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Effects of nano-scaled fish bone on the gelation properties of Alaska pollock surimi

Tao Yin[a, b]

Jae W. Park[b, *]

Jae.Park@oregonstate.edu

[a]College of Food Science and Technology, Huazhong Agricultural University, Wuhan, Hubei Province 430070, PR China

[b]Oregon State University Seafood Research and Education Center, 2001 Marine Drive #253, Astoria, OR 97103, United States

*Corresponding author. Tel.: +1 503 325 4531; fax: +1 503 325 2753.

Abstract

Gelation properties of Alaska pollock surimi as affected by addition of nano-scaled fish bone (NFB) at different levels (0%, 0.1%, 0.25%, 0.5%, 1% and 2%) were investigated. Breaking force and penetration distance of surimi gels after setting increased significantly as NFB concentration increased up to 1%. The first peak temperature and value of storage modulus (*G*′), which is known to relate to the unfolding and aggregation of light meromyosin, increased as NFB concentration increased. In addition, 1% NFB treatment demonstrated the highest *G*′ after gelation was completed. The activity of endogenous transglutaminase (TGase) in Alaska pollock surimi increased as NFB calcium concentration increased. The intensity of myosin heavy chain cross-links also increased as NFB concentration increased indicating the formation of more ε-(γ-glutamyl) lysine covalent bond by endogenous TGase and calcium ions from NFB.

Keywords: Surimi; Gelation; Nano-scaled fish bone; Transglutaminase; SDS–PAGE

1 Introduction

Surimi is an intermediate product produced from fish through heading, gutting, mincing, washing, dewatering, refining and blending with cryoprotectants. Gelation of protein is an important step in forming desired texture in many surimi-based products. Heat-induced gelation of surimi is a complex physicochemical process involving structural and functional changes of myofibrillar proteins. During gelation of salted surimi pastes, proteins unfold, exposing the functional group imbedded inside of myosin. These functional groups are subsequently involved in the formation of intra and intermolecular bonds during the aggregation process to form a three-dimensional network.

Four main types of chemical bonds are involved in protein gelation: (1) hydrogen bonds, (2) ionic linkages, (3) hydrophobic interactions, and (4) covalent bonds (Lanier, Carvajal, & Yongsawatdigul, 2005). The formation of intermolecular hydrophobic interactions among proteins, in addition to disulfide bonding that occurs during heating, is presently thought to be a primary mechanism of forming surimi gel (Gilleland, Lanier, & Hamann, 1997; Lanier et al., 2005).

Surimi subjected to incubation at lower temperature, from 5 to 40 °C, depending on species, for a period of time followed by heating at higher temperature (90 °C), can form a strong gel. The low temperature incubating process is called setting or suwari. It is generally accepted that endogenous transglutaminase (TGase) is responsible for inducing the setting effect. TGase (protein-glutamine γ-glutamyltransferase, EC 2.3.2.13) is capable of catalysing the acyl transfer reaction between the γ-carboxyl amide groups of glutamine and ε-amino groups of lysine, resulting in protein polymers via ε-(γ-glutamyl) lysine cross-links (Folk, 1980).

As endogenous TGase is a Ca^{2+}-dependent enzyme, addition of calcium compounds to surimi paste has been reported to activate TGase activity, and thus improve texture of surim gel (Lee & Park, 1998; Yongsawatdigul, Worratao, & Park, 2002). In addition to being endogenous TGase activator, Ca^{2+} has also been found to destroy the α-helical structure of myosin leading to enhancement of hydrophobic interactions and improvement of textural properties (Yongsawatdigul & Sinsuwan, 2007). Since Ca^{2+} is a divalent cation, a salt bridge might form between calcium and the negatively charged group of protein molecules, which contributes to gel texture improvement (Arfat & Benjakul, 2012).

Along with calcium chloride, calcium carbonate and calcium phosphate with relative lower solubility were reported to improve surimi gel texture when added at certain concentrations (Benjakul, Visessanguan, & Kwalumtharn, 2004; Lee & Park, 1998). Fish bone is rich in calcium, which is mainly in the form of hydroxyapatite (HA), and thus might possess the potential to be used as a functional ingredient in surimi products. However, the addition of fish bone powder in a large particle size (>600 μm) was not effective, according to our preliminary study, because large particles possibly disconnected protein–protein gel networks. Processing fish bone particles to a much smaller, nano scale could allow fish bone particles to be imbedded into fish myofibrillar protein gel networks without sacrificing gelling ability. However, effects of fish bone at the nano scale on gelation properties of surimi have not been investigated.

Captured fisheries and aquaculture supplied the world with about 148 million tons of fish in 2010, of which over 20 million tonnes was discarded as processing waste (FAOSTAT, 2012). Fish bone is the main solid in this waste portion, accounting for 10–15% of fish weight and is currently utilised as bone meal for animal feed (Kim & Mendis, 2006), which possesses a low economic value. Upgrading fish bone to be used in surimi seafood for calcium enrichment and gel texture enhancement would make our fisheries environmentally more sustainable and provide additional value for fish bone use in human food.

Our objectives were to evaluate the role of nano-scaled fish bone (NFB) to improve the gelling properties of surimi while achieving calcium enhancement.

2 Material and methods

2.1 Materials

Alaska pollock surimi (FA grade), approximately 2–3 month frozen, was obtained from American Seafoods (Seattle, WA, USA). Surimi was cut into about 1000 g blocks, vacuum-packaged, and stored in a freezer (–18 °C) throughout the experiments. Dried fish bone made from Pacific whiting was obtained from Trident Seafoods (Newport, OR, USA). Fish bone was soaked in alkaline solution (pH 12) for 2 h to remove myofibrillar proteins and rinsed with tap water three times before being dried in an oven (105 °C) over night. Dried fish bone was further processed to a nano-scaled fish bone emulsion by Custom Processing Services (Reading, PA, USA) using a wet mill (Labstar, Netzsch Premier Technologies, Co., Exton, PA, USA). D_{50} of the fish bone particle in the emulsion was 280 nm (Fig. 1). Calcium concentration of the emulsion was 32.4 mg/g, which was analysed by Universal Testing Company (Quincy, IL, USA). The moisture content of the emulsion was 87.47% (AOAC, 2000).

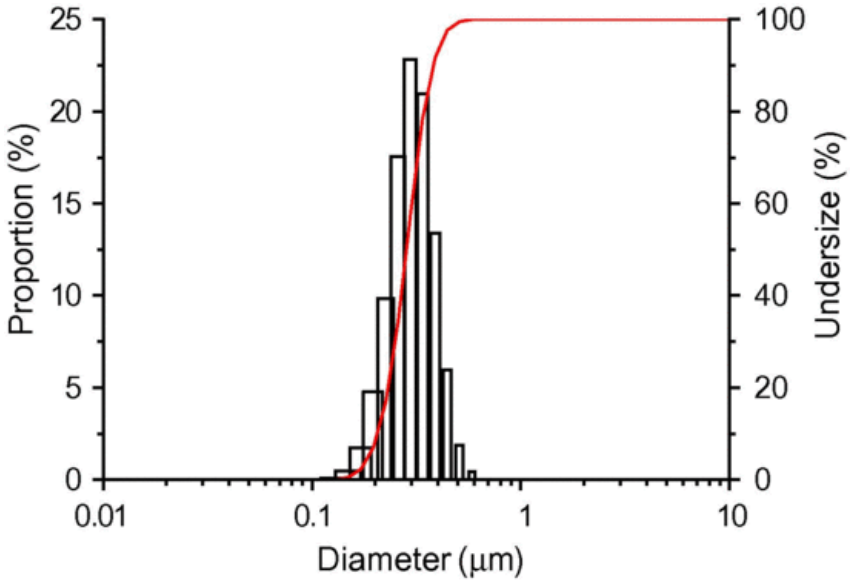

Fig. 1 Size distribution of fish bone particle in the emulsion.

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

2.2 Gel preparation

Frozen surimi was tempered at room temperature for 1 h before being cut into about 3 cm cubes. Surimi cubes were chopped at 1800 rpm for 1 min using a silent cutter (UM 5 universal, Stephan Machinery Corp, Columbus, OH, USA). Sodium chloride (2%) was added, and the surimi was chopped at 1800 rpm for 1 min. Nano-scaled fish bone emulsion, at 0%, 0.1%, 0.25%, 0.5%, 1.0%, and 2.0% of dried nano-scaled fish bone, was added into the salted surimi. Moisture content was adjusted to 77% using ice water (0 °C) and sugar before chopping at 1800 rpm for another 1 min. Sugar was added as an inert ingredient to substitute salt. For the final 3 min, chopping continued at 3600 rpm while a vacuum was maintained at 0.5–0.6 bar. During chopping, cold temperature (<5 °C) was maintained continuously using a NesLab chiller (NesLab, Portsmouth, NH, USA). The paste prepared above was packed into a polyethylene bag and subjected to a vacuum machine (Reiser VM-4142; Roescher Werke, Osnabrueck, Germany) to remove air pockets. The paste was stuffed into a nylon tube (Nylatron MC 907, Quadrant Engineering Plastic Products, Reading, PA, USA) with a 3 cm inner diameter and approximately 15 cm length using a sausage stuffer (The

Sausage Maker, Buffalo, NY, USA). The samples were heated with two different thermal treatments: (1) 90 °C for 30 min; (2) 25 °C for 3 h setting (pre-incubation) followed by 90 °C heating for 30 min. Gels were submerged in ice water for 15 min after cooking, and stored overnight in a refrigerator (4 °C).

2.3 Fracture gel evaluation

Fracture gel evaluation was performed using a TA-XT texture analyser (Stable Micro Systems, Surrey, UK) equipped with a spherical plunger (diameter 5 mm, crosshead speed of 60 mm/min). Cold gels (4 °C) were placed at room temperature for 2 h prior to gel testing. Cylinder-shaped samples (2.5 cm long) were prepared and subjected to fracture by penetration. Breaking force (g), indicating gel strength, and penetration distance (mm), denoting deformability, were recorded.

2.4 Oscillatory dynamic measurement

Surimi paste was subjected to a temperature sweep to monitor heat-induced gelation using a CVO rheometer (Malvern Instruments Ltd., Worcestershire, UK). Samples were applied between cone (4° and 40 mm diameter) and plate with a gap of 150 μm. Samples were covered by a trapper with moistened sponge to avoid sample drying out during heating. Samples was subjected to temperature sweep (20–90 °C) at a heating rate of 2 °C/min, a fixed frequency of 0.1 Hz, and shear stress of 100 Pa found in the linear viscoelastic region through shear stress sweep.

2.5 Transglutaminase (TGase) activity

TGase activity was assayed by the method of Yongsawatdigul et al. (2002) with slight modifications. Surimi sample (5 g) was homogenised in 4 volumes of extraction buffer (10 mM NaCl and 10 mM Tris–HCl, pH 7.5). The homogenate was centrifuged at 16,000*g* (Sorvall, DuPont Co., Newton, CT, USA) at 4 °C for 30 min. The supernatant was used as crude extract. The assay mixture consisted of 1.0 mg/ml N,N′-dimethylated casein (DMC), 15 μM monodansylcadaverine (MDC), 3 mM dithiothreitol (DTT), and 50 mM Tris–HCl (pH 7.5). Fish bone emulsion or $CaCl_2$ solution was added into the mixture and vortexed immediately. Two levels of $CaCl_2$ (0.025 and 0.25 mM) were introduced to compare their effectiveness with the calcium released from NFB. The mixture was incubated at 25 °C for 5 min. One hundred microliters of crude enzyme were added and further incubated at 25 °C for 10 min. After incubation, EDTA solution was added to a final concentration of 20 mM 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). One unit of TGase activity was defined as the amount of enzyme that catalysed the incorporation of 1 nmol of MDC into DMC per min. TGase activity was expressed as unit/ml extract.

2.6 SDS–PAGE

Protein patterns of all samples were studied using SDS–PAGE described by Laemmli (1970). Solubilisation of gel samples with 5% sodium dodecyl sulfate solution (90 °C) was carried out according to Morrissey, Wu, Lin, and An (1993). Stacking and separating gels were made using 4% (w/v) and 10% (w/v) acrylamide, respectively. Gels were fixed and stained with 0.125% Coomassie brilliant blue R-250 (Bio-Rad, Richmond, CA, USA), and destained in a solution containing 50% methanol and 10% acetic acid. The molecular weight of the protein bands were determined by comparing the relative mobility of a protein standard (Kaleidoscope precision plus, Bio-Rad Laboratories, Hercules, CA, USA).

To investigate the polymerisation of myosin heavy chain in surimi gels, the continuous SDS–PAGE system described by Weber and Osborn (1969) was used. Samples were prepared according to Kamath, Lanier, Foegeding, and Hamann (1992). The surimi gel samples were cut into small pieces (0.4 g) and solubilised in 7.5 ml of a buffer made of 2% SDS-8 M urea, 2% β-mercaptoethanol, and 20 mM Tris–HCl (pH 8.0). The samples were heated at 100 °C for 2 min and stirred continuously for 24 h at room temperature (~25 °C). Homogenates were centrifuged at 10,000*g* (Sorvall, DuPont Co., Newton, CT, USA) at room temperature for 20 min. The protein concentration of supernatants was measured by the method described by Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (Sigma Chemical Co., St. Louis, MO, USA) as a standard. Gels were prepared using 3% (w/v) polyacrylamide and 6 M urea. GelBond PAG film (FMC Bioproducts, Rockland, ME, USA) was used to support the polyacrylamide gel system. Phosphate buffer (0.06 M NaH_2PO_4, 0.14 M Na_2HPO_4, 0.002% (w/v) SDS, pH 7.0) was diluted with water (1:2) and used as a running buffer. A constant current of 80 mA was applied. Gels were fixed and stained with 0.125% Coomassie brilliant blue R-250 (Bio-Rad, Richmond, CA, USA), and destained in a solution containing 50% methanol and 10% acetic acid.

2.7 Statistical analysis

A split plot design was applied to evaluate effects of nano-scaled fish bone (NFB) on gelation properties of Alaska pollock surimi. The six levels of emulsion corresponding to 0%, 0.1%, 0.25%, 5%, 1% and 2% of dried NFB were assigned as a main plot factor. Analysis of variance (ANOVA) was conducted using the SAS program (SAS Institute Inc., Carry, NC, USA). Differences among mean values were established using the Duncan multiple range test (DMRT) at $p < 0.05$.

3 Results and discussion

3.1 Gel texture evaluation

Effects of NFB added at different concentrations on the breaking force and penetration distance of the surimi gels with and without setting are illustrated in Fig. 2. A marked increase in the breaking force and penetration distance of Alaska pollock

surimi gel was observed after setting ($p < 0.05$). Endogenous enzyme was proven to play an important role in determining the textural property of surimi gel with setting (Kamath et al., 1992; Yongsawatdigul et al., 2002). It is generally accepted that endogenous transglutaminase (TGase) is responsible for inducing the setting effect. During setting, endogenous TGase catalyses the formation of ε-(γ-glutamyl) lysine cross-links among actomyosin molecules, resulting in gel texture improvement (Yongsawatdigul et al., 2002). There are two contradictory phenomena (endogenous protease-induced degradation vs. endogenous TGase induced-aggregation of myosin) affecting gel texture during setting depending on time and temperature (Yongsawatdigul, Park, Virulhakul, & Viratchakul, 2000). Depending on which process dominates at a given setting condition, weaker or stronger gels can be formed, respectively. In general, protease activity in Alaska pollock was low (Yongsawatdigul & Park, 1996), while its TGase activity and reactivity were reported as high (Araki & Seki, 1993). Thus, the texture of gels made from high grade pollock surimi was greatly improved after setting. Additionally, gradual alignment and aggregation via hydrophobic interactions and disulfide bonds during setting could have contributed to a stronger gel compared to directly cooked gel (90 °C for 30 min) (Chan, Gill, Thomphson, & Singer, 1995). Heat-denatured proteins align in an ordered fashion to form a fine gel network when aggregation is slow with respect to denaturation (Yongsawatdigul et al., 2000).

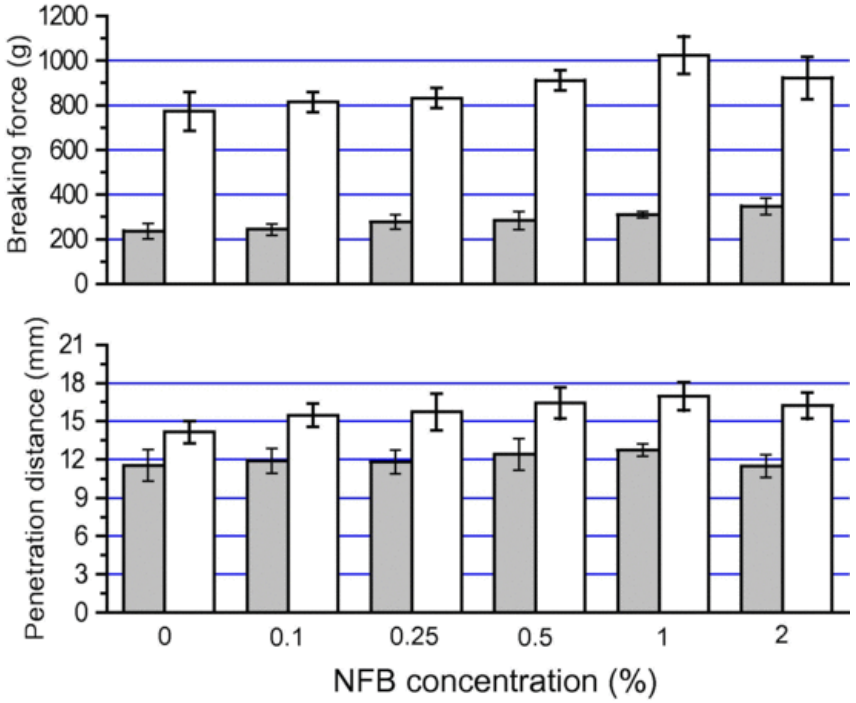

Fig. 2 Breaking force and penetration distance of Alaska pollock with different NFB concentration.

: Gels cooked at 90 °C for 30 min.

: Gels incubated at 25 °C for 3 h followed by heating at 90 °C for 30 min.

Breaking force and penetration distance of gels with setting increased as the concentration of NFB ($p < 0.05$) increased up to 1%. This improvement is probably due to the role of endogenous TGase, which is Ca^{2+}-dependent and activated as NFB concentration increased. The addition of 1% NFB (corresponding to ~0.3% calcium) resulted in increased breaking force and penetration distance of Alaska pollock surimi gel by approximately 25% and 14%, respectively. The results were similar to the report by Lee and Park (1998), which indicated that shear stress of Alaska pollock gel increased by 34% after the addition of 0.2% calcium carbonate. Benjakul et al. (2004) also reported that breaking force and penetration distance of bigeye snapper surimi gel increased significantly with the addition of calcium carbonate ranging from 0.5 to 1.5%. According to Hemung and Yongsawatdigul (2005) and Yongsawatdigul and Sinsuwan (2007), Ca^{2+} ion, in addition to being a TGase activator, induced the unfolding of myosin at 10–100 mM as evidenced by the increase of surface hydrophobicity and loss of α-helical structure. As a result, more exposure of those reactive residues was reported to favour a TGase mediated reaction, which improves gel texture.

Breaking force of surimi gel without setting increased slightly with NFB ($p < 0.05$). However, no significant changes in penetration distance were observed. Since cross-linking reaction catalysed by TGase occurred to a limited extent in gel without setting, the enhancement of breaking force was caused somewhat more by forming a 'salt bridge' between proteins. As was postulated, myofibrillar proteins carry an overall net negative charge at the normal pH of surimi. Calcium ions, having a divalent positive charge (Ca^{2+}), can thus form ionic linkages between negatively charged residues on two adjacent proteins (Lanier et al., 2005). Divalent metal ions were also reported to cause changes in protein conformation, leading to enhancement of hydrophobic interactions and improvement in textural properties (Arfat & Benjakul, 2012).

3.2 Oscillatory dynamic rheology

Changes in rheological properties of Alaska pollock surimi with different NFB concentrations during temperature sweep are shown in Fig. 3. Of the rheological parameters assessed, storage modulus (G') was used to evaluate gel formation. An increase in storage modulus (G'), which represents energy recovered per cycle of sinusoidal shear deformation, indicated an increase in rigidity of the sample associated with the formation of an elastic gel structure (Egelandsdal, Martinsen, & Autio, 1995). G' of Alaska pollock surimi paste started to increase at 33.4 °C and reached the first peak between 37 and 40 °C. This formation was suggested to relate to the unfolding and cross-linking of light meromyosin chain (LMM) (Reed & Park, 2011). In addition, the shift of the first peak temperature to slightly higher temperatures was noted as indicated by T_2 (Fig. 3).

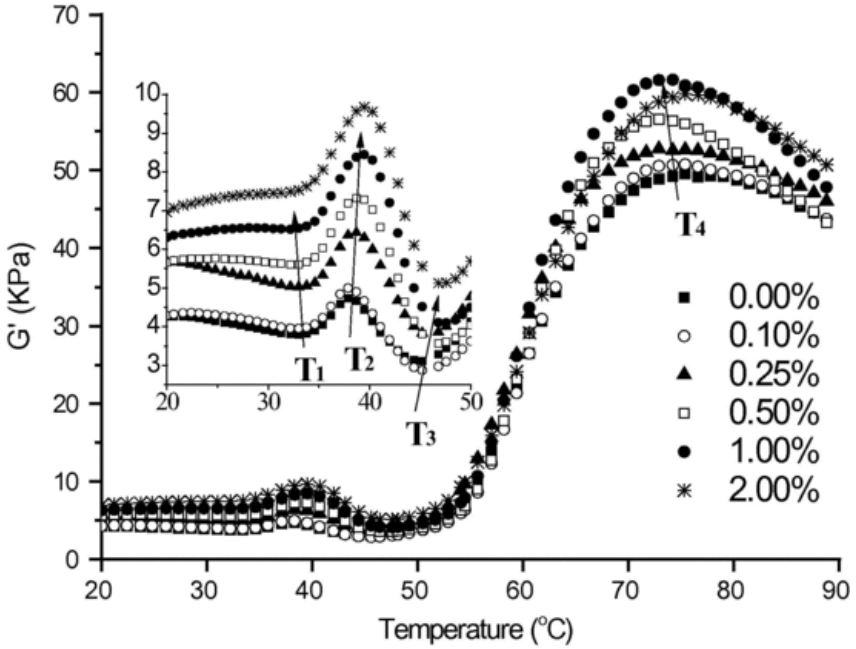

Fig. 3 Storage modulus of Alaska pollock surimi paste with different NFB concentrations (%). T_1–T_4 denotes the transition points during temperature sweeps.

G' at the first peaks increased as NFB concentration increased, which might be related to the formation of more intermolecular 'salt bridge' by Ca^{2+} between partially dissolved and unfolded protein molecules after chopping ((Bryant & McClements, 2000). LMM is made up of the α-helical coiled-coil tail. The content of α-helical from myosin were reported to decrease markedly during setting with increased Ca^{2+} concentration (Hemung & Yongsawatdigul, 2005; Yongsawatdigul & Sinsuwan, 2007). Thus, during the constant temperature rise, more LMM of Alaska pollock was disassociated as NFB concentration increased and LMM was simultaneously aggregated among intermolecular proteins, resulting in an increase of G'. In addition, increasing NFB concentration resulted in an increase of endogenous TGase activity, which caused the formation of more covalent cross-links of LMM, thus contributing to an increase in G'.

The slightly decreased G' between 39 and 47 °C was postulated to be due to the helix-to-coil transformation of myosin, which leads to a large increase in fluidity of the semi-gel and may disrupt some of the protein network already formed (Sano, Noguchi, Tsuchiya, & Matsumoto, 1988). The onset of the second peak in G' was noticed at 44–47 °C. G' started to rise constantly and reached its maximum value at 73–75 °C. This maximum was influenced by both an increase in the number of cross-links between protein aggregates and a deposition of additional denatured proteins in the existing protein networks to strengthen the gel matrix (Xiong, 1997). Normally the higher G', at the point where gelation is completed, determines the higher gel strength. Surimi gel with 1% NFB showed the highest G' (Fig. 3), which was consistent with breaking force of the surimi gel (Fig. 2).

3.3 TGase activity induced by NFB

Effect of NFB on endogenous TGase activity in Alaska pollock surimi was monitored by means of the incorporation MDC into DMC (Fig. 4). Activities of crude TGase extract incubated in assay with 0, 0.1, 0.25, 0.5 1 and 2 mM NFB calcium were 7.5, 8.3, 8.9, 11.8, 14.9 and 17.1 unit/ml, respectively, while activities of crude TGase extract incubated in assay with 0.025 and 0.25 mM $CaCl_2$ were 14.1 and 28.1 unit/ml, respectively. Active site region of TGase includes a catalytic triad of Cys-His-Asp or Cys-His-Asn. During the acyl-transfer reaction, thiol from cysteine reacts with a glutamine side chain of a protein or peptide substrate to form a thioester intermediate from which the acyl group is transferred to an amine substrate (Folk, 1980). The catalytic cysteine was reported to be located in a hydrophobic tunnel, bridged by two tryptophan residues. Glutamine accesses the catalytic cysteine from one side of the active-site channel, lysine approaches the catalytic cysteine from the other side and keeps the lysyl ε-amino group in the hydrophobic channel (Pinkas, Strop, Brunger, & Khosla, 2007). It was postulated that calcium ion activates endogenous TGase by inducing the conformational changes of the enzyme, which consequently exposes two key tryptophan residues that control substrate access to the active site (Nozawa, Cho, & Seki, 2001).

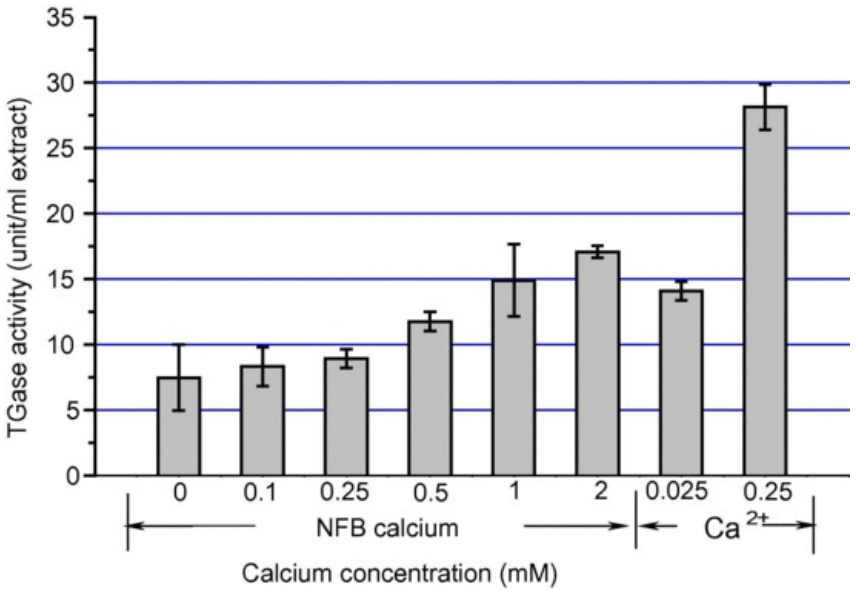

Fig. 4 Effects of calcium from NFB and $CaCl_2$ on the activity of endogenous TGase from Alaska pollock surimi. NFB calcium (mM) was calculated based on 32.4 mg/g emulsion. Ca^{2+} 0.025 and 0.25 mM were made using $CaCl_2$.

The results clearly demonstrated the remarkable increase of TGase activity with NFB concentration was related to activation of TGase by an increased concentration of calcium ions. As shown in Fig. 4, TGase activity of surimi with NFB calcium was much lower than that with calcium chloride at the same calcium concentration. This was possibly due to NFB calcium's lower solubility compared to calcium chloride. Thus, a much higher calcium concentration of NFB was needed to provide sufficient calcium ion for inducing endogenous TGase.

The Ca^{2+} concentration for full activation of endogenous TGase varies with fish species used for surimi processing. It was reported that optimal Ca^{2+} concentrations for crude TGase extract from carp, threadfin bream, and tilapia were at 5, 5, and 1.25 mM, respectively (Kishi, Nozawa, & Seki, 1991; Piyadhammaviboon & Yongsawatdigul, 2009; Worratao & Yongsawatdigul, 2005). However, Benjakul, Visessanguan, and Kwalumtharn (2004) reported the optimal Ca^{2+} concentrations for purified TGase from carp and tilapia were at 50 and 20 mM, respectively. Thus, optimal Ca^{2+} concentration for inducing TGase is also dependent on TGase purity.

3.4 Covalent cross-linking induced by NFB

In order to clarify the contribution of NFB concentration to the cross-linking of myosin heavy chains (MHC) during gel formation, surimi gels were analysed using SDS–PAGE. Comparing to gel without setting (Fig. 5a), myosin heavy chain of Alaska pollock surimi gel markedly disappeared after setting (Fig. 5b). Gradual reduction of MHC from the gel with setting was observed as NFB concentration increased, while that of gel without setting was not significant. However there was a trend of slight, but gradual reduction in the density of MHC (Fig. 5a), probably due to the fact that 30 min heating at 90 °C for a sample with 3 cm diameter still induces slow heating. Decreased MHC corresponded well with an increase of gel texture (Fig. 3). This emphasised the importance of myosin as a major component responsible for gel-forming ability of surimi.

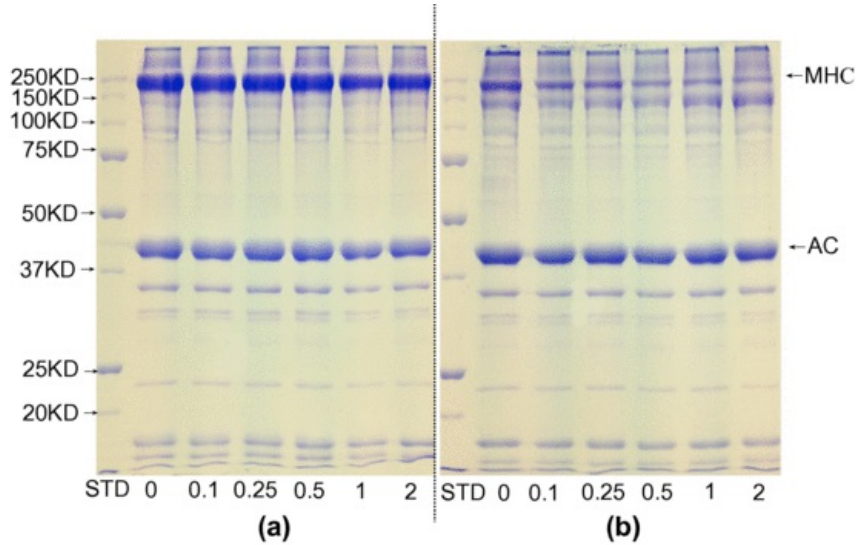

Fig. 5 SDS–PAGE patterns of Alaska pollock surimi gel without (a) and with (b) setting. Samples were applied to 10% polyacrylamide gels at 20 μg protein/lane. Numbers designate NFB concentration (%). STD = Kaleidoscope protein standard, MHC = myosin heavy chain, AC = actin.

MHC cross-links of surimi gel with NFB were also investigated on 3% acrylamide gels (Fig. 6). As the intensity of MHC band gradually decreased up to 1% NFB, the combined intensity of cross-links (MHCXL) and myosin heavy chain dimer (MHC_2) noticeably increased (Fig. 6b).

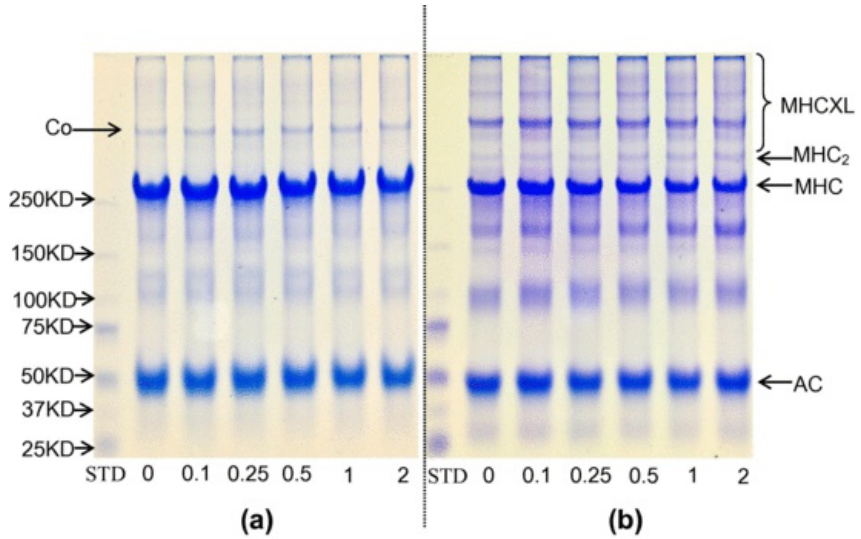

Fig. 6 SDS–PAGE patterns of Alaska pollock surimi gels without (a) and with (b) setting. Samples were applied to 3% polyacrylamide gels at 50 μg protein/lane. Numbers designate fish bone concentration (%). STD = Kaleidoscope protein standard, MHCXL = cross-links of myosin heavy chain, MHC = myosin heavy chain, MHC_2 = myosin heavy chain dimer, AC = actin, Co = β-connectin.

4 Conclusions

Gelation properties of Alaska pollock surimi with setting were significantly affected by NFB. Both LMM and MHC were aggregated to a larger extent as NFB concentration increased. Gel texture was improved with up to 1% NFB

primarily by the function of calcium released from NFB and endogenous transglutaminase. In addition, the size of bone particles made a positive contribution to gel texture: Nano-scaled particles were too small to disconnect the myofibrillar protein gel network. Addition of NFB into surimi seafood will contribute to both calcium enrichment and gel texture enhancement.

Acknowledgements

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Highlights

- Addition of nano-scaled fish bone (NFB) improved texture of Alaska pollock surimi gel significantly.
- Optimum gel texture was obtained with 1% NFB and setting at 25 °C for 3 h.
- NFB improved gel texture as calcium compound activated endogenous transglutaminase in surimi.
- Increased intensity of MHC cross-links supported texture improvement by the addition of NFB.

Queries and Answers

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