained with the addition of calcium

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compounds to enhance TGase-mediated setting (Benjakul & Visessanguan, 2003; Lee & Park, 1998).

Thailand is one of the largest surimi producers in Southeast-Asia. At present, 12 surimi factories are located in Thailand, with a total production of about 60,000 metric tons per year (Morrissey & Tan, 2000). Normally, threadfin bream (*Nemipterus* spp.), bigeye snapper (*Priacanthus* spp.), croaker (*Pennahia* and *Johnius* spp.) and barracuda (*Sphyraena* spp.), are species commonly used for surimi production in Thailand. Recently, high-temperature setting at 40 °C, for an appropriate time has been reported to enhance gel strength of surimi from some tropical fish (Benjakul, Visessanguan, & Chantarasuwan, submitted for publication). Also, high-temperature setting is widely used in Thailand, due to the short time required. Although setting has been used in surimi industries in Thailand for many decades, there is little information about the role of endogenous TGase in cross-linking of surimi during the setting process of surimi produced from tropical fish, particularly those commonly used for manufacturing in Thailand. The objective of this investigation was to study the influence of endogenous TGase activator and inhibitors on gel-forming ability and cross-linking of myofibrillar proteins in suwari gels of surimi from four tropical fish species.

2. Materials and methods

2.1. Chemicals

Ethylenediaminetetraacetic acid (EDTA), ammonium chloride, *N*-ethylmaleimide (NEM) and calcium chloride were purchased from Sigma Chemical Co. (St Louis, MO, USA). Sodium dodecyl sulfate (SDS) and *N,N,N′,N′*-tetramethyl ethylene diamine (TEMED) were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Surimi and surimi gel preparation

Frozen surimi, grade A, produced, from threadfin bream (*Nemipterus bleekeri*), bigeye snapper (*Priacanthus tayenus*), and baracuda (*Sphyraena jello*) and surimi grade AA from bigeye croaker (*Pennahai macrophthalmus*), were purchased from Man A Frozen Foods Co., Ltd., Songkhla, Thailand. Surimi was kept at −20 °C until used.

To prepare the gel, frozen surimi was tempered for 30 min in running water (25 °C). The surimi was then cut into small pieces with an approximate thickness of 1 cm. The surimi was placed in a mixer (National model MK-K77, Tokyo, Japan). The moisture was adjusted to 80% and 2.5% salt was added. To obtain a homogeneous paste, the mixture was chopped for 5 min at 4 °C. The temperature of surimi sol was kept below 10 °C.

To study the effect of additives on suwari gel properties, calcium chloride (10, 20, 50, 80 or 120 mmol/kg), EDTA (5 or 10 mmol/kg), NEM (5 and 10 mmol/kg) and ammonium chloride (0.5 or 1.0 mole/kg) were added and mixed thoroughly prior to setting. The surimi sol with and without additives was stuffed into polyvinylidine casing (2.5 cm diameter) and both ends were sealed tightly. Suwari gels were prepared by setting sol at 40 °C for 2, 1, 1.5 and 3 h for surimi from bigeye snapper, threadfin bream, barracuda and croaker, respectively, in a temperature controlled water bath (Benjakul et al., in preparation). After setting for the designated time, the gels were cooled immediately, using iced water, and stored at 4 °C for 24 h before analysis. The gels obtained were referred to as 'suwari gels'.

2.3. Texture analysis

Texture analysis of suwari gels was performed using a texture analyser, model TA-XT2 (Stable Micro Systems, Surrey, England). Gels were equilibrated and tested at room temperature. Five cylinder-shaped samples (2.5 cm in length; 2.5 cm in diameter) were prepared. The breaking force (gel strength) and deformation (elasticity/deformability) were measured using the texture analyser equipped with a cylindrical plunger (5 mm diameter; 60 mm/min deformation rate).

2.4. Solubility studies

Solubilities of suwari gels in 20 mM Tris–HCl, pH 8.0, containing 1% (w/v) SDS, 8 M urea and 2% (v/v) β-ME, were determined as described by Chawla, Venugopol, and Nair (1996). The sample (1 g) was homogenized in 20 ml of solution for 1 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia). The homogenate was heated in a boiling water (100 °C) for 2 min and stirred at room temperature for 4 h. The resulting homogenate was centrifuged at 10,000 *xg* for 30 min, using a Sorvall model RC-B plus centrifuge (Newtown, CT, USA). Protein in the supernatant (10 ml) was precipitated by the addition of 50% (w/v) cold TCA to a final concentration of 10%. The mixture was kept at 4 °C for 18 h and then centrifuged at 10,000 ×*g* for 30 min. The precipitate was washed with 10% TCA and solubilized in 0.5 M NaOH. To obtain the total amount of protein, gels were directly solubilised in 0.5 M NaOH. The protein content was measured using the Biuret test (Robinson & Hodgen, 1940). The solubility was expressed as percent of the total protein.

2.5. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein patterns of suwari gels were analysed on SDS–PAGE according to the method of Laemmli

(1970). To prepare the protein sample, 27 ml of 5% (w/v) SDS solution heated to 85 °C was added to the sample (3 g). The mixture was then homogenised using a homogeniser (IKA Labortechnik, Selangor, Malaysia) for 2 min. The homogenate was incubated at 85 °C for 1 h to dissolve total proteins. The samples were centrifuged at 3500 $\times g$ for 20 min to remove undissolved debris. Protein concentration was determined according to the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard. SDS–PAGE gel was made of 10% running gel and 4% stacking gel. After separation, the proteins were fixed and stained with Coomassie Blue R-250.

2.6. Statistical analysis

Analysis of variance (ANOVA) was performed and mean comparisons were run by Duncan's multiple range test (Steel & Torrie, 1980).

3. Results and discussion

3.1. Effect of calcium chloride on suwari gel textural properties

Breaking force of suwari gels, from all surimi, increased as the calcium chloride concentration increased, especially with concentrations up to 20 mmol/kg ($P<0.05$) (Fig. 1). With addition of calcium chloride at a level of 20 mmol/kg, breaking forces of suwari from bigeye snapper, threadfin bream, barracuda and bigeye croaker increased by 256.8, 24.7, 67.3 and 29.9%, compared with the control (without calcium chloride), respectively. Without calcium chloride added, suwari gel from different surimi exhibited different breaking forces. Breaking force of suwari gel from bigeye snapper, threadfin bream, barracuda and croaker was 214.8, 393.8, 342.8, and 688.8 g, respectively. It was noticeable that bigeye croaker suwari gel (grade AA) had the highest breaking force. This result suggested that higher grade surimi possibly had an appropriate alignment of protein molecules as well as a high cross-linking activity induced by endogenous TGase during setting at 40 °C. From the results, the breaking forces of surimi from different fish of the same grade were different ($P<0.05$). Suwari from bigeye snapper had the lowest breaking force ($P<0.05$). This result indicated a different aggregation of muscle protein during setting between fish species. Setting at 40 °C possibly caused different changes in protein conformation, resulting in differences in the exposure of reactive groups (glutamine and lysine) for the cross-linking reaction via TGase. Two substrate protein molecules and the enzyme must become associated in a highly oriented and conformation-dependent fashion at some stage of the catalytic process (Folk & Chung, 1973). The methylene group of glutamine residues is necessary to confer substrate properties and is essential for interaction with a hydrophobic region near the active site of the enzyme (Folk & Chung, 1973). Differences in TGase activity and its reactivity toward the protein substrates may occur in different surimi on calcium chloride addition even at the lowest concentration (10–20 mmol/kg). However, a considerable increase in breaking force was found with suwari gel from bigeye snapper. Therefore, it was presumed that TGase in bigeye snapper was very reactive toward calcium ion. As a result, TGase from bigeye snapper surimi became fully activated, leading to increased cross-linking with a concomitant increased breaking force. Also, a high level of TGase activity in this species is suggested. Fish TGases have differing sensitivities to calcium ion (Ashie & Lanier, 2000). Walleye pollack TGase required 3 mM calcium ion, while carp muscle TGase required 5 mM calcium ions for full activation (Kishi et al., 1991). Therefore, effectiveness of calcium chloride in enhancing setting response might depend on fish species. From our results, addition of excessive amount of calcium chloride, especially 120 mmol/kg, resulted in decreases in breaking force in all surimi tested ($P<0.05$). At high levels of calcium chloride, calcium or chloride ion might cause changes in protein conformation. Ions interact with oppositely charged groups on protein molecules to form a double layer of ionic groups, which decreases electrostatic interactions between protein molecules (Vojdani, 1996). Since NaCl was added to dissolve the myofibrillar protein, the addition of excessive amount of calcium ions may cause salting-out, leading to loss in solubility of protein. Thus, an appropriate amount of calcium chloride is needed to maximise the setting of surimi.

Deformation of suwari gels from all surimi tested exhibited similar trends (Fig. 1). With the addition of calcium chloride at a level of 20 mmol/kg, deformation of suwari gel, of surimi from bigeye snapper, threadfin bream, barracuda and bigeye croaker, increased by 54.3, 8.8, 22.8 and 2.8%, compared with those of the control, respectively. From the result, deformation, indicating elasticity, of suwari gel could be much improved for bigeye snapper and barracuda surimi.

3.2. Effect of calcium chloride on solubility of suwari gel

Solubility of suwari gels of surimi from different species with calcium chloride added at different levels is shown in Table 1. A progressive decrease in solubility of suwari gels from all surimi, except that from bigeye croaker, was observed as the calcium chloride concentration increased up to 20 mmol/kg. A further slight increase in solubility was found with the addition of calcium chloride at a level above 20 mmol/kg. However,

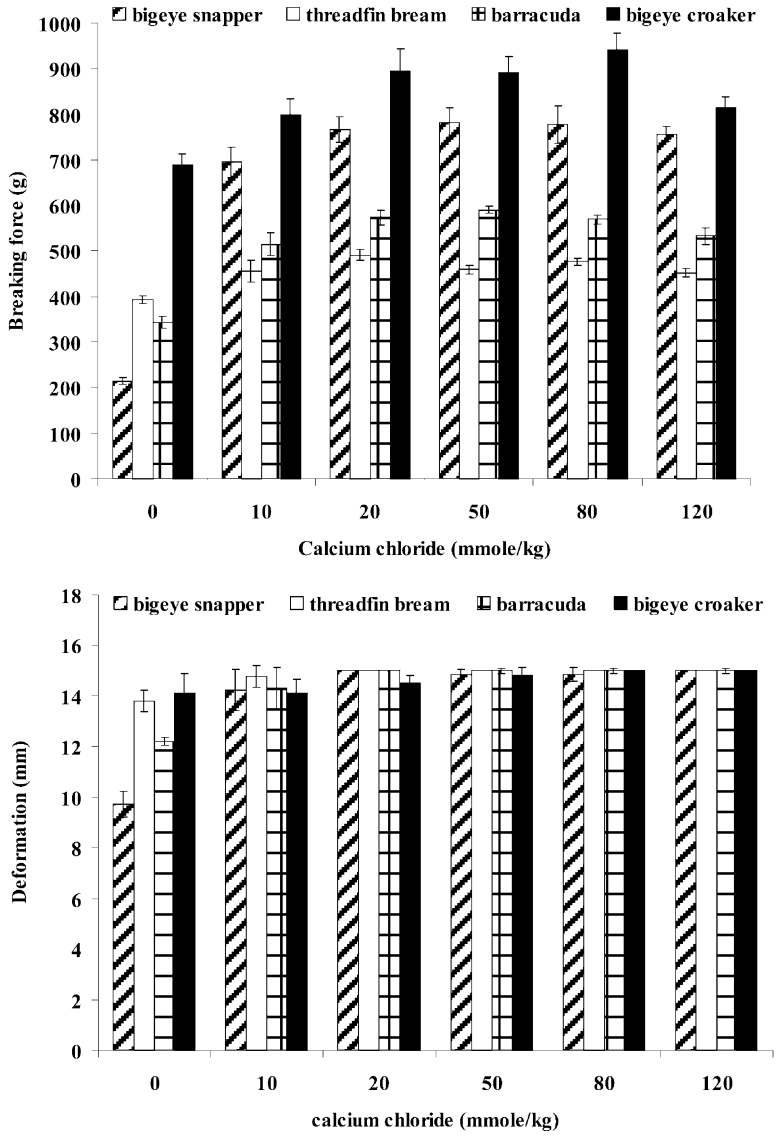

Fig. 1. Breaking force and deformation of suwari gels of surimi from four tropical fish with addition of calcium chloride at different levels. Bars indicate the standard deviation from five determinations.

the solubility of suwari gel from bigeye croaker surimi grade AA was lowest, even without calcium chloride addition. The decrease in solubility indicated the presence of non-disulfide cross-links formed during setting at high temperature. Decreases in solubility coincided with increased breaking force and deformation. The subsequent increase in solubility was also associated with a decrease in breaking force. A solution containing SDS, urea and β-mercaptoethanol was used to solubilise protein by destroying all bonds, except non-disulfide covalent bonds, particularly the ε-(γ-glutamyl) lysine linkage (Benjakul, Visessanguan, & Srivilai, 2001). Thus, decreased solubility indicated non-disulfide covalent bond formation induced by endogenous TGase. TGase has been known to play an essential role in ε-(γ-glutamyl) lysine linkage formation in surimi gel (Kumazawa et al., 1995). The lowest solubility in suwari gel from bigeye croaker surimi suggested that cross-linking occurred to the greatest extent, possibly due to either high TGase activity or the reactive myofibrillar protein in this species toward TGase induced reaction.

3.3. Effect of calcium chloride on protein pattern of suwari gel

Protein patterns of suwari gels of surimi from bigeye snapper, threadfin bream, barracuda and bigeye croaker, with and without addition of calcium chloride at different levels, are depicted in Fig. 2. Even without calcium chloride addition, no myosin heavy chain (MHC) was observed in suwari gels of any fish species tested. This indicated that the setting at 40 °C, for a proper time, effectively resulted in the cross-linking of MHC, especially via non-disulfide covalent bonds. At

higher amounts of calcium chloride added, actin band intensity tended to decrease, especially at a concentration of 120 mmol/kg. The results suggested that MHC was a preferred protein substrate for polymerisation induced by TGase, compared with actin. However, actin could be polymerised after MHC was totally cross-linked. The rate of actin polymerisation varied among species. Barracuda actin was found to be more reactive toward TGase-induced reaction, compared with actin from other species. This result was in agreement with Nakahara, Nozawa, and Seki (1999), who found that carp endogenous TGase preferentially polymerised MHC rather than other myofibrillar proteins.

3.4. Effect of TGase inhibitor on suwari gel textural properties

Breaking force and deformation of suwari gels of surimi from four fish species decreased in the presence of NEM, ammonium chloride and EGTA (Fig. 3). In general, breaking force and deformation decreased to a higher extent when concentration of TGase inhibitors

Table 1
Solubility of suwari gels of surimi from some tropical fish with addition of calcium chloride at different concentrations

Calcium chloride (mmol/kg)	Solubility (%)[a]			
	Bigeye snapper	Threadfin bream	Barracuda	Bigeye croaker
0	72.19±1.44e[b]	65.37±0.74a	63.15±0.36abc	33.73±0.33e
10	53.59±0.36ab	59.53±0.74b	58.64±2.74a	31.03±0.17d
20	52.30±0.36a	51.31±1.10c	58.38±2.37a	31.62±0.04d
50	55.36±0.36bc	60.16±0.18b	59.67±2.34ab	29.86±0.17c
80	59.31±0.91d	71.75±2.03c	63.79±0.55bc	27.52±0.16a
120	56.89±0.02c	73.96±0.06c	67.27±1.46c	29.16±0.50b

[a] Mean±S.D. from triplicate determinations.
[b] Different letters in the same column denote significant differences ($P<0.05$).

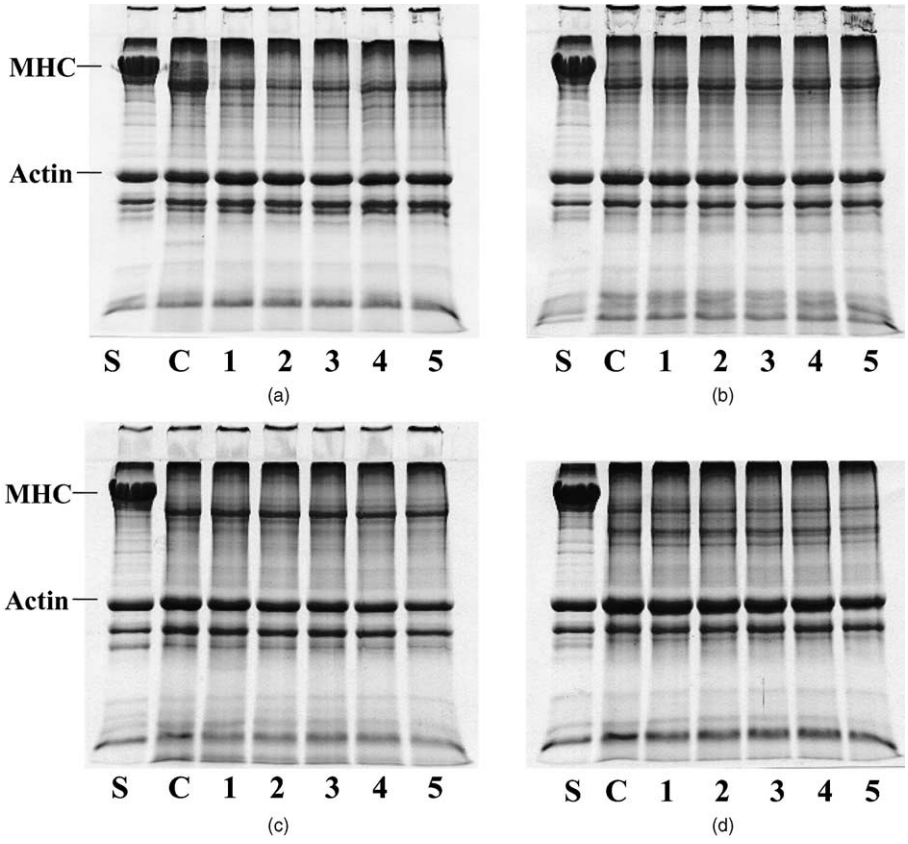

Fig. 2. SDS–PAGE patterns of suwari gels of surimi from bigeye snapper (a), threadfin bream (b), barracuda (c) and bigeye croaker (d) treated with different levels of calcium chloride. S: surimi sol; C: suwari gel without calcium chloride. Numbers 1, 2, 3, 4 and 5 designate suwari gel added with calcium chloride at level of 10, 20, 50, 80 and 120 mmol/kg, respectively.

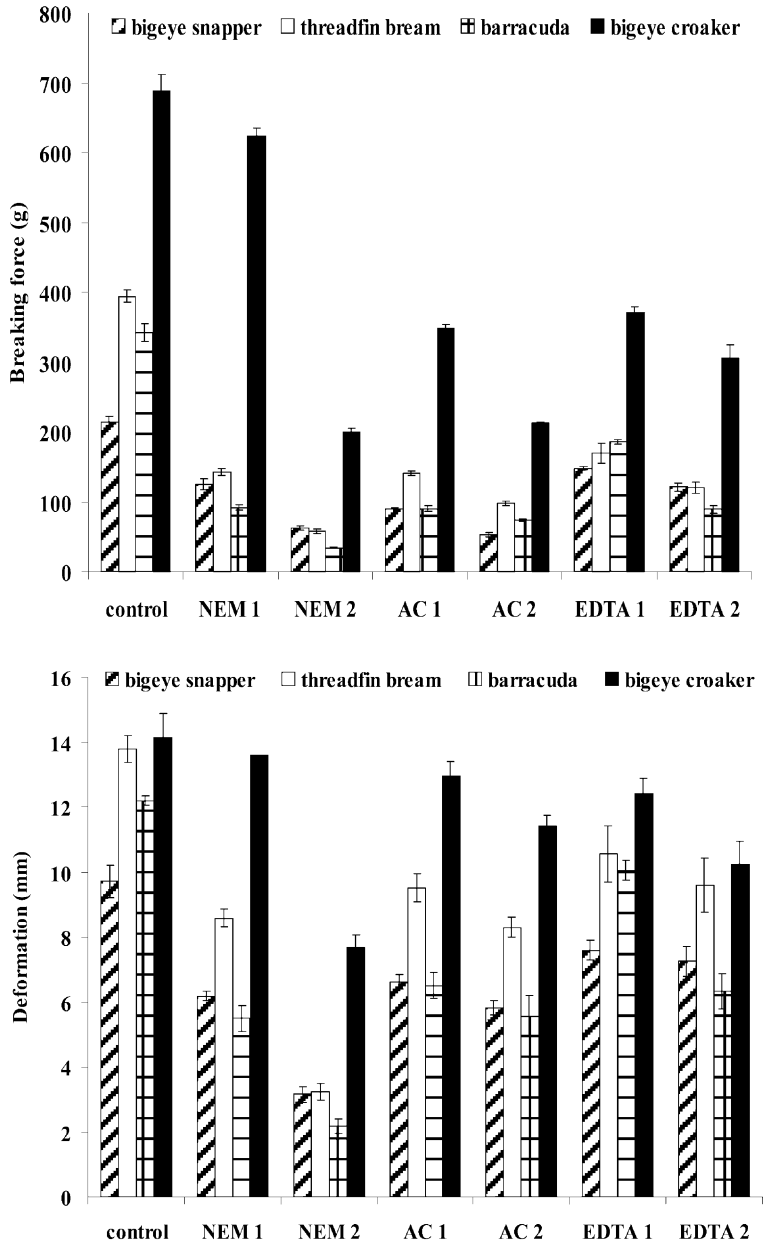

Fig. 3. Force and deformation of suwari gels of surimi from four tropical fish with addition of various transglutaminase inhibitors at different levels. Bars indicate the standard deviation from five determinations.

increased. When NEM at a level of 10 mmol/kg was added, the breaking force of suwari gel from bigeye snapper, threadfin bream, barracuda and bigeye croaker decreased by 71.1, 85.4, 90.1 and 70.9%, compared with the control (without inhibitors), respectively and deformation decreased by 67.5, 76.5, 82.1 and 45.6%, respectively. NEM, a sulfhydryl blocking agent, was presumed to block the reactive sulfhydryl group in the active site of TGase, leading to lower activity (Ashie & Lanier, 2000). Apart from an inhibitory activity of NEM on TGase itself, blocking of sulfhydryl groups in myofibrillar proteins also probably occurred. This would result in the suppression of disulfide bond formation between myosin molecules in the gel matrix and would be associated with the decreased breaking force and deformation (Benjakul & Visessanguan, 2003).

The addition of ammonium chloride resulted in decreased breaking force and deformation of suwari gel of all surimi, particularly at a high concentration ($P<0.05$). With the addition of ammonium chloride at a level of 1 mol/kg, breaking force of suwari gel of surimi from bigeye snapper, threadfin bream, barracuda and bigeye croaker decreased by 57.1, 75.0, 78.7 and 69.1%, compared with the control, respectively and deformation decreased by 31.9, 32.6, 46.1 and 19.2%, respectively. Ammonium chloride suppressed Alaska pollack surimi gel formation by reduction of the ε-(γ-glutamyl) lysine isopeptide (Kumazawa et al., 1995).

Ammonia is generated during the acyl transfer reaction between γ-carboxyamide groups of glutamine residues and primary amines and the excess amount of ammonium ion prevents further progress of the reaction (Ashie & Lanier, 2000; Takagi, Saito, Kikuchi, & Inada, 1986). Therefore, ammonium chloride added was presumed to inhibit TGase activity, resulting in less cross-linking with a concomitant decrease in breaking force and deformation.

At a level of 10 mmol EDTA/kg, breaking force and deformation of suwari gel of surimi from bigeye snapper, threadfin bream, barracuda and croaker decreased by 34.4, 56.8, 73.9 and 55.7%, compared with the control, respectively and deformation decreased by 25.4, 30.4, 48.1 and 20.4%, respectively. From the result, EDTA, effectively suppressed the setting, as shown by decreases in both breaking force and deformation. Fish TGase has been found to be Ca^{2+}-dependent (Ashie & Lanier, 2000). Therefore, reduced availability of calcium ion, by the addition of EDTA, caused the lower activity of TGase, which was associated with decreased gel strength. Kuwazawa et al. (1995) reported that gel formation of Alaska pollack surimi was totally inhibited in the presence of 5 mmol EDTA/kg, while Wan, Kimura, Satake, and Seki (1994) found that no increase in gel strength of walleye pollack surimi occurred without calcium ion.

From our results, all TGase inhibitors showed a detrimental effect on setting of all surimi, by lowering the gel-forming ability of suwari gels, while calcium chloride was shown to increase the setting response of all surimi. Thus, it was inferred that TGase was involved in the setting of all surimi produced from tropical fish.

3.5. Effect of TGase inhibitors on solubility of suwari gel

In the presence of TGase inhibitors, solubility of suwari gel prepared from all surimi increased (Table 2). However, no marked differences in solubility were observed with suwari to which two different levels of TGase inhibitors had been added. This indicated that non-disulfide covalent bonds were formed to a smaller extent when TGase inhibitors were added. Among all surimi, a considerable increase in solubility was found in suwari gel from bigeye croaker surimi grade AA in the presence of TGase inhibitors. TGase activity in bigeye croaker surimi may be most susceptible to those inhibitors added, resulting in a marked decrease in gel strength and a sharp increase in solubility. The ε-(γ-glutamyl) lysine formation in Alaska pollack gel was decreased by the addition of TGase inhibitors (Kumazawa et al., 1995). Therefore, the results reconfirmed that TGase played an important role in non-disulfide covalent bond formation, which strengthened the gel matrix of suwari gels.

3.6. Effect of TGase inhibitors on protein pattern of suwari gels

Protein patterns of suwari gel with and without various TGase inhibitors at different concentrations are shown in Fig. 4. In the presence of TGase inhibitors, MHC was more retained, especially as the concentration of TGase inhibitors increased. This result shows that more MHC remained in the presence of EDTA, when compared with other inhibitors. MHC from threadfin bream and bigeye croaker surimi was almost recovered by the addition of EDTA at a level of 10 mmol/kg. This reconfirmed the importance of calcium ions for cross-linking activity induced by TGase. The result was in accordance with Kuwazawa et al. (1995), who reported that no decrease in MHC was found and ε-(γ-glutamyl) lysine formation was completely suppressed by the addition of EDTA. The differences in MHC recovery with the addition of TGase inhibitors, were presumed to be due to the differences in TGase activity and the different susceptibilities to inhibitors between species. It was noted that no differences in actin band intensities were observed in the presence of all TGase inhibitors tested at different levels. The results

Table 2
Solubility of suwari gels of surimi from some tropical fish with addition of various inhibitors at different concentrations

Inhibitors	Concentration mmol/kg	Solubility (%)[a]			
		Bigeye snapper	Threadfin bream	Barracuda	Bigeye croaker
Control	–	72.19±1.44a[b]	65.37±0.74a	63.15±0.36a	33.73±0.33a
NEM	5 mmol/kg	80.41±0.00cd	77.23±0.89bc	69.85±2.55c	70.02±1.65bc
	10 mmol/kg	81.43±0.36d	78.13±2.95bcd	69.59±1.10c	70.38±0.50c
NH_4Cl	0.5 mol/kg	80.15±0.72c	75.00±0.37b	68.25±0.37b	67.57±0.50b
	1 mol/kg	81.05±0.18b	76.96±2.40bc	69.85±1.82b	69.33±0.33bc
EDTA	5 mmol/kg	77.61±0.36d	79.69±0.06cd	75.39±3.10b	69.09±0.04c
	10 mmol/kg	79.39±0.02cd	81.12±0.18d	77.97±0.54b	70.38±0.50c

[a] Mean±S.D. from triplicate determinations.
[b] Different letters in the same column denote significant differences ($P<0.05$).

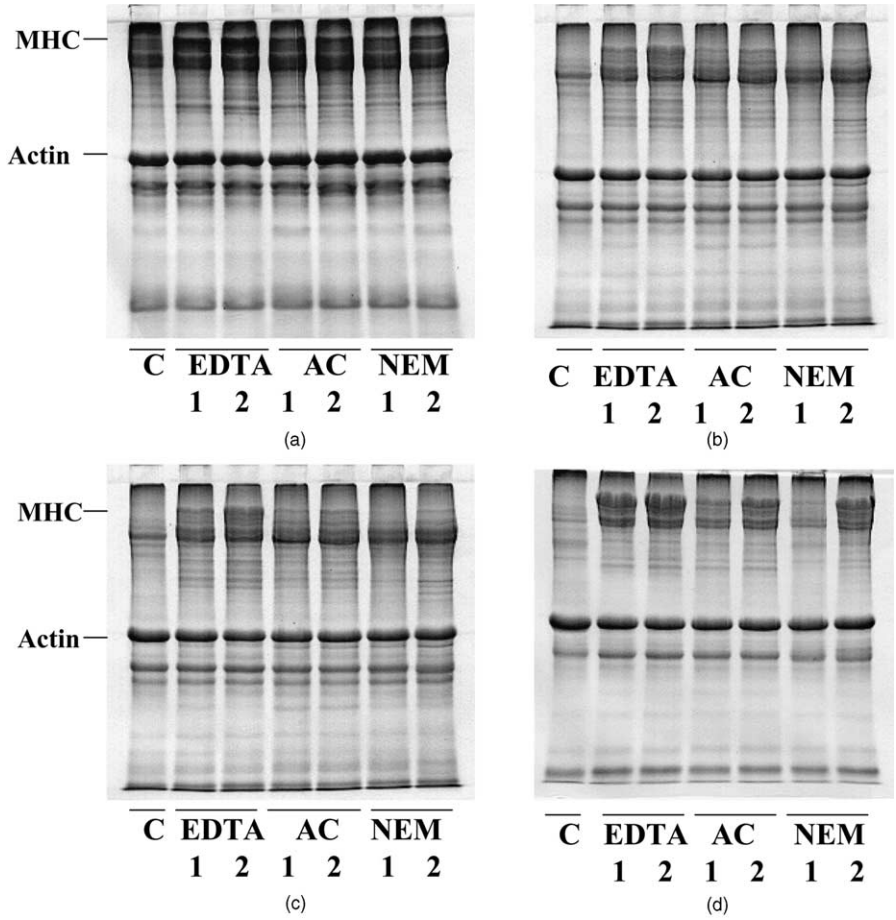

Fig. 4. SDS–PAGE patterns of suwari gels of surimi from bigeye snapper (a), threadfin bream (b), barracuda (c) and bigeye croaker (d) treated with various transglutaminase inhibitors at different levels. C: suwari gel without inhibitors The numbers 1 and 2 designate 5 and 10 mmol/kg for EDTA and NEM and 0.5 and 1 mol/kg for ammonium chloride, respectively.

reflected the contribution of endogenous TGase to cross-linking of myofibrillar proteins, particularly MHC during setting of surimi from tropical fish.

4. Conclusion

High temperature setting of surimi from tropical fish was mediated by endogenous TGase. Suwari gel formation was enhanced by calcium chloride, TGase activator, but was suppressed by EGTA, NEM and ammonium chloride, TGase inhibitors. Thus, setting is a promising means to improve gel quality of surimi from tropical fish, especially by maximising endogenous TGase activity.

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