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Comparative study on acid-induced gelation of myosin from Atlantic cod (*Gardus morhua*) and burbot (*Lota lota*)

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Abstract

Physicochemical and rheological properties of myosin from Atlantic cod and burbot during acid-induced gelation at room temperature (22–23 °C) by D-gluconic acid-δ-lactone (GDL) were monitored. Turbidity and particle size of both myosins increased and salt soluble content decreased when pH decreased, suggesting the formation of protein aggregates caused by acidification. The formation of disulphide bonds in myosin gelation was induced by acid. Ca^{2+}-ATPase activity of myosin decreased ($p < 0.05$), while surface hydrophobicity increased during acidification ($p < 0.05$). Furthermore, the decreases in maximum transition temperature (T_{max}) and the denaturation enthalpies (ΔH) were found in both myosins. During acidification, the increases in storage modulus (G') and loss modulus (G'') of myosin were observed ($p < 0.05$), revealing the formation of elastic gel matrix. Thus, gelation of myosin from Atlantic cod and burbot could take place under acidic pH via denaturation and aggregation. However, myosin from Atlantic cod was generally more favourable to gelation than was burbot myosin.

Keywords: Myosin; Acid-induced gelation; Aggregation; Atlantic cod; Burbot

1. Introduction

Myosin is generally important for gel development of meat and fishery products. Myosin is the most abundant of the myofibrillar proteins and comprises approximately 50% of the total protein in muscles (Xiong, 1997). Myosin molecule has a unique structure comprising two 200 kDa heavy chains and two sets of two types of light chains components with sizes about 20–27 kDa (Xiong, 1997). Myosin consists of two globular head regions, each having two non-covalently bound light chains, and a rodlike tail portion that is a coiled-coil of α-helix (Harrington & Rodgers, 1984). The characteristic properties of myosin vary with specific portions of the molecule; the active site of ATPase and the binding site with actin are at the head portion, whereas the solubility characteristics and filament-forming ability are at the tail portion (Harrington & Rodgers, 1984). Among all muscle proteins, myosin is the most important in the formation of thermally induced fish muscle gels (Wicker, Lanier, Knopp, & Hamann, 1989). Network formation and rigidity development of fish muscle gels mainly result from the formation of three-dimensional structure, constructed by the intermolecular binding of myosin. Such formation was obtained by unfolding of the myosin molecule and subsequent interactions such as the interaction between non-polar groups in the hydrophobic regions or patches (Wicker et al., 1989).

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Acid-induced gelation is another important process associated with the textural development and unique characteristics of some meat and fish products. The acid-induced gelation of muscle protein in sausage during fermentation process has been reported (Riebroy, Benjakul, Visessanguan, & Tanaka, 2007). Slow lowering of pH could induce gel formation of myosin (Fretheim, Egelandsdal, Harbitz, & Samejima, 1985). The mechanism of myosin gels at high salt is strongly dictated by its dual fibrous-globular structure, in which head-to-head interactions are believed to initiate gel network formation on heating, followed by cross-links of the rods forming a strand-like gel network (Xiong, 1997). The mechanism of acid-induced gel formation could be explained by aggregation of proteins at pH close to isoelectric point, in which the aggregation clusters can form the gel network (Totosaus, Montejano, Salazar, & Guerrero, 2002). Since the long rod of myosin is composed of many charged residues, the functionality of the protein is highly sensitive to changes in pH and ionic strength (Kristinsson & Hultin, 2003). The solubility properties of the protein are largely determined by pH and ionic strength (Zayas, 1997). The lowering pH of muscle protein could contribute to the textural formation of some fermented fish products, particularly gel-like products. Nevertheless, a little information is available on the acid-induced gelation of muscle protein. Therefore, the objective of this study was to elucidate the physicochemical and rheological changes of myosin from Atlantic cod, a marine fish, and burbot, a freshwater fish which have become an economically important species, during acid-induced gelation at high ionic strength condition.

2. Materials and methods

2.1. Reagents

Adenosine-5′-triphosphate (ATP), 8-anilino-1-naphthalenesulfonic acid (ANS), D-gluconic acid-δ-lactone (GDL), guanidine thiocyanate, ethylene glycol-bis (β-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1-(L-*trans*-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), sodium hydrogen sulfite, and Tris–maleate were procured from Sigma Chemical Co. (St. Louis, MO, USA). Potassium chloride, sodium chloride, sodium azide, ethylenediaminetetraacetic acid (EDTA), sodium bicarbonate, magnesium chloride, magnesium acetate, calcium chloride, ammonium molybdate and trichloroacetic acid were purchased from Merck (Darmstadt, Germany). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from WAKO Pure Chemical Industries (Tokyo, Japan). Phenylmethane-sulfonyl fluoride (PMSF) and EPON®812 were obtained from Fluka (Buchs, Switzerland).

2.2. Fish samples

Atlantic cod (*Gardus morhua*) with the size of 2.3–2.5 kg/fish and off-loaded approximately 12 h after capture during August and November, 2006 were purchased from fish market in Trondheim, Norway. Burbot (*Lota lota*) with a size of 0.8–1.2 kg/fish were caught from a fish pot of Snåsa Lake in Central Norway. After capture, the burbot were immediately subjected to exsanguinations in ice. Fish were stored in ice with fish/ice ratio of 1:2 in the insulated boxes and transported to the Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway within 24 h. Upon the arrival, the fish were washed immediately and manually filleted. Fish flesh was stored in ice until used for myosin preparation.

2.3. Myosin preparation

Myosin from Atlantic cod and burbot flesh was extracted according to the method of Martone, Busconi, Folco, Trucco, and Sanchez (1986) as modified by Visessanguan, Ogawa, Nakai, and An (2000). All steps were performed at 0–4 °C to minimise proteolysis and protein denaturation. Fish flesh was minced by using a grinder (Illico, Moulinex, France). Minced fish was added with 10 volumes of buffer A (0.10 M KCl, 1 mM PMSF, 10 μM E-64, 0.02% NaN_3, and 20 mM Tris–HCl, pH 7.5). After incubation in ice for 10 min with occasional stirring, the washed muscle was recovered by centrifugation at 1000*g* for 10 min using a Sorvall Model RC-5B plus (Mandel Scientific, Newton, CT, USA). The pellet was suspended in five volumes of buffer B (0.45 M KCl, 5 mM βME, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA and 20 mM Tris–maleate, pH 6.8), and adenosine 5′-triphosphate (ATP) was added to obtain a final concentration of 10 mM. The mixture was kept in ice for 1 h with occasional stirring and centrifuged at 10,000*g* for 15 min. The supernatant was collected and treated slowly with 25 volumes of 1 mM $NaHCO_3$, followed by incubation for 15 min on ice. Precipitated myosin was collected by centrifugation at 12,000*g*, resuspended gently with 5 volumes of buffer C (0.50 M KCl, 5 mM βME and 20 mM Tris–HCl, pH 7.5), and treated with 3 volumes of 1 mM $NaHCO_3$. $MgCl_2$ was also added to obtain a final concentration of 10 mM. The mixture was kept overnight on ice prior to centrifugation at 22,000*g* for 15 min. Myosin recovered as pellet was used immediately. Myosin pellet was dissolved in 0.5 M NaCl, pH 7.0 and then centrifuged at 5000*g* for 15 min. The supernatant was collected and protein content was determined by the Biuret method using bovine serum albumin (BSA) as a standard (Copeland, 1994). Myosin with purity greater than 90%, determined by SDS–PAGE (Laemmli, 1970) and densitometric analysis (GS-700 Imaging Densitometer, Bio-Rad Laboratories, Hercules, CA, USA), was used for further study.

2.4. Acid-induced gelation of myosin

Myosin solution from both Atlantic cod and burbot (2 mg/ml, 50 ml) had finely ground GDL powder (7.5 mg) added every 12 h up to 48 h. Total GDL amount of 30 mg was used to obtain a final pH of 4.6. Myosin without GDL

addition was used as the control. The mixture was incubated at room temperature (22–23 °C). The samples were taken every 6 h for analyses during incubation of 48 h. To acidify myosin at high protein concentration, myosin (40 mg/ml, 30 ml) was added with GDL (0.18 g) to obtain pH 4.6 within 6 h of incubation. Acidified myosin was used for rheology, thermal denaturation, and electron microscopic studies. To retard microbial growth during incubation, sodium azide (0.01%) was added in all myosin samples.

2.5. pH

The pH value of sample was measured using a MP220 pH meter (Metler Teledo GmbH, Schwerzenbach, Switzerland).

2.6. Turbidity measurement

The turbidity of myosin solution was analyzed according to the method of Visessanguan et al. (2000). Diluted myosin solution (1 mg/ml in 0.5 M NaCl) was placed in a cuvette (path length of 1 cm). Turbidity was measured as absorbance at 350 nm using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England). Results were reported as absorbance per protein concentration (A_{350}/mg/ml).

2.7. Particle size measurement

The particle size of the sample was analyzed by a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The diluted myosin in 0.5 M NaCl (1 mg/ml, 2 ml) was placed in the disposable sizing cuvette. The cuvette containing the sample was placed into the cell area at 22 °C. A laser at the wavelength of 633 nm was used to illuminate the sample particle. A detector at 173° was used to measure the intensity of the scattered light. Average particle size was calculated using Dispersion Technology Software version 4.2 (Malvern Instruments, Worcestershire, UK).

2.8. Determination of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was determined according to the method of Benjakul, Seymour, Morrissey, and An (1997). Prior to assay, myosin in 0.5 M NaCl (2 mg/ml) was 2-fold diluted using deionised water. The diluted sample (1 ml) was added to 0.6 ml of 0.5 M Tris–maleate, pH 7.0. The mixture was added with $CaCl_2$ solution to obtain a final concentration of 10 mM $CaCl_2$ with the total volume of 9.5 ml. To initiate the reaction, 0.5 ml of 20 mM ATP was added. The reaction was conducted for exactly 10 min at 25 °C and was terminated by adding 5 ml of chilled 15% (w/v) trichloroacetic acid. The reaction mixture was centrifuged at 3500*g* for 5 min and the inorganic phosphate liberated in the supernatant was measured by the method of Fiske and Subbarow (1925). Ca^{2+}-ATPase activity was expressed as μmole inorganic phosphate released/mg protein/min. A blank solution was prepared by adding chilled trichloroacetic acid prior to the addition of ATP.

2.9. Determination of solubility

Myosins in 0.5 M NaCl (5 ml) with and without GDL addition were taken every 6 h during the incubation up to 48 h. The samples were centrifuged at 5000*g* for 15 min at 4 °C. The supernatant was collected and protein content was measured by the Biuret method using BSA as a standard. Salt soluble protein content was expressed as the percentage in comparison with that obtained before GDL addition.

2.10. Determination of sulphydryl (SH) and disulphide bond contents

Total and surface reactive SH contents were determined according to the method of Ellman (1959). To 1 ml of diluted myosin solution in 0.5 M NaCl (1 mg/ml), 9 ml of 0.2 M Tris–HCl buffer, pH 6.8 containing 8 M urea, 2% SDS and 10 mM EDTA were added. After incubation with 0.4 ml of 0.1% DTNB in 0.2 M Tris–HCl buffer, pH 6.8 at 40 °C for 25 min, the absorbance at 412 nm was measured using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England). The total SH content was calculated using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}. Surface reactive SH was determined by incubating 1 ml of myosin solution in 3 ml of 0.2 M Tris–HCl, pH 6.8, containing 2% SDS and 10 mM EDTA. After incubation with 0.4 ml of 0.1% DTNB in 0.2 M Tris–HCl buffer, pH 6.8 at 5 °C for 25 min, the absorbance at 412 nm was measured. Surface reactive SH content was calculated from the absorbance at 412 nm using the molar extinction coefficient of 14,150 M^{-1} cm^{-1} as described by Riddles, Blakeley, and Zerner (1983). A blank was prepared by replacing sample with 0.5 M NaCl (pH 7.0). Both total and surface reactive SH contents were calculated and expressed as moles per 10^5 g protein. Disulphide bond content in samples was determined using 2-nitro-5-thiosulfobenzoate (NTSB) assay as described by Thannhauser, Konishi, and Scheraga (1987). To 2 ml of myosin solution, 3 ml of freshly prepared NTSB assay solution were added. The mixture was incubated in the dark at room temperature (22–23 °C) for 25 min. Absorbance was then measured at 412 nm. The disulphide bond content was calculated using a molar extinction coefficient of 13,900 M^{-1} cm^{-1} and expressed as moles per 10^5 g protein.

2.11. Determination of surface hydrophobicity

Protein surface hydrophobicity was measured according to the method of Benjakul et al. (1997). The myosin solution was diluted to 0.125, 0.25, 0.5 and 1 mg/ml in 10 mM phosphate buffer, pH 6.0, containing 0.5 M NaCl. The diluted myosin solution (2 ml) was then mixed with 10 μl of 10 mM ANS in 50 mM phosphate buffer, pH 7.0.

Fluorescence intensity of ANS-protein conjugates was measured using a Luminescence spectrophotometer (LS50B, Perkin–Elmer, Ueberlingen, Germany) at an excitation wavelength of 374 and an emission wavelength of 485 nm, respectively. Surface hydrophobicity was calculated from the initial slope of plot of fluorescence intensity against protein concentration and was referred to as S_0ANS.

2.12. Dynamic rheological analysis

Rheological changes in myosin during acidification with GDL were monitored by using a Rheologica StressTech rheometer (StressTech, Rheologica instruments, Lund, Sweden). The rheometer was equipped with 40 nm, 4° slope cone and plate geometry with a gap of 1 mm. Myosin in 0.5 M NaCl added with GDL as previously described (3 ml) was applied to the rheometer and covered with low viscosity silicone oil (BDH silicone Products, KeboLab, 10-cSt at 20 °C). The measurements were carried out at a fixed frequency of 0.1 Hz with a strain control of 0.005 Pa. The condition was determined to give a linear response in the viscoelastic region. The measurements were run at 22 °C for 48 h. Dynamic rheological properties of the samples were described in terms of storage modulus (G'), loss modulus (G'') and phase angle (δ).

2.13. Differential scanning calorimetry (DSC)

DSC of myosin without and with acidification using GDL was carried out using a Micro-DSC VII (Setaram, Caluire, France). The system was calibrated using naphthalene (T_m 80.11 °C, ΔH 148.14 J/g). The myosin in 0.5 M NaCl (700–750 mg) with and without GDL addition was accurately weighed into the DSC vessel. Milli-Q water was used as a reference. Samples were thermally scanned from 2 to 95 °C at 1 °C/min. Nitrogen gas was used to eliminate water condensation in and under the DSC vessels. The peak of maximum transition temperature (T_{max}) was recorded. To estimate the enthalpy of denaturation (ΔH), a baseline was constructed by drawing a straight line from the starting point to the end point of transition, and the peak area was then integrated to give the value of ΔH by using the Setsoft2000 program version 1.6 (Setaram, Caluire, France). All samples were tested in four replications.

2.14. Transmission electron microscopy (TEM)

The acidified myosin was prepared by adding GDL (0.18 g) to myosin (40 mg protein/ml) dissolved in 0.5 M NaCl and the mixture was allowed to stand for 6 h. The acidified sample was then subjected to TEM as described by Ngapo, Wilkinson, and Chong (1996) with a slight modification. All specimens were cut into 4 mm × 4 mm × 4 mm using a razor blade and fixed with 2.5% glutaraldehyde and 3% formaldehyde in 0.1 M phosphate buffer, pH 7.2 at room temperature for 4 h. The samples were then washed three times with cacodylate buffer at room temperature and post fixed in 2% osmium tetroxide in 1.5% potassium ferrocyanide at room temperature for 2 h. The samples were then washed five times with distilled water and were negatively stained with 1.5% uranyl acetate. After washing with distilled water, the samples were dehydrated by a graded ethanol series (70–100%) for 10 min each. Dehydrated samples were embedded with resin EPON-812. After mixing, the mixture was loaded into capsules and allowed to polymerize at 60 °C for 24 h. Thin section (90 nm) was cut using a diamond cutter with an ultramicrotome (model MTXL, RMC, AZ, USA) and collected on copper grids. Thin sections were stained with 5% uranyl acetate for 10 min at room temperature followed by staining with 0.3% lead citrate for 5 min at room temperature. The specimens were visualized in a JEOL JEM-2010 electron microscope (JEOL Ltd., Tokyo, Japan) at an accelerated voltage of 160 kV.

2.15. Statistical analysis

Data were subjected to analysis of variance (ANOVA) and mean comparison was performed by Duncan's multiple-range test (Steel & Torrie, 1980). A student's *t*-test was used to compare means between species. The statistical analysis was performed using the SPSS statistic program version 11.0 for Windows (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Changes in myosin during acidification induced by GDL

3.1.1. pH value

The pH values of myosin from Atlantic cod and burbot with and without GDL addition during incubation at room temperature are depicted in Fig. 1. A continuous decrease in pH was obtained in myosin from both fish with added GDL up to 48 h of incubation ($p < 0.05$). Ngapo et al. (1996) reported that GDL (0.7%) can be used for lowering pH of bovine myosin during incubation at 4 °C. Decreasing rate of pH of myosin added with GDL was similar to the acidification rate of naturally fermented fish sausage (Riebroy et al., 2007). The simple pH lowering of myosin by dialysis with GDL in phosphate buffer was reported (Fretheim et al., 1985). Additionally, the use of an acidulant agent, glucono-δ-lactone, is very common in acid-induced food gels (Totosaus et al., 2002). GDL was hydrolyzed to form gluconic acid (Dziezak, 1990). The gluconic acid contributes to a gradual but continuous decrease in pH values (Dziezak, 1990; Fretheim et al., 1985). However, no changes in pH values of the control (without GDL addition) of both myosins were observed throughout the 48 h of incubation ($p > 0.05$). From the result, GDL could decrease the pH of myosin.

3.1.2. Turbidity and particle size

Changes in turbidity and average particle size of myosin without and with GDL addition during incubation at room

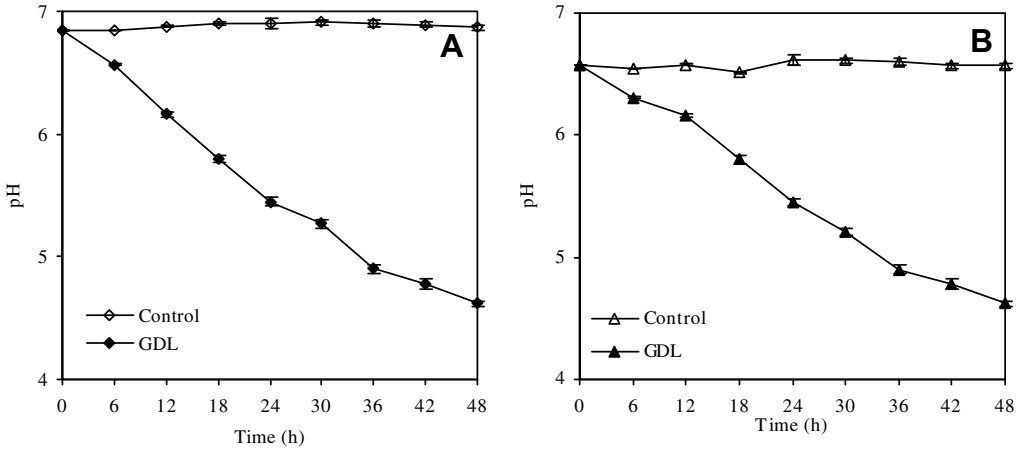

Fig. 1. Changes in pH values of myosin from burbot (A) and Atlantic cod (B) added without and with GDL during incubation at room temperature. GDL and Control represent sample with and without GDL addition, respectively. Bars represent standard deviation from triplicate determinations.

temperature (22–23 °C) are shown in Fig. 2. Myosin from both fish species added with GDL began to aggregate at 6 h (pH 5.9) with a gradual increase in average particle size ($p < 0.05$). Thereafter, turbidity and average particle size of both myosins continuously increased up to 48 h ($p < 0.05$). After acidification (48 h, pH 4.6), myosin from Atlantic cod had higher turbidity and greater average particle size than that from burbot ($p < 0.05$). Absorbance reading was used to monitor the extent of protein–protein interactions and the relative size of protein aggregates (Visessan-

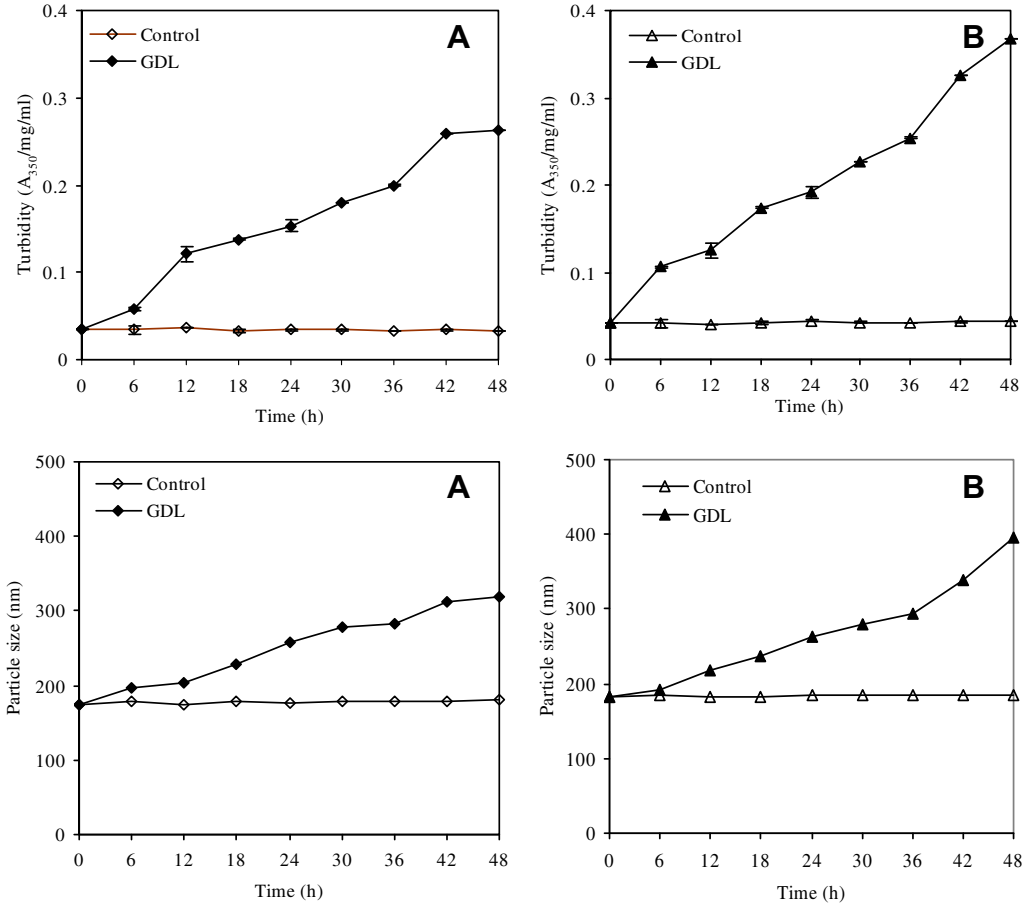

Fig. 2. The turbidity and particle size of myosin from burbot (A) and Atlantic cod (B) added without and with GDL during incubation at room temperature. GDL and control represent sample with and without GDL addition, respectively. Bars represent standard deviation from triplicate determinations.

guan et al., 2000). The increase in absorbance was due to the continuous formation of protein aggregates, which were large enough to cause light scattering. From the result, both myosins exhibited the same general pattern of the increase in turbidity and particle size changes. However, the rate and extent of aggregation induced by acid formed were species specific. The formation of aggregated particles resulted in the increased turbidity of myosin solution (Fretheim et al., 1985). When pH decreased to the isoelectric point (p*I*), the pH at which the net charge is zero, the repulsion between protein molecules was minimized and protein aggregation took place. When spherical particles of determined radius move by Brownian motion, they can aggregate when they encounter each other (Totosaus et al., 2002). From the result, the formation of myosin aggregates increased by the lowering of pH at room temperature. This contributed to the development of protein network under acidic pH.

3.1.3. Salt soluble protein and Ca^{2+}-ATPase activity

The changes in salt soluble protein content and Ca^{2+}-ATPase activity of myosin from Atlantic cod and burbot during acidification at room temperature are shown in Fig. 3. During acidification, the losses of salt soluble protein were observed in both myosins ($p < 0.05$). No changes in salt soluble protein of the control myosin from both fish species were found ($p > 0.05$). Generally, the protein solubility in salt has been widely described as the salting-in phenomenon (Zayas, 1997). A high concentration of sodium chloride is required to solubilise myofibrillar proteins, particularly myosin and actomyosin (Totosaus et al., 2002). At neutral pH, most proteins are negatively charged and a few are positively charged (Zayas, 1997). The pH lowering might enhance the electrostatic interaction of protein via charge neutralization. The electrostatic interaction phenomenon is the modification of protein molecules by attractive and repulsive forces, affecting protein–protein and protein–solvent interactions (Zayas, 1997). These electrostatic interactions are promoted by changes in ionic strength or pH (Totosaus et al., 2002). At low pH values, some proteins have a net positive charge and negatively charged chloride ions from NaCl bind with protein, thereby decreasing the electrostatic repulsions as well as leading to protein precipitation (Zayas, 1997). From the result, the lowered pH with high ionic strength resulted in the decrease in solubility of myosin.

A marked decrease in Ca^{2+}-ATPase activity of both myosins was observed during acidification ($p < 0.05$). Within 18 h of acidification, the greater decrease in

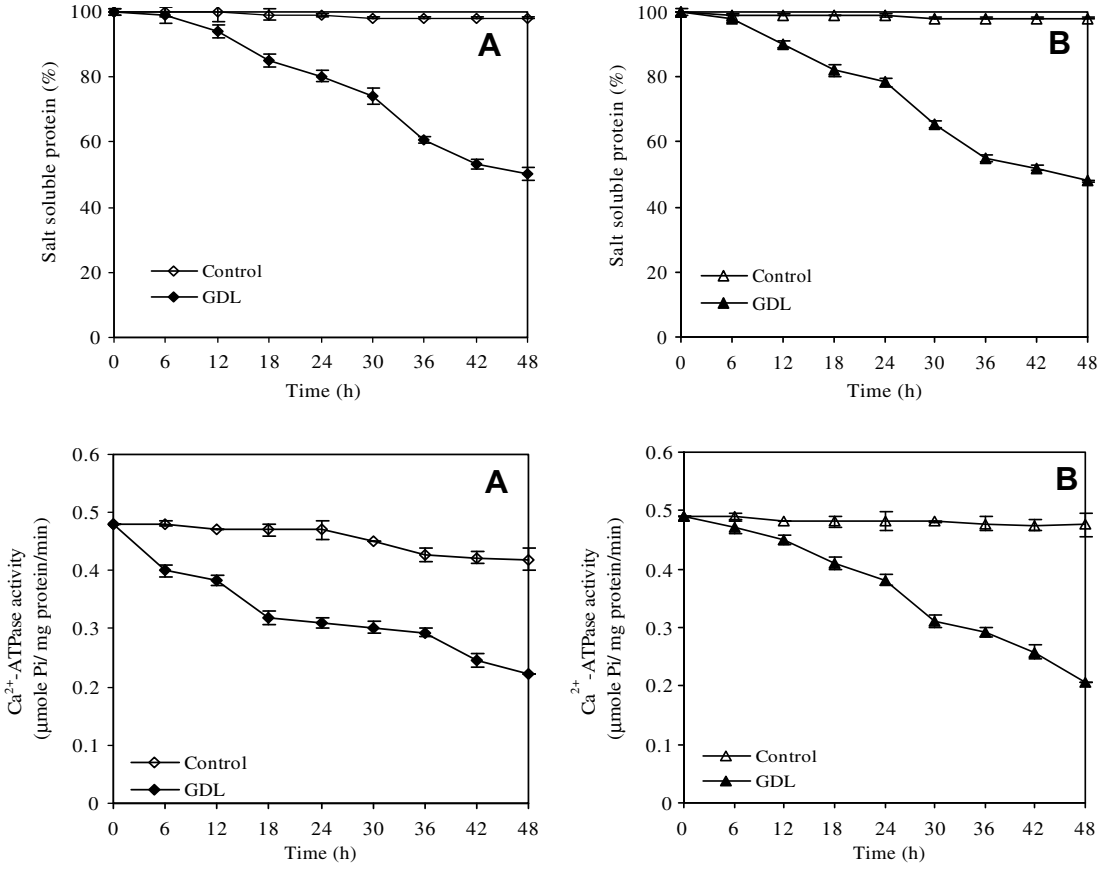

Fig. 3. Salt solubility and Ca^{2+}-ATPase activity of myosin from burbot (A) and Atlantic cod (B) added without and with GDL during incubation at room temperature. GDL and control represent sample with and without GDL addition, respectively. Bars represent standard deviation from triplicate determinations.

Ca^{2+}-ATPase was found in burbot myosin, compared with that of Atlantic cod myosin. Nevertheless, the greater decrease in activity was noticeable in Atlantic cod myosin during 24–48 h. Ca^{2+}-ATPase activity is normally used as an index of myosin denaturation (Benjakul et al., 1997). The decrease in fish myofibrillar ATPase activities was reported to be a direct function of pH (Benjakul et al., 1997). Denaturation of protein leads to aggregation and decreased solubility (Xiong, 1997). Myosin molecules, particularly head portion with ATPase activity, might undergo the conformational changes via charge modification at lowered pH. Thus, the decrease in Ca^{2+}-ATPase activity in acidified myosin indicated that the denaturation of myosin occurred at acidic pH.

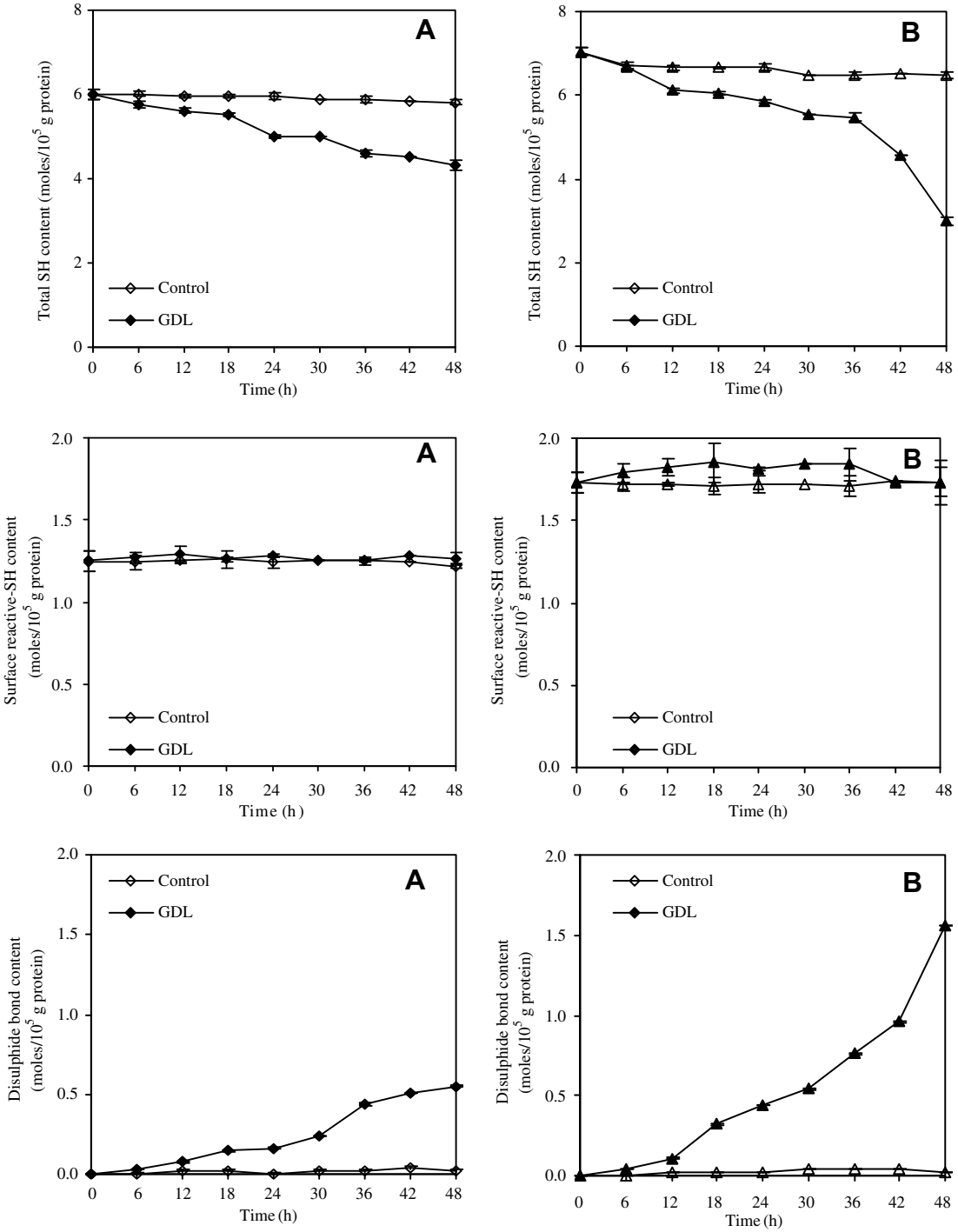

Fig. 4. Total, surface reactive sulfhydryl groups and disulphide bond contents of myosin from burbot (A) and Atlantic cod (B) added without and with GDL during incubation at room temperature. GDL and control represent sample with and without GDL addition, respectively. Bars represent standard deviation from triplicate determinations.

3.1.4. Reactive SH and disulphide bond contents

Changes in total and surface reactive SH contents of myosin from Atlantic cod and burbot without and with GDL addition during incubation at room temperature are depicted in Fig. 4. During acidification, the total SH content of both myosins decreased up to 48 h of incubation ($p < 0.05$). A greater decrease in total SH content in Atlantic cod myosin during acidification was found ($p < 0.05$), compared with burbot counterpart, particularly during 36–48 h of incubation. Total SH content of myosin from Atlantic cod and burbot was estimated to be 7.0 and 6.0 moles/10^5 g protein, respectively. Lin and Park (1998) reported that the total SH content of salmon myosin was 6.5 moles/10^5 g protein. Myosin has approximately 42 SH groups, of which 12 or 13 are in each globular head (Buttkus, 1971). The surface reactive SH content of Atlantic cod and burbot myosins was 1.7 and 1.2 moles/10^5 g protein, respectively, constituting 24% and 20% of the total SH contents, respectively. During the first 18 h of incubation, the increase in surface reactive SH content was observed in Atlantic cod myosin with GDL addition ($p < 0.05$) and it remained constant up to 36 h, followed by the slight decrease up to 48 h. However, no changes in surface reactive SH content were observed in myosin from burbot without and with GDL addition up to 48 h ($p > 0.05$).

In the presence of GDL, the increase in disulphide bond content in myosin from both fish species was observed during incubation ($p < 0.05$) (Fig. 4). This was coincidental with the decreases in total SH group content. The formation of disulphide bonds was most likely through the oxidation of SH groups or disulphide interchanges (Hayagawa & Nakai, 1985). From the result, the marked increase in disulphide bond was found in Atlantic cod myosin after 36 h of acidification, which was in agreement with the coincidental sharp decrease in total SH group content. Smyth, Smith, and O'Neill (1998) reported that the disulphide bond formation was not a prerequisite for the gelation of chicken breast myosin, but intermolecular disulphide bonds, especially from thiol groups on subfragment-1 (S-1), contributed to gel network formation. Covalent disulphide bonds among polypeptide chains involved in protein gelation, increasing the apparent chain length of polypeptide, rather than acting as an initial network stabiliser (Totosaus et al., 2002). The decrease in SH groups observed during acidification not only indicated the formation of disulphide bonds during gel formation but also implied the role of myosin heads in aggregation (Taguchi, Ishizaka, Tanaka, Nagashima, & Amano, 1987). The result indicated that disulphide bond involved in myosin aggregation was induced by the acid. The oxidation of SH group in the head region might be associated with the loss in ATPase activity.

3.1.5. Surface hydrophobicity

Changes in the surface hydrophobicity of myosin from Atlantic cod and burbot during acidification at room temperature are shown in Fig. 5. The surface hydrophobicity of myosin from both fish species continuously increased as pH values decreased ($p < 0.05$). However, the surface hydrophobicity of Atlantic cod myosin remained constant after 30 h of incubation ($p > 0.05$). For the control (without GDL addition), no changes in surface hydrophobicity were observed for myosin from both species during the incubation period of 48 h. This indicated that no marked changes in conformation of myosin took place at the neutral pH ranges. Hydrophobicity is known to contribute largely to the stability and structural conformation of proteins (Niwa, Kaneohe, Nakayama, Wattage, & Hashimoto, 1989). Additionally, non-polar amino acids are grouped, forming a hydrophobicity nucleus surrounded by a polar residue layer in contact with the solvent, water, which plays an important role in protein organization (Totosaus et al., 2002). Due to the propensity of nonpolar amino acid residues to position themselves in the interior of the protein molecules in solution, thus avoiding contact with the aqueous surrounding; only a portion of them could be considered as being effective in hydrophobicity (Totosaus et al., 2002). As the pH decreased (Fig. 1), the myosin underwent

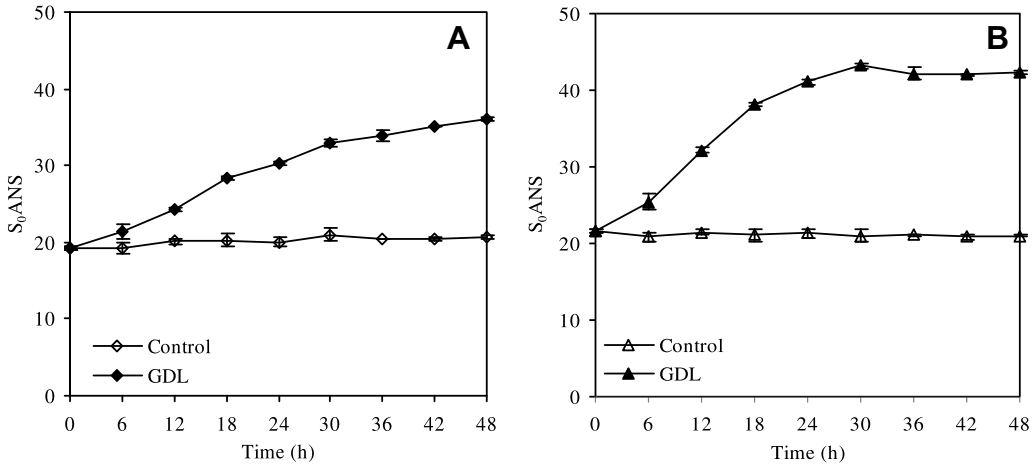

Fig. 5. Surface hydrophobicity of myosin from burbot (A) and Atlantic cod (B) added without and with GDL during incubation at room temperature. GDL and control represents sample with and without GDL addition, respectively. Bars represent standard deviation from triplicate determinations.

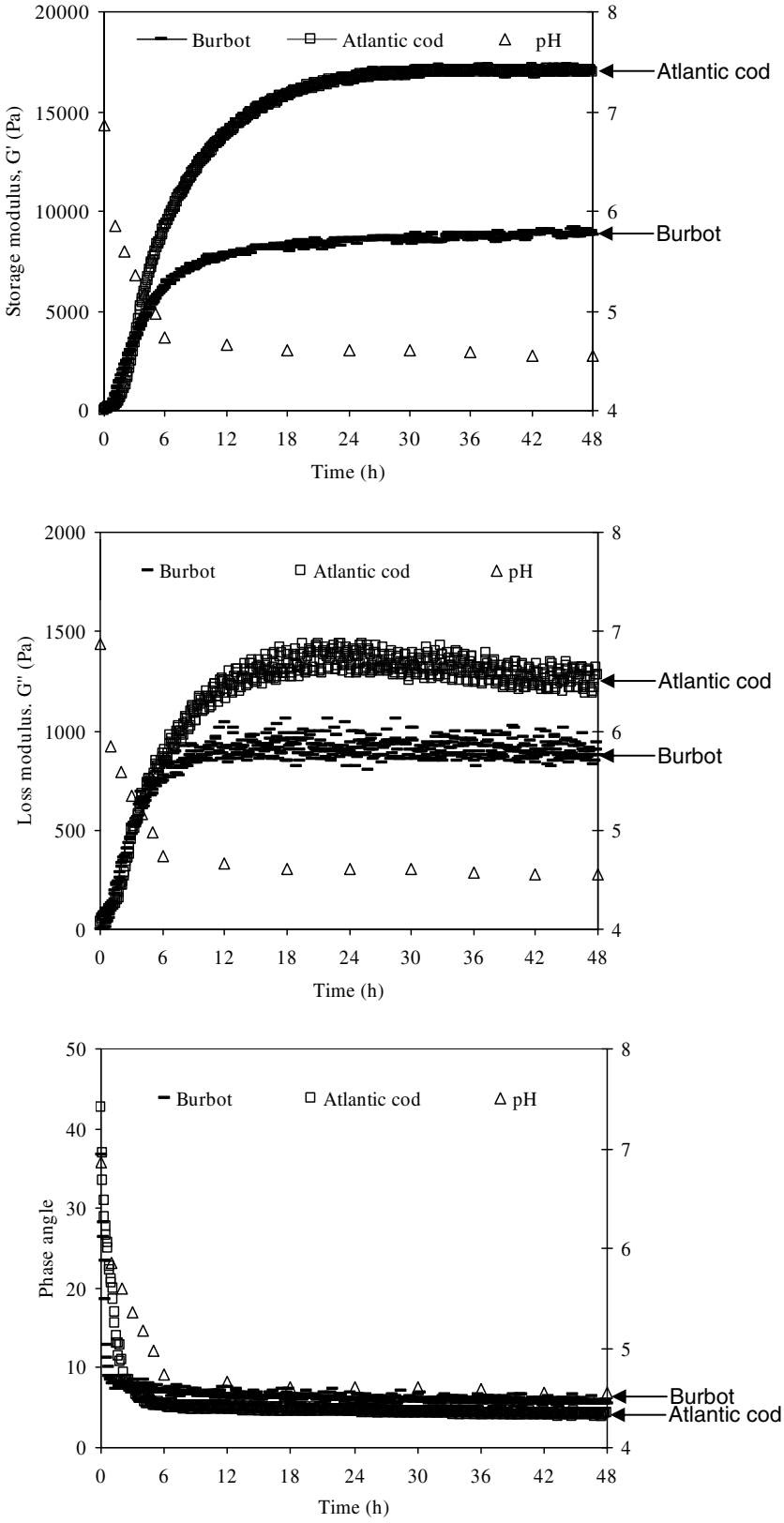

Fig. 6. Storage modulus, loss modulus and phase angle of myosin from burbot and Atlantic cod added without and with GDL during incubation at room temperature.

the conformational changes, in which the hydrophobic domains were exposed as evidenced by the increased surface hydrophobicity. The exposure of hydrophobic domains is suggested as a prerequisite for the formation of large myosin aggregates (Egelandsdal, Martinsen, & Autio, 1995). Fretheim et al. (1985) reported that the

conformation changes of acidified bovine myosin were involved in the acid-induced gelation. The increase in hydrophobicity was likely to be from the head region of myosin (Kristinsson & Hultin, 2003). From the result, protein conformation might be altered under acidic pH values. The constant surface hydrophobicity of Atlantic cod myosin after 30 h might reflect the balance between hydrophobic interaction and the exposure of hydrophobic proteins. Therefore, the differences in surface hydrophobicity during acidification between myosin from both species were caused by the differences in the rigidity of structure determined by varying bondings.

3.1.6. Dynamic rheological properties

Typical changes in rheological properties of myosin from Atlantic cod and burbot during acidification are shown in Fig. 6. With acidification, the storage modulus (G') and loss modulus (G'') of myosin from both the fish species increased ($p < 0.05$). The G' value of myosin sharply increased during the first 12 h of incubation, at which a significantly higher ($p < 0.05$) G' value was obtained for Atlantic cod (13,810 Pa) than for burbot (7879 Pa). The increase in G' of myosin acidified with GDL was caused by the entanglement of myosin molecules as well as the aggregation of myosin at pH values close to p*I*. Furthermore, disulphide bond and hydrophobic interaction could be formed between adjacent myosin molecules. The G' value of myosin from both fish species was generally higher than the G'' value during acidification, indicating that myosin became a more elastic gel. The poorer aggregation of burbot myosin during acidification correlated with the inferior elastic properties. An increase in G' value, a measure of energy recovered per cycle of sinusoidal shear deformation, indicated the increase in elasticity of the sample associated with the formation of elastic gel structure (Egelandsdal et al., 1995). G'' is a measure of the energy of dissipation per cycle of sinusoidal strain when different systems are compared at the same strain amplitude (Egelandsdal et al., 1995). G'' indicates the extent of viscous element in the sample. Egelandsdal et al. (1995) suggested that the initial increase in G' resulted from the cross-link between myosin molecules accompanying the denaturation of heavy meromyosin. The final increase in G' probably arose from the formation of irreversible gel network. Additionally, the rheological properties of protein are governed by composition as well as molecular shape, size, and charge, and are influenced by factors such as temperature, concentration, pH and ionic strength (Hamann, 1994). The protein–protein and protein–solvent interactions are influenced by many factors and affected by the type and properties of gels (Egelandsdal et al., 1995; Hamann et al., 1994). From the result, the increase in G' and G'' values of myosin during acidification indicated the formation of an elastic gel.

The phase angle value is used to describe viscoelastic behaviour (Steffe, 1996). This parameter is directly related to the energy loss per cycle. During the first 6 h of incubation, the phase angle of myosin rapidly decreased. Thereafter, it remained constant up to 48 h ($p > 0.05$). After 48 h of the incubation, the phase angle values of myosin from Atlantic cod and burbot were 4.3 and 5.6, respectively. The use of phase angle to evaluate network characteristics has the advantage of incorporating the contribution of G' and G'' into a single parameter to evaluate the final network (Egelandsdal et al., 1995). Changes in the phase angle reflected a transition of the viscous myosin sol to the elastic myosin gel, which correlated with the changes of G' observed during acid-induced gelation.

3.2. Effect of GDL acidification on thermal property and microstructure of myosin

3.2.1. Thermal denaturation

Myosin from Atlantic cod and burbot showed the typical thermogram with one major endothermic peak (Fig. 7).

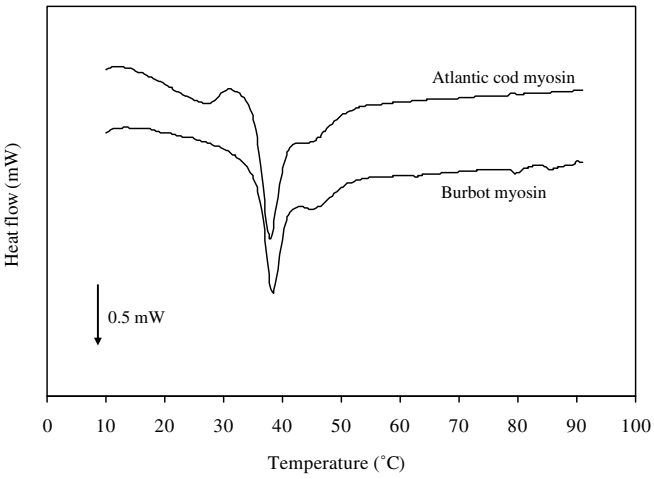

Fig. 7. DSC thermogram of myosin from burbot and Atlantic cod heated from 2 to 95 °C at a rate of 1 °C/min.

Table 1
T_{max} and ΔH of Atlantic cod and burbot myosins before and after acidification with GDL*

Samples	Peak 1	
	T_{max} (°C)	ΔH (J/g dry sample)
Atlantic cod-myosin	37.63 ± 0.92a** B***	3.39 ± 0.09aA
Atlantic cod-myosin-salt	35.71 ± 1.22bB	3.02 ± 0.01bB
Atlantic cod-myosin-salt-GDL (pH 4.6)	32.08 ± 0.59cB	2.22 ± 0.00cB
Burbot myosin	38.84 ± 1.02aA	3.27 ± 0.09aB
Burbot myosin-salt	37.12 ± 1.24bA	3.18 ± 0.04bA
Burbot myosin-salt-GDL (pH 4.6)	35.55 ± 1.32cA	2.84 ± 0.01cA

* Mean ± SD from four determinations.
** Different letters in the same column within the myosin from the same fish species indicate significant differences ($p < 0.05$).
*** Different letter cases in the same column between the myosin from different species under the same treatment indicate significant differences ($p < 0.05$).

The maximum transition temperature (T_{max}) of myosin from Atlantic cod and burbot were observed at 37.6 and 38.8 °C, respectively (Table 1). T_{max} is a temperature at which protein undergoes the conformational changes, resulting in the subsequent protein–protein interactions and aggregate formation (Ishioroshi, Samejima, & Yasui, 1979). The observed T_{max} was within the temperature range of 25–46 °C found among various fish species (Ogawa, Ehara, Tamiya, & Tsuchiya, 1993) and closely related to those of other cold-water fish including cod and herring (Angsupanich, Edde, & Ledward, 1999). However, it was lower than those of myosins extracted from chicken, turkey, beef, and pork, of which transition normally occurred at 45–55 °C. The results indicated that both myosins were highly unstable to heat and its thermal susceptibility might be related to the habitat temperature of the living animal. Angsupanich et al. (1999) reported that the endothermic transition of cod myosin was found at 34 °C. Visessanguan et al. (2000) reported that thermal denaturation of myosin was due to the disruption of α-helical structure.

After acidification, the decreases in T_{max} and ΔH were generally observed in myosin from both fish species ($p < 0.05$) (Table 1). The lowered pH directly affected the thermal stability of myosin. Wright and Wilding (1984) also reported that myosin denaturation enthalpy decreased when pH value was lowered to 5.5. The prolonged exposure to low pH was associated with the conformational alteration and aggregation, in which lower temperature and enthalpy were required for complete denaturation. Additionally, salt might induce the dissociation of actomyosin complex (Beas, Wagner, Crupkin, & Anon, 1990). From the result, myosin peak exhibited the lower T_{max} and ΔH in the presence of 0.5 M sodium chloride ($p < 0.05$). Therefore, the decrease in thermal stability of myosin was caused by acidification using GDL.

3.2.2. Microstructure

Microstructures of acidified myosin from Atlantic cod and burbot are shown in Fig. 8. After acidification, myosin exhibited the network formation with inter-connecting fibrous strands. A highly interconnected and finer network structure was observed in acidified Atlantic cod myosin. The network of acidified Atlantic cod myosin was denser than acidified burbot myosin. Gelation is the result of protein denaturation, which leads to intermolecular covalent and non-covalent interactions, including disulphide bonds and hydrophobic interactions (Totosaus et al., 2002). The pH lowering produces enough protein denaturation to cause the interactions and the formation of a network structure (Totosaus et al., 2002). Fretheim et al. (1985) reported that the myosin solution formed gels at 5 °C when the pH decreased slowly to the range of 2.5–5.5. At pH 4.0, myosin filaments can interact spontaneously and form a gel network without heating (Hermansson, Harbitz, & Langton, 1986). Protein–protein interactions of myosin are dependent upon the temperature and pH (Totosaus et al., 2002). The conformational changes in myosin head during acidification might initiate the cross-linking via various bondings and the interconnection of tail portion also took place. As a consequence, a gel matrix was formed. Thus, the decrease in pH could induce the protein–protein interaction, in which protein aggregate or network could be formed.

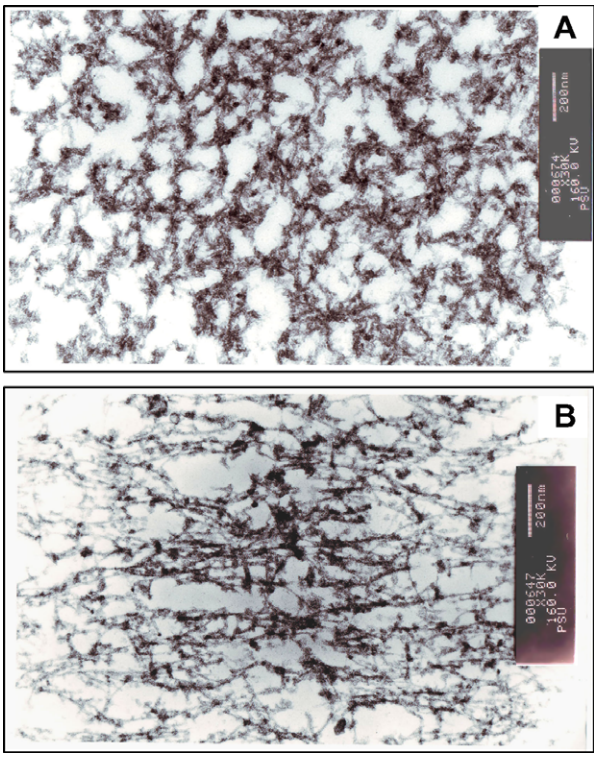

Fig. 8. Transmission electron micrograph of acidified burbot (A) and Atlantic cod (B) myosin at the 30,000× magnification.

4. Conclusion

Gelation of myosin from burbot and Atlantic cod was induced by GDL at room temperature. Decrease in pH induced the denaturation and aggregation of myosin molecules via electrostatic interaction, hydrophobic interaction and disulphide bond formation. However, gel characteristics varied with species. Therefore, the uses of mixture between different fish species could be a promising means to improve the acid-induced gelation of those having the poor gel forming ability. Further investigation should be carried out to obtain the acid-induced fish protein gel with superior quality and high acceptability.

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