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ORIGINAL PAPER

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Gel-forming ability of small scale mud carp (*Cirrhiana microlepis*) unwashed and washed mince as related to endogenous proteinases and transglutaminase activities

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Abstract Gel-forming ability of small scale mud carp (*Cirrhiana microlepis*) mince and washed mince was investigated with respect to their proteinase and transglutaminase (TGase) activities. Proteinases in sarcoplasmic fluid showed the optimum activity at 65 °C and pH 9, whereas autolytic activity was maximum at 70 °C and pH 10, indicating the presence of heat-stable alkaline proteinases. When mince was washed with three volumes of water twice, TGase and proteinases were mainly removed in the first washing cycle, resulting in a decreased autolytic activity of washed mince. Breaking force of the single washed mince gels was greater than the twice washed mince and the unwashed mince gels ($p<0.05$). Pre-incubation of mince pastes at 40 °C for 1 h prior to cooking (90 °C/30 min) increased breaking force of all samples, particularly the single washed mince ($p<0.05$). This coincided with an increase of higher molecular weight polymers observed on SDS-PAGE. Washing did not completely eliminate proteinases as it was evident by an increased trichloroacetic acid (TCA)-soluble oligopeptides of washed gels pre-incubated at 55 °C. Whiteness values of washed mince gels were greater than that of mince gels.

Keywords Small scale mud carp · Mince · Proteinase · Transglutaminase · Gel

Introduction

Gelation is one of the important functionalities of fish muscle protein. Endogenous enzymes in fish muscle, namely proteinases and transglutaminase, are important factors that partly govern the gel-forming ability of fish protein. The presence of heat-activated proteinases has detrimental effect on the textural properties of fish protein gel. Degradation of myofibrillar proteins, particularly myosin, limited a proper gel network formation. When proteinases are contained in the sarcoplasmic fraction, they can be effectively eliminated by washing and dewatering as it is typically practiced in surimi processing. However, some proteinases are tightly bound to myofibril, which cannot be removed by washing. In this case, washing removes soluble proteins and concentrate myofibril-bound proteinases, resulting in more severe proteolysis of myofibrillar proteins.

Transglutaminase (TGase) (*R*-glutaminyl-peptide: amine γ-glutamyltransferase; EC 2.3.2.13) is an enzyme catalyzing acyl-transfer reaction, resulting in ε-(γ-glutamyl)lysine cross-links [1]. TGase is present in most mammalian tissues and body fluids and is also found in walleye pollock liver [2], Japanese oyster [3], and muscle of tilapia [4]. TGase activity varied with fish species [5]. Endogenous TGase has been reported to be responsible for "setting" phenomenon of fish protein gels prepared from various species, resulting in an increased gel strength [6]. Although TGase in threadfin bream mince was removed during washing, substantial residual activity remained in surimi, which contributed to isopeptide cross-linking of myosin and improved gel elasticity [7].

Freshwater fish is also an important source of food protein. Total world freshwater fish production excluding salmonoids was estimated to be 7.6 million metric tons in 2003 [8]. Utilization of freshwater fish mince and washed mince is rather limited despite of its large production and low value. Gel-forming ability of surimi made from some freshwater species has been reported [9–11]. However, gelation of freshwater fish mince has not been thoroughly investigated, particularly in relationship to endogenous proteinase and TGase activities. Small scale mud carp (*Cirrhiana microlepis)* is one of the most prevalent aquacultured species with an annual production of 514,662 metric tons worldwide [8]. The objectives of this study were to investigate the changes of endogenous proteinase and TGase activities in small scale mud carp mince as influenced by washing cycle. In addition, to study the effect of setting

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temperature and the number of washing cycles on the gel-forming ability of small scale mud carp mince.

Materials and methods

Samples and chemicals

Fresh small scale mud carps (*Cirrhiana microlepis*; body length: 35.5 ± 1.6 cm; weight: 438.6 ± 59.5 g) were purchased from the freshwater fish wholesale market, Nakhon Ratchasima, Thailand. Fish were about 12 h postmortem. Fish were packed in a polystyrene box, filled with ice, and immediately transported to the Suranaree University laboratory. Sample preparation was done immediately upon arrival.

N–,*N*′-Dimethylated casein (DMC) and monodansylcadaverine (MDC) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Dithiothreitol (DTT) was purchased from Bio Basic Inc. (Markham, Ontario, Canada). Reagents used for gel electrophoresis were obtained from Bio-Rad (Hercules, CA, USA). All other chemicals were of analytical grade.

Preparation of mince and washed mince

Fish was manually beheaded, gutted, and filleted. Fish fillets were ground using a meat grinder (Biro 8-22, Biro Manufacturing, Marblehead, Ohio, USA) with a screen size of 1.5 mm perforation. The washing was performed with a 1:3 (w/w) ratio of mince to cold distilled water (4 °C), and continuously stirred for 5 min. The homogenate was filtered through four layers of cheesecloth, subsequently dewatered using a manual pressing machine, and referred to as the first washed mince. The second washing step was carried out using 0.5% NaCl solution with mince to NaCl solution ratio of 1:3 (w/w). Sample was referred to as the second washed mince after dewatering.

TGase activity

TGase activities of first washed mince, second washed mince and wash water were determined. Mince samples were homogenized in four volumes of extraction buffer (10 mmol l^{-1} NaCl, 5 mmol l^{-1} EDTA, 20 mmol l^{-1} Tris–HCl, pH 8.0). The homogenates were centrifuged at 16,000 × *g* at 4 °C for 20 min (Rotor GSA, Sorvall RC-5C plus, Dupont, Delaware, USA). The supernatants were filtered through glass wool and used as a crude extract. Wash water samples were centrifuged at 16,000 × *g* at 4 °C for 20 min (Rotor GSA, Sorvall RC-5C plus, Dupont, Delaware, USA) and used for activity assay.

TGase activity was assayed by the method of Takagi et al. [12] with slight modifications. The assay mixture contained 1.0 mg ml^{-1} DMC, 15 μmole l^{-1} MDC, 5 mmol l^{-1} $CaCl_2$, 3 mmol l^{-1} DTT, 50 mmol l^{-1} Tris–HCl (pH 8.0), and 100 μl of crude enzyme. After incubation at 37 °C for 10 min, a time period found to be in a linear range from a preliminary study, EDTA solution was added to a final concentration of 20 mmol l^{-1} to stop the reaction. The fluorescence intensity was measured with excitation and emission wavelengths of 350 and 480 nm, respectively, using a Shimadzu spectrofluorometer (RF-1501; Shimadzu Co., Kyoto, Japan). The enhancing factor, indicating the degree of fluorescence enhancement of the dansyl group after incorporation into DMC, was determined from our study to be 1.63. One unit of TGase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of MDC into DMC per minute.

Proteinase activities

Temperature and pH profiles of proteinase activity of wash water were determined by the method of An et al. [13]. For the temperature profile, the reaction mixture containing 1.6 mg ml^{-1} casein, 0.112 mol kg^{-1} Tris–HCl (pH 9.0), and 250 μl wash water was incubated at 40, 50, 60, 65, 70, and 80 °C for 60 min. For the pH profile, casein substrate and wash water with appropriate buffers as described above were incubated at 65 °C, the optimum temperature of the crude enzyme, for 60 min. When the incubation time was attained, 50% trichloroacetic acid (TCA) solution was added to terminate the reaction. TCA-soluble oligopeptides content was determined by Lowry method [14] using tyrosine as a standard. One unit activity was defined as nanomoles of tyrosine released min^{-1}. Specific activity was expressed as the amount of activity per milligrams protein of the crude extract.

Temperature and pH profiles of autolytic activity in mince were determined at varied temperatures and pHs as in wash water. Autolytic activities of first washed and second washed mince were determined according to Yongsawatdigul et al. [15]. Three grams of the sample was incubated at 70 °C, the optimum temperature of autolysis, for 1 h. Subsequently, 5% cold trichloroacetic acid (TCA) solution was added to 27 ml. The mixture was homogenized using an IKA homogenizer (IKA Works Asia, Bhd, Malaysia) and centrifuged at 6000 × *g* (PK 121R, ALC International Srl. Cologna Monzese, Italy) for 15 min at 4 °C. Supernatant was analyzed for oligopeptide content using Lowry's assay method [14] with tyrosine as a standard. Total soluble protein of the samples was determined by solubilizing 3 g of samples in hot 5% SDS solution as detailed by Yongsawatdigul et al. [15]. TCA-soluble oligopeptide content was expressed as nanomoles of tyrosine per milligrams of total soluble protein.

Gel preparation

Mince and washed mince were chopped in a Stephan vacuum cutter (UM5, Stephan Machinery Co., Columbus, Ohio, USA). Sodium chloride and sodium tripolyphosphate were added at 2.2% and 0.3% of the total weight, followed the typical surimi gel preparation. Based on

preliminary results, addition of 0.22% $CaCl_2$ increased gel strength of mud carp mince, therefore, it was added during chopping. The moisture content of all samples was adjusted to 83–84%. The raw paste was stuffed into a 3 cm-diameter casing and pre-incubated at 40, 55, and 65 °C for 1 h prior to heating at 90 °C for 30 min. High setting temperatures were chosen to investigate the effect of both TGase and proteinase activities on textural properties. The control sample was heated at 90 °C for 30 min without pre-incubation. Gels were chilled in ice water and kept in a refrigerator (∼5–8 °C) overnight before analyzing TCA-soluble oligopeptides content as previously described and measuring texture as well as color.

Texture and color measurement

Texture Analyzer (Stable Micro System, Surrey, England) was used to evaluate the textural properties of the gels. Gel samples were cut into pieces of 3 cm length. Breaking force (*g*) and deformation (*mm*) were determined using a 5 mm spherical plunger probe at a test speed of 1 mm/s.

Color of cooked gels was measured using a Chroma Meter (CR 300, Minolta Corp., Osaka, Japan). The instrument was equipped with a D65 illuminant and 2° observer optical position. Color values were expressed using the International Commission on Illumination L^*, a^*, and b^* values. Whiteness was calculated using the equation $L^* - 3b^*$, according to Park [16]. Gels were equilibrated to room temperature before measurement.

SDS-PAGE

Sample solubilization was carried out using 5% SDS solution as detailed by Yongsawatdigul et al. [15]. Thirty micrograms of protein were loaded on to 10% (w/v) polyacrylamide gel according to the method of Laemmli [17]. Gels were run at a constant voltage setting of 120 V. Gels were stained with 0.125% Coomassie Brilliant Blue *R*-250 and destained in a solution containing 25% ethanol and 10% acetic acid.

Statistical analysis

The experiment was repeated twice using two different lots of fish. All enzyme assays were carried out in duplicate for each replication. Statistical analysis of TGase and proteinase activities was carried out using one-way analysis of variance (ANOVA). The effect of washing cycle and setting temperature on textural and color properties was analyzed as a split–split plot. The washing cycle (mince, single-washed mince, and twice-washed mince) was assigned as a split plot factor and the three heating treatments as a split–split plot factor. In each treatment, at least five gel specimens were measured to obtain the average of breaking force, deformation and color values. Duncan's multiple range test (DMRT) was used to determine differences between mean at $p<0.05$. Statistical analysis was performed using Statistical Analysis System (SAS) software Version 6.08 (SAS Institute, Inc, Cary, NC, USA).

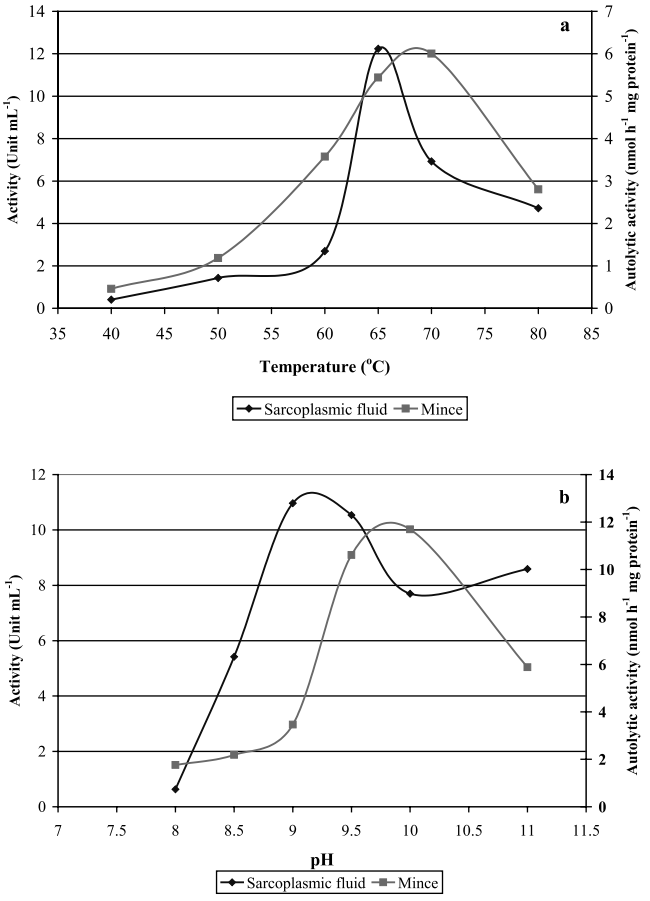

Fig. 1 Temperature **a** and pH **b** profiles of autolytic activity and proteinase activity from sarcoplasmic fluid of small scale mud carp mince

Results and discussion

Endogenous proteinase activities

Crude proteinase from sarcoplasmic fraction exhibited the maximum activity at 65 °C and pH 9.0, while autolytic activity was highest at 70 °C and pH 10.0 (Fig. 1a and b). Based on these results, heat-stable alkaline proteinase(s) appeared to be a predominant proteinase in both sarcoplasmic fluid and mince, and they could be different proteinases. Both acid and alkaline proteinases were found in carp (*Cyprinus carpio*) muscle [18]. Predominant alkaline proteinase in carp showed the optimum activity at 60–65 °C and pH 7.7–8. Kinoshita et al. [19] reported that proteinases in freshwater fish, crucian carp (*Carassius auratus cuvieri*), were tightly bound to myofibril and were not removed by washing. The myofibril-bound proteinase from carp (*Cyprinus carpio*) was purified and characterized as a serine proteinase with an optimum pH and temperature of 8.0 and 55 °C, respectively [20]. The myofibril-bound

Table 1 TGase and autolytic activities of unwashed and washed mince

Sample	TGase activity (unit/mg protein of mince)	Autolytic activity (nmol/h/mg protein of mince)
Mince	0.182 ± 0.022 a	9.252 ± 0.440 a
First washed mince	0.112 ± 0.008 b	4.720 ± 0.791 b
Second washed mince	0.093 ± 0.004 b	5.229 ± 0.217 b

serine proteinase was responsible for degradation of myosin heavy chain and actin of gel pre-incubated at 55–60 °C [21]. Autolytic activity of tilapia mince also was maximum at 65 °C [15]. Optimum temperature found in autolytic activity of small scale mud carp, 70 °C, was higher than those reported in other species, suggesting the presence of highly thermo-stable proteinases. It should be noted that proteinase activities of both sarcoplasmic fluid and mince were minimal at pH 7.0–7.5. Thus, proteolysis of mince during processing would be less problematic.

Effect of washing on TGase and proteinase activity

TGase activity of washed mince was lower than that of unwashed samples ($p<0.05$) (Table 1). Tissue TGase is present in cytosol and is easily removed when tissue is disintegrated and washed with a large amount of water [22]. TGase activity in units per milliliters of wash water from the first cycle was comparable to that of the second cycle ($p>0.05$) whereas specific activity of the former was lower than the latter ($p<0.05$) (Table 2). TGase and other sarcoplasmic proteins were mainly removed during the first washing cycle, resulting in a lower specific activity in the wash water from the first cycle. Removal of TGase by washing was also reported in threadfin bream surimi processing [7].

Washing also removed proteinase as autolytic activity of washed mince was lower than that of mince (Table 1). Proteinases in sarcoplasmic fluid were removed in the first and second washing cycle (Table 2). The second washing cycle did not further reduce autolytic activity in the washed mince. This could be because some proteinases were tightly bound to myofibrillar protein and could not be eliminated by washing. Therefore, extensive washing of small scale mud carp mince did not only eliminate desirable TGase activity but also concentrate myofibril-bound proteinases. Based on our results, a single washing cycle was sufficient to maintain TGase activity and reduce autolytic activity (Table 1).

Textural properties and SDS-PAGE patterns

Moisture content of raw mince was 78.89 ± 1.26% (wet basis), while that of first washed and second washed mince was 84.92 ± 1.18 and 85.72 ± 0.62% (wet basis), respectively. The final moisture content of gels ranged between 82.92–84.06% (wet basis). Washing significantly improved breaking force of small scale mud carp gel. Breaking force values of the first washed mince samples were highest ($p<0.05$), and those of second washed mince gels were higher than mince ($p<0.05$) (Fig. 2a). Pre-incubation at 40 °C resulted in higher breaking force than at 50 °C, while straight cooking at 90 °C showed the lowest values ($p<0.05$). Washing had no effect on the deformation values ($p>0.05$) (Fig. 2b). Deformation of gel was highest when pre-incubated at 40 °C ($p<0.05$), regardless of the number of washing cycles applied. Pre-incubation at 55 °C and straight cooking at 90 °C resulted in comparable deformation ($p>0.05$). Setting temperature appeared to have a greater effect on breaking force than on deformation. Ramirez et al. [23] also reported that setting affected shear stress (breaking force) to a larger extent than shear strain (deformation). Removal of sarcoplasmic proteins during washing resulted in an increased myofibrillar protein concentration. This could, in turn, increase breaking force of washed mince gels. Our results suggested that effective removal of proteinases in small scale mud carp mince could be achieved in a single washing cycle (Table 1). Although washing reduced autolytic activity (Table 1), TCA-soluble oligopeptide content of cooked mince gels were comparable to that of single washed mince gel (Fig. 3). In addition, reduction of MHC between two samples was similar without major degradation products (Fig. 4a and b). These results indicated that proteolysis during gel cooking occurred to a lesser extent than autolytic assay. This could be because incubating temperatures applied in gel setting (40–55 °C) were lower than the optimal autolytic activity (70 °C). Despite of the similar extent of proteolytic degradation, washed mince gels exhibited superior textural properties to the unwashed samples (Fig. 2a). Ko and Hwang [24] also reported that gel strength of the washed milkfish mince was about 1.4–2 times greater than that of mince.

When two washing cycles were applied, breaking force values were greater than those of mince ($p<0.05$), but lower than those of first washed gels. Further washing appeared to decrease breaking force (Fig. 2a). This may not be related to proteinase or TGase activities as TGase activity and TCA-soluble oligopeptide content of these two samples were comparable ($p>0.05$) (Table 1, Fig. 3). Moisture content of first washed mince gel was 82.92 ± 0.04% (wet basis) whereas that of the second washed mince gel was 84.06 ± 0.25% (wet basis). Since breaking force is greatly

Table 2 TGase and proteinase activities in wash water of small scale mud carp mince

Sample	TGase activity		Proteinase activity	
	Unit/ml	Unit/mg protein[a]	Unit/ml	Unit/mg protein[a]
First wash water	7.829 ± 1.199	0.944 ± 0.101	3.674 ± 0.959	0.448 ± 0.136
Second wash water	3.873 ± 0.534	1.409 ± 0.093	2.783 ± 0.472	0.984 ± 0.138

[a]Crude extract

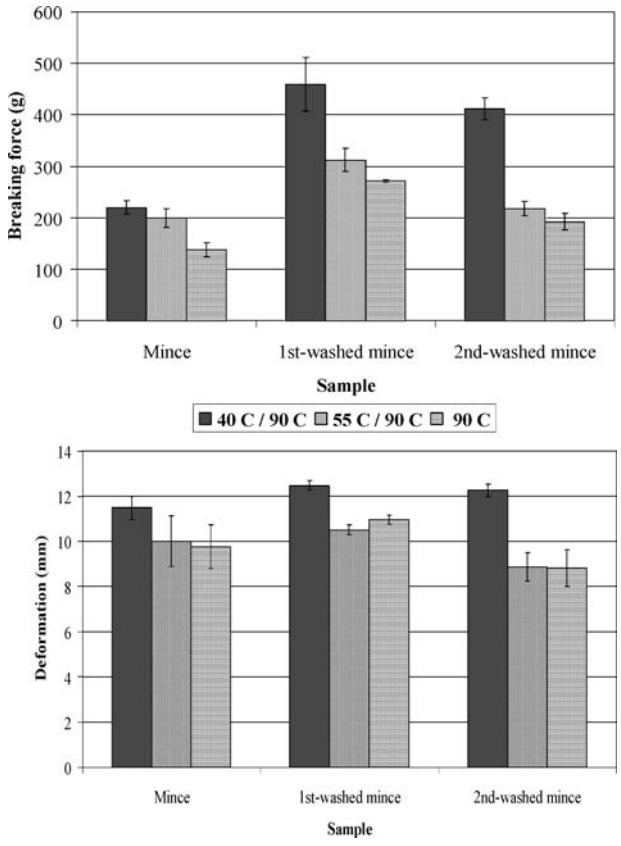

Fig. 2 Breaking force **a** and deformation **b** of mince and washed mince of small scale mud carp pre-incubated at various heating regimes

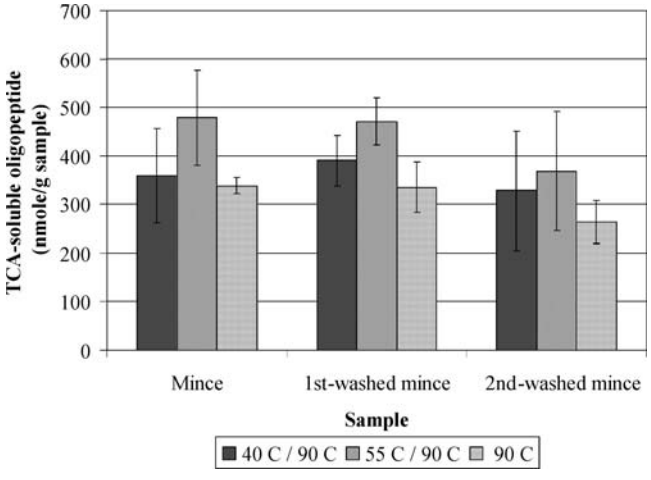

Fig. 3 Oligopeptide content of washed and unwashed mince gels pre-incubated at various temperatures

affected by moisture content, such a slight difference in moisture content could attribute to a lower breaking force value in second washed gel.

Setting temperatures at 40 and 55 °C were chosen in this study because they were lower than the optimum temperature of proteinase (65–70 °C). Pre-incubation at 70 °C resulted in inferior textural properties and therefore was not included in the study. Preliminary study also revealed that

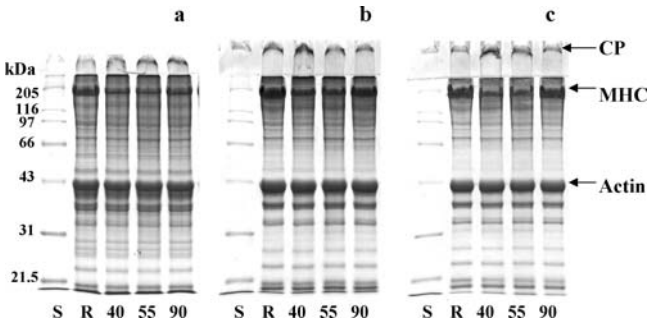

Fig. 4 SDS-PAGE patterns of small scale mud carp gels prepared from mince **a**, first washed mince **b**, and second washed mince **c** pre-incubated at various heating regimes. *R*: raw paste, 40 and 55: samples pre-incubated at 40 and 55 °C, respectively, 90: samples heated at 90 °C without pre-incubation, CP: cross-linked polymers, MHC: myosin heavy chain

low setting temperature (4, 25 °C) did not enhance the textural properties of small scale mud carp mince. This could be because small scale mud carp is a tropical fish species with muscle proteins exhibiting higher thermal stability. Unfolding of muscle proteins to expose reactive groups for TGase was minimal at low temperature setting. Pre-incubation of small scale mud carp gel at 40 °C showed the greatest improvement in textural properties. The effect of setting was more pronounced in the washed samples (Fig. 2a). It was likely due to an increased concentration of myofibrillar proteins. Endogenous TGase activity retained after washing could, therefore, catalyze the cross-linking reaction of myofibrillar protein to a greater extent. TGase in small scale mud carp tissue showed Ca^{2+}-dependent characteristic with the minimum activity in the absence of Ca^{2+} and highest activity at 1.25 mmol kg^{-1} (data not shown). Addition of 0.22% $CaCl_2$ in the mince paste in conjunction with pre-incubation at 40 °C promoted catalytic reaction of TGase by two folds. Firstly, Ca^{2+} and high temperature setting activated endogenous TGase activity. Secondly, these conditions promoted the unfolding of TGase substrate, myofibrillar protein, to expose more available reactive groups of glutamine and lysine [25]. These two combined effects would subsequently promote the cross-linking reaction in small scale mud carp gel.

Gels pre-incubated at 55 °C showed lower breaking force than those pre-incubated at 40 °C, but higher than control (90 °C/30 min) ($p<0.05$) (Fig. 2a). No obvious degradation of myosin heavy chain (MHC) was observed on SDS-PAGE at 55 °C (Fig. 4a–c). These results implied that proteolysis at 55 °C was not severe. Higher molecular weight cross-linked polymers (CP) that could not pass through 4% acrylamide were observed in concomitant with the reduced MHC intensity in the washed samples pre-incubated at either 40 or 55 °C (Fig. 4b and c). Among muscle proteins, TGase preferably catalyzed the cross-linking of MHC [26]. Therefore, CP of samples pre-incubated at either 40 or 55 °C resulted from the action of endogenous TGase. It should be noted that faint intensity of CP was also observed in the raw paste and 90 °C-heated gels, but with relatively high intensity of MHC. It was unlikely that CP of such samples were cross-linked MHC products induced by

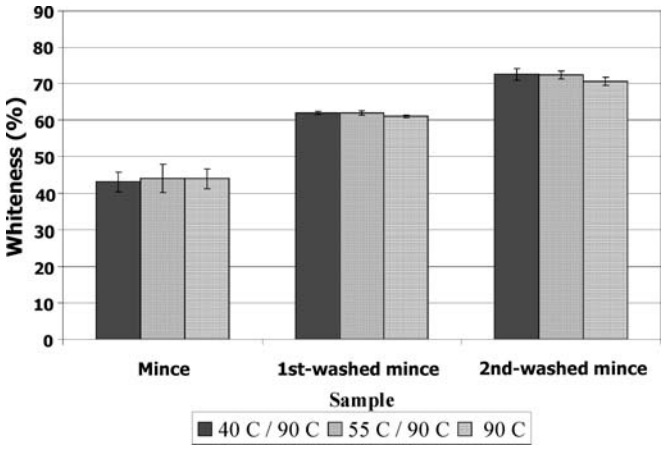

Fig. 5 Effect of washing on whiteness value

TGase. These might be high molecular weight scaffold proteins. Formation of CP might explain why washed mince pre-incubated at 55 °C exhibited reasonably good textural properties in spite of the evidence of proteolysis.

Based on our results, both cross-linking reaction induced by endogenous TGase and proteolysis catalyzed by endogenous proteinases simultaneously occurred during setting. The degree of proteolysis appeared to be less than the extent of cross-linked formation at both 40 and 55 °C. Washing appeared to promote the effect of TGase through reducing proteolytic activity and concentrating myofibrillar protein, a preferred substrate of TGase.

Washing also increased whiteness of small scale mud carp gel ($p<0.05$) (Fig. 5). Washing removed sarcoplasmic proteins, including myoglobin, and to some extent blood residues containing haemoglobin. Setting temperature had no effect on the color values of mince gel ($p>0.05$) as reported by Park [17]. When the whiteness is required, extensive washing is necessary to improve such an attribute.

Conclusions

The first washing cycle largely eliminated TGase and proteinases contained in sarcoplasmic fraction. Both cross-linking reaction catalyzed by endogenous TGase and proteolysis of muscle protein mediated by proteinases occurred simultaneously during setting. But the extent of the former was greater than the latter at 40 and 55 °C, resulting in an improved breaking force. The single washed mince of small scale mud carp exhibited the highest breaking force when pre-incubated at 40 °C. In addition, washing increased whiteness of mince gel.

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References

1. Folk JE (1980) Ann Rev Biochem 49:517–531
2. Kumazawa Y, Nakanishi K, Yasueda H, Motoki M (1996) Fish Sci 62:959–964
3. Kumazawa Y, Nakanishi K, Yasueda H, Motoki M (1997) J Agric Food Chem 45:604–610
4. Worratao A, Yongsawatdigul J (2005) Food Chem 93:651–658
5. Worratao A, Yongsawatdigul J (2003) J Food Biochem 27:35–51
6. Lanier TC, Carvajal P, Yongsawatdigul J (2005) Surimi gelation chemistry. In: Park JW (ed) Surimi and Surimi Seafood, 2nd edn. CRC Taylor&Francis, Bacon Raton, Florida, pp 435–489
7. Yongsawatdigul J, Worratao A, Park J (2002) J Food Sci 67:3258–3263
8. FAO Fisheries Department (2003) FAO Yearbook of Summary Statistics: Summary Table, FAO, Italy
9. Luo YK, Pan DD, Ji BP (2004) J Food Sci 69:E374–E378
10. Luo YK, Kuwahara R, Kaneniwa M, Murata Y, Yokoyama M (2001) J Food Sci 66:548–554
11. Lin D, Morrissey MT (1995) J Food Sci 60:1245–1247, 1253
12. Takagi J, Saito Y, Kikuchi T, Inada Y (1986) Anal Biochem 153:295–298
13. An H, Seymour T, Wu J, Morrissey MT (1994) J Food Sci 59:277–281
14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) J Biol Chem 193:265–275
15. Yongsawatdigul J, Park JW, Virulhakul P, Viratchakul S (2000) J Food Sci 65:129–133
16. Park JW (1995) J Food Sci 60:15–18
17. Laemmli UK (1970) Nature 227:680–685
18. Makidonan Y, Toyohara H, Ikeda S (1984) Comp Biochem Physiol 79B:129–134
19. Kinoshita M, Toyohara H, Shimizu Y (1990) Nippon Suisan Gakkaishi 56:1485–1492
20. Osatomi K, Sasai H, Cao MJ, Hara K, Ishiraha T (1997) Comp Biochem Physiol 116B:183–190
21. Cao MJ, Hara K, Osatomi K, Tachibana K, Isumi T, Ishihara T (1999) J Food Sci 64:644–647
22. Kimura I, Sugimoto M, Toyoda K, Seki N, Arai K, Fujita T (1991) Nippon Suisan Gakkaishi 57:1389–1396
23. Ramirez JA, Rodriguez-Sosa R, Morales OG, Vazquez M (2003) Food Chem 82:417–423
24. Ko WC, Hwang MS (1995) Fish Sci 61:75–78
25. Hemung B, Yongsawatdigul J (2005) J Food Sci 70:C455–460
26. Takeda H, Seki N (1996) Fish Sci 62:462–467