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Thermal Aggregation of Mixed Fish Myosins

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Cod and herring myosin solutions were mixed in five different ratios and heated to between 30 and 55 °C for 30 min. Extent of aggregation of the myosin solutions increased significantly ($p < 0.05$) with higher cod/herring myosin ratios at temperatures ≤40 °C. However, there was no significant difference ($p < 0.05$) in the extent of aggregation among the mixed and herring myosin samples above 45 °C. Two transition temperatures (T_m) were observed at 34 and 43 °C in cod myosin when the protein solution was heated from 25 to 56 °C at 1 °C/min, whereas herring myosin exhibited one T_m at 49 °C and the mixed myosin samples displayed an extra T_m at 39 °C which was absent in the pure cod or herring myosin samples. When a preheated herring myosin solution was mixed with unheated cod myosin in a ratio of one to one and the mixture was reheated at the same temperature, the extent of aggregation was significantly higher ($p < 0.05$) than the control heated at temperatures between 40 and 50 °C. Electrophoretic analysis revealed that more large myosin polymers were formed in the cod/preheated herring myosin samples than in the controls or in herring myosin alone, suggesting that preheated herring myosin and/or myosin aggregates interact with cod myosin, leading to the formation of larger mixed protein aggregates at temperatures between 40 and 50 °C.

Keywords: *Thermal aggregation; mixed fish myosins; noncovalent protein interactions*

INTRODUCTION

The recent dramatic drop of cod stocks in the North Atlantic suggests that more should be done to exploit some underutilized species. Herring (*Clupea harengus*) is one of the most abundant but underutilized fish species in North America and has potential for the production of surimi-based products. However, the textural properties of herring surimi are poor when compared with cod (*Gadus morhua*) and other white-fleshed species which form firm and elastic muscle gels (Spencer *et al.*, 1987). Chan *et al.* (1992a, 1993) compared the denaturation and aggregation behaviors of cod and herring myosins, and reported that the inferior gel forming ability of herring muscle proteins was related to the unfolding profile of interior hydrophobic domains when heated. Extent of aggregation for fish myosin seems to depend on the amount of hydrophobic surface exposed on the heated molecules (Chan *et al.*, 1992b; Wicker *et al.*, 1986); therefore, it is reasonable to assume that the extent of aggregation for poor gel-forming species, i.e. herring, would be improved by finding a strategy to increase the surface hydrophobicity of heated myosins. Heating cod and herring myosins together may increase the overall hydrophobic surface per myosin molecule which could enhance the formation of mixed myosin aggregates. The objectives of the present study were to investigate the thermal aggregation behavior of mixed cod and herring myosin solutions, and to test the hypothesis that the extent of aggregation for poor setting fish myosin could be enhanced by blending with myosin from a fish species that displays a higher surface hydrophobicity when heated.

MATERIALS AND METHODS

Preparation of Fish Myosin. Cod and herring were purchased from local fishermen on the same day of catch in May and June 1993. All fish used in this study were less than 24 h postharvest at the time of delivery to the laboratory, and

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storage temperature during transit did not exceed 4 °C. Fish myosin was prepared immediately upon delivery as described by Chan *et al.* (1992a). Extracted myosin was stored in 0.5 M KCl, 50 mM phosphate buffer (pH 7.0), and 50% glycerol at −30 °C. The purity of each myosin extract was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS−PAGE). The densitometric data showed that for the two species of fish the myosin was at least 90% pure.

Solution Preparation. Samples of myosin were prepared by precipitating frozen-stored myosin by dilution with 10 volumes of chilled, deionized distilled water prior to centrifuging at 6000 g for 15 min and dialyzing the pellet against 0.6 M NaCl and 50 mM imidazole (pH 6.5) overnight. Mixed myosin solutions (1 mg/mL) were prepared by mixing 1 mg/mL cod myosin and 1 mg/mL herring myosin solutions at three different ratios of 1:3, 1:1, and 3:1 (C/3H, C/H, and 3C/H) just prior to the heat treatments. Protein determinations were routinely performed by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

Turbidity Measurements. Turbidity was measured with a PU 8800 spectrophotometer (Pye Unicam, Cambridge, U.K.). Myosin samples (1 mg/mL, 4 mL) were heated isothermally at a temperature between 30 and 50 °C for 30 or 60 min or from 25 to 56 °C at a rate of 1 °C/min and the change in absorbance at 320 nm was measured continuously as heating proceeded. Temperature was controlled with a Haake PG10 temperature programmer and Haake N3 temperature control unit (Haake Mess-Technik GmbH, Berlin, Germany). For the preheating experiments, either cod or herring myosin solutions (1 mg/mL, 2 mL) were held at an experimental heating temperature for 30 min, and then the myosin solution from the other fish species (1 mg/mL, 2 mL) was added and the mixed sample held at the same temperature for another 30 min.

A transition temperature in the thermal scanning experiments was defined by the maximum exhibited in the first derivative plot of turbidity data. Extent of thermal aggregation of myosin samples, p, was defined as

$$p = (A_f/A_i - 1)/(A_f/A_i + 1)$$

where A_f and A_i were the absorbance of heat-treated and unheated myosin solutions, respectively (Lauffer, 1971). In our previous studies, the extent of aggregation was calculated as $(A_f - A_i)/A_i$, and it was demonstrated that $(A_f - A_i)/A_i$ was linearly correlated with the amount of aggregated myosin (Gill *et al.*, 1992). Similar patterns of myosin aggregation were observed for all samples in the present study when p or $(A_f - A_i)/A_i$ was plotted as a function of temperature. Timasheff (1981) derived equations for isodesmic self-association of proteins, and proved that p was the probability for monomeric units to form polymeric units in a system and p was also the same term used in the Flory gelation theory to describe the extent of gelation (Flory, 1936, 1941). Thus, in the present study p was substituted for $(A_f - A_i)/A_i$ to define extent of myosin aggregation, especially in attempting to relate myosin aggregation to the gelation process.

Electrophoretic Analysis. In the present study, the EDC/electrophoresis approach was adopted to monitor the thermal aggregation of mixed fish myosins. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) is a zero-length cross-linker which can buttress proteins covalently when interacting carboxyl and amino groups of adjacent peptides are juxtaposed (2−5 Å) and will not cross-link noninteracting proteins (Hoare and Koshland, 1967). Chan *et al.* (1992a, 1993) studied thermal aggregation of fish myosin by electrophoresis of the heat-treated, EDC cross-linked proteins and demonstrated that the extent of myosin aggregation as monitored by the EDC/electrophoretic and turbidimetric studies were generally in agreement.

Myosin samples (4 mg/mL, 0.5 mL) in 0.6 M NaCl and 50 mM imidazole (pH 6.5) were heated isothermally at a fixed temperature between 35 and 50 °C for 60 min. For the preheating experiments, 0.25 mL of herring myosin solution (4 mg/mL) was held at a temperature between 35 and 50 °C for 30 min, and then 0.25 mL of cod myosin solution (4 mg/mL) was added and the mixture further heated at the same temperature for another 30 min. Mixed samples (cod:herring = 1:1) that contained preheated herring or cod were designated C/H_h or C_h/H, respectively. Samples that were not preheated were designated C/H and were prepared by heating mixtures at a fixed temperature for 60 min. After the predetermined heating time, 0.1 mL of 36 mM EDC (Sigma E-7750) dissolved in the same buffer was added to the protein solution, and the mixture was held at the same heating temperature for an additional 5 min. The EDC solution was prepared fresh just prior to the cross-linking experiment because it is chemically unstable in aqueous solution (Gilles *et al.*, 1990). EDC cross-linking was stopped by adding 0.4 mL of a solution containing 8 M urea, 2.5% SDS, 0.54 M β-mercaptoethanol, and 25 mM Tris/HCl (pH 7.4), and the sample was subsequently heated at 90 °C for 20 min and exhaustively dialyzed against 0.1% SDS, 0.2 M β-mercaptoethanol, and 25 mM Tris/HCl (pH 7.4). The EDC cross-linked myosin samples (10−15 μg/lane) were electrophoresed on 2.5% acrylamide/agarose composite gels as described by Chan *et al.* (1992a). The gels were stained with Coomassie Brilliant Blue R250 and were scanned at 560 nm on the PU 8800 spectrophotometer (Pye Unicam Cambridge, England) with peak integration equipment.

Statistical Analysis. Analyses were performed in duplicate or triplicate from two different batches of myosin of each fish species. Tukey's test was used to compare means when effects were evaluated (Neter *et al.*, 1985).

RESULTS AND DISCUSSION

Isothermal Heating at Temperatures between 30 and 55 °C. Absorbance (320 nm) of myosin solutions increased with temperature due to the formation of myosin aggregates which were large enough to cause light scattering. The extent of aggregation for cod myosin doubled from 30 to 35 °C and increased linearly but to a lesser extent between 35 and 55 °C (Figure 1). The extent of aggregation for herring myosin also increased linearly with temperature and exhibited an inflection point at 45 °C, above which the extent of aggregation increased less sharply. In addition, the extent of aggregation for cod myosin was consistently higher than for herring at all heating temperatures (Figure 1). For the mixed myosin samples, the extent of aggregation increased nearly linearly with increasing temperature, but samples with a higher cod to herring myosin ratio, displayed a significantly higher extent of aggregation at temperatures ≤45 °C ($p < 0.05$). However, there was no significant difference ($p < 0.05$) between C/3H and herring myosin at 45 °C or above, or among C/H, C/3H and herring myosin at temperatures ≥50 °C. Chan *et al.* (1992b) reported that the denaturation temperatures of cod and herring myosins were 46 and 53 °C, respectively and the surface hydrophobicity for thermally denatured cod myosin molecules was significantly larger than for herring at temperatures ≤50 °C. Thus, it may be that the aggregation behavior of mixed myosin samples is related to the denaturation profile of the protein molecules. The superior aggregation ability displayed by the mixed myosin samples with higher cod/herring myosin ratios at the temperatures ≤45 °C may be due to more exposed hydrophobic surface on the cod myosin molecules, leading to the formation of larger protein aggregates. If this hypothesis were true, the less discernible difference in the extent of aggregation of mixed myosin samples at higher temperatures would be due to denaturation and unfolding of more hydrophobic surfaces of herring myosin molecules at 45 °C and above.

If aggregation of myosin of each fish species is an independent event, the extent of aggregation for mixed

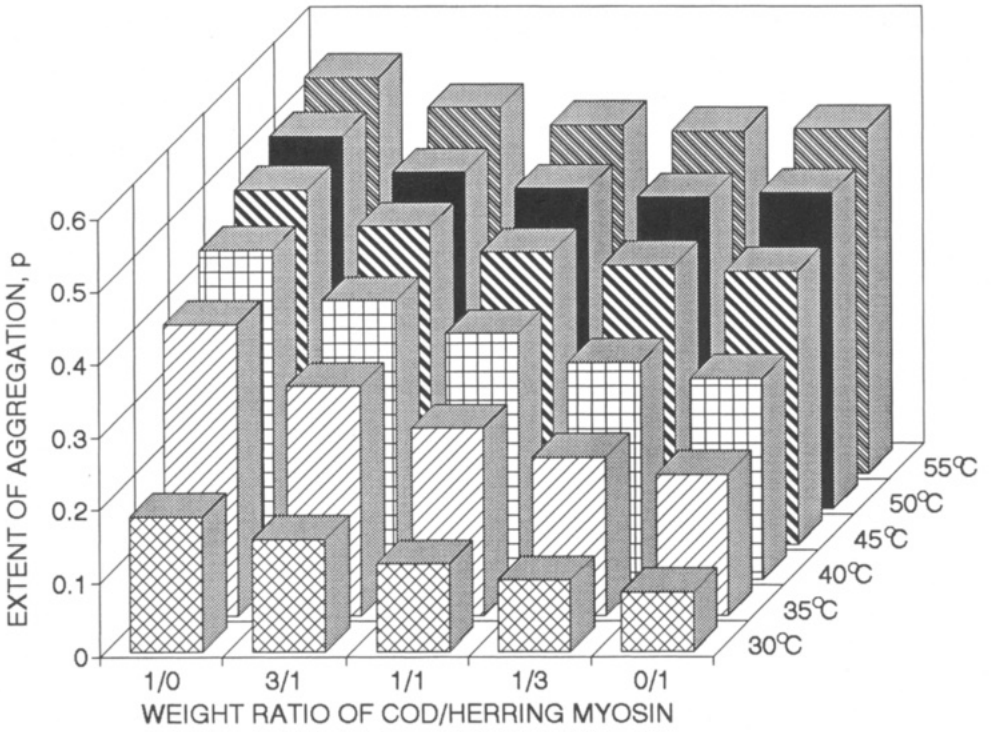

Figure 1. Extent of aggregation for mixed myosin solutions (1 mg/mL) heated at a set temperature between 30 and 55 °C for 30 min. The values were an average of four replicates.

myosin samples (i.e., cod:herring = 1:1, 1 mg/mL) would be equal to the arithmetic sum of the extent of aggregation for separately heated cod and herring myosin samples in a corresponding weight ratio (i.e., 0.5 mg/mL cod myosin, 0.5 mg/mL herring myosin). If there was a positive protein−protein interaction between cod and herring myosin molecules in the mixed samples, one would expect that the extent of aggregation would be greater than their arithmetic sum. In the present study, the extent of aggregation for all mixed myosin samples was significantly smaller ($p < 0.05$) and about 8 ± 3% less than the arithmetic sum of separately heated cod and herring myosin samples at all heating temperatures. Nishimoto *et al.* (1988) reported that there was no difference in breaking strength [Breaking strength of a product was measured with a rheometer with a 5 or 3 mm plunger and was evaluated as load value (g) from the break point.] and formation of myosin aggregates among the Alaska pollock, chum salmon, and mixed pollock and salmon muscle mince when heated at 25 or 40 °C. Thus, it is most likely that heating cod and herring myosins simultaneously does not accelerate the protein aggregation in the protein mixture.

Thermal Scanning from 25 to 56 °C. Myosin solutions exhibited a sigmoidal increase in the extent of aggregation with increasing temperatures and a rapid increase in the extent of aggregation at temperatures between 32 and 50 °C (Figure 2). The extent of aggregation was largest in cod, followed by 3C/H, C/H, C/3H, and then herring. The differences in "p" were significantly different from one another ($p < 0.05$) but only in the temperature range from 38 to 50 °C. The extent of aggregation for C/3H and herring appeared to be the same at temperatures below 38 °C and above 50 °C.

Transition temperature (T_m), as determined from the derivative plot of the turbidity data, has been suggested to be a temperature at which protein conformational changes result in protein−protein interactions and aggregate formation (Ishioroshi *et al.*, 1979). In addition, the presence of transition temperatures shown in the first derivative plot of turbidity data indicated that there was a temperature at which extent of myosin aggregation accelerated to a maximum. Compared with the results of thermal-unfolding profile of heat-treated myosin molecules (Chan *et al.*, 1992b), it is suggested that some conformational changes in myosin molecules seem to be the driving force for the acceleration of myosin-myosin interactions shown at the transition temperature.

Figure 3 shows the first derivative plot of the data from Figure 2. Cod myosin displayed two transition temperatures at 34 (T_{m1}) and 43 °C (T_{m3}). The transition temperature of herring myosin was rather ill-defined and was estimated to be around 49 °C (T_{m4}). The present data were similar to those reported earlier (Chan *et al.*, 1992b). The T_{m1} and T_{m3} were also observed in the mixed myosin samples of 3C/H and C/H but were ill-defined in the C/3H sample. In addition, the T_{m1} in the mixed myosin samples was 1 °C higher than that of pure cod myosin. The transition temperature of herring myosin, T_{m4}, was observed quite clearly only in the C/3H sample. It was notable that a new transition temperature (T_{m2}, 39 °C) was observed in all mixed myosin samples though the magnitude of T_{m2} decreased in the samples with lower cod/herring myosin ratios. The presence of only T_{m1}, T_{m3}, and T_{m4} in the mixed myosin samples would suggest that both cod and herring myosin would aggregate independently in the protein mixture during the heat treatment. However, all mixed myosin samples displayed T_{m2} indicating that there may be mixed aggregates forming between cod and herring myosins at 39 °C. The presence of only one extra, discernible transition temperature in all mixed myosin samples also implies that the interaction between cod and herring myosin is temperature specific, and the interaction most probably takes place only at the point where both cod and herring myosin molecules unravel "sufficient" interior hydrophobic surface area.

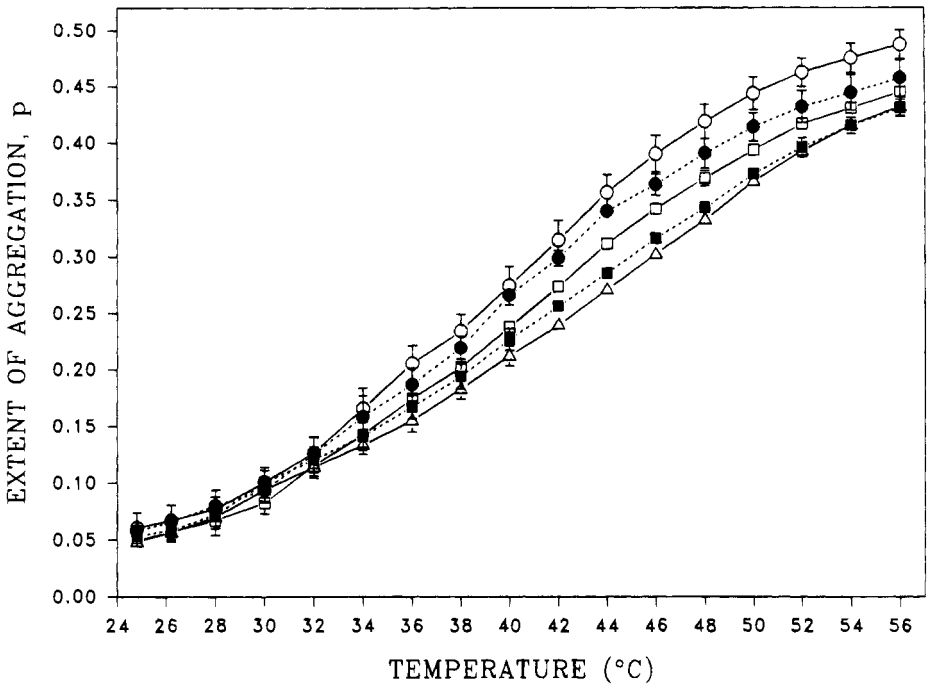

Figure 2. Effect of temperature on extent of aggregation of mixed myosin solutions (1 mg/mL). The protein solutions were continuously heated from 25 to 56 °C at a rate of 1 °C/min. (○) Cod myosin; mixed myosin samples,(●) 3C/H (cod myosin/herring myosin, 3/1 w/w); (□) C/H (cod myosin/herring myosin, 1/1 w/w); (■) C/3H, (cod myosin/herring myosin, 1/3 w/w); (△) herring myosin. Each point is the mean of three determinations, and error bars represent ±1 standard derivation.

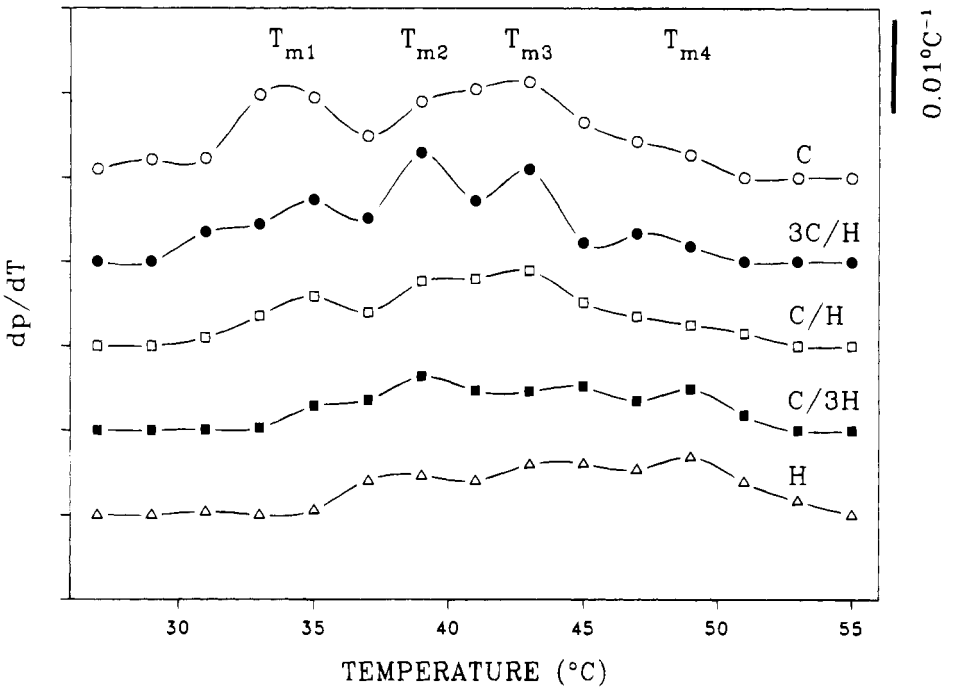

Figure 3. First derivative of turbidity data from Figure 2. The same legends are used as in Figure 2. Determination of the transition temperature (T_m) is described under Materials and Methods.

Preheated Mixed Myosin Samples. The thermal scanning data suggested the presence of mixed myosin aggregates. Therefore, an attempt was made to enhance this interaction by denaturing the fish myosin molecules first before thermal aggregation. Cod or herring myosin solutions (1 mg/mL, 2 mL) were heated at a fixed temperature between 30 and 55 °C briefly for 1–5 min. The preheated myosin solution was then mixed and further heated with myosin (1 mg/mL, 2 mL) from the other species at the same temperature for 30 min. There was no significant difference ($p < 0.05$) between the preheated mixed myosin samples and the appropriate controls (data not shown). On the other hand, the extent of aggregation for the mixed myosin samples containing herring myosin preheated for 30 min (C/H_h) was enhanced as compared to the C/H samples ($p < 0.05$) but only at fixed heating temperatures in the 40–50 °C range (Figure 4). The mixed myosin samples containing preheated cod myosin (C_h/H) appeared to display p values similar to the C/H samples for heating temperatures between 30 to 45 °C, and exhibited lower p values than C/H samples at temperatures ≥ 50 °C. The extent of aggregation for cod, herring, and mixed myosin samples (C/H) increased by about 10% at all

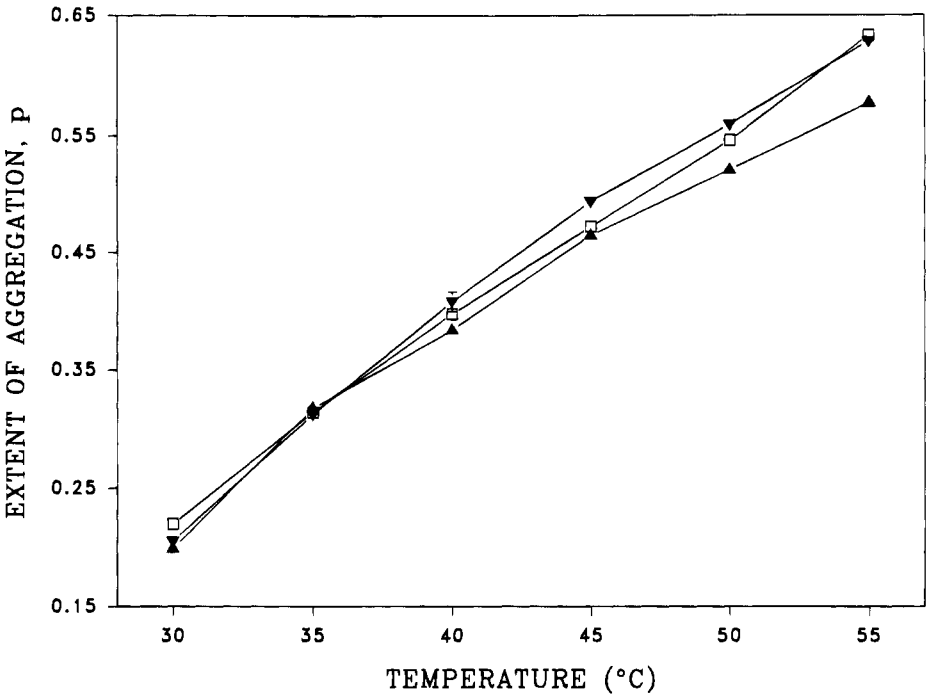

Figure 4. Extent of aggregation of the preheated mixed myosin solutions (1 mg/mL) which were heated at a set temperature between 30 and 55 °C for 60 min. The preheating condition is described under Materials and Methods. (▼) Preheated herring myosin samples (C/H_h); (□) mixed myosin samples (C/H); (▲) preheated cod myosin samples (C_h/H). Each point is the mean of three determinations, and error bars represent ±1 standard derivation.

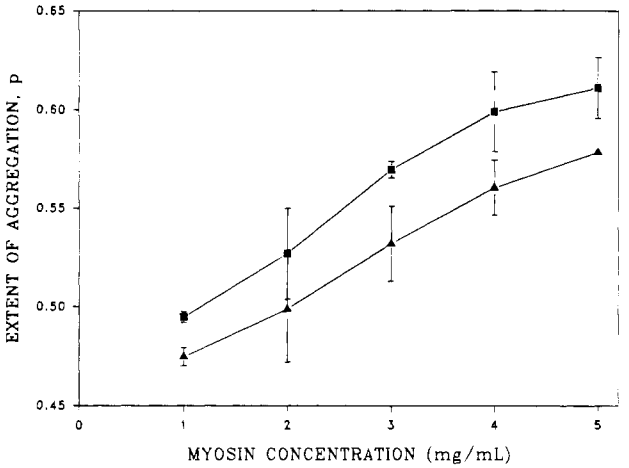

Figure 5. Effect of myosin concentration on change in extent of aggregation for preheated mixed myosin samples at 45 °C. (■) Preheated herring myosin samples (C/H_h); (▲) mixed myosin samples (C/H). Each point is the mean of three determination, and error bars represent ±1 standard derivation.

heating temperatures when the heating time increased from 30 to 60 min. If mixed aggregates were not formed with the preheated herring myosin, the extent of aggregation for C/H_h would be expected to be smaller than that of C/H. This is because the herring myosin in both C/H_h and C/H samples received the same heat treatment but the cod myosin in the C/H_h sample received 30 min less heating than that in the C/H sample. Therefore, the higher p values displayed by the C/H_h samples at temperatures between 40 and 50 °C confirm that preheating the herring myosin enhanced the formation of mixed aggregates in the cod/herring mixtures.

Figure 5 shows the effect of myosin concentration on extent of aggregation for C/H and C/H_h. Both samples were heated at 45 °C under the same preheating and reheating conditions as described previously. The extent of aggregation for C/H_h samples was larger than for C/H at all myosin concentrations. The results confirmed the idea that preheated herring myosin and/or myosin aggregates would interact with cod myosin and/or myosin aggregates, leading to the formation of larger myosin aggregates. It is known that fish myosin molecules denature rapidly when heated briefly, and tend to aggregate with prolonged heating (Connell, 1960; Kimura *et al.*, 1980). In the present study, preheating the herring myosin solutions for ≤5 min failed to enhance the overall extent of aggregation in the mixed myosin samples. Therefore, it is reasonable to assume that the protein–protein interaction observed in the C/H_h samples primarily involves herring myosin aggregates, instead of denatured herring myosin molecules. The similarity in the extent of aggregation between C/H_h and C/H samples heated to ≤40 °C suggests that the interaction between cod and herring myosin and/or myosin aggregates will take place only when herring myosin molecules unravel most of their interior hydrophobic surfaces. However, it is difficult to ascertain whether it is monomeric cod myosin heavy chain (MHC), or MHC aggregates, or both which become involved in the mixed polymers involving cod and preheated herring myosins. It is not known why preheated cod myosin failed to increase the extent of aggregation of C_h/H samples. It is most likely that it is necessary for both components in the mixed myosin aggregates to expose a "sufficient" hydrophobic surface simultaneously and herring myosin requires more heat input for this to occur.

Electrophoretic Analysis. In the present study, SDS–PAGE coupled with EDC was used to examine non-covalent interactions and to estimate the relative amounts of myosin aggregates formed in the heat-treated samples. When myosin samples were heated and reacted with the EDC cross-linking reagent, a group of myosin polymers (n = 2–5) was observed between the top of 2.5% acrylamide/agarose composite gels and

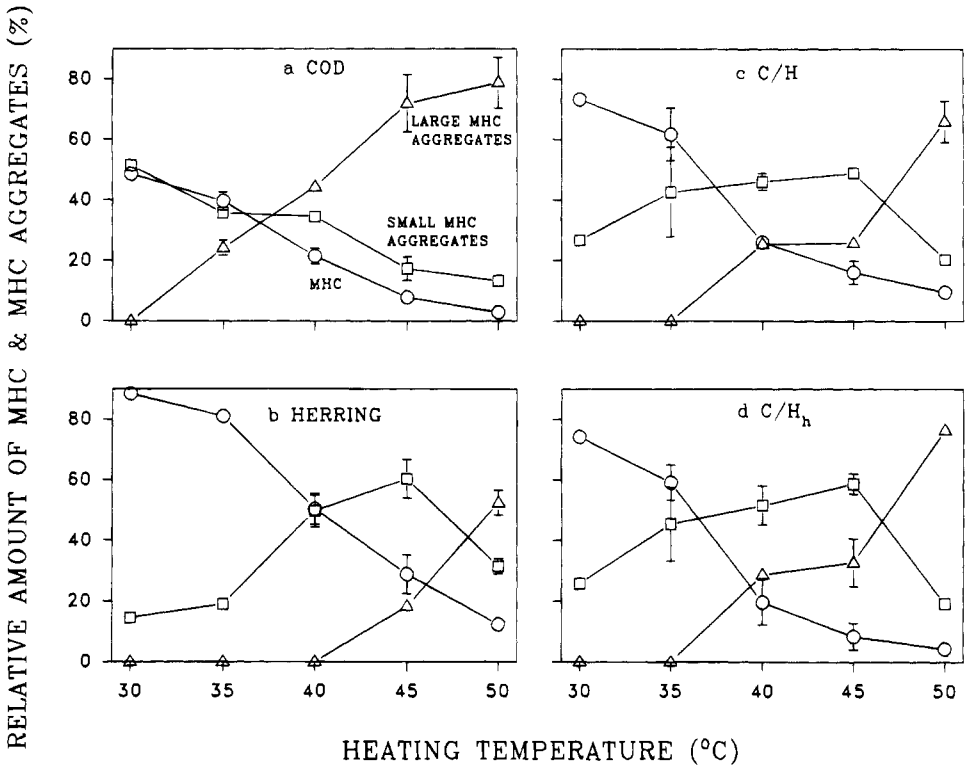

Figure 6. Distribution of MHC and MHC polymers in the mixed myosin samples as determined by EDC/SDS–PAGE. The protein solutions (4 mg/mL) were heated at a set temperature between 30 and 50 °C for 60 min: (a) cod myosin; (b) herring myosin; (c) mixed myosin sample (C/H); (d) preheated herring myosin sample (C/H$_h$); (○) MHC; (□) small MHC polymers (n = 2–5); (△) large MHC polymers (n > 5). Each point is an average of three determinations, and error bars represent ±1 standard derivation.

the MHC band. Another group of high molecular weight MHC polymers (n > 5) was also observed and remained on the top of the composite gels in some heat-treated myosin samples. The relative amount of MHC and each group of MHC polymers was calculated from the densitometric data for each heating experiment. Figure 6a shows that monomeric cod MHC and small MHC polymers (n = 2–5) decreased at higher heating temperatures, whereas large MHC polymers (n > 5) increased rapidly between 30 and 45 °C and increased slightly at the temperatures ≥45 °C. In contrast, herring myosin molecules were cross-linked and formed only small MHC polymers (n = 2–5) at the temperatures <45 °C. In addition, far fewer herring myosin molecules were involved in the thermal aggregation when compared with those of cod (Figure 6b). At temperatures ≥40 °C, large myosin polymers (n > 5) were observed in the herring myosin samples and increased but less than cod at higher temperatures. Parts a and b of Figure 6 illustrate the progressive two-step cross-linking for fish myosin molecules during thermal aggregation. First, the MHCs form small aggregates and then large protein aggregates are observed. In addition, the formation and distribution of myosin polymers appears to depend on the temperature at which fish myosin molecules denature.

The distribution of MHCs and MHC polymers in C/H and C/H$_h$ samples seems to be intermediate between that of the pure cod and herring myosin samples (Figure 6c,d). There were more MHC polymers (n = 2–5 and n > 5) observed in C/H$_h$ than in C/H samples, but only at the temperatures between 40 and 50 °C. This confirmed the turbidimetric results which suggested protein–protein interactions between cod and preheated herring myosins, leading to the formation of larger protein aggregates. It remains to be seen as to the actual proportions of cod and herring MHCs present in the protein aggregates.

In conclusion, the results of the present study suggest that aggregation behavior of herring myosin would be improved by preheating the herring myosin first and then reheating with cod myosin at the temperatures between 40 and 50 °C. It may be that not only the extent of aggregation but the gelation properties of muscle proteins from a poor setting fish species could be enhanced by blending with muscle proteins from a good setting species at a temperature at which myosin molecules of the poor setting species have been unfolded at their interior hydrophobic surface. In addition, it remains to be seen whether or not the gelation properties of herring surimi would be improved when mixed with gadoid surimis.

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