Effect of ultrasonication on secondary structure and heat induced gelation of chicken myofibrils

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Abstract Ultrasonication has been suggested as a new promising technique to improve the quality of meat and other meat products. In this study ultrasonication at low frequency (20 kHz) was carried out to investigate the effect on structural and biochemical properties of myofibril proteins. The possible implications between ultrasonication-induced structural changes and gelation properties were also investigated. Structural changes were investigated by ATPase activity, SDS-PAGE, circular dichroism and fluorescence spectroscopy. Microstructural changes in heat induced gels were observed by SEM and water holding capacity was determined by centrifugation. Ultrasonic treatment for 30 min significantly reduced the Ca^{2+} -ATPase activity. Moreover significant change in structure of proteins at secondary level, as indicated by marked decrease in α -helicity, was observed. Marginal change in fluorescence at 10 min was followed by significant increase at 20 and 30 min reflecting exposure of hydrophobic residues on surface during unfolding. Microstructural analyses of gels showed marked improvement in regular three dimensional network at 20 and 30 min of sonication. WHC at 20 min and 30 min were significantly higher than control. Our results suggest that ultrasonication at low frequency (20 kHz) can prove beneficial for improving functional properties of meat and meat products.

Keywords Chicken myofibrils · CD spectroscopy · Fluorescent spectroscopy · Scanning electron microscopy · SDS-PAGE · Ultrasonication

Introduction

Over the past few decades, ultrasonication as an emerging technique has been paid special attention to modify the biochemical and functional attributes of meat or meat products for consumers satisfaction. Available reports suggest that ultrasound improves water retention capacity, textural properties and cohesiveness of meat and meat products by facilitating the release of myofibrillar proteins (Reynolds et al. 1978; Vimini et al. 1983; McClements 1995; Dolatowski et al. 2000). Ultrasound was found effective in disrupting the architecture of myofibrils and thereby facilitating the solubilisation of more than 80 % of myofibrillar proteins in water (Ito et al. 2003). Moreover, reports suggest that high power ultrasound can change the degree of meat tenderness (Jayasooriya et al. 2004) either by breaking integrity of muscle cells or by promoting enzymatic reactions (Chemat and Khan 2011). Many researchers suggest that ultrasound can be used to increase the mass transfer (Cárcel et al. 2007; Siró et al. 2009) by accelerating the diffusion of salts and simultaneous extraction of myofibrillar proteins during brining of meat (McDonnell et al. 2014).

Above cited reports on the benefits of ultrasound in modulating physicochemical and functional attribute of muscle proteins are very convincing. As demand for promising techniques to improve sensory and functional properties of meat are continuously growing (Petracci and Cavani 2012), therefore to understand the effect of ultrasonication on structural and functional properties of

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myofibrils is very important for obtaining desirable functional meat products and stable meat qualities. Li et al. (2014, 2015) has successfully demonstrated the association between structural changes induced by ultrasound and gelation properties of chicken meat. However, these reports describe the application of ultrasound in structured whole muscle where influence of ultrasonication (in presence of various factors) on gelling properties of myofibrils is not clearly understood. In order to limit the number of factors and to decrease the complexity, myofibrils as simplified model system, is advantageous to understand the effect of ultrasound. Published literature do not show any report on the effect of ultrasonication on structure and functional properties (more specifically, on gelation) of 'extracted myofibrils'. Therefore, the present study was carried out to understand the possible association between ultrasonication and gelation property of isolated myofibrils keeping the following two objectives: Firstly, to determine the effect of ultrasound (20 kHz) on structure of isolated myofibrils of broiler chicken and secondly, to assess the impact of these structural changes on heat-induced gelation.

Materials and methods

Materials

Acrylamide, adenosine 5'-triphosphate disodium salt (ATP), ammonium per sulphate, bis-acrylamide, phenylmethanesulfonyl fluoride (PMSF), tris buffer, osmium tetroxide and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were procured from Sigma-Aldrich chemicals Pvt. Ltd. 1-amino-2-naphthol-4-sulphonic acid, potassium chloride, bovine serum albumin and Perchloric acid were purchased from SRL, India. All other chemicals and reagents used were of analytical grade. Broiler chicken (*Gallus gallus*) aged between 14 and 16 weeks were used in this study. Breast meat was obtained from freshly sacrificed broiler chickens and transported to lab from local market in ice box. *Pectoralis major* from breast muscles were excised out and were manually trimmed and cleaned from connective tissues and fats.

Methods

Extraction of myofibrils

Myofibrils from breast (*Pectoralis major*) muscles of freshly sacrificed chicken were prepared by the method of Perry (1955) with slight modifications. Briefly, the muscles were excised out and finely chopped and minced with sharp knife. Finely minced muscles were first

washed two times in 5 mM Tris-maleate (pH 7.2) at 5000 rpm to remove water soluble sarcoplasmic proteins. Then, washed minced muscles were repeatedly washed 4 times with a solution containing 20 mM KCl and 10 mM Tris-maleate (pH 7.2). After washing the minced muscles were suspended in a solution containing 50 mM KCl, 5 mM MgCl₂, 2 mM EGTA, 2 mM PMSF and 20 mM Tris-maleate (pH 7.2) for 30 min. The suspension was filtered through two layer cotton gauze to remove connective tissue. The filtrate was centrifuged at 2000 rpm for 10 min and saved as extracted myofibrils. The preparation of myofibrils was screened by SDS-PAGE and protein concentration was determined by the method of Lowry et al. (1951).

Ultrasonication

Extracted myofibrils (4.5 mg/ml) dissolved in 0.1 M NaCl (20 mM Tris-maleate, pH 7.2) were exposed to low frequency ultrasound in a beaker surrounded by crushed ice. Probe type ultrasonic processor (Ralco, India Ltd.) was used to administer ultrasonic treatment at constant frequency of 20 kHz and power of 120 W. The probe of the sonicator (12 mm) was immersed in middle of the solution and ultrasonic burst of 30 s followed by 10 s cooling lag was given. Four aliquots of equal volume at the interval of 5, 10, 20 and 30 min were collected and saved for future analyses.

Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was utilized to monitor the changes in polypeptide composition of sonicated myofibrils. The gels were prepared and run according to the method of Laemmli (1970). Briefly, resolving gels (10 %) and stacking gels (4 %) were prepared from the stock solution (acrylamide: bis-acrylamide = 29.2:0.8). Resolving gel contain 15 % glycerol where as all buffers including acrylamide gels contain 0.1 % SDS. Samples were prepared in SDS containing 5 % β-mercaptoethanol and boiled for 5 min. After the run was over, the gels were washed in solution containing 30 % (v/v) methanol and 10 % (v/v) acetic acid. The washed gels were stained with CBBR-250 and destained with 5 % acetic acid. Stained gels were documented using digital camera (SONY CYBERSHOT; Zoom-4X; 14.1 Megapixels) or by direct scanning on an all-in-one HP Deskjet (F370) computer assembly. Best contrast prepared on Photoshop (Adobe Photoshop 7.0) was selected and densitometry was done by Scion Imaging software programme (Scion Corporation: Beta release 4.0).

ATPase activity

Ca^{2+} -ATPase activity of control and sonicated myofibrils were determined by the method of Hasnain et al. (1979) with slight modifications. Briefly, 2 ml reaction mixture at final concentration contained: 25 mM Tris-HCl buffer (pH 7.5), 5 mM CaCl_2 , 50 mM KCl and 0.2 ml protein sample. After pre-incubating for 2 min to allow proper mixing, the reaction was initiated by ATP (1 mM final). The reaction initiated by ATP was conducted precisely for 4 min at 20 °C and terminated by adding equal volume of trichloroacetic acid (15 % w/v). Blank was prepared by adding trichloroacetic acid (15 %) to reaction mixture before the addition of ATP. Liberated inorganic phosphate (Pi) was determined by the method of Fiske and Subarow (1925).

CD spectroscopy

The CD spectra of sonicated and control protein samples were recorded by using Chirascan spectrometer (Applied Photophysics) at 20 °C in 20 mM phosphate buffer (pH 7.2). The protein sample was diluted to 0.1 mg/ml and transferred to quartz cell with 1 mm path length. CD spectra in the range of 200–260 nm were recorded with resolution of 0.1 nm at 50 nm/min scan rate having response time of 5 s. Three scans were recorded and average was corrected for the solvent signal. Alpha helicity (%) was calculated from mean residue ellipticities at 222 nm $[\theta]_{222}$ according to equation given below (Ogawa et al. 1995).

$$\text{Alpha-helicity (\%)} = 100 \times \{\text{MRE}/-40,000\}$$

The mean residue ellipticity (MRE) at 222 nm was calculated by the following equation (Kelly et al. 2005).

$$\text{MRE} = 115 \times [\theta]_{222\text{nm}}/10 \times d \times c$$

where, 115 is mean residue molecular weight of myosin, $[\theta]_{222\text{nm}}$ is the observed ellipticity (degrees) at wavelength 222 nm, d (cm) and c (g/ml) are the path length and concentration, respectively.

Determination of surface hydrophobicity

The surface hydrophobicity of myofibrils was measured using 1-anilinonaphthalene-8-sulphonic acid (ANS) as a probe according to the method of Benjakul et al. (1997) with some modifications. Protein concentration of myofibril sample was adjusted to 1 mg/ml with 0.6 M NaCl phosphate buffer (pH 7.0). The assay was carried out in 2 ml myofibril sample by adding 10 µl of ANS (15 mM in 0.1 M phosphate buffer, pH 7.0). After 20 min at 25 °C, the fluorescence was measured by using excitation

wavelength of 390 nm and an emission wavelength in the range of 400–600 nm with fluorescence spectrophotometer (Hitachi F-2700).

Heat-induced gelation

Control and sonicated myofibrils were placed in glass tubes and pre heated at 40 °C for 5 min followed by heating at 70 °C for 15 min in water bath. The glass tubes were immediately cooled under flowing tap water for 15 min. The gels were stored at 4 °C for 8–12 h for estimation of WHC and observation under SEM.

Scanning electron microscopy of gels

Heat induced gels of control and sonicated myofibrils were cut into 2–4 mm cubes and fixed in 2.5 % glutaraldehyde containing phosphate buffer, pH 7.2 for 3 days. After fixation, the gels were rinsed three times with distilled water and were exposed to 1 % OsO_4 for 4 h. After 3 washings in 0.1 M phosphate buffer (pH 7.0) the specimens were dehydrated in increasing series of ethanol for 2 h. Finally the specimens were dried with liquid CO_2 by critical point dryer (Leica, EM CPD 300). The dried specimens were mounted on aluminium stubs, coated with gold and observed under scanning electron microscope (JEOL JSM 6510).

Water holding capacity (WHC) measurement by centrifugation

WHC of gel was measured by centrifugation. The gel (2gm) was centrifuged at 10,000 rpm for 10 min and supernatant was discarded. The total weight of centrifuge tube and gel was taken before and after centrifugation. WHC was expressed as the ratio of gel weight after centrifugation to the initial gel sample weight

$$\text{WHC (\%)} = (W_2 - W)/(W_1 - W) \times 100$$

where W_2 and W_1 respectively are the total weight (g) of centrifuge tube with gel after and before centrifugation, W is the weight (g) of centrifuge tube.

Statistical analysis

The experiments performed in random design were repeated three times ($n = 3$) and all analyses were carried out in triplicates. One-way analysis of variance was performed using SPSS 16.0 software (SPSS Inc., USA). Significant differences were defined at $p < 0.05$ and comparisons of means were determined using Duncan's multiple range test.

Results and discussion

ATPase activity

Ca^{2+} -ATPase activity of myofibrils was monitored at 5, 10, 20 and 30 min of ultrasonication (Fig. 1). A significant ($p < 0.05$) decrease in Ca^{2+} -ATPase activity of myofibril was observed during ultrasonication. Maximum decrease was observed between 5 and 10 min followed by gradual decrease up to 30 min of sonication. At 5 min, with respect to control, marginal activation in Ca^{2+} -ATPase activity was noted due to low frequency ultrasonic treatment. Overall, 29 % (± 1.97 %) decrease in enzymatic activity of myofibrils was estimated during ultrasonication for 30 min. Decrease in ATPase activity of various myofibrillar proteins under ultrasonic treatment is also observed by many workers (Barany et al. 1963; Ahmad and Hasnain 2013; Saleem et al. 2015a). Ca^{2+} -ATPase activity was functionally correlated with the myosin molecule integrity (Benjakul et al. 1997; Saleem et al. 2015b). Globular heads of myosin are mainly responsible for Ca^{2+} -ATPase activity. Decrease in Ca^{2+} -ATPase activity during ultrasonication indicates possible conformational changes of myosin specifically in head region. As globular head region is more susceptible to ultrasonic treatment (Barany et al. 1963) and conformational changes in the same region was responsible for decrease in ATPase activity (Okada et al. 1986), we suggest that ultrasonication at low frequency causes changes in native conformation of globular head region of myosin.

Fig. 1 Change in Ca^{2+} -ATPase activity of myofibrils from breast muscles of broiler chicken due to ultrasonication at low frequency. *Significance at $p < 0.05$ with respect to control

Electrophoresis

The effect of low frequency ultrasound on polypeptide composition of myofibrils from breast muscles was analysed by SDS-PAGE, shown in Fig. 2. Myofibril contains myosin and actin as major proteins which are naturally associated with troponin and tropomyosin (Benjakul et al. 2001). Electrophoretic profile of myofibrils reveal that bands of major polypeptides like myosin heavy chain (~ 200 kD), alpha-actinin (around 97.4 kD), actin (~ 42 – 44 kD), tropomyosin (~ 36 kD) were intact and no fragmentation was observed. With respect to unsonicated control, the intensity of polypeptide bands of sonicated samples showed no variation with increasing time of sonication. Similar results showing absence of any cleavage product in SDS-PAGE under the influence of ultrasonication were also reported by Ito et al. (2003). Moreover, SDS-PAGE results of this study were in confirmation with our previous reports (Ahmad and Hasnain 2013; Saleem et al. 2015a) and thereby it is reasonable to assert that low frequency ultrasonication under the present experimental conditions does not cause any fragmentation of major myofibrillar proteins.

Fig. 2 SDS-PAGE (10 %) of myofibrils treated with low frequency ultrasound (20 kHz). Lane M molecular weight marker, Lane C control, Lane 5, 10, 20 and 30 represent myofibrillar samples sonicated for 5, 10, 20 and 30 min respectively

Circular dichroism (CD)

The change in secondary structure of myofibrils under the influence of ultrasonication was determined by CD spectroscopy as illustrated in Fig. 3. Circular dichroism spectra in far-UV wavelength region are routinely used to monitor the changes in conformation of myofibrillar proteins (Choi and Ma 2007). As shown in Fig. 3, the CD spectrum showed two minima at 210 and 222 nm, indicating the presence of α -helical structure (Greenfield 2007). As myosin constitutes major portion of myofibrillar proteins, the α -helix conformation is predominantly determined by supercoiled α -helix structure of myosin tail (King and Lehrer 1989; Kristinsson and Hultin 2003). Structurally, myosin is composed of two globular heads and a rod-like tail (Harington and Rodger 1984). The tail portion of myosin is almost 100 % helical, whereas the globular head region has less than 50 % α -helix. The far-UV CD spectra of myofibrils treated with low frequency ultrasonication are distinctly different from unsonicated control samples. It indicates that myofibrils were significantly perturbed at secondary structural level by ultrasonication. At 5 min of sonication, the magnitude of far UV CD spectra was noticeably reduced indicating distinct decrease in α -helical content. When ultrasonic treatment was further increased, the magnitude of far-UV CD spectra showed significant

Fig. 3 Far-UV circular dichroism (CD) spectra of myofibrils treated with low frequency (20 kHz) ultrasound

reduction reflecting distinct decrease in α -helicity of myofibrils under the influence of ultrasonication. By calculations from CD spectra, 14 % decrease in α -helix fraction was noted by 30 min of sonication with respect to control. Reduction in α -helix fraction clearly indicates that secondary structure of myofibril proteins is distinctly affected by ultrasonication at low frequency. Gulseren et al. (2007) also pointed out that structural changes in proteins under the influence of ultrasonic radiations are concomitant with the decrease in α -helicity of proteins. Our findings are in agreement with those of Li et al. (2015) where ultrasound has been reported to cause decrease in α -helical content of proteins present in chicken meat batters. Since the secondary structure of proteins is mainly stabilised by electrostatic interactions and hydrogen bonding that occurs between carbonyl oxygen and amino hydrogen of polypeptide chain (Satoh et al. 2006), it is quite reasonable to accentuate that sonication may cause disruption of these interactions leading to change in secondary structure.

Surface hydrophobicity

Changes in surface hydrophobicity of myofibrils under the influence of ultrasonication at low frequency (20 kHz) are illustrated in Fig. 4. The graph showed marginal increase in surface hydrophobicity by 10 min of sonication, whereas marked increase in surface hydrophobicity was observed at 20 and 30 min of sonication. Since 1-Amino-2-naphthol-4-

Fig. 4 The change of surface hydrophobicity of myofibrils treated with low frequency ultrasonication

sulphonic acid (ANS) has been found to bind hydrophobic amino acids on unfolded myosin molecule (Benjakul et al. 1997), surface hydrophobicity can be used as an indicator of conformational changes occurring in myofibril proteins, specifically in myosin. Moreover, it is reported that myosin head is richer in hydrophobic residues (Maita et al. 1991), therefore marked increase in hydrophobicity observed at 20 min and 30 min indicated significant exposure of hydrophobic residues in head region of myosin. A significant decrease in Ca^{2+} -ATPase activity (as an indicator of myosin integrity) also demonstrates that 20 min and 30 min of ultrasonic exposure significantly perturb the conformation of myosin head. As reported, unfolding of myosin is accompanied by increased hydrophobicity (Hemung et al. 2008) due to exposure of deeply buried hydrophobic amino acids, present in native state, to the surface of protein (Riebroy et al. 2008). The results of our study indicate that hydrophobic regions of unsonicated native myofibrils were located deep within the interior of protein complex, while the ultrasonication associated cavitation might have exposed some of the hydrophobic regions to the surface. Samejima et al. (1981) proposed that heat induced gelation of myosin consists of two steps: (1) aggregation of globular head and (2) network formation of the unfolded helical tail segment. With increased exposure of hydrophobic sites, their interactions become inevitable and it can be inferred that improved gelation of myofibrillar proteins by ultrasound is probably facilitated by the increased exposure of hydrophobic sites in head region.

Heat-induced gelation

The change in microstructure of heat induced gels of sonicated myofibrillar proteins from chicken breast muscle is shown in Fig. 5. The solid grey matter in electron micrographs represents the protein filaments whereas dark spots denote the presence of water entrapping spaces. We observe that three dimensional network of heat-induced gels of sonicated myofibrils is markedly different from the gels of unsonicated control myofibrils. The gels from control samples of myofibrils possess dense aggregates with minute pores. Variation in architecture of heat-induced gels of chicken meat batter due to ultrasonication was also reported by Li et al. (2014, 2015). The gels of myofibrils sonicated for 5 min possess disordered structure with large number of dense aggregates having irregularly arranged large pores. When ultrasonication was carried for 10–30 min, the distribution of pores showed marked uniformity in gels. In addition, the size of pores decreased and thereby imparting honey-comb like appearance to gels. At 20 and 30 min, more filaments with increased number of small cavities having homogenous distribution were seen.

These results are in good agreement with hydrophobicity and may be accounted for better protein–protein interaction. Myofibrillar proteins play an important role in controlling the functional properties of meat products such as gelation, water holding capacity and texture (Puolanne and Halonen 2010). Gelation of myofibrillar proteins involves partial denaturation followed by irreversible aggregation of myosin heads through disulfide bonds formation (Samejima et al. 1981). Helix-coil transition of myosin tail is mainly responsible for the formation of three dimensional networks (Samejima et al. 1981). The heat induced gelation occurs in two phases: (1) at low temperature (<55 °C) the major events are changes in conformation and (2) at high temperature (>55 °C) involves the aggregation of myosin rod (Chan et al. 1992). Distinct change in conformation of myofibrils, as depicted by CD spectra, and surface hydrophobicity indicates positive correlation between gelling properties and structural changes under the influence of ultrasound. Exposure of ultrasound before heating might have facilitated the denaturation and aggregation of myofibrillar proteins during heat induced gelation to form homogenous and fine network of gels.

Water holding capacity (WHC)

Water holding capacity is one of the most significant functional properties of heat-induced protein gels (Puolanne and Halonen 2010). The change in WHC of sonicated myofibril gels due to ultrasonication is shown in Fig. 6. We observed non-significant ($p > 0.05$) increase in WHC at 5 and 10 min of sonication. However ultrasonic exposure beyond 10 min showed marked increase in WHC. Ultrasonic exposure of 30 min leads to 26 % (± 2.13 %) increase in WHC of myofibril gels. WHC of gel reflects the ability of association between gel proteins and water. The observed variation in WHC showed almost parallel correlation with change in microstructure of myofibril gels caused by ultrasonication. It appears that ultrasonication promoted the formation of stable three dimensional networks with improved water retention capacity. The gels at 5 and 10 min depict dense and irregular aggregates whereas at 20 and 30 min, the structure of gel is more regular with negligible aggregates. Protein gels with homogenous and fine arrangement had comparatively higher WHC than gels with non-homogenous structure (Samejima et al. 1981). Moreover ultrasonication has been shown to optimise the gelation properties of chicken meat resulting in improved water holding capacity and structure of heat induced gels (Li et al. 2014, 2015; Saleem and Ahmad 2016). Structural changes under ultrasonication, as indicated by CD spectra and hydrophobicity profile, leads to increased exposure of hydrophobic residues. The exposure of hydrophobic residues and their mutual interactions

Fig. 5 Scanning electron micrographs of heat induced gels of myofibrils from breast muscles sonicated at low frequency. **a** Control, **b**, **c**, **d** and **e** represent microscopic structure of gels of myofibrils sonicated for 5, 10, 20 and 30 min respectively

have been suggested to determine properties of the protein gels (Chan et al. 1992; Benjakul et al. 2001).

Conclusion

The results of our study indicate that ultrasonication at low frequency (20 kHz) causes significant changes in structure of myofibrillar proteins. The alteration in structure of myofibrillar proteins, more specifically of myosin component, leads to change in gelation properties. The ultrasonication not only decreases dense aggregation, but also

positively enhances the water retention capacity of heat induced gels of myofibrils and can also improve water holding capacity of muscle proteins. Several researches came out with interesting applications of high power ultrasound for modifying properties of meat and meat products. The present findings can be directly or indirectly correlated with the physical and/or chemical properties of meat as the myofibrils are the principal constituent of muscle. Hence, ultrasonic treatment can also be utilized for tenderizing meat as a new method alternative to salts because addition of salts may cause serious health hazards. Ultrasonication effects on biochemical and functional

Fig. 6 Change in water holding capacity (WHC) of heat induced gels of myofibrils due to ultrasonication. *Significance at $p < 0.05$ with respect to control

properties of isolated major myofibrillar proteins like actomyosin and myosin demand future investigations.

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