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Full Length Research Paper

Nano-structural analysis of fish collagen extracts for new process development

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Nano and micro imaging were used to evaluate effects of pretreatment solution on gelatin. Four types of pretreatment solution were used during fish gelatin extraction in this study. They are acetic acid (A), acetic acid-NaCl (SA), acetic acid-NaOH (BA) and acetic acid-NaOH-NaCl (SBA). Different patterns were observed for different gelatins pretreated. Results show that each pretreatment gave different nano imaging patterns: A (fibril), SA (zig-zag cracks), BA (straight rods) and SBA (cross-linked rods). Cross-linked rods observed in SBA denote adequate removal of non-collagen content of the fish skin and increased its surface area. SBA is suggested as the best pretreatment for perch fish gelatin. The result of viscosity was also highest for sample SBA (0.0245 ± 0.0001 pas), while viscosity for others are 0.0155 ± 0.0002, 0.0123 ± 0.0001 and 0.0025 ± 0.0001 pas for samples BA, SA and A, respectively. SDS-PAGE analysis showed the presence of α and β in samples SBA and BA.

Key words: Fish gelatin extraction, pretreatment solution, viscosity.

INTRODUCTION

Gelatin from fish has been identified as the most safe and acceptable due to its halal status and also its epidemic free nature as compared to other counterpart sources, that is, porcine, bovine and pig. Although, mammalian gelatin is still in its wide spread, a lot of challenges threaten its future usage. Not so long ago, bovine was threatened with bovine spongiform encephalopathy (BSE) in Europe which questioned its safety for gelatin production. In the recent time, the case of swine flu combined with rejection by Islamic world and Jews cum vegetarian populace remain a stumbling block for pig gelatin (Karim and Rajeev, 2008).

Nanoscience and nanotechnology entail comprehensive understanding and allow modification/manipulation of materials at the atomic, molecular and macromolecular scales. The greater surface area per mass when compared with larger-sized particles of the same chemistry renders nanosized particles more active biologically (Oberdorster et al., 2005). Nanotechnology has the potential to impact many aspects of food and agricultural systems (Weiss and Terech, 2006; Weiss et al., 2006). Applications of nano technology in food include creation of rapid detection methods such as sensors for food safety and quality, design of high performing packaging materials, development of processing technology and development of novel delivery system that better protect functional ingredient and allow for control release of encapsulated compound (Betty and Meryl, 2007).

Different optimal conditions have been used to extract gelatin from fish skin: 0.115 M (84 min) acetic acid (Wang and Yang, 2009), 0.115 M acetic acid plus 0.2 M (3 h) NaOH (Wang and Yang, 2009), acetic acid plus ~0.1 M NaCl (Montero and Gomez-Guillen, 2000) and acetic acid, NaOH and NaCl (Gomez-Guillen and Montero, 2000). Comparative studies at nano scale of effect of different pretreatment sets have only been done for acetic and acetic-NaOH (Wang and Yang, 2009). Comparing different sets of pretreatment methods is a worthwhile study in order to determine the best pretreatment method for gelatin extraction.

Some recent works for nano structural analysis of gelatin involve the use of atomic force microscopy (AFM), transmission X-ray microscopy and scanning electron microscopy (SEM) (Wang and Yang, 2009; Jiang et al.,

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2010; Wangtueai and Noomhorm, 2009). The interaction used in AFM are the local attractive and repulsive forces occurring between a tip attached to a flexible cantilever and the sample surface, while SEM involves bombardment of sample surface with electron beams which are reflected to give the surface pattern (Kumar, 2006). Field emission scanning electron microscopy (FESEM) is more advanced as it uses cold electron sources, better image quality as its size of electron beam is 1000 times smaller than in normal microscopy and can also be used to explore the nanostructure of materials. Very little success has been achieved in order to correlate micro and nano structure of a gelatin to the complex macroscopic physical properties. Microscopic structure was also related to transparency of gelatin. More opaque was associated with increase in concentration of triacetin when Jiang et al. (2010) studied physical and microscopic properties of gelatin and triacetin mixture. The aim and objectives of this study were to investigate different pretreatment methods available for fish gelatin extraction and to evaluate the nano structure of dried gelatin in order to provide useful evident for structure-physical properties correlation.

MATERIALS AND METHODS

Gelatin extraction

Frozen perch fish was obtained from a local supermarket (Kompleks Idaman, Gombak Selangor) and transported in frozen condition to the laboratory where their skins were peeled, washed with water (4°C) and cut into small sizes (2 to 3 cm squares). Pretreatment solutions (0.115 M acetic acid, 0.2 M NaOH and 0.1 M NaCl) were prepared and kept at ~4°C. The skins were divided into four groups, each groups weighing at least 30 g, namely A, BA, SA and SBA. Group A was pretreated with only acetic acid, BA pretreated with NaOH and then acetic acid, AS pretreated with acetic NaCl and then acid, while SBA was pretreated with NaCl followed by NaOH and then acetic acid. Pretreatment timing are: NaCl (24 h), NaOH (3 h) and acetic acid (84 min). After every pretreatment, the skin was adequately washed (3 times) with water and drained using four layers of cheesecloth. Ratio of skin to solutions/water was 1:6 (w:v) while temperature of solutions and water was ~4°C. Extraction was performed with distilled water in a water bath at 55°C for 3 h. The solution was filtered through four layers cheesecloth and the filtered solution was kept at -20°C. Some portions from each group were lyophilized.

Microscopic imaging of fish collagen skin

After adequate scaling and removal of adhered tissues from the fish skin, the layers of collagen were slightly scraped and peeled off from each other. Smaller pieces were then made to attach on glass slide and viewed under a light microscope. The image of each sample was viewed under 10 and 40x eye and objective lens, respectively, and captured with the aid of a digital camera connected to a desktop computer.

Field emission scanning electron microscopy (FESEM) imaging

Powdered sample of lyophilized gelatin was subjected to prefixing by cold slush nitrogen (cryo-fixation) for 30 min. Then, each were mounted on field emission scanning electron microscopy (FESEM, JEOL JIM-5600), and then viewed for their nano structures with the aid of computer.

Determination of viscosity

The concentrations of extracted gelatin solutions were adjusted to 3.3% with distilled water. Then the viscosity of 50 ml of diluted solutions was determined using a Visco Tester at 27 ± 1°C. The instrument was allowed to run for 30 min for each run.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Gelatin was first dissolved at 5 mg/ml in distilled water at 60°C and divided into two parts. The first part of each sample was then mixed with two-fold-concentrated loading buffer containing β-mercaptoethanol. One part was not diluted. Protein samples were heat-denatured for 25 min at 90°C and analysed by SDS-PAGE (Laemmli, 1970) using 4% stacking gel and 9% resolving gel in a Mini Protean II unit (Bio-Rad Laboratories, Hercules, CA) at 25 mA/gel. The loading volume was 15 µl for each sample well. Protein bands were stained with Coomassie brilliant Blue R250.

RESULTS AND DISCUSSION

Microscopic image of fish skin layers

Figure 1 shows the microscopic images of cleaned fish layer. Transparent regions were observed due to the transparent nature of fish collagen which allows light rays to pass through. Thus, a well processed collagen should be transparent. Also, thin connective tissues that appeared opaque to light rays were visibly observed. These are non-collagenous substances that adhered to fish skin collagen. The connective tissues are interwoven and embedded in and out of the collagen layers. During gelatin extraction, it is important that these non-collagenous substances be properly removed in order to avoid lowering interaction between collagen molecules. In addition, fibril patterns of collagen were still retain since the skin had not been treated with any solution that can cause partial denaturization. This is in agreement with report of Mentero and Gomez-Guillen (2000) that collagen has fibril patterns.

Nano imaging techniques

Scanning electron imaging requires bombardment of electron on surface of material to be characterized. Hence, surface fixation is important for any material to be characterized in order to disallow alteration of the surface of materials during imaging. Fixation can be done by either chemical or physical means. Because microstructure of materials are very sensitive to changes in composition, addition of compounds such as a stain or fixative, can cause chemical reaction between the fixative

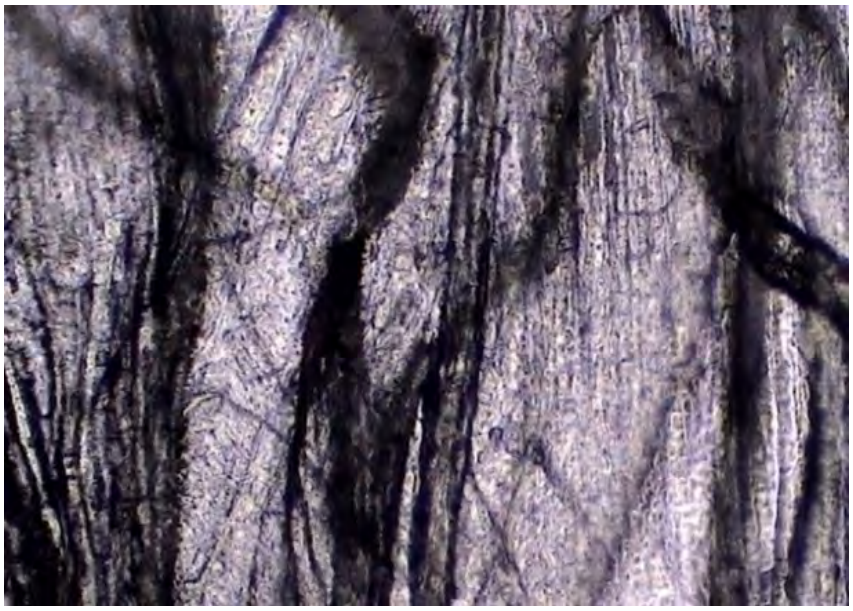

Figure 1. Microscopic images of untreated fish skin collagen layer (magnification 400x).

and the specimen, and often by drying the sample which may alter the original structure of the studies system. This is the reason why chemical fixation is unacceptable for the study of materials like gelatin. Hence, the method that was adopted when analyzing gelatin using FESEM is thermal fixation (ultra-fast cooling of the specimens into a vitrified or quasi-solid state). This is achieved by rapidly plunging or contact with cryogen. In addition, thermal diffusivities are larger than mass diffusivities; therefore, thermal fixation is much more rapid than with chemical.

Nano structural

Examination of impacts of all known pretreatments solutions for gelatin production was studied at nano scale. Experiment was designed in such a way that single effect of these solutions will be understood alongside with their effects when combined. Four pretreatment designs were formulated with the first set pretreated with only acetic acid (A), second with acetic acid and NaOH (AB), third with acetic acid and NaCl (AS) and forth with acetic acid, NaOH and NaCl (ABS).

Figure 2 represents the structure of lyophilized gelatin which was pretreated with only acetic acid and fibril pattern was observed. This fibril pattern is present in the parent material (collagen) meaning that it was not lost during conversion to gelatin. Hence, acetic acid did not affect bonds responsible for formation of fibril pattern. The fibril pattern noticed in acid pretreatment also agrees with the result of Jiang et al. (2010). This fibril pattern was related to partial formation or retention of collagen helitical structure (Mentero and Gomez-Guillen, 2000). Although, while conducting the experiment, swelling was noticed when samples were soaked in acetic acid, possibly due to penetration of water into the skin structure, application of acid, which aids increase in H^+, favours the access of water to collagen fibers which is held in by electrostatic forces between charged polar groups (electrostatic swelling) or hydrogen bonding between uncharged polar groups and negative atoms (lyotropic hydration) (Gustavson, 1956).

Figure 3 represents nano structure of gelatin when pretreated with acetic acid and then NaOH solutions with rod like shapes which are overlapping; comparing this pattern with that of Figure 2, additional effects of NaOH could be studied. NaOH solution was probably responsible for separation of the rods attachment from each other, making overlapping layers to separate from one another. Yoshimura et al. (2000) reported that alkaline attacks predominantly the telopeptide region of the collagen molecule during pretreatment; thus alkaline solution can be used to solubilize collagen. Also, rod-rod side attachment was also affected by revealing each rod as an entity. This means that bonds holding the rods and layer together have been probably affected. These separated rods were the fibrous patterns observed in acid and alkaline pretreated gelatin as reported by Yang et al. (2008). Slight swelling occurred (while sample was in NaOH) which was due to creation of space for water to penetrate and become absorbed. Likewise, any non-collagenous substances initially entrapped within collagen matrix were easily released; thus, making migration of non-collagenous substance from the interior region of skin possible (Cho et al., 2005). Consequently, this made the NaOH solution to change from colorless to light

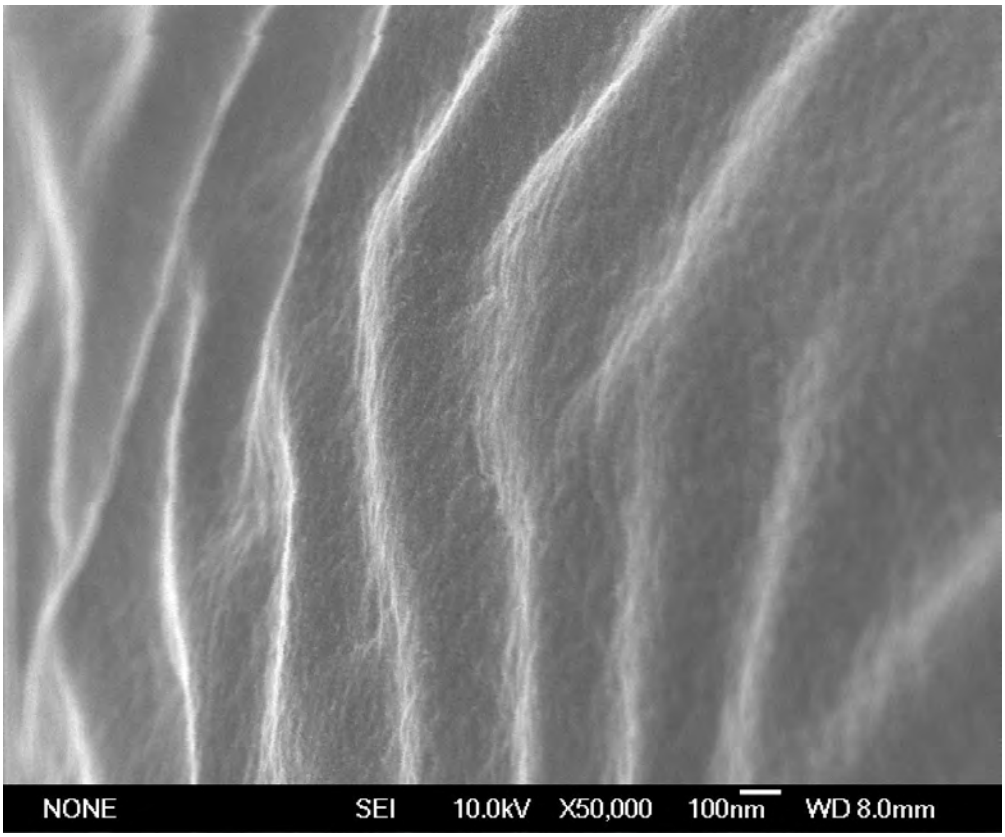

Figure 2. Nano-structure of gelatin at 100 nm pretreated with only acetic acid.

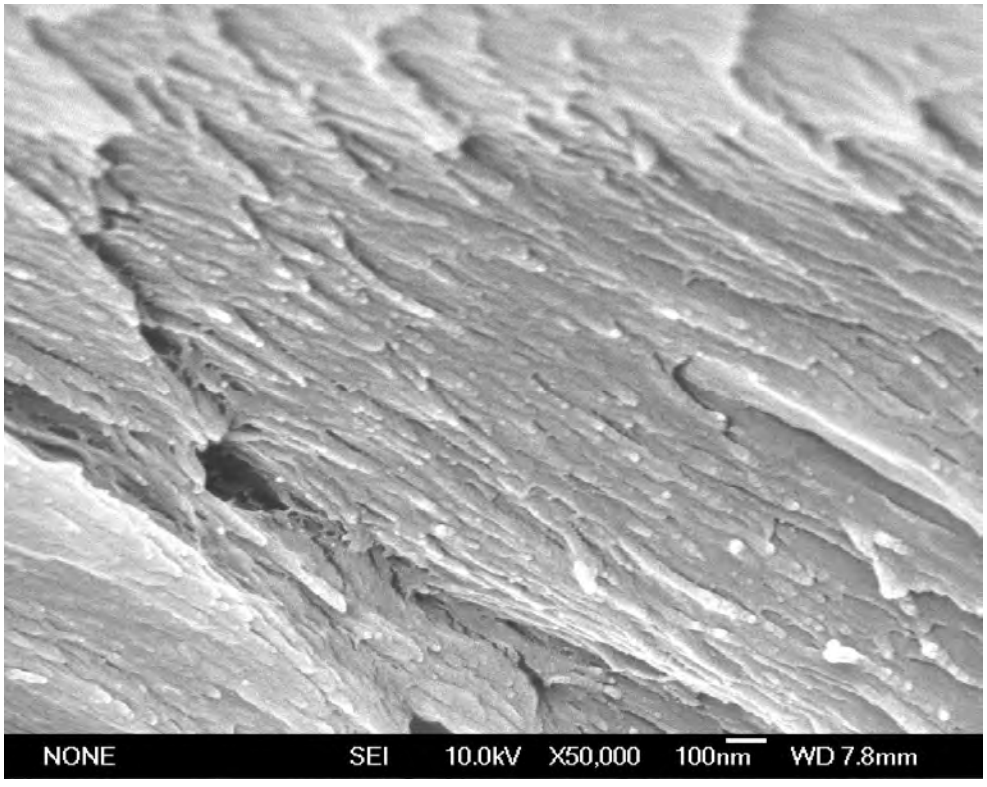

Figure 3. Nano-structure of gelatin at 100 nm pretreated with acetic acid and NaOH.

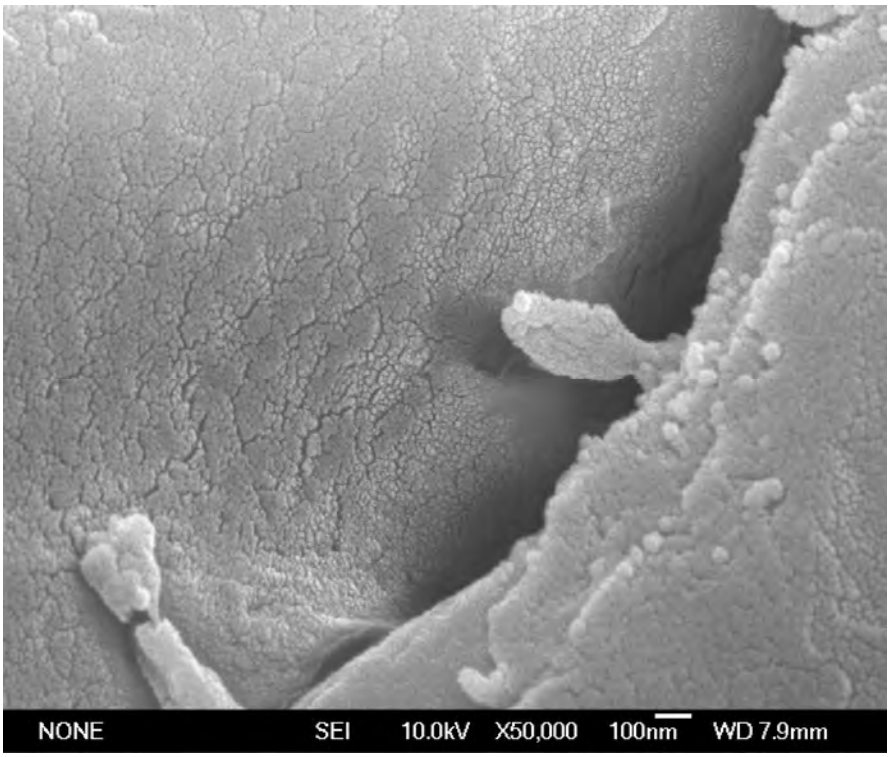

Figure 4. Nano-structure of gelatin at 100 nm pretreated with acetic acid and NaCl.

brown solution during the cause of pretreatment. In addition, the removal of non-collagenous materials and rod formation were responsible for creation of spaces in the gelatin and allow water percolation during rehydration. Spaces or spores were also observed in the nano structure of gelatin, as reported by Yang et al. (2008), when gelatin was pretreated with alkaline.

Figure 4 shows zig-zag cracks patterns when pretreatment involves acetic acid and NaCl solutions. As compared to application of acetic acid alone (Figure 2), NaCl caused breaking bonds responsible for retention of fibril structure. Likewise, some peptide bonds might be affected resulting to formation of cracks (Figure 4). This can be achieved by either binding directly to the peptide backbone of collagen, or affecting collagen folding indirectly by interacting with structurally bounding-water molecules (Asghar and Henrickson, 1982). Lyotropic hydration occurred when ions of neutral salts disrupt non-ionic bonds (e.g hydrogen bonds) of collagen. So salt electrolytes, acting as lyotropic agent, consequently altered water structure around collagen molecules, interrupt internal hydrogen bonds, or interact with internal hydrophobic bonds by direct bindings at same sites of protein chain (Asghar and Henrickson, 1982).

Figure 5 shows cross-linked tubular structures when combination of acetic acid, NaOH and NaCl was applied for pretreatment solution during gelatin production. These rods were no longer in an organized manner as compared to sample pretreated with acetic acid and NaOH solution (Figure 3). Also, adequate spaces are available between the rods. Since NaCl was applied before NaOH, more cracking would have been done on the skin collagen followed by separation of rods and layers by NaOH. Hence, efficient degradation was achieved by combination of NaOH and NaCl with acetic acid. These openings definitely allow adequate release of non-collagenous adhered substances and more room for water penetration during rehydration. These nano tubes will have added advantage of large space to volume for interaction during gelation. Large surface area will be available for Van Daal wall interaction between molecules (tubes) and more protein junctions will also be formed which will lead to increase gel strength. According to Yang et al. (2008), continuous sponge-like patterns at nano scale gave higher macroscopic textural properties than that observed with the irregular separated aggregates.

These cross link rods observed in Figure 5 for sample SBA was an indication that combination of NaCl and NaOH with acetic acid was able to create nano sized protein tubes. This exhibits another potential method of producing protein nanomaterials and gelatin (sample ABS) with high functionalities. Protein nanoparticles have lots of added advantages since they are biodegradable, metabolizable and can also be easily amenable for surface modification and covalent attachment of drugs and ligands. High surface area of sample ABS will enhance force of attraction between molecules during

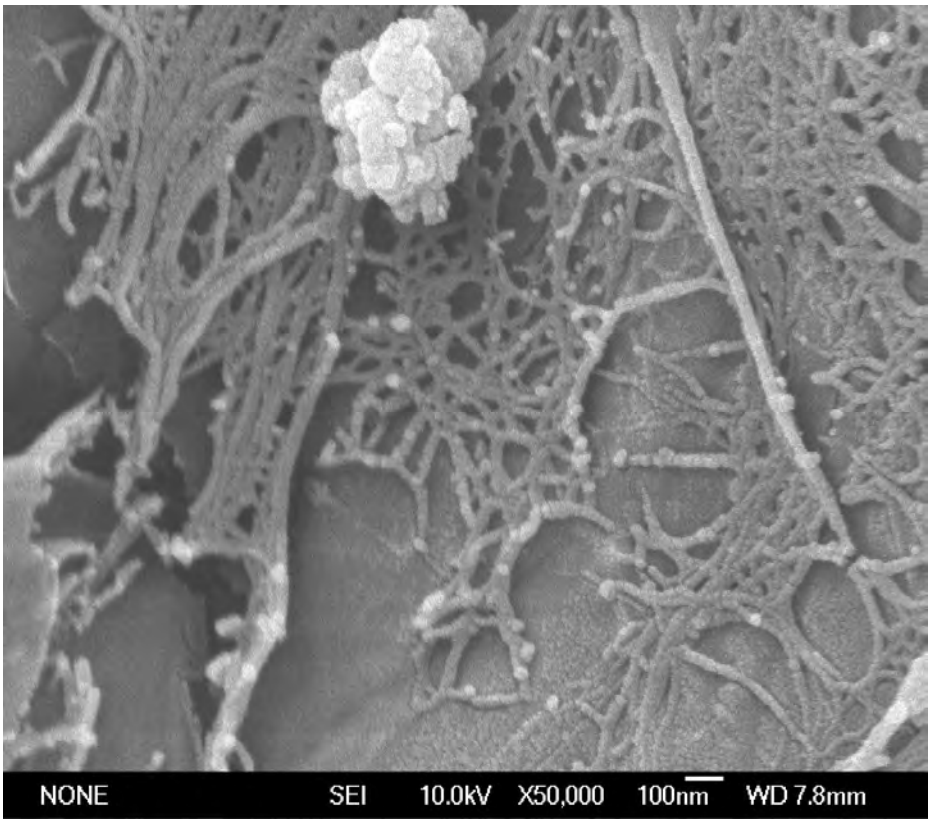

Figure 5. Nano-structure of gelatin at 100 nm pretreated with acetic acid, NaOH and NaCl.

gelation and provide wide surface for attachment of bioactive components. Since drug compounds can be entrapped into the matrix of carrier, sample ABS with meshes of protein tubes will definitely allow easy penetration of bio active materials and become embedded.

Viscosity

Viscosity is a very important parameter in process design and product development. Sample SBA (0.0245 ± 0.0001 pas) has the highest viscosity value followed by sample BA (0.0155 ± 0.0002 pas) and then sample SA (0.0123 ± 0.0001 pas), while sample A (0.0025 ± 0.0001 pas) exhibited the lowest viscosity. All samples behave like Newtonian fluid as the rate of change of shear stress and rate are constants. The highest viscosity of sample SBA strongly suggests that intermolecular interactions (van der Waals and hydrogen bonds) are high between its molecules which are responsible for its resistance to flow. High viscosity is an added advantage in application of gelatin as an ingredient in food industries as it contributes to improvement of product mouth feel and texture.

SDS-PAGE interpretation

Figure 6 shows the results from SDS-PAGE for the four samples in duplicate. The four well known protein bands for gelatin were present namely alpha (α_1 and α_2), beta (β) and gamma (γ) (Karim and Rajeev, 2008). The bands for each sample were duplicated so as to enhance our observation in this analysis.

Apart from these four bands, some low molecular weight proteins were present mostly in sample SA, some traces were also observed for samples BA and sample SBA. Obviously, the intensities of these bands are different from each other. Lowest bands intensity for α, β and γ sized protein, were observed for sample SA, while that of sample A did not even show any of the bands. The result for sample A correlated with that of Gimenez et al. (2005a) that gelatin pretreated with acetic acid alone lack α, β and γ bands, except for the presence of lower band of about 100 kD reported. This difference can be due to variation in concentration of CH_3COOH of 0.115 M as compared to 0.05 M.

Sample SA have very faint bands for α, β and γ proteins, as could be observed from run labeled SA_1. Meaning that some gelatin molecules were released when pretreatments stage include salt. This also agrees with the report of Gimenez et al. (2005b), that salt washing contribute to increase in α, β and γ of gelatin. Salt washing (NaCl or KCl) may enhance opening of collagen structure and facilitate swelling and penetration of acetic acid, thus, allow release of higher molecular weight poly-

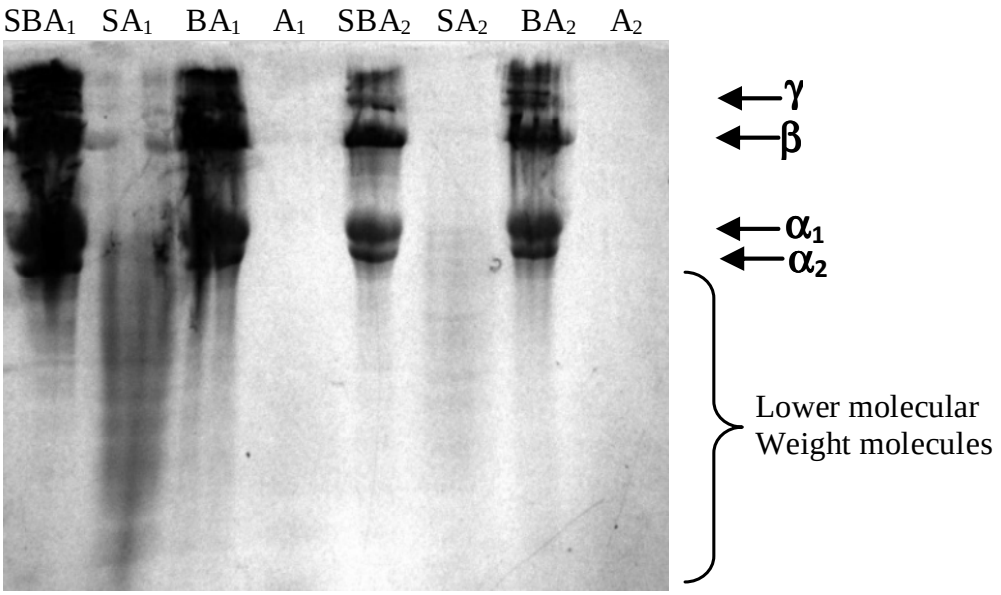

Figure 6. Molecular weight distribution of gelatins from SDS-PAGE analysis. * All samples with sub-script 1 are at higher concentration as compared to those with sub-script 2.

mers more easily during extraction (Gimenez et al., 2005a). However, if salt was not removed from the solution properly, it could still have impact on the collagen structure itself, thus reducing some of collagen molecules to smaller molecular size protein noticed earlier. Consequently, α, β and γ bands of sample SA are not as intense as that of sample AB and SBA.

Addition of alkaline (NaOH) to sample BA and SBA contribute immensely to release of higher molecular sized proteins as these protein bands were conspicuously more than the other two samples. This corresponds with the finding of Zhou and Regenstein (2005). Non-collagenous protein are usually being removed by alkali (NaOH) (Zhou and Regenstein, 2005), so, gelatin (α, β and γ sized molecules) are less affected. This resulted in higher intensity observed for them. Also, combination of CH_3COOH and NaOH has been reported to create a condition that will favor breaking of collagen cross-link rather than peptide bond of collagen structures (Zhou and Regenstein, 2005).

Comparing both sample SBA and sample BA, they are very analogous to each other except that sample SBA has higher band intensity at α_1 and β bands. This might be due to release of some higher sized protein, not affected by NaOH and salt. Molecular weight distribution has great impact on the functional properties of gelatin, most especially gel strength and viscosity.

Conclusion

Dried gelatin powders were evaluated using FESEM which reveals impact of different pretreatment on gelatin. Mechanism and impacts of processing were studies from the structural analysis and SBA was suggested as the best pretreatment for production of high value fish gelatin.

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