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Effect of pH on the formation of edible films made from the muscle proteins of Blue marlin (*Makaira mazara*)

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Abstract

Whole meat of Blue marlin (*Makaira mazara*) was used to prepare edible films. Protein solubility in film-forming solutions was high at acidic and alkaline pHs, while that at neutral pH was close to zero. Acidic and alkaline pHs improved the tensile strength while the effects of pH, on elongation at break, water vapour permeability and light transmission of the films, were not significant. From the film solubility in various protein denaturants it was revealed that the main interaction responsible for the formation of acidic and alkaline pH films was hydrophobic interaction, while that for neutral pH films was ionic bonding.

Keywords: Edible film; Fish muscle proteins; Mechanical properties; Blue marlin

1. Introduction

The total fishery products consumption in Japan is about 10 million tons per year and world fish production has been increasing (Ministry of Agriculture, Forestry & Fisheries, Japan, 2003). However, the most valuable fish stocks are now over-exploited or depleted, despite efforts to identify maximum sustainable yields and introduce quota systems for many fish species (Venugopal & Shahidi, 1995). On the other hand, approximately 30% of total landings are considered to be underutilized by-catch. Moreover, about 50% of the total catch is discarded during processing (Shahidi, 1994). These waste materials are now mainly used for the production of feeds and fertilizers. Therefore, it is necessary to increase value-added utilization of fisheries wastes and process-discarded materials, especially because of their ecological, economical, and socio-cultural impacts (Clucas, 1997).

The interest in biopolymer films has been increasing during the past decade, because they are environmentally friendly alternatives to synthetic and non-biodegradable films. Although it is not feasible to entirely replace synthetic packaging films instantly, biopolymer films have the potential to reduce and replace chemically synthetic films in some applications in the future. Basically, biodegradable/edible films are prepared from polysaccharides, proteins, and lipids. Among these materials, proteins have been extensively studied because of their relative abundance, film-forming ability, and nutritional qualities (Gennadios, McHugh, Weller, & Krochta, 1994; Krochta, 2002).

In our laboratory, the preparation of edible/biodegradable films, based on sarcoplasmic and myofibrillar proteins of Blue marlin (*Makaira mazara*) meat, has been studied. Sarcoplasmic or water soluble proteins of Blue marlin comprise 44% of the total protein (Wahyuni, Ishizaki, & Tanaka, 1998). They are globular proteins which, in general, have to be thermally denatured to form a continuous matrix (Iwata, Ishizaki, Handa, & Tanaka, 2000). Films formed from sarcoplasmic proteins are flexible and transparent, and can be formed over the pH ranges 3–6 and 9.5–12 (Iwata et al., 2000). On the other hand, myofibrillar or salt soluble proteins comprising of

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54% of the total protein (Wahyuni et al., 1998) are fully stretched and closely associated with each other in a parallel structure (Cuq, Aymard, Cuq, & Guilbert, 1995). It is found that films prepared from myofibrillar proteins are also flexible but semi-transparent and their mechanical properties are altered with shifting pH. The main interaction involved in the structure of both sarcoplasmic and myofibrillar protein films are hydrophobic interactions (Shiku, Hamaguchi, & Tanaka, 2003).

Although films could be successfully formed from both sarcoplasmic and myofibrillar proteins, the procedures for preparing and extracting the proteins from fish meat are tedious and time-consuming, which makes films not economically feasible. Therefore, in this study the whole muscle of Blue marlin, without any extraction treatment, was used to prepare edible films. The idea to use the whole muscle of fish was first proposed by Brazilian researchers, who worked with Thai and Nile tilapia muscles under acidic pH conditions with thermal treatment (Garcia & Sobral, 2005; Paschoalick, Garcia, Sobral, & Habitante, 2003; Sobral, Santos, & Garcia, 2005). In this study, a simple way to prepare the edible film is proposed and, to obtain the optimal physicochemical conditions for the film formation, attention is focussed on the overall effect of pH on the mechanical and chemical properties on edible films.

2. Materials and methods

2.1. Preparation of film-forming solutions

Flesh of Blue marlin (*Makaira mazara*) was obtained as a frozen block from Misaki, Kanagawa Prefecture, Japan. The block of Blue marlin was diced into 1 cm^3 and homogenized with distilled water in a food mixer (MX-X103, Matsushita Denki Co., Osaka, Japan) for 2 min. The obtained suspension was filtered by using two sieves (mesh size 4 and 1 mm) to remove stromal proteins. The protein content of the film-forming solution was adjusted to 2% and glycerol was added as a plasticizer at 50% (w/w) of protein. The concentration of protein was determined by a Bio-Rad *DC* protein assay method (Lowry method, Bio-Rad Lab., Hercules, USA). The film-forming solution thus prepared was stirred using a magnetic stirrer for 30 min, then the pH was adjusted from 1 to 12, at 1 pH unit intervals, with 1 M HCl or 1 M NaOH and the whole dispersed thoroughly with a glass homogenizer (Sibata Scientific Tech. Ltd., Tokyo, Japan). A Hybrid Mixer (HM-500; Keyence Co., Tokyo, Japan) was used to remove air bubbles of the final solution before casting. Four milliliters of the prepared film-forming solutions with different pHs were centrifuged at 9620*g* for 30 min and the protein contents in the supernatants were measured by the Lowry method to determine the protein solubility in the film-forming solutions.

2.2. Determination of surface hydrophobicity

Surface hydrophobicity of the film-forming solutions was determined by a hydrophobic fluorescence probe method, using 8-anilino-1-naphthalene sulfonic acid (ANS) (Hayakawa & Nakai, 1985). The protein concentration was adjusted to 0.01% and 0.4 ml of 0.04% ANS (Wako Pure Chemical Industries Ltd., Tokyo, Japan) was added to 4 ml of the diluted film-forming solutions. After keeping at room temperature for 15 min, fluorescence intensities of ANS–protein conjugates were measured using a spectrofluorometer (Model RF-1500; Shimadzu Co., Kyoto, Japan) at an excitation wavelength of 365 nm and emission wavelength of 470 nm. Data express the change in surface hydrophobicity relative to the film-forming solution at pH 7 which was assigned a value of 1.

2.3. Film casting and drying

The prepared film-forming solution (4 ml) was cast onto a rimmed silicone resin plate (50 × 50 mm) sitting on a level surface and dried in a ventilated oven (Environmental Chamber model H110K-30 DM; Seiwa Riko Co., Tokyo, Japan) at 25 ± 0.5 °C and $50 \pm 2\%$ relative humidity (RH) for 24 h. After the water had evaporated, the resulting films were manually peeled off.

2.4. Measurements of film thickness

The film thickness was measured using a micrometer (Dial Pipe Gauge; Peacock Co., Tokyo, Japan) to the nearest 0.005 mm at six random locations of the film. Precision of the thickness measurements was ±5%.

2.5. Mechanical properties

Prior to testing the mechanical properties, the films were conditioned for 72 h at 25 ± 0.5 °C and $50 \pm 2\%$ RH. Tensile strength (TS) and percentage elongation at break (EAB) were determined using a Tensipresser (TTP-508X II, Taketomo Electric Inc., Tokyo, Japan), operated according to the ASTM standard method D 882-22 (ASTM, 1989). Two rectangular strips (width 20 mm; length 45 mm) were prepared from each film to determine their mechanical properties. Initial grip separation and mechanical crosshead speed were set at 30 mm and 0.5 mm/s, respectively. TS (MPa) was calculated by dividing the maximum load (N) necessary to pull the sample apart by cross-sectional area of the sample film (m^2). The average thickness of the film strip was used to estimate the cross-sectional area of the sample. EAB (%) was calculated by dividing film elongation at the moment of rupture by initial grip length (30 mm) of samples multiplied by 100. A total of 10 samples were tested for each film type.

2.6. Water vapour permeability (WVP)

WVP was measured using a modified ASTM method reported by Gontard, Guilbert, and Cuq (1992). Sample film was sealed on a glass permeating cup containing silica gel (0% RH) with silicone vacuum grease and a plastic band to hold the film in place. The cups were placed in a desiccator with distilled water (100% RH) at 30 °C. The cups were weighed at 1 h intervals over 12 h periods and WVP of the films was calculated as follows: WVP $(\times 10^{-10}\ \mathrm{g\ m^{-1}\ s^{-1}\ Pa^{-1}}) = w \times x \times A^{-1} \times t^{-1} \times (P_2 - P_1)^{-1}$, where w is the weight gain (g), x is film thickness (m), A is the area of exposed film (m^2), t is time of gain(s), and $(P_2 - P_1)$ is the vapour pressure differential across the film (Pa). This entire procedure was repeated twice for a total of five tests on each film type.

2.7. Hydrolysis by protease

Ground film powder (20 mg) was suspended in 20 ml of enzyme solution (0.2 mg α-chymotrypsin/ml of 40 mM Tris–HCl buffer (pH 7.6)). The protease-substrate suspension was then incubated at 37 °C for 120 min (Yildirim & Hettiarachchy, 1998). Hydrolysis was stopped by adding 1 ml of 50% TCA to 4 ml suspension. After these solutions were centrifuged at 9620*g* for 30 min, the supernatant was removed and 4 ml of 0.5 NaOH were added to dissolve precipitates. The amount of protein in the NaOH solutions was determined by the Lowry method. Hydrolysis by protease was calculated by the following equation: PD $(\%) = \{1 - (w_2/w_1)\} \times 100$, where w_1 is the protein content in the precipitate before incubation and w_2 is the protein content after incubation.

2.8. Light transmission and transparency

The ultraviolet and visible light barrier properties of the films were measured at selected wavelengths, from 200 to 800 nm, using a UV–visible recording spectrophotometer (UV-160, Shimadzu Co., Kyoto, Japan). The light transmission of film was measured by a modified version of the ASTM method D 1746-92 (Fang, Tung, Britt, Yada, & Dalgleish, 2002). The transparency of the films was calculated as follows (Han & Floros, 1997): Transparency $= A_{600}/x$ or $(-\log T_{600})/x$, where $A600$ is absorbance at 600 nm, T_{600} is transmittance (%) at 600 nm, and x is film thickness (mm).

2.9. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to the method of Laemmli (1970). A 7.5% polyacrylamide gel (AE-6000, NPU-7.5L PAGEL, ATTO Co., Tokyo, Japan) was used. Films were dissolved in 2% SDS–8M urea–2%mercaptoethanol–20 mM Tris–HCl (pH 8.8). Gels were stained with 0.05% Coomassie Brilliant Blue R-250 (Tokyo Kasei Co., Tokyo, Japan) in methanol/acetic acid/water (5:10:85%, v:v:v), then destained in methanol/acetic acid/water (30:10:60%, v:v:v). The standard protein mixture (Sigma Chemical Co., St. Louis, USA), ranging in molecular mass from 14.2 to 97.2 kDa, was used.

2.10. Film solubility in various protein denaturant solutions

The films were solubilized in four different solutions: 0.6 M NaCl (S1), 1.5 M urea + 0.6 M NaCl (S2), 8.0 M urea + 0.6 M NaCl (S3), 0.5 M 2-β-mercaptoethanol + 8 M urea + 0.6 M NaCl (S4), according to Perez-Mateos, Lourenco, Montero, and Borderias (1997). Ground film powder (25 mg) was immersed in 5 ml of each solution (S1, S2, S3, or S4) and gently shaken overnight at room temperature by using a reciprocal shaker. The solution was centrifuged at 9620*g* for 30 min and the protein concentration of the supernatant was determined in triplicate by using the Lowry method.

2.11. Statistical analysis

Statistical analysis, on a completely randomized experimental design, was performed using the General Linear Model procedure in the SPSS computer programme (SPSS Statistical Software, Chicago, USA). One-way analyses of variance (ANOVA) were carried out and mean comparisons were run by Duncan's multiple range test (Stell & Torrie, 1980).

3. Results and discussion

3.1. Preparation of fish muscle protein films

The predominant protein of Blue marlin meat is myofibrillar protein. It was reported that myofibrillar proteins can be dispersed, not only in salt solution, but also in very low ionic strength solutions under neutral conditions (Cuq et al., 1995). Formation of fish muscle protein films was successfully achieved in the pH ranges of 1–4 and 7–12. Films could not be formed at pH 5 and 6, because it was close to the isoelectric point where most of proteins were coagulated and casting of the film-forming solution was impossible. In the same manner, sarcoplasmic protein films and myofibrillar protein films from Blue marlin meat could not be formed from pH 7 to 9 or 4 to 6, respectively (Iwata et al., 2000; Shiku et al., 2003). It is of relevance to note that films based on muscle proteins of Blue marlin could be prepared from the film-forming solutions with a wider range of pH.

3.2. Properties of films

TS and EAB of muscle protein films are summarized in Table 1. The lowest TS of films were observed at pH 7. TS became higher with decreasing or increasing pH value of the film-forming solutions. However, TS of muscle protein films is considered to be lower than those of sarcoplasmic

Table 1
Effect of pH on tensile strength (TS), elongation at break (EAB), water vapour permeability (WVP) and protease digestibility (PD) of fish muscle protein films*

pH	TS (MPa)	EAB (%)	WVP ($\times 10^{-10}$ g m^{-1} s^{-1} Pa^{-1})	PD (%)
1	2.58 ± 0.56^{c}	58.3 ± 9.7^{a}	1.63 ± 0.06^{b}	80.0 ± 1.4^{a}
2	2.94 ± 0.61^{c}	85.2 ± 9.8^{b}	1.49 ± 0.05^{a}	83.1 ± 1.3^{a}
3	3.10 ± 0.58^{c}	89.5 ± 9.4^{b}	1.55 ± 0.08^{ab}	88.8 ± 2.8^{ab}
4	2.23 ± 0.46^{bc}	78.6 ± 6.3^{ab}	1.63 ± 0.05^{b}	84.6 ± 1.4^{a}
7	1.64 ± 0.54^{a}	83.0 ± 2.1^{b}	1.59 ± 0.05^{b}	87.5 ± 2.7^{ab}
8	1.81 ± 0.61^{ab}	81.0 ± 8.0^{b}	1.56 ± 0.03^{ab}	89.0 ± 2.8^{b}
9	1.94 ± 0.29^{b}	80.6 ± 5.8^{b}	1.60 ± 0.04^{b}	90.0 ± 2.0^{b}
10	1.96 ± 0.53^{b}	74.6 ± 7.4^{ab}	1.49 ± 0.03^{a}	85.4 ± 2.1^{a}
11	2.98 ± 0.35^{c}	74.3 ± 8.1^{ab}	1.50 ± 0.09^{a}	93.2 ± 1.5^{b}
12	2.81 ± 0.51^{c}	81.7 ± 4.3^{b}	1.47 ± 0.07^{a}	89.3 ± 1.8^{b}

* Means ± SD. Any two means in the same column followed by the same letter are not significantly different ($p > 0.05$).

and myofibrillar protein films from the same fish species (Iwata et al., 2000; Shiku et al., 2003). It was reported by Hamm (1960) that the solubility of myofibrillar proteins is affected by shifting pH and higher solubility is usually observed under extreme pH conditions. Improved TS of muscle protein films, prepared under acidic and alkaline conditions, seemed to be due to solubilized muscle proteins in the film-forming solutions (Fig. 1), which subsequently interact with each other during drying. On the other hand, no significant effect of pH was observed on EAB of muscle protein films. Relatively flexible films were prepared at all pHs. This tendency was quite similar to that of sarcoplasmic protein films of the same fish species (Iwata et al., 2000), but myofibrillar protein films were less flexible (Shiku et al., 2003), suggesting that the flexibility of muscle protein films is more or less dependent on the presence of sarcoplasmic proteins.

Fish muscle is known for its hydrophilic characteristics; therefore water vapour barrier properties of protein films are expected to be poor compared to those from other non-hydrophilic materials, such as paraffin wax, carnauba wax and beeswax. WVP of fish muscle protein films are presented in Table 1. Water vapour barrier properties of muscle protein films were not influenced by the pH value of the film-forming solutions. These results are not consistent with the previous reports on protein films from wheat gluten and soy protein isolates (Gennadios, Brandenburg, Weller, & Testin, 1988). Wheat gluten films had the lowest WVP at pH 2–3, whereas soy protein isolate films had lower WVP at pH values above the isoelectric point than at acidic conditions (pH 1–3). WVPs of muscle protein films are comparable to those of sarcoplasmic protein films but higher than those of myofibrillar protein films prepared from Blue marlin meat (Iwata et al., 2000; Shiku et al., 2003).

Hydrolysis of muscle protein films by α-chymotrypsin is shown in Table 1. It is obvious that the digestibility, by α-chymotrypsin, of muscle protein films prepared in this study was more than 80%. It was close to that of sarcoplasmic protein films (Iwata et al., 2000) and was more than that of myofibrillar protein films (Shiku et al., 2003). As a whole, it is quite apparent that fish muscle protein films can easily be digested, even if they are ingested.

Table 2 presents the light transmission, at selected wavelengths from 200 to 800 nm, and transparency (A_{600}/mm) of fish muscle protein films. It is clear that light transmission of the films is not affected by pH. It is worth noting that muscle protein films prepared in this study have excellent barrier properties against UV light in the range 200–280 nm, regardless of pH. Protein films are considered to have very good UV barrier properties, owing to their high content of aromatic amino acids that absorb UV light. By contrast, muscle protein films, prepared under neutral pH conditions, block most light in the UV–visible range, from 350 to 800 nm, because of their poor transparency. The muscle protein films became more transparent at lower or higher pHs. The average transparency of muscle protein films at pH 1–4 and 11–12 was 6.6 ± 1.4 (A_{600}/mm), which was higher than sarcoplasmic films (Iwata et al., 2000) but was close to myofibrillar films (Shiku et al., 2003). These results indicate that muscle protein films from Blue marlin meat are transparent and clear enough for use as see-through packaging.

3.3. Mechanism of film formation

Film formation, by the casting method, requires dissolution of muscle proteins by adjusting the pH of film-forming

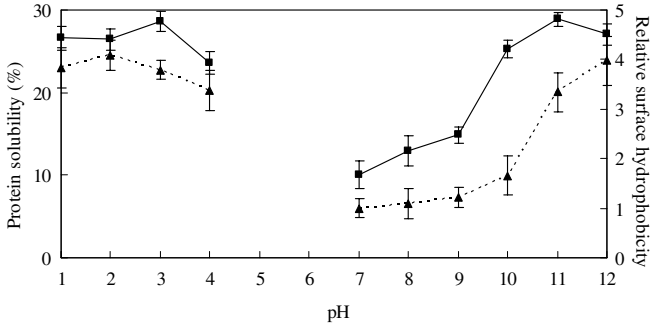

Fig. 1. Effect of pH on protein solubility (%, ■) and surface hydrophobicity (▲) of film-forming solutions.

Table 2
Effect of pH on light transmission (%) and transparency (A_{600}/mm) of fish muscle protein films

pH	Light transmission at different wavelengths (nm)							Transparency
	200	280	350	400	500	600	800	
1	0.3	0.3	34.2	45.0	51.0	52.6	53.7	5.8
2	0.3	0.3	53.3	60.4	65.9	67.3	63.3	5.1
3	0.3	0.3	34.8	46.2	51.0	52.8	54.0	6.0
4	0.3	0.3	38.4	59.5	64.0	65.3	66.2	6.9
7	0.3	0.3	22.2	26.3	30.4	31.7	33.0	12.3
8	0.3	0.3	34.4	39.3	44.9	46.9	48.7	13.0
9	0.3	0.3	34.0	39.1	44.6	46.5	48.0	11.3
10	0.3	0.3	25.9	48.3	53.4	55.2	56.8	9.6
11	0.3	0.3	22.5	46.0	51.5	54.8	57.9	6.8
12	0.3	0.3	28.3	57.6	62.6	65.1	67.7	6.0

solutions. The protein solubility, in film-forming solutions, reached a maximum value at around 30% under acidic and alkaline conditions (Fig. 1). Proteins in Blue marlin muscles were insoluble at pH 5–6 and slightly soluble at pH 7. At pH 5–6, muscle proteins of blue marlin were coagulated rather than dispersed, thereby not allowing for casting of film-forming solutions. At pH above or below the isoelectric point, electrostatic repulsion between protein molecules increased, resulting in high protein solubility. Furthermore, increased ionic strength of film-forming solutions under acidic and alkaline conditions seemed to improve the protein solubility (Lin & Park, 1998). A similar trend was observed in surface hydrophobicity of muscle proteins in film-forming solutions (Fig. 1). Increasing or decreasing pH value from the isoelectric point caused a concomitant increase in surface hydrophobicity. From the results of Fig. 1, it can be concluded that muscle protein molecules in film-forming solutions, under acidic and alkaline conditions, are partially unfolded due to the protein denaturation, and their hydrophobic groups are exposed (Kristinsson & Hultin, 2003).

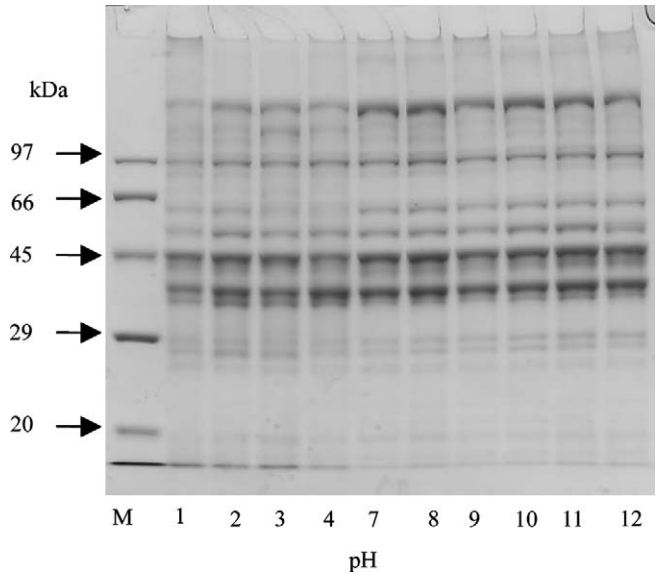

Fig. 2. SDS–PAGE patterns of fish muscle protein films as affected by pH. M, standard molecular weight mixture.

Fig. 2 depicts SDS–PAGE patterns of films prepared from Blue marlin meat with different pH values. Various protein bands were observed in films prepared under neutral, acidic and alkaline conditions. It is clear that myosin heavy chain (MHC) in acidic films were degraded, while the band intensity of MHC in neutral and alkaline films was higher than that in acidic films. The results agree with earlier findings by Cuq et al. (1995) who worked on myofibrillar protein films based on sardines. They reported that proteolytic enzymes, such as cathepsins are active under acidic conditions, causing the degradation of MHC to smaller peptides. The larger surface hydrophobicity observed in the acidic film-forming solutions (Fig. 1) could be partially due to degradation of MHC molecules.

Film formation from muscle proteins of Blue marlin meat is believed to proceed through the formation of a three-dimensional network of protein molecules by ionic, hydrogen, hydrophobic, and disulfide bonds. Therefore, the solubility of the films in the following four different denaturing solutions was determined to reveal the main associative force involved in the film formation (Fig. 3). The solutions employed in this study were 0.6 M NaCl solution (S1) that disrupts ionic bonds, 0.6 M NaCl and 1.5 M urea solution (S2) that disrupts hydrogen bonds, 0.6 M NaCl and 8 M urea solution (S3) that disrupts hydrophobic interactions, and 0.6 M NaCl, 8 M urea and 0.5 M mercaptoethanol solution (S4) that disrupts disulfide bonds. In the case of muscle protein films prepared at neutral pH which had lower TS values (Table 1), the main

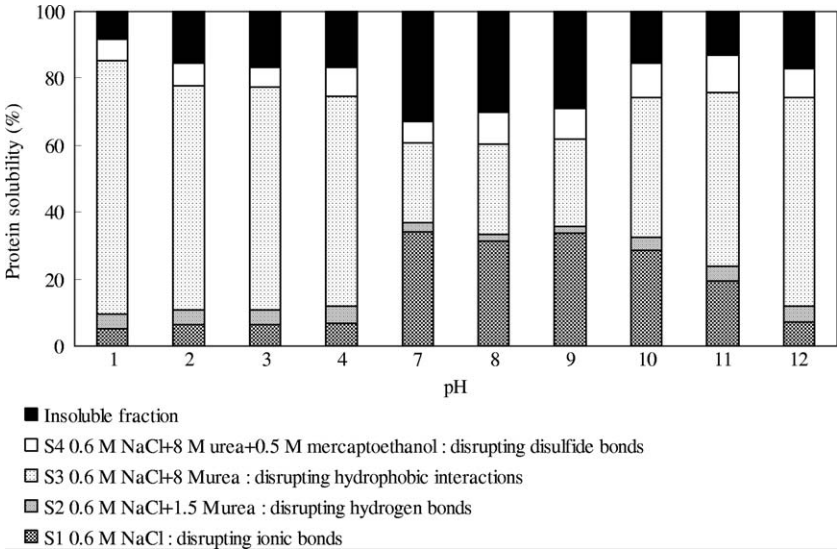

Fig. 3. Effect of pH on protein solubility of films in various protein denaturant solutions.

force involved in the formation of film structure was ionic bonds and a significant amount of insoluble proteins was present because most muscle proteins were not solubilized in the film-forming solution (Fig. 1). On the other hand, hydrophobic interactions were the main associative force for the formation of muscle protein films under acidic conditions. The present results are supported by the evidence of increased protein solubility and surface hydrophobicity (Fig. 1), together with escalated degradation of MHC (Fig. 2). In the case of muscle protein films prepared under alkaline conditions, with a similar TS level to films prepared under acidic conditions, the contribution of ionic bonds in the film structure became smaller with increasing pH while that of hydrophobic interactions became predominant. It is apparent from Fig. 3 that disulfide bonds did not play an important role, regardless of pH values of film-forming solutions.

Above all that can be concluded from all of the aforementioned studies (Table 1 and Figs. 1–3) is that the main associative force for the formation of muscle protein films of Blue marlin meat is that of hydrophobic interactions. This leads to the following hypothesis for the film formation. At pH values distant from the isoelectric region, partially unfolded protein molecules (due to the protein denaturation) expose more hydrophobic groups to water. This exposure of hydrophobic groups could be enhanced by degradation of MHC under acidic conditions. Exposed hydrophobic groups extend out of the water into air at the water/air interface. As water, in film-forming solutions, evaporates from the surface, the protein concentration increases and the protein–protein interactions (mainly hydrophobic interactions) can be accelerated. As a result of this procedure, muscle protein films with greater TS are believed to be formed under acidic and alkaline conditions.

4. Conclusions

Flexible and semi-transparent films were successfully prepared from Blue marlin meat under acidic and alkaline conditions. The solubility of proteins in film-forming solutions increased in the pH ranges 1–4 and 10–12. The hydrophobic groups exposed to water led to the formation of stronger and transparent films. EAB, WVP and light transmission were not significantly influenced by pH. It is of relevance to note that muscle protein films were prepared, even under neutral conditions, although they were less transparent and had poorer mechanical properties. These films can be used as edible packaging materials since they contain only proteins and glycerol. We are now continuing a series of experiments to improve the functional properties of these films by incorporating natural antimicrobials and antioxidants.

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