**Gelling Properties of Atlantic Croaker Surimi Processed
by Acid or Alkaline Solubilization**

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ABSTRACT

Textural properties of gels from Atlantic croaker surimi recovered by isoelectric precipitation, following acid (AC) or alkaline (AL) solubilization, were compared to that of conventionally washed (CW) surimi. Gel strengths were in the order of AL>AC>CW, with higher gel strength and deformability induced by microbial transglutaminase (MTGase) addition. None of the surimi treatments evidenced endogenous TGase activity. Salt (2% NaCl) addition had variable effects. Proteolysis was evident by SDS-PAGE in gels from all treatments when preincubated at 30 or 40 °C.

Key words: transglutaminase, surimi, acid, alkaline, solubilization

35 **Introduction**

Croaker is an abundant species in Atlantic waters, but has limited marketability in the traditional forms of fish products desired by western consumers. Surimi made from croaker is highly valued in Japan, but a limiting factor in the traditional method of surimi manufacture is the relatively low yield resulting from removal of water-soluble proteins.

40 Because the Atlantic croaker fishery is not amenable to the economies of scale possible with surimi manufacture from species such as Alaska pollock, a more efficient recovery process would enhance the feasibility of surimi production from this species.

Acid and alkaline processing are new alternatives to manufacturing surimi (Hultin and
45 Kelleher 1999, 2000; Cortés-Ruiz and others 2001; Choi and Park 2002; Yongsawatdigul and Park 2001; Undeland and others 2002). One distinguishing attribute of these processes is that the sarcoplasmic protein fraction of the meat is retained. While sarcoplasmic proteins were previously thought to interfere with gelation of the dominant myofibrillar fraction, several studies indicate they can enhance gel strength (Morioka and
50 Shimizu 1990; Morioka and others 1992; Delamballerie and others 1993; Ko and Hwang 1995). Nowsad and others (1995) found that part of the positive effect was due to increased 'suwari' (gel strengthening resulting from crosslinking by endogenous transglutaminase).

55 Transglutaminase (TGase) is an enzyme of the sarcoplasmic fraction of fish muscle (Lanier 2000) that catalyzes the crosslinking of proteins via formation of nondisulfide covalent bonds between glutamine and lysine residues. This endogenous enzyme is responsible for gelation of surimi pastes at low temperatures (5-40 °C), termed 'setting', which manifests upon cooking as enhancement of gel strength (Seki and others 1990).

60 A microbially derived TGase (MTGase) approved for food use is also known to strengthen surimi gels (Lee and others 1997; Motoki and Seguro 1998; Ashie and

Lanier 2000); its activity might also be affected by acid- or alkaline-solubilization of the proteins.

65 The present study was conducted to determine the influence of acid- and alkaline-
processing on the heat-induced gelling properties of surimi from Atlantic croaker, since
these properties in large part determine the commercial value of any surimi.
Additionally, the effect of both endogenous TGase as well as added MTGase was
evaluated.

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Materials and Methods

Stabilized mince preparation

Atlantic croaker (*Micropogon undulatus*) was harvested off the North Carolina coast.
Fresh, net-caught fish were headed, eviscerated, and thoroughly washed. Skinless mince
75 was obtained by passing these through a Yanagiya mini belt deboner, having drum
perforations of 5 mm diameter. Ten percent sucrose and 0.3% sodium tripolyphosphate
w/w (BK Ladenburg Corp., Simi Valley, Cal.) were added as cryoprotectants and mixed
for 2 min in a large dough mixer. The stabilized mince (MacDonald and others 1990) was
portioned (1 kg) and vacuum-packed in oxygen-impermeable bags (Cryovac® CN-590
80 cook-in material bags; Cryovac Division of W. R. Grace and Co., Duncan, S.C.), then
frozen and stored at -20 °C until needed (within 2 mo).

Processing into surimi

Frozen stabilized mince (600 g) was tempered at room temperature briefly before cubing
85 with a knife while still frozen, then homogenized with 8 parts deionized cold water (0-6 °C)
for 1 min in a blender at maximum speed (Model CB-6/34BL22, Waring Products Division,
Dynamics Corporation of America, New Hartford, Conn.). The pH was adjusted from
about 5.8 (native) to 2.5 using 1 N cold HCl for the acid-aided process (AC), or to pH 10

with 1 N cold NaOH for alkaline-aided surimi (AL). The homogenates were centrifuged at
90 8,000 x *g* for 30 min at 4 °C. The top layer (containing neutral lipid) and bottom layer
(containing insoluble lipid membranes and connective tissue) were discarded. The
remaining layer containing soluble protein, of low viscosity, was filtered through 4 layers of
cheesecloth, then adjusted to pH 5.5 using 1 N cold NaOH or 1 N cold HCl with slow
stirring. The average time for pH adjustment was around 10 min per batch. The resulting
95 precipitate (surimi) was collected by centrifugation at 8,000 x *g* for 30 min at 4 °C and
excess moisture was removed by squeezing manually between “chiffon” mesh fabric.
Moisture content was determined and surimi was kept overnight in frozen storage (-20 °C)
at pH 5.5.

100 For comparison with the above treatments, conventional surimi (CW) was also
prepared in the laboratory by a typical water washing method. The mince was washed
in cold water (0-6 °C) in a 3:1 proportion (solution:mince) and dewatered by
centrifuging at 8,000 x *g* for 30 min at 4 °C. The mince was washed and dewatered this
way 3 times, adding 0.2% NaCl in the last wash to enhance subsequent removal of
105 water. Excess water after centrifugation was removed by squeezing manually between
chiffon mesh fabric. Before freezing at -20 °C, 10% sucrose and 0.3% sodium
tripolyphosphate were added as cryoprotectants as in the traditional surimi processing.

Gel preparation

110 Frozen surimi was tempered at room temperature briefly before cubing with a knife
while still frozen. Sodium chloride (0% or 2% w/w) and ice sufficient to achieve 78%
(w/w) moisture were added to the tempered surimi. Because the pH of the
conventionally washed surimi was near 7.0, 1 N cold NaOH or 1 N HCl (between 5 and
10 mL) was added to adjust the pH to 7.0 for the AC and AL surimi types, respectively,
115 and the mixture was chopped in a food processor (Model LPP; Cuisinart, East Windsor,

N.J.) to a final temperature of 5 °C (about 6 min). To some pastes 0.2% of a commercial preparation of microbial transglutaminase (MTGase) derived from *Streptovorticillium* (Activa™ TI, containing 100 units of MTGase/g dispersed in maltodextrin; Ajinomoto USA, Inc., Teaneck, N.J.) was added, yielding 20 units of MTGase activity per 100 g surimi paste (Lee and others 1997).

The pastes were extruded into open-ended (ends cut off) polypropylene centrifuge tubes (9 cm length, 1.4 cm i.d.), previously sprayed inside with a lecithin-based release agent to prevent gel adhesion. The tubes were capped on both ends with serum sleeve stoppers clamped with metal thumbscrew tube clamps. They were then heated by one of three water bath treatments: (1) 90 °C for 20 min; (2) 30 °C for 2 h followed by 90 °C for 20 min; (3) 40 °C for 2 h followed by 90 °C for 20 min. Immediately after heating, the tubes were cooled in ice water. Gels were then removed from the tubes and held at 4 °C overnight in sealed plastic bags.

Puncture test

Gelled samples were cut into cylindrical specimens (1.4 cm long, 1.4 cm i.d.) and briefly tempered at 20 °C. A round stainless steel probe (3.175 mm dia ball) penetrated gels to their breaking point (or to a maximum 70% penetration into sample) on a Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA) at a penetration speed of 1.1 mm/s. Breaking force (g) and deformation (penetration depth, mm) were determined on at least 6 specimens per treatment.

Due to the small amount of surimi generated by laboratory preparation of each treatment, this punch testing protocol utilized a smaller sample size and probe diameter than that used for standardized measurement of surimi gel texture (Lanier 1992). Thus the fracture

values obtained by the punch test are much lower than would be typically expected by standard test methods.

145 **SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

The activity of endogenous or added MTGase, resulting in covalent crosslinking of proteins, as well as the activity of endogenous proteolytic enzymes can be evidenced by SDS-PAGE of the solubilized protein from cooked surimi gels (Lee and others 1997; Choi and Park 2002). Surimi gels (0.8 g) were solubilized in test tubes with 15 mL urea buffer
150 (20 mM Tris, 8 M urea, 2% sodium dodecyl sulfate (SDS), and 2% β -mercaptoethanol; pH 8) after a heat treatment in boiling water for 2 min followed by continuous shaking for 16-24 h at room temperature. The supernatant, recovered after centrifugation at 10,000 x g for 20 min, was assayed for protein concentration using the microassay procedure (Bio-Rad Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Hercules, Calif.)
155 based on the method of Bradford (1976). Solubilized samples were prepared following the recommendations developed for NuPAGE[®] Bis-Tris high-performance electrophoresis (Invitrogen, Carlsbad, Calif.) using 4X LDS sample buffer and 0.5% dithiothreitol (DTT) as a reducing agent (Engelhorn and Updyke 1996). Protein was applied at 6 μ g per lane. Electrophoresis was carried out on a NuPAGE[®] 3-8% polyacrylamide, tris-acetate
160 gradient gel on a mini-vertical gel system (XCell Sure Lock; Invitrogen) using a constant voltage of 150 V at ambient temperature followed by staining in Coomassie brilliant blue (colloidal blue staining; Invitrogen), and destaining in water. A protein mixture (Mark12[™]: 2.5-200 kDa; Invitrogen) was used for molecular weight markers. Densitometer scans were made on gels using an Alphamager[™] (Alpha Innotech Corporation, San Leandro,
165 Calif.). Protein was quantified as percentages of the total protein in each lane. Three regions were measured for each lane: the sum of bands above myosin heavy chain (MHC), sum of bands below MHC, and the MHC band itself. These data were used to determine statistical differences between treatments and are not included in the results.

Statistical analysis

170 Data were subjected to analysis of variance and planned, paired comparisons were made
using the Student's t-test with significance of difference set at $p < 0.01$ unless otherwise
indicated in the text.

Results and Discussion

175 Gels made without added MTGase

Gels made with acid-aided (AC) and alkaline-aided (AL) surimi with and without NaCl
exhibited higher punch force than those made with the conventional (CW) surimi (Figure
1). Deformation values were equal or higher in the AL surimi gels, but equal or less in the
AC gels. CW gels without NaCl (90 °C cook) were reasonably strong and deformable for
180 all cooking methods. These CW gels were comparable to gels with a torsional fracture
stress of 27 kPa and fracture strain of 2.4 as determined by a preliminary comparison of
conventionally washed surimi gels tested by both a standard torsion method (Lanier 1992)
and the punch test protocol used in this study (data not shown). Because the CW surimi
contained cryoprotectants, where the AC and AI surimi did not, a lower gel strength for
185 these gels would be expected due to their lower protein content.

The superior effect on gelation from the alkaline processing is consistent with the findings
of others who noted alkaline-processed surimi made from Pacific whiting (Kim and others
2001), rock fish (Yongsawatdigul and Park 2001), croaker, mullet, Spanish mackerel
190 (Demir and Kristinsson 2003), and catfish (Theodore and Kristinsson 2003) produced gels
with higher breaking force and deformation than surimi prepared by both the conventional
water leaching process and the acid process. Kristinsson and Hultin (2003) reported that
acid- or base-induced solubilization leads to substantial changes in the conformation and
structure of fish proteins, such that upon refolding these proteins have different and
195 sometimes better properties than the untreated proteins. Their related studies suggest

that dissolution of muscle structure by acid or alkaline processing may also play a key role in better distributing proteins for heat-induced gel formation.

200 The ability of CW croaker surimi to gel without NaCl was surprising, although this has been documented in other species before. Hennigar and others (1988) found that satisfactory gels could be prepared from the washed minced muscle tissue of red hake and to some extent cod without the addition of NaCl. Additionally, Bakir and others (1994) reported higher stress and nearly equal strain values for surimi gels without added NaCl made from Atlantic cod or dab compared to those made with 3% added NaCl. They also
205 reported excellent gelling ability without the addition of NaCl for other species including Atlantic pollock, red hake, and yellowtail flounder.

AC surimi also gelled equally or better without NaCl addition. The AL surimi gelled much better with the addition of 2% NaCl for the 90 °C and the 30/90 °C cooking treatment,
210 while for the 40/90 °C heating treatment, NaCl addition to AL surimi produced gels that were equal in strength but 52% less deformable than those made without NaCl.

Significant myosin degradation for all of the gels cooked at 40/90 °C, but especially for the AC and AL surimi gels with NaCl, was evident by the increase in bands located below
215 MHC shown in the electrophoresis gels (Figure 2, a, b, c: lane 5). Since myosin is the main structural protein involved in muscle gelation, its breakdown would result in gels with lower strength.

Choi and Park (2002) also showed substantial breakdown of myosin (as well as actin)
220 in acid-solubilized surimi made from Pacific whiting. Although they showed that significant cathepsin L and cathepsin B activity remained in their AC surimi compared to the conventionally washed surimi (about 3 times as much), they were unable to

determine whether the myofibrillar protein breakdown was due to these cathepsin proteases, perhaps enhanced by the acidic conditions of the production process, or to acid hydrolysis during processing.

Undeland and others (2002) followed the degradation of myosin during the production process for both acid- and alkaline-solubilized herring surimi. They found that the longer the myofibrillar protein remained under acidic conditions, the more myosin was broken down. However, the length of time these proteins were under alkaline conditions did not affect the myosin, even after 20 h of alkaline conditions.

Cheng and others (1979) reported the presence of an endogenous heat-stable alkaline protease in Atlantic croaker with a maximum activity around pH 8.0-8.5 and 60 °C. It also had significant activity (about 23% of maximum) at 40 °C and pH 7.5. Likely this same protease contributed to the myosin breakdown seen in the present study for the surimi preincubated at lower temperatures. Sodium chloride seemed to increase its activity, with higher breakdown products seen in surimi gels containing 2% NaCl (Figure 2) compared to those without NaCl (Figure 3). Olsen and others (2002) showed that the addition of NaCl to salmon mince pretreated with either acid or alkali increased proteolytic activity particularly that of the alkaline proteases. Increases in proteolytic activity due to NaCl addition have also been reported in washed squid muscle (Konno and Fukazawa 1993). They showed increased myosin breakdown in squid muscle containing up to 1.0 M NaCl compared to muscle with no NaCl (25 °C). The highest proteolytic activity occurred in muscle with 0.3 M NaCl and activity decreased at higher levels. They suggested that the NaCl disassembled the myosin filament into monomeric myosin, which was more susceptible to proteolytic cleavage.

Gels made from surimi prepared by all 3 methods failed to display setting (increase of gel strength due to low-temperature preincubation) (Figure 1). For the CW surimi, this was

unexpected since Kamath and others (1992) showed significant setting in conventionally washed croaker surimi with optimum activity at 40 °C. Absence of setting was also apparent from SDS-PAGE of these gels (Figures 2,3; a, b, c: lane 5) in that no increase in bands of a molecular weight (MW) greater than myosin heavy chain (MHC) (indicating myosin polymerization) were evidenced in the 40 °C preincubated treatments. Setting in conventional surimi is catalyzed by a calcium-dependent endogenous transglutaminase. The lack of setting indicates that the endogenous transglutaminase of the croaker was either absent (washed away) or inactivated by the particular surimi process. It is also possible, although unlikely, that cross-linking of myosin heavy chains occurred, but due to extensive breakdown of other parts of the molecule, the protein fragments were unable to form a strong gel.

The high level of proteolytic activity and lack of setting at 40 °C for the CW treatment are hard to explain since fish were well handled and in good condition at the time of processing into stabilized mince. Fish used in this study were eviscerated while very fresh, and scrubbed to minimize residual kidney tissue high in protease activity (Lin and Lanier 1980). Possibly the preparation of surimi from frozen stabilized mince, a procedure documented to be equivalent to use of fresh fish for surimi production from other species (MacDonald and others 1990; Simpson and others 1994), is not an effective method for croaker surimi production. This could be due to a higher activity of proteolytic enzymes in this fish compared to the species used by these other researchers.

Since the CW surimi did not demonstrate setting, it is not surprising that AL and AC surimi gels did not set when preincubated at 40 °C. In addition, Bergamini and others (1999) reported that human erythrocyte transglutaminase could be heat-inactivated easier under acidic (pH=6.1) and alkaline (pH=9.0-9.5) conditions than at neutral pH (pH=7.5). They also reported that the inactivation was different between the two: at acid pH, the

whole structure of the transglutaminase was lost irreversibly with massive aggregation, whereas at alkaline pH less aggregation occurred and secondary structure was lost depending upon the ionization state of crucial lysine residues.

Addition of MTGase

When MTGase was added during gel preparation, gels with higher gel strength (punch force at breakage) and deformability were attained for each washing treatment in the general order AL>AC>CW, particularly for those gels preincubated at 40 °C before cooking (Figure 1). For the CW and AC treatments, the addition of NaCl increased the setting effect caused by the MTGase.

In conventionally washed pollock surimi, NaCl is needed for setting to occur. Wan and Seki (1992) showed no setting induced by endogenous TGase in unsalted Atlantic pollock surimi after incubation for up to 6 h at 25 °C. As NaCl was added, breaking strength of set only, and set and cooked gels increased up to 0.2 M NaCl addition and then decreased as more NaCl was added. Using MTGase as the enzyme source, Téllez-Luis and others (2002) showed a lack of setting when NaCl was absent and an increase in the setting effect as NaCl was increased from 0 to 2 % in silver carp surimi gels. It is thought that the NaCl helps to open up, or unfold, the myosin molecules making them more reactive for self-association. Bouraoui and others (1997) showed through Raman spectroscopy that the addition of NaCl to Pacific whiting surimi caused slight decreases in α -helical content with slight increases in random coil content, indicating such unfolding.

The addition of MTGase to the AL-processed gels resulted in a much larger setting effect than in the CW and AC gels, but the addition of NaCl decreased this effect. Even without preincubation, the gel strength of AL gels with MTGase (especially those without NaCl) was surprisingly high (Figure 1). The deformability of AL gels made without NaCl and

305 containing MTGase were also quite high such that they did not break when the punch probe was fully extended. In a puncture test of this geometry, the fracture force and deformability are geometrically coupled (Hamann and Lanier 1987); that is, a high response for one attribute can result in a falsely high value being measured for the other. This may explain the very high fracture force measured for the 90 °C AL sample with
310 MTGase. When, in a separate test, the sample height was in effect doubled by placing one specimen on top of another, it was found that all samples did fracture, and that the 90 °C sample now exhibited a fracture force about 50% lower than those preincubated at 30 or 40 °C.

315 The enormous effect of MTGase on AL surimi gels compared to CW and AC surimi gels suggest that the myosin in AL surimi is conformationally different than that in CW and AC surimi; having more primary amines and glutamyl carboxyamide groups in close proximity to each other for crosslinking. Joseph and others (1994) suggested that, at any given temperature, the transglutaminase-mediated setting reaction in surimi is limited more by
320 the conformation of the substrate (namely myosin) than by that of the enzyme. The authors explained that the availability of reactive groups on the myosin surface is focal in determining the transglutaminase-mediated cross-linking of myosin.

Kristinsson and Hultin (2003) reported greater surface hydrophobicity in purified cod
325 myosin (0.45 mg/mL 600 mM KCl) processed by the alkali- (158%) and acid- (121%) method compared with the pH 7.5 control. Interestingly, Choi and Park (2002) reported greater surface hydrophobicity for conventionally washed Pacific whiting surimi compared with acid-processed surimi. This contradiction is likely due to the presence of additional proteins in the surimi system compared to the purified myosin model system. These
330 reports, however, do confirm that protein conformational changes result from alkali- and acid- processing.

In the present study, SDS-PAGE results for surimi gels containing MTGase are deceiving. Although AL surimi with added MTGase had excellent gelling ability, no dimers or other
335 oligomers of myosin heavy chain are evident in the SDS-PAGE gels. For these treatments, incomplete solubilization of the cooked surimi gel in the urea buffer was evident, with an insoluble gelatinous material formed during overnight stirring. This material, therefore, could not be included in the SDS-PAGE samples, so the electrophoretic separations for these treatments were on an incomplete set of proteins.
340 This phenomenon has occurred before in our laboratory and it was confirmed that this fraction had a high degree of myosin crosslinking (Lee and others 1997)

When incomplete solubilization occurs, evidence of polymerization can be assumed by loss in MHC as long as concomitant breakdown of myosin is not indicated (Lee and others
345 1997). So, for AL surimi gels without NaCl containing added MTGase and precooked at 30 or 40 °C, MHC polymerization can be assumed by loss of MHC (Figure 3c; lanes 4 and 6). For AL surimi gels with added NaCl and added MTGase, breakdown products are evident in the SDS-PAGE gels, so polymerization of MHC cannot be simply assumed. The increased gel strength compared to AL surimi gels without MTGase as well as the
350 insoluble portion observed during sample preparation suggest that some polymerization did occur. The lower gel strength and deformability seen with NaCl addition to MTGase-containing AL surimi gels was likely due to the sodium chloride's effect on the enzymatic breakdown of myosin (like that seen in other NaCl-containing gels in the study) rather than to its effect on TGase activity.

355 **SDS-PAGE results for CW and AC surimi gels were inconclusive for myosin polymerization since reductions in MHC were seen in samples that did not show increased gel strength by the puncture method. For samples with added**

MTGase preincubated at 40 °C (with or without the addition of NaCl), the
360 reduction in MHC is so significant that polymerization of MHC is certainly
suggested.

In summary, gelling properties of fish proteins were altered by a pH shift-induced
solubilization and reprecipitation recovery process. Both acid and alkaline processes
365 seem to render the muscle proteins more accessible as a substrate to MTGase and to
protein-protein interactions, which lead to enhancement of gel strength and
deformability. The alkaline process seems to impart stronger gelling properties overall,
which are generally enhanced by NaCl addition except when MTGase is added.
Considered along with the higher protein recovery yields that should be possible by
370 such a process for surimi manufacture (Hultin and Kelleher 1999; Kim and others
2001), such a process could considerably increase the value of surimi manufactured
from Atlantic croaker. However, stability of this new material in frozen storage must be
established and organoleptic evaluation conducted to demonstrate equivalent
palatability to conventionally processed surimi. The parameters of the solubilization
375 and reprecipitation process should also be optimized. For example, in a similar
alkaline process for recovery of soy protein isolate, Salt and others (1982) found that
the rate at which pH changes occur can affect the nature of the association, and
therefore the functionality, of the recovered proteins.

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Figure 1-Comparison of punch test values for surimi gels made with 3 surimi types: conventionally washed (CW), acid-solubilized (AC) and alkaline-solubilized (AL). Gels were made with or without 2% NaCl, and with or without 0.2% added microbial transglutaminase (TGase). 90 indicates that gels were cooked at 90 °C for 20 min, 30/90 and 40/90 indicate gels incubated at 30 °C and 40 °C, respectively, followed by cooking at 90 °C. Error bars represent the standard deviation of 6-10 samples.

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Figure 2- Comparison of the effect of MTGase and 2% NaCl on muscle proteins in surimi gels made with 3 surimi types: conventionally washed (a), acid-solubilized (b) and (c) alkaline-solubilized. Lanes 1,3,5 of each SDS-PAGE gel show results for surimi gels made with no added MTGase; lanes 2,4,6 of each SDS-PAGE gel show results for surimi gels made with MTGase. Lanes 1&2, 3&4, and 5&6 show results for surimi gels cooked at 90 °C for 20 min, 30 °C for 2 h followed by 90 °C for 20 min, and 40 °C or 2 h followed by 90 °C for 20 min, respectively.

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Figure 3-Comparison of the effect of MTGase on muscle proteins in surimi gels made with the 3 types of surimi and without the addition of NaCl. Location of treatment combinations is the same as those in Figure 2.

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