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Effect of Fish Meat Quality on the Properties of Biodegradable Protein Films

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Fish muscle protein films were prepared from blue marlin (*Makaira mazara* Jordan & Evermann) meat which had been stored at 30℃ to intentionally lower the meat quality. In this study, the effects of meat quality and pH on the formation of these protein films were investigated. Moreover, ε-polylysine was added to the film-forming solutions to reduce the microbial population of films. The mechanical properties of the films were slightly affected by acidic and alkaline pHs. However, the water vapor permeability of muscle protein films was not affected by either the quality of the fish meat or the pH of the film-forming solutions. SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel electrophoresis) showed the degradation of myosin heavy chain in acidic films and polymerization in alkaline films. It was revealed that biodegradable films can be produced even from very low quality fish meat, and that the bacterial population of films could be drastically reduced by the addition of ε-polylysine.

Keywords: Biodegradable protein films, Mechanical properties, Blue marlin

Introduction

World fish production has been rapidly increasing over the past 15 years, reaching 150 million metric tons in 2004 (Ministry of Agriculture, Forestry and Fisheries of Japan, 2006). However, there are numerous fish species which cannot be harvested on a sustainable basis due to innovative fishing technologies and international competition. Moreover, it is estimated that 30～35 million tons of the global catch are lost due to improper handling, inadequate fishing practices, and various other reasons (FAOSTAT, 2005); a similar amount is discarded during the processing of seafood products. The majority of discards are comprised of heads, skins, viscera, scales, frames (spinal bones with adhering meat), and trimmings (pieces cut from the fillets during processing). These losses represent a very large resource that is not being properly or responsibly utilized. Most fishery wastes are presently used to produce fish meal, fertilizer, fish oil and pet food, but the economic value of these products is usually low. Therefore, it is necessary to develop new techniques to convert and utilize more of those wastes as valuable products (Venugopal and Shahidi, 1995).

Biopolymer films based on discarded materials could be used as an alternative packaging material, because they are environmentally-friendly compared to synthetic and non-degradable films. Basically, biodegradable/edible films can be formed from proteins, polysaccharides and lipids; among these materials, proteins have been extensively used because of their relative abundance, film-forming ability, and nutritional qualities (Gennadios *et al.*, 1994; Krochta, 2002).

In our previous studies, edible/biodegradable films based on the meat proteins of blue marlin were prepared and characterized. In the first series of studies, edible films were formed from the sarcoplasmic proteins of blue marlin, which comprise 44% of meat proteins (Iwata *et al.*, 2000). These films, which are formed at pH ranges of 3 to 6 and 9.5 to 12, are flexible and transparent. In the second study series, myofibrillar proteins consisting of 54% of the total proteins were used to prepare edible films, and the effect of pH on their mechanical properties was investigated (Shiku *et al.*, 2003). The main associative force involved in the formation of film structures of sarcoplasmic and myofibrillar protein films was revealed to be hydrophobic interactions. Although films were successfully formed from both proteins, the procedures for preparing and extracting the proteins from fish meat are tedious and time-consuming, and render these films economically unviable. Therefore, in the third study series, we developed a simple method of preparing biodegradable/edible films from whole blue marlin muscle without any extraction treatment (Hamaguchi *et al.*, 2007). Semi-transparent films possessing the intermediate tensile strength and elongation properties of sarcoplasmic and myofibrillar protein films were successfully prepared at neutral pH. In the present study, we examined the effect of meat quality on the physicochemical properties of biodegradable films derived from samples of whole blue marlin meat of varying quality, as the freshness and qual-

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ity of fishery processing discards was expected to be low.

Materials and Methods

Preparation of meat samples with different qualities Blue marlin (*Makaira mazara* Jordan & Evermann) meat was obtained as a frozen block from Misaki port (Kanagawa, Japan). The frozen meat was diced into 1-cm^3 pieces and placed in storage at 30℃ for 1 h to completely thaw. This was considered the 0 h sample; 12, 24, and 36 h samples were then prepared by keeping them in storage at 30℃.

Biochemical analyses

Total volatile basic nitrogen (TVBN) Total volatile basic nitrogen (TVBN) was determined as a quality index of fish meat freshness according to the Conway's microdiffusion method (Conway and Byrne, 1936). Three g of minced meat was homogenized with 30 ml of 5% trichloroacetic acid (TCA) solution and centrifuged for 5 min at 3000 *g* (H-108 NA; Kokusan Denki Co., Ltd., Shizuoka, Japan). After the supernatant was collected, the precipitate was again extracted with TCA. The supernatants were combined in a 100-ml solution, which was then filtered with a cellulose acetate disposable syringe filter of 0.45-μm pore size (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and used for analysis. TVBN was released by the addition of saturated K_2CO_3 and diffused into a 1% boric acid solution. Titration of the solution was performed with 0.02 N H_2SO_4, and the amount of TVBN was calculated using the following equation.

$$\mathrm{TVBN(mg/100\,g\,meat)} = 0.28 \times (T - B) \times 100 \times A^{-1} \quad [1]$$

where T is the titration volume for the extracted sample (ml), B is the titration volume of blank (ml), and A is the weight of muscle sample (g).

Total viable cell counts (TVC) One g of blue marlin meat was homogenized in a stomacher bag (Eiken Kizai Co., Tokyo, Japan) for 2 min with 99 ml of 0.1% peptone and 0.85% NaCl solution (pH 7). Bacterial counts of fish homogenates were determined by plating triplicate decimal dilutions of samples onto the plate count agar (pH 7.1; Pearl Core, Eiken Kizai Corp., Tokyo, Japan) and incubated at 30℃ for 48 h. The results were expressed as log colony-forming unit per g (log CFU/g).

Determination of ATP- related compounds (K value) Fish meat samples (about 500 mg) were minced with 0.5 ml of 0.5 M perchloric acid in a test tube using a glass rod, and the extracted solution was then filtered using a mixed cellulose acetate disposable syringe filter of 0.45 μm pore size (Advantec MFS, Inc., Tokyo, Japan). An aliquot of 2 μl was injected into a high-performance liquid chromatograph DGU-12A (875-UV, Shimadzu Co., Kyoto, Japan) to determine the amount of ATP and its related compounds (Shirai *et al.*, 1996). The K value was calculated as follows:

$$\mathrm{K\ value(\%)} = [(\mathrm{HxR} + \mathrm{Hx}) / (\mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP} + \mathrm{IMP} + \mathrm{HxR} + \mathrm{Hx})] \times 100 \quad [2]$$

where ATP is adenosine triphosphate, ADP is adenosine diphosphate, AMP is adenosine monophosphate, IMP is inosine monophosphate, HxR is inosine, and Hx is hypoxanthine.

Preparation of film-forming solutions Fish meat samples with different qualities were homogenized with distilled water in a food mixer (Model MX-X103; Matsushita Denki Co., Osaka, Japan) for 2 min. The obtained suspension was passed through two sieves (mesh sizes: 1 and 4 mm) to remove stromal proteins. Protein content of the film-forming solution was adjusted to 2% and glycerol was added as a plasticizer at 50% (w/w) of protein. Following the addition of plasticizer, 0.1% ε- polylysine (Chisso Co., Yokohama, Japan) was added as an antimicrobial agent. The concentration of protein was determined by a Bio-Rad *DC* protein assay method (Lowry method; Bio-Rad Lab., Hercules, CA, USA). The film-forming solution thus prepared was stirred using a magnetic bar for 30 min; the pH was then adjusted to 3 with 1 N HCl and 10 with 1 N NaOH. The solution was then 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 from the final solution before casting.

Casting and drying The prepared film-forming solution (4 ml) was cast onto a rimmed silicone resin plate (50 × 50 mm) setting 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℃ and 50±2% relative humidity (RH) for 24 h. After the water had evaporated, the resulting films were manually peeled off.

Measurements of film thickness Film thickness was measured using a micrometer (Dial Pipe Gauge; Peacock Co., Tokyo, Japan) to the nearest 0.005 mm at 6 random locations on the film. Precision of the thickness measurements was±5%.

Mechanical properties Prior to testing the mechanical properties, the films were conditioned for 72 h at 25± 0.5℃ and 50±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 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 at a 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.

Water vapor permeability (WVP) WVP was measured using a modified ASTM method reported by Gontard *et al.* (1992). Sample films were sealed on a glass permeating cup containing silica gel (0%RH) with silicone vacuum grease and an O-ring to hold the film in place. The cups were placed in a desiccator with distilled water (100% RH) at 30℃. The cups were weighed at 1 h intervals over a 12 h period and WVP (g m^{-1} s^{-1} Pa^{-1}) of the films was

calculated as follows (McHugh *et al.,* 1993):

$$WVP = w \times x \times A^{-1} \times t^{-1} \times (P_2 - P_1)^{-1} \quad [3]$$

where w is the weight gain (g), x is the film thickness (m), A is the area of exposed film (m^2), t is the time of gain(s), and (P_2-P_1) is the vapor pressure differential across the film (Pa). This entire procedure was repeated twice for a total of 5 tests on each film type.

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). The 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), and were destained in methanol/acetic acid/water (30: 10: 60%, v: v: v). Page Ruler™ Protein Ladder (Fermentas Life Science, Hanover, MD, USA) ranging from 10 to 200 kDa was used as a standard protein marker.

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

Results and Discussion

Quality evaluation of blue marlin meat The quality of blue marlin meat was intentionally lowered by storing the samples at 30℃ for 12, 24, and 36 h. Biochemical analyses such as TVBN, TVC and K value were carried out to evaluate the quality of the fish meat.

Changes in TVBN of blue marlin meat are presented in Table 1. TVBN of blue marlin meat immediately after thawing (0 h) was 11.1 mg N/100 g meat. It increased with increasing storage time at 30℃, reaching the maximum value of 33.0 mg N/100 g meat after 36 h storage. The level of TVBN for fish meat is generally considered to be fresh when TVBN is less than 20 mg N/100 g sample. When TVBN reaches 30 mg N/100 g sample or more, it is considered as unfit for consumption (Connell, 1995). It is clear from this table that blue marlin meat stored at 30℃ for 24 and 36 h, which had a putrid odor, could not be consumed due to high level of TVBN.

The TVCs of blue marlin meat with different storage periods are listed in Table 1. The initial total bacterial load of blue marlin meat was 4.4 log CFU/g. TVC values increased with storage time, reaching 8.5 and 9.0 log CFU/g after 24 and 36 h, respectively. The TVC level generally accepted for the safe consumption of fish is below 6.0 log CFU/g (ICMSF, 2002), suggesting that blue marlin samples stored for 24h at 30℃ are not acceptable for human consumption due to the dense populations of bacteria.

Changes in the K values of blue marlin meat are shown in Table 1. The K value of thawed blue marlin meat was approximately 12%. After 12 h storage at 30℃, the K value reached about 31%, and increased drastically to more than 90% thereafter. The K value for raw consumption of fish meat is considered to be less than 20% (Ehira *et al.,* 1970), demonstrating that the quality of blue marlin meat stored more than 12 h at 30℃ was quite poor.

Table 1. Quality changes of blue marlin meats during the storage at 30℃.

Storage time at 30℃ (h)	TVBN (mg N/100g)*	TVC (log CFU/g)*	K-value (%)*
0	11.1 ± 1.0	4.4 ± 0.4	12 ± 2
12	14.6 ± 1.2	7.2 ± 0.2	31 ± 4
24	26.0 ± 1.6	8.5 ± 1.7	96 ± 1
36	33.0 ± 2.2	9.0 ± 0.1	98 ± 1

*Means±standard deviation from 3 determinations.

Table 2. Tensile strength (TS), elongation at break (EAB), and water vapor permeability (WVP) of muscle protein films prepared from blue marlin meats with different qualities*.

Storage time at 30℃	pH	TS (MPa)	EAB (%)	WVP ($x10^{-10}gm^{-1}s^{-1}Pa^{-1}$)
0h	3	3.10 ± 0.58^{b}	89.5 ± 9.4^{e}	1.55 ± 0.08^{a}
	7	2.30 ± 0.28^{a}	66.2 ± 9.7^{d}	1.59 ± 0.05^{ab}
	10	1.96 ± 0.53^{a}	74.6 ± 7.4^{d}	1.49 ± 0.03^{a}
12h	3	3.60 ± 0.55^{bc}	13.0 ± 9.0^{a}	1.67 ± 0.19^{b}
	7	2.39 ± 0.44^{a}	40.7 ± 9.4^{bc}	1.77 ± 0.10^{c}
	10	3.01 ± 0.52^{b}	41.2 ± 6.2^{bc}	1.79 ± 0.09^{c}
24h	3	3.66 ± 0.28^{bc}	10.5 ± 1.6^{a}	1.52 ± 0.08^{a}
	7	2.37 ± 0.29^{a}	58.2 ± 8.1^{cd}	1.75 ± 0.16^{c}
	10	3.13 ± 0.30^{b}	36.2 ± 4.6^{b}	1.53 ± 0.05^{a}
36h	3	3.63 ± 0.41^{bc}	16.2 ± 8.3^{ab}	1.67 ± 0.08^{b}
	7	2.40 ± 0.43^{a}	47.7 ± 10.0^{c}	1.69 ± 0.06^{bc}
	10	3.30 ± 0.41^{b}	26.6 ± 6.4^{b}	1.81 ± 0.08^{d}

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

Properties of films Film-forming solutions were prepared from blue marlin meat samples with different qualities (0, 12, 24, and 36 h incubation time at 30℃) in acidic (pH 3), neutral (pH 7), and alkaline (pH 10) conditions. The mechanical and chemical properties, such as TS, EAB and WVP, of prepared films were analyzed. All prepared films did not have any putrid odor, regardless of the meat quality, because the volatile putrid odor was evaporated during the drying process of films.

Protein films prepared from blue marlin meat of varying qualities in acidic conditions showed the highest TS, while films from neutral conditions possessed the lowest TS (Table 2). These results are in accordance with those obtained by Iwata *et al.* (2000) and Shiku *et al.* (2003), who studied the effect of pH on the sarcoplasmic and myofibrillar proteins of blue marlin, respectively. However, the quality of meat did not influence TS of films prepared at pH 3 or 7. In the case of films prepared at pH 10, TS became larger compared to the control film when blue marlin meats were stored at 30℃ for 12, 24, and 36 h.

EAB of muscle protein films prepared in acidic conditions significantly decreased with increasing storage time

(Table 2), while EAB of films prepared in neutral conditions was not markedly influenced by the storage time. In the case of films prepared in alkaline conditions, EAB gradually lowered during storage at 30℃. According to Shiku *et al.* (2004), the flexibility of surimi films also decreased with increasing denaturation of myofibrillar proteins. The marked decrease of EAB could be explained in part by the bacterial consumption of the glycerol used as a plasticizer during the casting and drying steps of film preparation.

In Table 2, WVP of muscle protein films from blue marlin meat with different qualities are presented. WVP of muscle protein films was not affected by the quality of fish meat or the pH of the film-forming solutions. Films from low quality fish meats had WVP at the same magnitude of sarcoplasmic and myofibrillar protein films (Iwata *et al.,* 2000; Shiku *et al.,* 2003).

SDS-PAGE of protein films Figure 1 shows the SDS-PAGE patterns of protein films prepared from blue marlin meat of varying quality and pH. Various protein bands were observed in films prepared at acidic, neutral and alkaline conditions. It is obvious from this figure that there was no change in protein bands during the storage at 30℃ for up to 12 h. On the other hand, it is clear that myosin heavy chain (MHC, 200 kDa) in the acidic film was degraded with a formation of new band of 150 kDa. The results agree with earlier findings by Cuq *et al.* (1995) who worked on myofibrillar protein films from sardine meat. They reported that proteolytic enzymes like cathepsins are active in acidic conditions, causing the degradation of MHC to smaller peptides. It should be noted that the intensity of band on the top of the acrylamide gel became intense at pH 10, suggesting the occurrence of protein polymerization in alkaline conditions irrespective of fish meat quality.

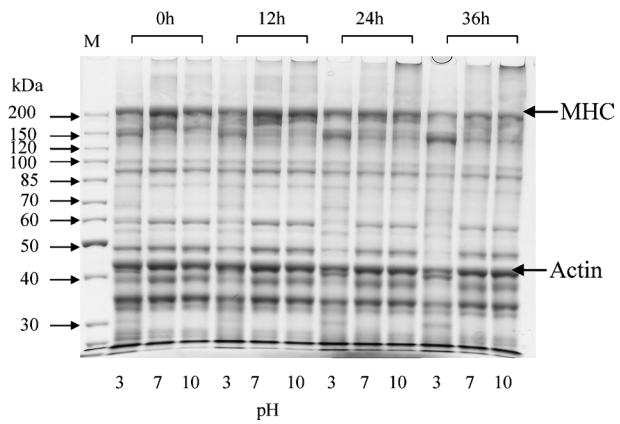

Fig. 1. SDS-PAGE patterns of muscle protein films prepared from blue marlin meats of varying pH and quality.
M : Standard molecular weight mixture, MHC : Myosin heavy chain.

Effect of ε-polylysine added to films From the results obtained above, it appears that muscle protein films can be successfully prepared even from blue marlin meat of very poor quality. However, films prepared from low quality meat were not suitable for use as packaging materials due to the dense population of bacteria. Therefore, an antimicrobial agent was added to films to reduce the bacterial population. In this study, the antimicrobial agent ε-polylysine was used because of its strong inhibitory action against both Gram-positive and Gram-negative bacteria (Shima and Sakai, 1997). According to ADME (absorption, distribution, metabolism and excretion) studies, the use of ε-polylysine as a preservative in food is considered safe (Hiraki *et al.,* 2003). The results of antimicrobial addition to films are shown in Fig. 2. The TVC of control films increased from 2.4 to 7.2 log CFU/cm^2 during 36 h storage at 30℃, whereas that of films containing 0.1% ε-polylysine increased from 0.6 to 3.0 log CFU/cm^2. Total bacterial cell counts for all films containing ε-polylysine were drastically reduced regardless of meat quality.

The antimicrobial protein films prepared in this study were not suitable for use as food packaging, but the results elucidate the potential development of active packaging for foods using ε-polylysine. To control undesirable microorganisms in foods, antimicrobial substances such as ε-polylysine can be incorporated into or coated onto food packaging materials. The principle action of antimicrobial films is based upon the release of antimicrobials, some of which could pose a safety risk to consumers if the release is not firmly controlled within

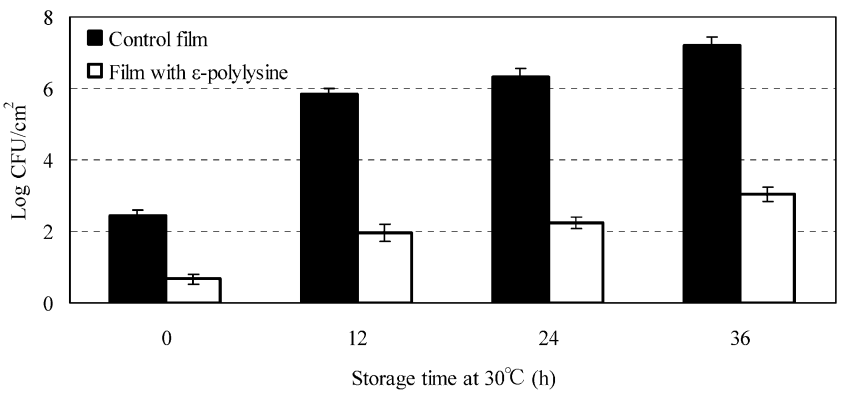

Fig. 2. Total viable cell counts (log CFU/cm^2) of muscle protein films containing 0.1% ε-polylysine. Control film did not contain ε- polylysine.

the packaging materials. Protein packaging films prepared in manner used this study could act as reservoirs and release antimicrobial agents to maintain a relative high and constant inhibitory effect on food surfaces.

Conclusions

Semi-transparent and flexible protein films were prepared from blue marlin meat, including samples of very poor quality. The effect of pH on various properties of these films was similar to those of the muscle protein films and myofibrillar/sarcoplasmic protein films obtained from the same fish species (Iwata *et al.,* 2000; Shiku *et al.,* 2003; Hamaguchi *et al.,* 2007). The properties of films and the mechanism of film formation were not significantly affected by the quality of the meat. Thus, it is relevant to note that muscle protein films, which have the potential to be utilized as biodegradable films, can be prepared from the discarded meats of seafood processing plants.

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