Study on the glass transition for several processed fish muscles and its protein fractions using differential scanning calorimetry

TOMOKO HASHIMOTO, TORU SUZUKI,* TOMOAKI HAGIWARA AND RIKUO TAKAI

Department of Food Science Technology, Tokyo University of Marine Science and Technology, Minato, Tokyo 108-8477, Japan

ABSTRACT: The glass transition behavior of processed fish muscles (bonito, tuna, mackerel, sea bream, cod) and its muscle protein fractions (sarcoplasmic and myofibrillar proteins) were studied using differential scanning calorimetry. Each dried processed fish muscle and the extracted protein fractions showed clear glass transition phenomenon. The T_g values of muscles and myofibrillar proteins from red muscle fishes tended to be lower than those from white muscle fishes though there was no difference on T_g of sarcoplasmic proteins. The T_g value of whole muscle was considerably lower than that of extracted protein fractions because of the plasticizing effects of low molecular weight materials contained in the muscle.

KEY WORDS: **differential scanning calorimetry, fish muscle, glass transition, protein fraction.**

INTRODUCTION

The glass transition concept has made a deep impact in the field of food science because any type of food properties related to molecular mobility, including texture and shelf-life, are affected strongly by its glass transition behavior.[1,2] Below the glass transition temperature (T_g), several deterioration reactions of foods, texture loss, enzymatic spoilage, and flavor release, are significantly reduced because the molecular motions of glassy material are strictly prohibited. Over the last decade, much research has been reported about the importance of glass transition for a huge variety of food materials and food ingredients. Also, in the case of fishery products, the glass transition about frozen fishes, such as tuna,[3–6] cod[3,7,8] and mackerel,[9] have been reported for the association with frozen storage technique. Unfortunately, research regarding processed fish products is relatively scarce though there are so many processed foods manufactured from marine products all over the world. In a previous study,[10] we studied the glass transition of *Katsuobushi* (boiled and smoke-dried bonito fish stick), which is a well-known Japanese traditionally preserved food, using differential scanning calorimetry (DSC) and dynamic mechanical analysis and demonstrated that *Katsuobushi* is in a glassy state when it is kept under the ambient temperature, and its glass transition temperature depresses as the moisture increases. This result suggested that several properties of *Katsuobushi*, such as high storage stability, glass-like appearance, and change of state with increased temperature or moisture, are a reflection of its glassy nature. This result also implied the possibility that several fish products processed by a similar way can be in the same glassy state as *Katsuobushi*. If the glass transition concept could be applied commonly for every dried fish product, the information about its glass transition behavior will provide useful information to determine the optimum process and storage condition. For that purpose, however, a larger variety of fishes must be examined and the characteristics of its glass transition behavior understood.

The objectives of the present study are to generalize the glass transition concept to a wide variety of dried fish products and to improve the understanding about its glass transition behavior. Thus, we attempted to perform DSC analysis of several processed fish muscles (bonito, tuna, mackerel, cod, sea bream) and its muscle protein fractions (sarcoplasmic and myofibrillar proteins) to examine the generality of the glass transition concept and the contribution of two types of muscle pro-

*Corresponding author: Tel: 81-3-5463-0623. Fax: 81-3-5463-0585. Email: toru@s.kaiyodai.ac.jp
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tein fractions to the glass transition behavior of fish whole muscle.

MATERIALS AND METHODS

Sample preparations

Several dried fishes

Fresh bonito (*Katsuwonus pelamis*), bigeye tuna (*Thunnus obesus*), mackerel (*Scomber japonicus*), cod (*Gadus macrocephalus*), and sea bream (*Paglus major*) were used as samples for the determination of the glass transition temperatures. These fishes were purchased at a local store in the frozen state and thawed before measurement. The relation between fish freshness and its glass transition behavior are still unknown at the present time. Therefore, the effect of fish freshness on the glass transition was not discussed in this study. The dorsal muscles of those fishes were chopped by a knife into small brocks (1 cm × 1 cm) and boiled in water for 15 min at 100°C. Boiled samples were freeze-dried for 72 h and kept in a vacuum desiccator over P_2O_5 for 1 week for further drying. At the same time, chopped bonito and cod dorsal muscles were freeze-dried without boiling before drying in order to examine the heat treatment effect on its glass transition behavior. After that, these were hand-powdered using a mortar and pestle. These were stored in a refrigerator at –30°C before using.

Extraction of myofibrillar and sarcoplasmic protein fractions from bonito and cod muscles

Fresh bonito and cod were used as samples to examine the glass transition behavior for muscle protein fractions. Myofibrillar and sarcoplasmic protein fractions were prepared according to the procedures described by Marphy *et al.*[11] Homogenized muscle samples (400 g) were thawed and blended in 1600 mL buffer solution (0.05 M NaCl, 0.05 M potassium phosphate, 5 mM EDTA, pH 7.0) for 4 min at maximum speed at 2–4°C. The suspension was mixed with a propeller at 30 rpm for 4 h followed by filtration through a strainer at 4°C. The suspension was centrifuged at 7000 ×g for 30 min. The supernatant (sarcoplasmic protein fraction) was retained. The resulting pellet was resuspended in 1600 mL buffer four times, each followed by centrifugation at 7000 ×g for 30 min at 2–4°C. Lipid accumulation at the top of the bottles during each centrifugation was discarded. Final resulting pellet was collected as myofibrillar protein fraction. The extracted sarcoplasmic protein fraction were dialyzed against deionized water for 24 h twice to remove the low-molecular weight substances, and freeze-dried. The myofibrillar proteins were washed with deionized water six times before being freeze-dried.

The extracted sarcoplasmic and myofibrillar protein fractions were subjected to SDS-PAGE as described by Craeys and others.[12] A total of 5–20% gradient acrylamide gel (Atto Co. Ltd, Tokyo, Japan) was used. The molecular weights of protein components were determined by comparison of their relative mobility and order of migration with those of protein molecular weight standards (6500–205 000 dalton protein molecular weight standards, Molecular Probes, Inc., USA).

Adjustment and determination of moisture content

Different moisture content samples were prepared by equilibrating them in separate chambers of different relative humidity (RH) for 10 days. Saturated salt solutions of LiBr, LiCl, CH_3COOK, $MgCl_2$, K_2CO_3, NaBr, NaCl, KCl giving RH of 6.6, 11.3, 23, 33, 44, 58, 75.5, 85.5, respectively, were used in this study. The lowest moisture content samples were obtained by dehydrating in vacuum desiccators over P_2O_5 for 1 week. Actual moisture content of samples were determined by placing the samples in a drying oven at 110°C until it reached a constant weight. Four samples were used for the calculation of the sample moisture contents.

Differential scanning calorimetry

A Shimadzu DSC-50 differential scanning calorimeter (Shimadzu CO., Ltd, Kyoto, Japan) fitted with a LTC-50 cooling system (Shimadzu CO., Ltd) was used for this study. The temperature calibration was performed with indium (melting point, 156.6°C; ΔH_m, 28.5 J/g) and distilled water (melting point, 0.0°C; ΔH_m, 333 J/g). α-Alumina powder was used as a reference. N_2 at a flow rate of 20 mL/min was used as carrier gas. Samples of approximately 20 mg were weighed and hermetically sealed into aluminum pans by using a sealer. Samples were cooled with liquid nitrogen as a cooling medium and scanned from –50°C to 160°C at heating rate 5°C min^{-1}. All DSC scans were performed three times for the same sample in aluminum pan, namely first run, second run and third run after cooling the sample with liquid nitrogen to check the reversibility of heat capacity change. Thermal analysis software TA-60WS (Shimadzu Co., Ltd) was used to analyze the experimental data. The

glass transition temperature was determined from the midpoint temperature of the step-wise change in heat capacity.

RESULTS AND DISCUSSION

Several fish muscles

Typical DSC curves of boiled and freeze-dried bonito muscle with 7.7% moisture content are shown in Fig. 1. A small endothermic peak overlapping stepwise change in heat capacity was observed at 60°C in the first scanning curve. This peak is attributed to the phenomenon called 'enthalpy relaxation' that is one of the important characteristics of glassy material.[13] This feature was on the first scanning and was quite similar to the reported results for *Katsuobushi*.[10,14] In many cases,[15–18] this relaxation peak makes it difficult to determine the exact glass transition temperature even if it is in the glassy state. In this study, therefore, the first scanning was done only for the purpose of elimination of the relaxation peak. In the second scanning curve, a clear base-line shift in heat capacity, that was an indicator of the glass transition, was observed at 70°C. Furthermore, the reversibility of this heat capacity change, which is one important characteristic of the glass transition,[19] was confirmed in the third scanning curve. The other processed (boiled and freeze-dried) fish muscles used in this study, tuna, mackerel, cod and sea bream, also showed the same tendency as bonito muscle in their DSC curves (data not shown), suggesting that processed fish muscles can be in the glassy state just the same as *Katsuobushi*.

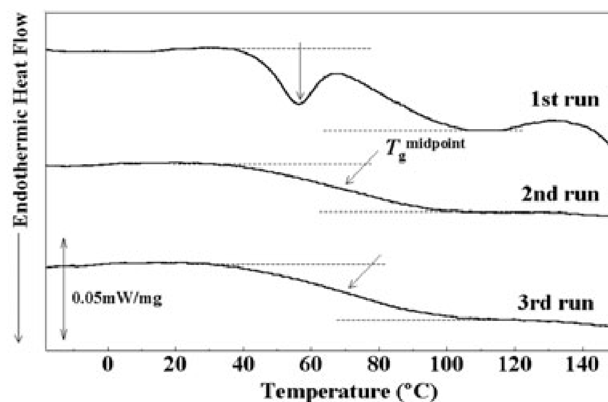

Fig. 1 Typical differential scanning calorimetry thermograms of boiled and freeze-dried bonito muscle at moisture content 7.7%. The sample was cooled with liquid nitrogen and heated at 5°C/min three times. The first, second and third heating scans are named first-run, second-run and third-run. The first heating scan was only performed to eliminate relaxation peak and the glass transition temperature (T_g) value was determined from the midpoint temperature of step-wise heat capacity change in the second scanning curve.

Figure 2 shows typical DSC curves of only freeze-dried bonito muscle with 5.7% moisture. This sample can be considered to have been in a non-denatured state before scanning since it was not heat-treated before freeze-drying. In the first scanning curve, two endothermic peaks were observed at 55°C and 120°C. It is known that fresh fish muscle contains over 80% moisture, and generally shows three endothermic peaks in the DSC curve in the temperature range of 40–80°C, which are caused by the heat denaturation of its constituent proteins,[2] and the denaturation temperature shifts to the higher temperature range as the sample moisture decreases. In contrast, an endothermic peak at 55°C observed in this study was not likely due to heat denaturation of muscle protein because it is expected that such a low moisture content sample would show higher denaturation temperature than 55°C. This endothermic peak should be considered as an enthalpy relaxation peak rather than a denaturation peak. However, a base-line shift caused by glass transition could not be detected in the first scanning curve differently from the sample boiled before drying. Judging from such knowledge, it can be considered that an endothermic peak at 120°C indicated the heat denaturation of muscle proteins. The difference in the number of denaturation peaks may be attributed to the difference in moisture content, heating rate in DSC measurement, and sample sources. In the second scanning

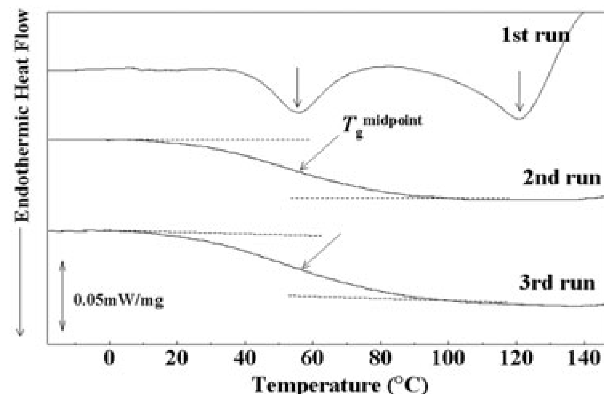

Fig. 2 Differential scanning calorimetry thermograms of freeze-dried bonito muscle without heat-treatment before drying at moisture content 5.7%.

curve, a clear glass transition could be detected at 60°C and its reversibility was also confirmed in the third scanning curve. The DSC curves for freeze-dried cod muscle also showed quite similar to bonito muscle (data not shown). These experimental results demonstrated that there is an apparent difference in the glass transition behavior between non-denatured and heat denatured samples; non-denatured fish muscle did not show the detectable glass transition but heat-denatured muscle showed clear glass transition in DSC curves. Such differences suggest that the glass transition behavior of fish muscle is related to the heat denaturation process of its muscle proteins. The relation between protein denaturation and its glass transition phenomenon has already been reported for several proteins and protein-rich products.[20–22] In the case of freeze-dried soy protein powder,[21] the glass transition of non-denatured samples was difficult to detect because the heat-capacity change in the DSC curve is relatively small. In contrast, the glass transition of heat-denatured samples was easy to detect because heat capacity change become larger than that of non-denatured samples. Such similarity between soy protein and our result for fish muscle suggests that there exists a common mechanism of the effect of heat denaturation on the glass transition behavior of proteins. However, it was not elucidated clearly since further discussion could not be performed from the present result. At least, however, our experimental results demonstrated that any dried fish muscle after heat denaturation can be in the glassy state.

Typical DSC curves of several boiled and dried fish muscles (bonito, tuna, mackerel, cod and sea bream) at different moisture contents are shown in Fig. 3. These were second scanning curves after eliminating the relaxation peak on the first scanning. In each curve, a clear glass transition phenomenon was observed. With the increase in moisture content, the glass transition temperature dropped down significantly to a lower temperature, reflecting the plasticizing effect of water. Figure 4 shows the moisture dependence behavior of the glass transition temperatures of boiled and dried fish muscles. Furthermore, the glass transition temperatures of dried bonito and cod muscles that were heated in the dry state at the first heating scan were also plotted in Fig. 4. From this figure, it was found that there were two interesting characteristics in the glass transition behaviors of fish muscles.

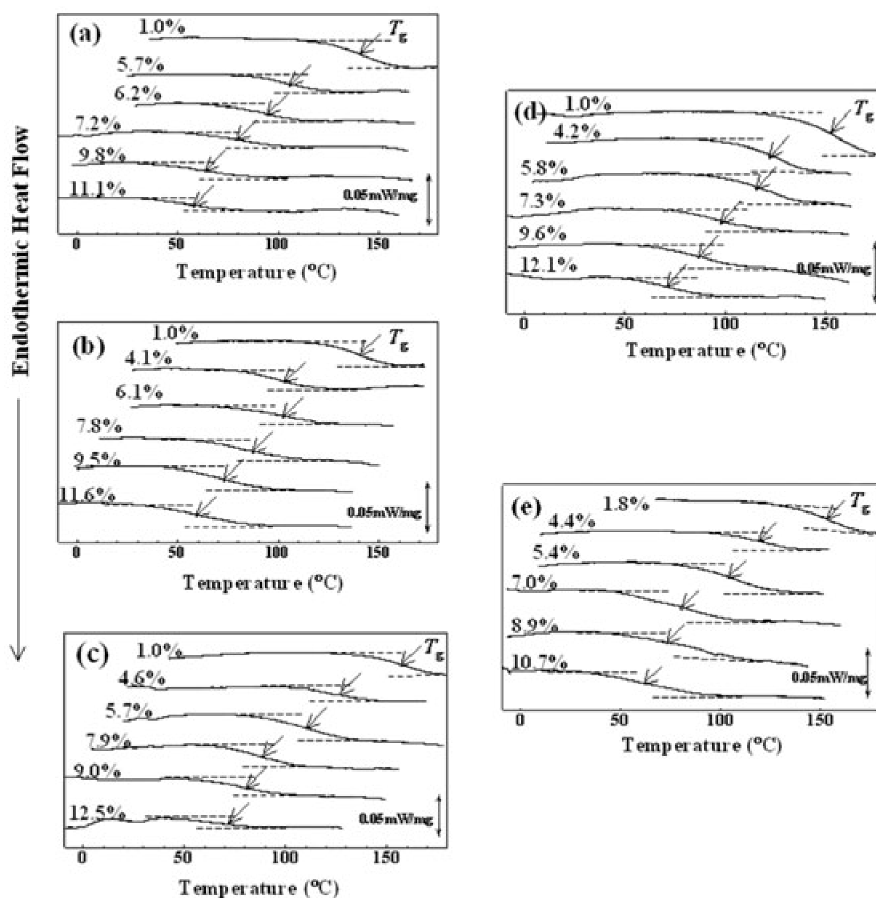

Fig. 3 Differential scanning calorimetry thermograms of several freeze-dried fish muscles boiled before drying at different moisture content: (a) bonito; (b) tuna; (c) mackerel; (d) cod; and (e) sea bream. These were second-run curves to eliminate relaxation peaks.

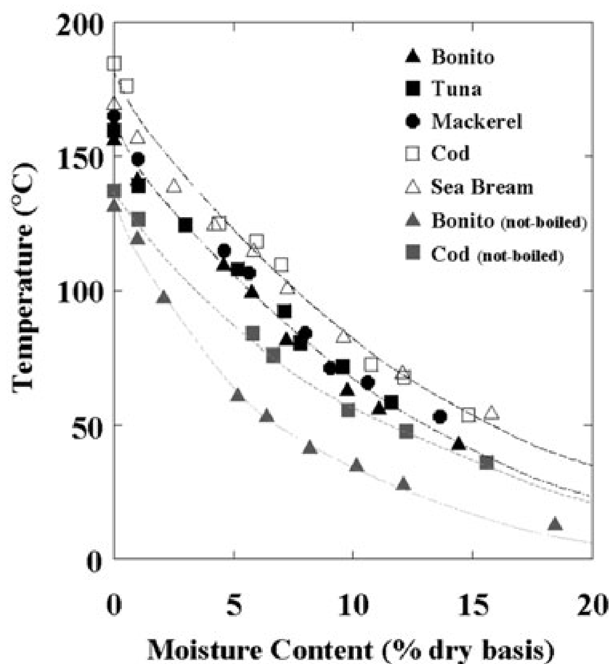

Fig. 4 The relation between moisture content and the glass transition temperature values of several boiled and freeze-dried fish muscles; bonito, tuna, mackerel, cod and sea bream. The glass transition temperatures of freeze-dried bonito and cod muscles heated in dry state in differential scanning calorimetry pans were also plotted. Plotted T_g values were determined from the midpoint temperature of step-wise change in heat capacity.

One is the difference in T_g values between boiled and non-boiled sample; there was a tendency that the sample boiled before drying showed a higher T_g value than the sample heated in the dry state after drying. The difference in T_g value was over 30°C in the case of bonito muscle. This result suggests that heat treatment before drying is more effective to elevate the glass transition temperature of fish muscle than heat treatment after drying. There is a possibility that the difference in T_g value caused by the difference in the heating process is related to heat denaturation mechanism of muscle protein. For more discussion about this point, however, more intimate researches will be required.

The other interesting finding is that there was a difference in T_g value between red muscle fishes and white muscle fishes. White muscle fishes (sea bream, cod) showed higher T_g values than red muscle fishes (bonito, tuna, mackerel). For example, the T_g values of red muscle fishes at 10% moisture content were in the range of 40–45°C. While, the T_g values of white muscle fishes were in the range of 60–65°C at the same moisture content. This result suggests that the difference between red muscle fishes and white muscle fishes may affect the T_g value. There are two reasons to explain such difference in T_g between red and white muscle fishes, one is due to the difference in lipid content and the other is to the difference in composition ratio of muscle protein fractions. Red muscle fishes used in this study have generally higher lipid content than white muscle fishes. However, we considered that the effect of such difference in lipid content is negligible because it was known that the lipid in food materials has generally no or a relatively small plasticizing effect on the T_g of proteins.[23,24] Therefore, it should be considered that the difference in composition ratio of muscle proteins and its thermal properties are more important. Generally, white muscle fish contain more myofibrillar protein than red muscle fish. In contrast, red muscle fish contain more sarcoplasmic protein than white muscle fish. Furthermore, it is well-known that several thermal properties between red and white muscle fishes, such as gel forming ability[25–29] and thermal stability,[30–33] are governed by the difference in thermal properties of sarcoplasmic and myofibrillar proteins. Therefore, it can be considered that such differences in composition ratio and thermal properties of muscle proteins may contribute to the difference in T_g values between red and white muscle fishes, the same as the gel-forming ability.

Sarcoplasmic and myofibrillar protein fraction extracted from bonito and cod muscles

Figure 5 shows the SDS-PAGE patterns of sarcoplasmic and myofibrillar protein fractions extracted from bonito and cod muscles. The electrophoretic pattern of myofibrillar protein fraction showed two major bands as myosin heavy chain and actin at 200 kDa and 50 kDa, respectively, and these bands were not observed for sarcoplasmic protein fraction. This result proved that the extracting of protein fractions was performed well without admixture.

Figures 6 and 7 show typical DSC curves for freeze-dried sarcoplasmic and myofibrillar protein fractions extracted from bonito and cod muscles. Moisture contents of these samples were 5.5% and 4.9% for bonito and 6.1% and 5.6% for cod, respectively. In the first run curves, each sample showed only one endothermic peak at 137°C and 140°C for bonito and 123°C and 123°C for cod, and no baseline shift that is an indicator of the glass transition could be detected. Of course, the endothermic peaks on the first scanning are considered due to heat denaturation of its constituent proteins. In contrast, in the second run curves, all samples showed a clear base-line shift caused by the glass

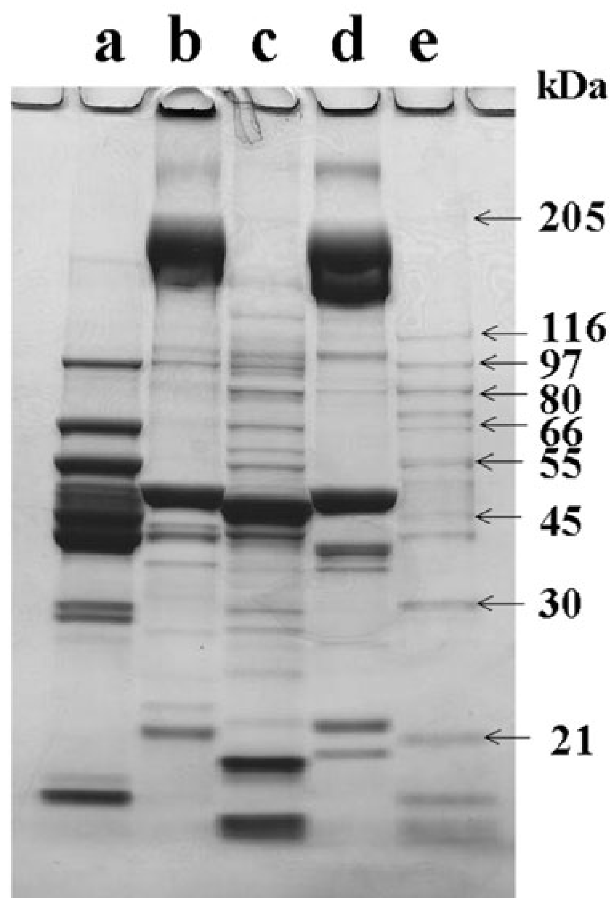

Fig. 5 The SDS-PAGE patterns for sarcoplasmic and myofibrillar protein fractions extracted from bonito and cod muscles: (a) sarcoplasmic protein of bonito; (b) myofibrillar protein of bonito; (c) sarcoplasmic protein of cod; (d) myofibrillar protein of cod; and (e) molecular standards.

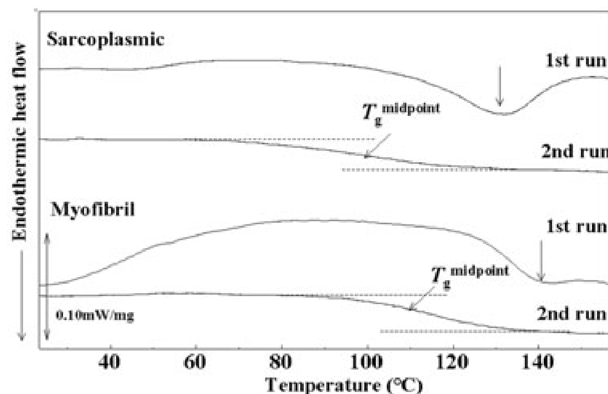

Fig. 6 Typical differential scanning calorimetry thermograms of freeze-dried sarcoplasmic and myofibrillar protein fractions extracted from bonito muscle adjusted at the same relative humidity (RH = 6.6%). The moisture content of sarcoplasmic and myofibrillar proteins was 5.5% and 4.9%, respectively.

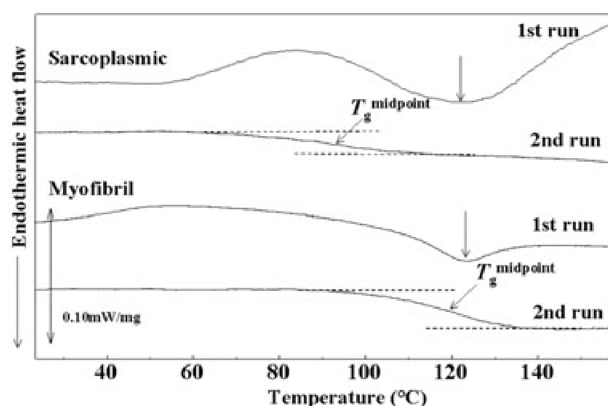

Fig. 7 Typical differential scanning calorimetry thermograms of freeze-dried sarcoplasmic and myofibrillar protein fractions extracted from cod muscle adjusted at the same relative humidity (RH = 6.6%). The moisture content of sarcoplasmic and myofibrillar proteins was 6.1% and 5.6%, respectively.

transition phenomenon. The glass transition temperature of sarcoplasmic and myofibrillar proteins were 100°C and 107°C for bonito and 93°C and 118°C for cod, respectively. These DSC results for extracted protein fractions were quite similar to those for dried bonito and cod whole muscles as described in the previous section, which demonstrates that both sarcoplasmic protein and myofibrillar proteins turned into the glassy state after heat denaturation. Therefore, it can be considered that these two types of protein fractions play a dominant role for the glass transition of processed fish products.

Figure 8 shows DSC curves of sarcoplasmic and myofibrillar protein fractions of bonito and cod at different moisture contents. These were second scanning curves after heat denaturation at first heating scan. The glass transition temperature of these heat-denatured samples shifted to the lower temperature range with the increase in moisture content, reflecting the plasticizing effect of moisture. Figure 9 shows the relation between the moisture content and the T_g values for sarcoplasmic and myofibrillar protein fractions extracted from bonito and cod muscles. For comparing, the T_g values for whole muscle obtained from the experiment mentioned in the previous section were also plotted in the same figure. Our experimental result demonstrated that whole muscle showed considerably lower T_g value over 30°C than that of extracted proteins. This result suggests that the glass transition behavior of fish muscle could not be described as a simple water–protein mixture

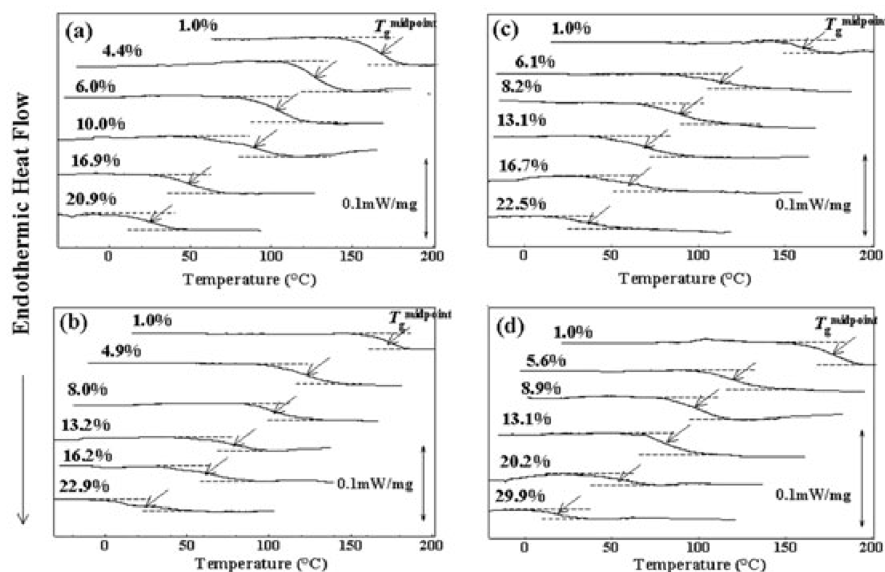

Fig. 8 Differential scanning calorimetry thermograms of freeze-dried sarcoplasmic and myofibrillar protein fractions extracted from bonito and cod muscles at different moisture content: (a) sarcoplasmic protein of bonito; (b) myofibrillar protein of bonito; (c) sarcoplasmic protein of cod; and (d) myofibrillar protein of cod. These were second-run curves after heat denaturation at the first heating scan.

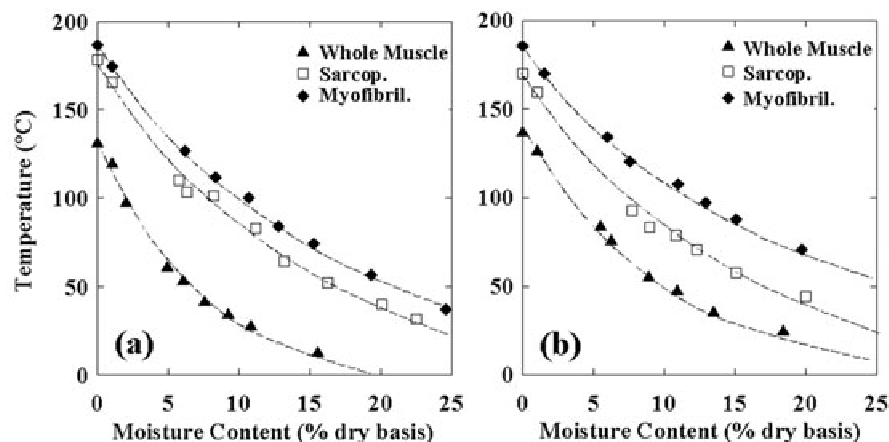

Fig. 9 The relation between the moisture content and the glass transition temperatures of freeze-dried and heat-denatured whole muscle, sarcoplasmic protein and myofibrillar protein: (a) bonito; and (b) cod.

system. In the case of extracted proteins, the small solutes (e.g. salts, sugars, minerals) contained in muscle were already removed by a dialysis during the extraction process, while these still remained in the whole muscle sample because no treatment was performed before drying. So, it can be considered the small solutes contained in muscle may give the plasticizing effect on T_g of muscle proteins. Also, in the case of gluten, it has been reported that the presence of several sugars (sucrose, fructose or glucose) strongly depresses the T_g value of gluten, especially in the low moisture region.[16] It should also be noted that the T_g of myofibrillar protein was higher than that of sarcoplasmic protein for both bonito and cod muscle. The difference in T_g values was relatively small for bonito and for cod. It can be considered that the difference in each nature between sarcoplasmic and myofibrillar proteins contributes to the difference in their glass transition temperature.

Figure 10 shows the moisture dependence behavior of T_g for whole muscle and protein fractions separately in order to discuss the difference between bonito and cod. Whole muscle and myofibrillar protein showed similar tendency; cod showed higher T_g value than bonito. However, T_g for sarcoplasmic proteins of bonito and cod showed almost the same moisture dependences. This result implies that the difference in T_g value between bonito and cod muscle is closely affected by its myofibrillar proteins rather than sarcoplasmic proteins. It is known that myofibrillar protein of red muscle fish is rich in slow twitch fiber and myofibrillar protein of white muscle fish is rich in fast-twitch fiber.[34–36] Boyer *et al.*[29] pointed out the possibility of such a difference in muscle type between red and white muscle fish affecting several important thermal properties of fish muscle such as gel-forming ability. Therefore, there is a possibility that the difference in glass transition

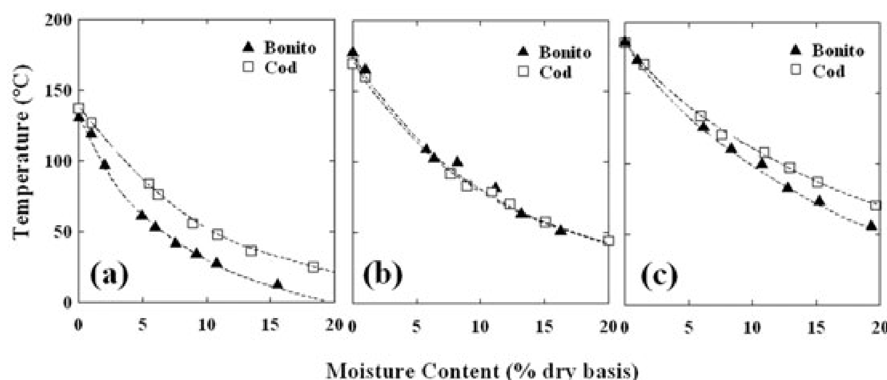

Fig. 10 The comparative difference in the glass transition temperatures between bonito and cod: (a) whole muscle; (b) sarcoplasmic proteins; and (c) myofibrillar proteins. All samples were freeze-dried and heat-denatured.

behavior between red and white muscle fishes may also be caused by the difference in its muscle type. Though, how the effect of muscle type has on the glass transition is not clear at the present time, it is interesting that the glass transition phenomenon showed muscle type dependence to be the same as the gel-forming ability. Clarification about the reason for the difference in the glass transition behavior between red and white meat fishes is an issue for future research.

CONCLUSION

Several dried fish muscles preboiled before freeze-drying, such as bonito, tuna, mackerel, cod and sea bream showed clear glass transition phenomenon in DSC curves. In contrast, bonito and cod muscles freeze-dried without any heat-treatment did not show the detectable glass transition, but after heat denaturation, these muscles showed clear glass transition. These experimental results demonstrated that the glass transition is a general characteristic for any dried and heat-treated fish muscles and the heat denaturation process gives an important effect for its glass transition behavior. Furthermore, it became clear that the glass transition of fish muscles is affected by the difference in fish species; the T_g of white meat fishes (cod and sea bream) tended to be higher than that of red meat fishes (bonito, tuna and mackerel). Such difference in T_g value dependent on fish species was caused by the difference in the properties of protein fractions, especially for myofibrillar protein. Furthermore, it became clear that the T_g values of whole muscle were considerably lower than that of extracted protein fractions because of the plasticizing effect of low molecular materials contained in the muscle.

Though the importance of the glass transition has been recognized for so many low-moisture food materials, the research about fishery products has not been reported. However, the present study demonstrated the importance of how the glass transition concept could be applicable to a large variety of fish products. The experimental results obtained in this study will provide useful information for a deeper understanding about the glass transition of many low-moisture fish products. We hope the importance of the glass transition concept will be recognized widely, especially in the fishery industry.

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