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Differences in Polymer Formation through Disulfide Bonding of Recombinant Light Meromyosin between White Croaker and Walleye Pollack and Their Possible Relation to Species Specific Differences in Thermal Unfolding

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Fast skeletal light meromyosins (LMMs) of white croaker and walleye pollack were prepared in our expression system using *Escherichia coli* and determined for their polymer-forming ability and thermodynamic properties by using sodium dodecyl sulfate polyacrylamide gel electrophoresis and differential scanning calorimetry (DSC), respectively. White croaker LMM formed dimer by heating at 80 °C and showed only a single peak at 32.1 °C of temperature transition in DSC. On the other hand, walleye pollack LMM hardly formed polymer and showed four peaks at 27.7, 30.5, 35.8, and 43.9 °C. When Cys525 of white croaker LMM was replaced by alanine, this point-mutated LMM showed no change in its DSC profile but formed no dimer upon heating, suggesting a possible role of Cys525 in dimer formation. On the other hand, walleye pollack LMM where Cys491 was substituted by alanine changed its DSC profile, showing four peaks at 27.9, 29.1, 38.4, and 43.9 °C. However, this point-mutated LMM formed no dimer upon heating as in the case of native LMM. These results suggest that cysteine residue(s) participates in thermal gel formation of LMM when it locates in a suitable position of the sequence.

KEYWORDS: Disulfide bond; DSC; polymer formation; light meromyosin; recombinant protein; walleye pollack; white croaker

INTRODUCTION

Myosin is the most abundant myofibrillar component and plays an important role in the muscle contractile process (*1*). Its molecule consists of two heavy chains of about 200 kDa each and four light chains of about 20 kDa each (*2*). The N-terminal half of each heavy chain is folded into a globular head or myosin subfragment 1 (S1), which contains the actin- and adenosine triphosphate (ATP)-binding sites. The remainder participates in the formation of a rodlike coiled coil α-helical structure with a light meromyosin (LMM) region responsible for thick filament formation and a subfragment 2 (S2) region connecting S1 and LMM. Limited proteolysis is achieved chiefly in two regions. A cleavage in the first region produces S1 and rod, whereas a cleavage in the second region results in the separation of heavy meromyosin (HMM) containing S1 and S2 from LMM (*3*). S2 can be produced by further digestion of HMM or rod (*4*).

Fibrous proteins of the coiled coil α-helices, such as myosin rod, tropomyosin, and paramyosin, consist of a series of 28 amino acid repeats, which themselves are composed of a unit of seven amino acid residues, a−g (*5*−*7*). While hydrophobic residues predominate at positions a and d, which lie at the interface between the two α-helices (*5*), their hydrophobic interactions form the basis for the coiled coil structure of two α-helices. Positions e and g, where charged residues predominate, form a salt bridge between the two α-helices and further stabilize the coiled coil structure. On the other hand, residues in the outermost positions, b, c, and f, are highly charged with repeating negative and positive patches spaced 14 residues apart. In addition to these charged groups, a 29 amino residue region near the C terminus, which is named the assembly competence domain, is considered to mediate the packing of the myosin molecules into filaments, where the interactions between neighboring molecules are largely electrostatic (*8*−*11*).

It is generally accepted that fish myosins are much more unstable than mammalian counterparts (*12*−*16*). Moreover, the stability of fish myosins also differs considerably among fish species (*17*). Thermal stability of myosin (*18*), very often examined as a component of actomyosin Mg^{2+}−ATPase (*18*, *19*) and myofibrillar Ca^{2+}−ATPase (*20*), strongly suggests that it is closely associated with environmental temperatures. It has been reported that carp, inhabiting a wide range of environmental temperatures, express three types of myosin heavy chain isoforms with different thermal stabilities in relation to environmental temperatures (*22*−*24*). Although the complete amino

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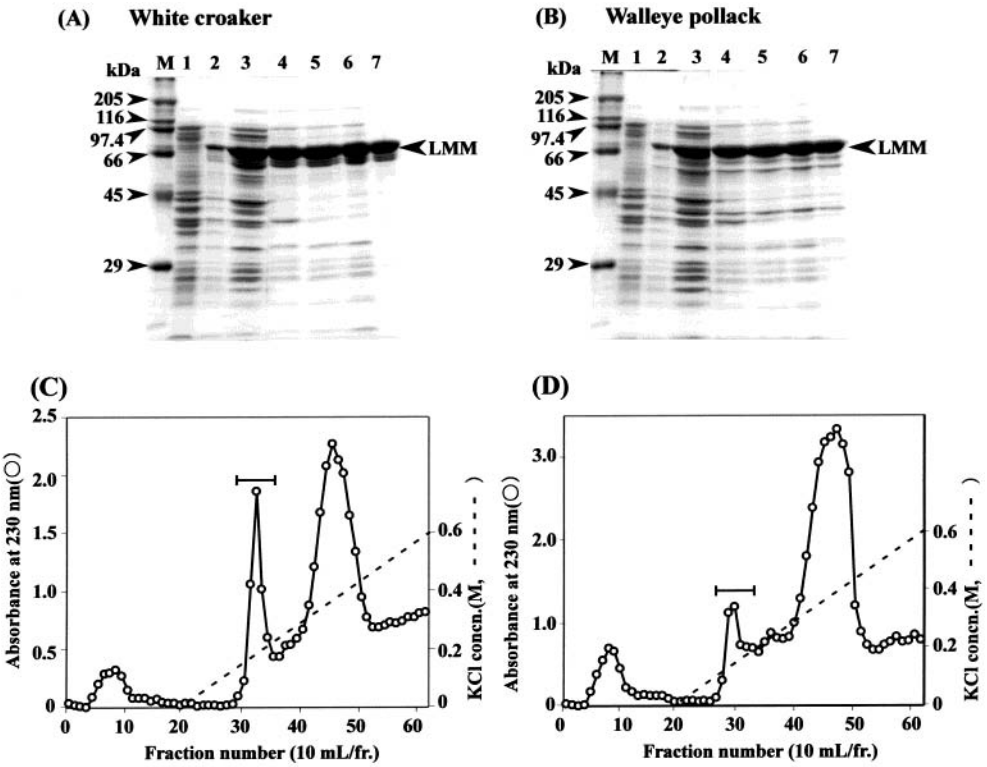


Figure 1. Purification of white croaker and walleye pollack LMMs expressed by recombinant DNA and their ion exchange chromatographic profiles. Coomassie Blue-stained 12.5% polyacrylamide gels for white croaker (**A**) and walleye pollack (**B**) recombinant LMMs at different purification steps are shown. Proteins of 10 μg/lane were applied. Lane M, molecular weight markers; lane 1, the total cell lysates before isopropyl-β-D-thiogalactopyranoside (IPTG) induction; lane 2, the total cell lysates after IPTG induction; lane 3, the supernatant of the total cell lysates; lane 4, the precipitate formed during dialysis against a low salt buffer; lane 5, LMM fraction after treatment with low and high salt buffers; lane 6, LMM fraction after sequential treatments with low, high, and low salt buffers; lane 7, the purified LMM after ion exchange chromatography. LMM fractions for white croaker (**C**) and walleye pollack (**D**) after sequential treatments with low, high, and low salt buffers were subjected to a DEAE–Toyopearl 650M ion exchange column (1.4–26 cm) equilibrated with 20 mM sodium pyrophosphate (pH 7.5) containing 30 mM KCl, 1 mM DTT, and 1 mM EDTA. The horizontal bars represent purified LMM fractions.

acid sequences for these myosin heavy chains have been determined (*22*), a possible explanation for the relationship between stability and primary structure is still on the way (*25*).

Myosin is a very important protein for the food industry, because it is the most abundant protein in the edible part of meat and extremely responsible for the heat-induced gel-forming ability of meat (*26*, *27*). Walleye pollack and white croaker meat are processed to surimi in Japan. Walleye pollack meat is much more popular as a material for surimi but does not necessarily have high quality. On the other hand, white croaker meat is superior to walleye pollack meat in terms of gel-forming ability, but the supply of the former fish is very limited. We compared previously differences in the thermal gel formation between walleye pollack and white croaker myosins by measuring viscoelastic parameters (*17*). As a result, walleye pollack and white croaker myosin showed marked differences in gel-forming ability. Moreover, their gel-forming processes upon heating were different from each other, especially at low temperatures in a range of 20−40 °C. It has been reported that fish myosin forms a thermal gel in which rod reacts at lower temperatures and S1 reacts at higher temperatures (*28*, *29*). Therefore, our previous observation suggests that the differences in myosin gel formation between walleye pollack and white croaker are possibly attributed to the species specific structural properties of the LMM region, a part of the myosin rod (*17*).

Preparation of LMM through a conventional proteolytic method is difficult, because this method also cleaves a C-terminal region, thus changing the thermodynamic properties of LMM (*30*). On the other hand, the *Escherichia coli* expression system (*22*, *23*, *25*, *30*) can easily engineer LMM and facilitate the preparation of highly pure LMM and in addition possible identification of the amino acid residue(s) or sequence(s), which may be responsible for thermal gel formation (*31*).

The objective of this study was to examine polymer-forming ability and species specificity in thermodynamic properties of the LMM region from white croaker and walleye pollack. First, white croaker and walleye pollack LMMs were heated at 80 °C and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) for examination of their polymerization. Second, these two LMM fragments were subjected to differential scanning calorimetry (DSC) analysis to determine possible relationships of their thermodynamic properties with heat-induced polymerization. Third, to identify the amino acid residue(s) that is responsible for polymer formation and changes in the secondary structure, the site-directed mutagenesis was employed, focusing on Cys525 for white croaker and Cys491 for walleye pollack.

MATERIALS AND METHODS

Materials. The expression vector plasmid pET-11a and *E. coli* strain BL21(DE3)pLysS were purchased from Novagen (Madison, WI). The expression vector pET-wpLMM, which encodes walleye pollack LMM, was previously established (*30*).

Bacterial Expression and Protein Purification. The expression vector of white croaker LMM, pET-wcLMM, was constructed as follows. The cDNA clone that encodes white croaker LMM was

amplified by polymerase chain reaction (PCR) with white croaker fast skeletal muscle cDNA library (*32*) as a template and a set of primers, P-wcF (5′-dATGGCTAGCAGATCTAAGTATGAAACTGAT-3′) and P-wcR (5′-dCACAGGATCCAGTTACTCAGCTGCGTCTTT-3′) containing *Nhe*I and *Bam*HI recognition sites, respectively. The PCR product was digested with *Nhe*I and *Bam*HI restriction endonucleases and ligated to a *Nhe*I−*Bam*HI site of pET-11a.

Site-directed mutagenesis was carried out with U.S.E. Mutagenesis Kit (Pharmacia) using expression vectors pET-wcLMM and pET-wpLMM as templates and target mutagenesis primers P-wcC/A (5′-dCTTCCTGGCCTTGGACAGGTG-3′) and P-wpC/A (5′-dCTGTGACTGGTGACCGGTCAACCAAGTCATTCT-3′), together with a selected primer P-vm (5′-AAGAAGAATGGCGCCAGACTTCAGGATCTTGTT-3′). The constructed expression vectors, pET-wcC525A and pET-wpC491A, encoded point-mutated LMMs where Cys525 of white croaker and Cys491 of walleye pollack were replaced by an alanine residue. The sequences of mutated DNA fragments in expression vectors were confirmed by DNA nucleotide sequencing.

LMMs were expressed in *E. coli* and purified as described by Kakinuma et al. (*22*, *23*). Briefly, crude LMM treated with low and high ionic strength buffers was dissolved in 20 mM sodium pyrophosphate (pH 7.5) containing 30 mM KCl, 1 mM dithiothreitol (DTT), and 1 mM ethylenediaminetetraacetic acid (EDTA). After dialysis, the solution was applied to a DEAE−Toyopearl 650 M ion exchange column (1.4−26 cm) equilibrated with the same buffer, whereas proteins adsorbed were eluted with a linear gradient of 30−600 mM KCl. Fractions containing LMMs were concentrated by ultrafiltration. Protein concentrations were determined by the biuret method (*33*) using bovine serum albumin as the standard.

SDS−PAGE. SDS−PAGE was performed according to the method of Laemmli (*34*) using 10 or 12.5% polyacrylamide gels containing 0.1% SDS. The gel was stained with a solution containing 0.05% Coomassie Brilliant Blue R-250, 50% methanol, and 10% acetic acid and destained with a solution containing 25% methanol and 7% acetic acid. The samples were heated alone to 80 °C at an increasing rate of 60 °C/h. Thereafter, the samples were treated for SDS−PAGE.

Coiled Coil Prediction. Coiled coil prediction of LMM was performed using a computer soft, PROTANUS for win (Kyoritu, Tokyo). Its algorithm, Coils, is based on Lupas (*35*). A window of 28 amino acids was used to generate the profile.

DSC Analysis. DSC was performed with a MicroCal microcalorimeter model VP-DSC (MicroCal, MA) equipped with a personal computer system. The solvent used was 50 mM Tris-HCl (pH 8.0) containing 0.6 M KCl, 5 mM $MgCl_2$, and 1 mM DTT. The concentration of protein solution was prepared in a range of 1.0−1.5 mg/mL. DSC scans were performed at an increasing rate of 60 °C/h in a range of 5−70 °C under air pressure at 2.7 Pa. DSC data were analyzed according to Nakaya et al. (*16*).

RESULTS AND DISCUSSION

Preparation of White Croaker and Walleye Pollack LMM. We confirmed the expression in *E. coli* of white croaker and walleye pollack LMMs by determination of molecular weight using SDS−PAGE and N-terminal amino acid sequencing (data not shown). The N-terminal amino acid sequence contained two additional amino acid residues, alanine and serine, derived from the expression vector. The first supposed amino acid residue, methionine, was excluded as reported previously (*36*, *37*). SDS−PAGE patterns for white croaker and walleye pollack LMM at various purification steps are shown in **Figure 1A**,**B**. The first purification step for LMM was based on its solubility in different ionic strength buffers. LMM was precipitated and dissolved alternatively in low and high ionic strength buffers, respectively (*38*), and finally purified by DEAE−Toyopearl 650 M ion exchange column chromatography (**Figure 1C**,**D**). The chromatography gave two main peaks when eluates were monitored by UV absorption at 230 nm. The first peak shown by a horizontal bar corresponded to the purified LMM, whereas the second peak was supposed to contain DNA (*30*). Although several minor components are seen in lane 7 of **Figure 1A**,**B**, the purity of LMM was at an extremely high level. Such purity has been demonstrated to be enough for DSC analysis (*22*, *23*, *30*).

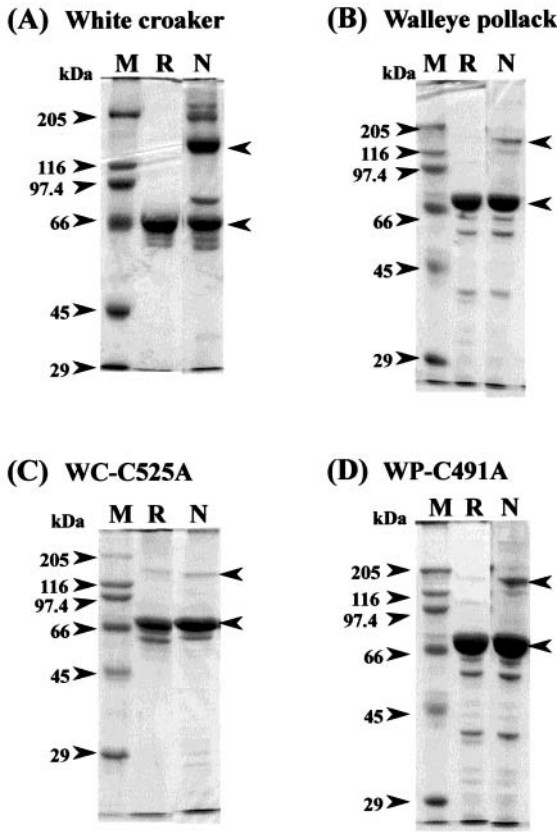
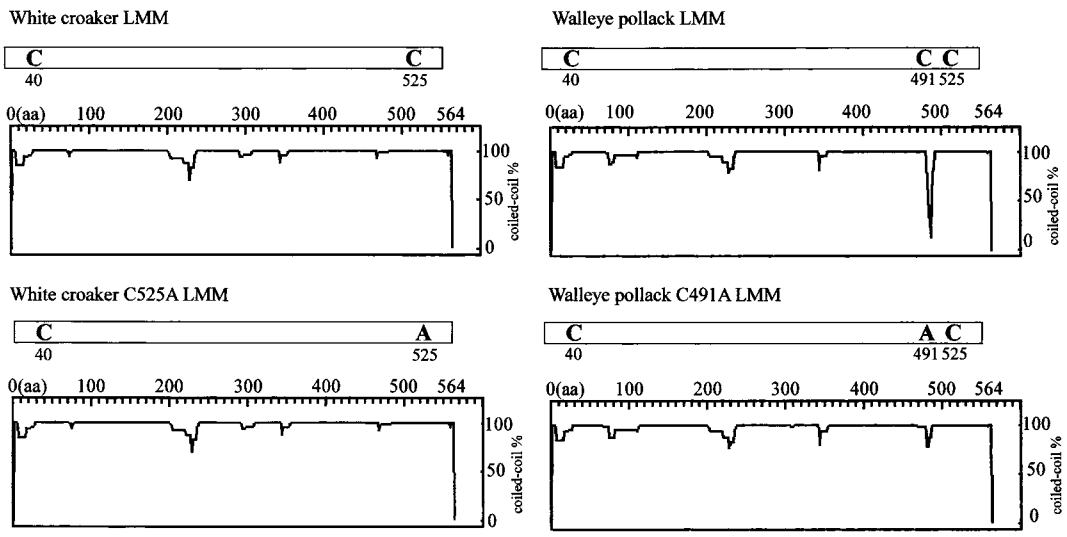


Figure 2. SDS−PAGE patterns for white croaker LMM (**A**), walleye pollack LMM (**B**), and point-mutated LMMs of white croaker (**C**) and walleye pollack (**D**). WC-C525A LMM represent white croaker point-mutated LMM where Cys525 was replaced by alanine. WP-C491A LMM represent walleye pollack point-mutated LMM where Cys491 was replaced by alanine. Polyacrylamide gels (10 and 12.5%) were used for **A** and **B** and for **C** and **D**, respectively. Proteins of 10 μg/lane were applied. Two arrowheads indicate bands with the molecular weight of dimer and monomer LMM. Abbreviation: M, molecular weight markers; R, LMMs under reducing conditions in the presence of 2-mercaptoethanol; N, LMMs heated at 80 °C under nonreducing conditions without 2-mercaptoethanol.

Polymerization Analyses by SDS−PAGE. SDS−PAGE patterns for white croaker and walleye pollack LMMs under reducing and nonreducing conditions are shown in **Figure 2**. Upon heating under nonreducing conditions, white croaker LMM formed dimer and additionally small quantities of components of higher polymerization (**Figure 2A**). On the other hand, walleye pollack LMM hardly formed polymer (**Figure 2B**). Because white croaker LMM only slightly formed polymer under reducing conditions but markedly formed polymer under nonreducing conditions, it was thought that disulfide bonding through cysteine residues would be important in polymer formation.

The coiled coil prediction algorithm Coils (*35*) was employed for white croaker (*32*) and walleye pollack LMMs (*39*) as shown in **Figure 3** together with their comparison in amino acid sequences deduced from cDNA clones. White croaker and walleye pollack LMMs include 564 amino acids and show 51 amino acid substitutions, most of which are irregularly located in the C-terminal half (*32*, *39*). Cysteine residues, which may form disulfide bonds, exist at the 40th and 525th positions in white croaker LMM, whereas walleye pollack LMM contains

(A)



(B)

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          defgabc defgabc defgabc defgabc  defgabc defgabc defgabc defgabc
          *   *   *   *   *   *   *   *    *   *   *   *   *   *   *   *
croaker                    RSKYET DAIQRTEE LEEAKKK LAQRLQD AEEQIEA VNSKCAS   42
pollack                    ...... ........ ...S... ......E ....... .......

croaker   LEKTKQR LQSEVED LMIDVER ANGLAAN  LDKKQRN FDKVLAE WKQKYEE GQAELEG   98
pollack   ....... ..G.... ..V.... .......  ....... ......D ....... .......

croaker   AQKEARS LGTELFK MKNSYEE SLDQLET  MKRENKN LQQEISD LTEQIGE TGKSIHE  154
pollack   SL..... .S..... .....K. T..H...  ....... ....... ....... .......

croaker   LEKAKKQ VETEKSE IQTALEE AEGTLEH  EESKILR VQLELNQ VKGEVDR KLAEKDEE 211
pollack   ...S... .....T. ..S.... .......  ....... ....... I...... ........

croaker   MEQIKRN SQRVIDS MQSTLDS EVRSRND  ALRIKKK MEGDLNE MEIQLSH ANRQAAE  267
pollack   ....... ....... ..A.... .......  ....... ....... ....... .......

croaker   SQKQLRN VQAQLKD AQLHLDD AVRAQDD  FKEQAAM VERRNGL MMAEIEE LRVALEQ  323
pollack   A...... ..G.... ....... ....AE.  L...... ....... .V..... ..A....

croaker   TERSRKV AEQELVD ASERVGL LHSQNTS  LMNTKKK LEADLVQ IQGEVDD TVQEARN  379
pollack   ...G... ....... ....... .......  .L.S... ..S.... V...... S......

croaker   AEEKAKK AITDAAM MAEELKK EQDTSSH  LERMKKN LEVAVKD LQHRLDE AENLAMKG 436
pollack   ....... ....... ....... .......  ....... ...T... ....... ........

croaker   GKKQLQK LESRVRE LEAEVEA EQRRGGD  AVKGVRK YERRVKE LTYQTEE DKKNVAR  492
pollack   ....... ..A...D ..S..DN .....AE  .I..... ....... ....... ....GC.

croaker   LQDLVDK LQLKVKG YKRQAEE AEEQANV  HLSRCRK IQHELEE AEERADI AETQVNK  548
pollack   ....... ......A ....S.. ......S  Y..K... ......A ....... .......

croaker   LRVKSRDIGKGKDAAE                                               564
pollack   ..A.T..S....ET..
```

Figure 3. Coiled coil prediction for white croaker LMM and its point-mutated LMM of WC-C525A as well as walleye pollack LMM and its point-mutated LMM of WP-C491A (**A**) and comparison in the amino acid sequence between white croaker and walleye pollack LMMs (**B**). (**A**) The prediction was performed by the Coils program (*35*), where the *x*-axis denotes amino acid numbers from the N terminus and *y*-axis, the probability of forming a coiled coil. A window of 28 amino acids was used to generate the profiles shown. (**B**) Amino acid sequences of LMMs were cited from Yoon et al. (*32*) and Togashi et al. (*39*). The top line is a seven residue repeat (a–g), and asterisks indicate positions a and d, which are in the interface of coiled coil. Dots on walleye pollack LMM denote amino acids identical to those of white croaker LMM. Boxes in the amino acid sequences indicate cysteine residues to be substituted for point-mutated LMMs.

three cysteine residues at the 40th, 491st, and 525th positions. White croaker LMM had high probabilities of over 71% to form a coiled coil throughout the molecule. On the other hand, walleye pollack LMM showed considerably low probability of 13% between the 482nd and the 487th residues, while the other residues showed high probability of over 77%. The 482nd to 487th residues of white croaker LMM showed high probabilities of over 99%. Interestingly, this low probability was recovered by the replacement of 491st cysteine residue to alanine, while the 491st cysteine residue showed a 100% probability. Therefore, this cysteine residue was considered to be important to maintain the secondary structure and thermostability. However, the replacement of Cys525 to alanine residue in white croaker LMM resulted in no change in the coiled coil structure. We prepared these point-mutated LMMs and used them for further analyses as described below.

White croaker C525A LMM where Cys525 was replaced by alanine and walleye pollack C491A LMM where Cys491 was replaced by alanine were prepared with the same protocol as that for preparation of white croaker and walleye pollack LMMs. SDS−PAGE patterns for white croaker C525A and walleye pollack C491A LMM under reducing and nonreducing conditions are shown in panels **C** and **D** of **Figure 2**. In contrast to native white croaker LMM forming dimers and higher polymers

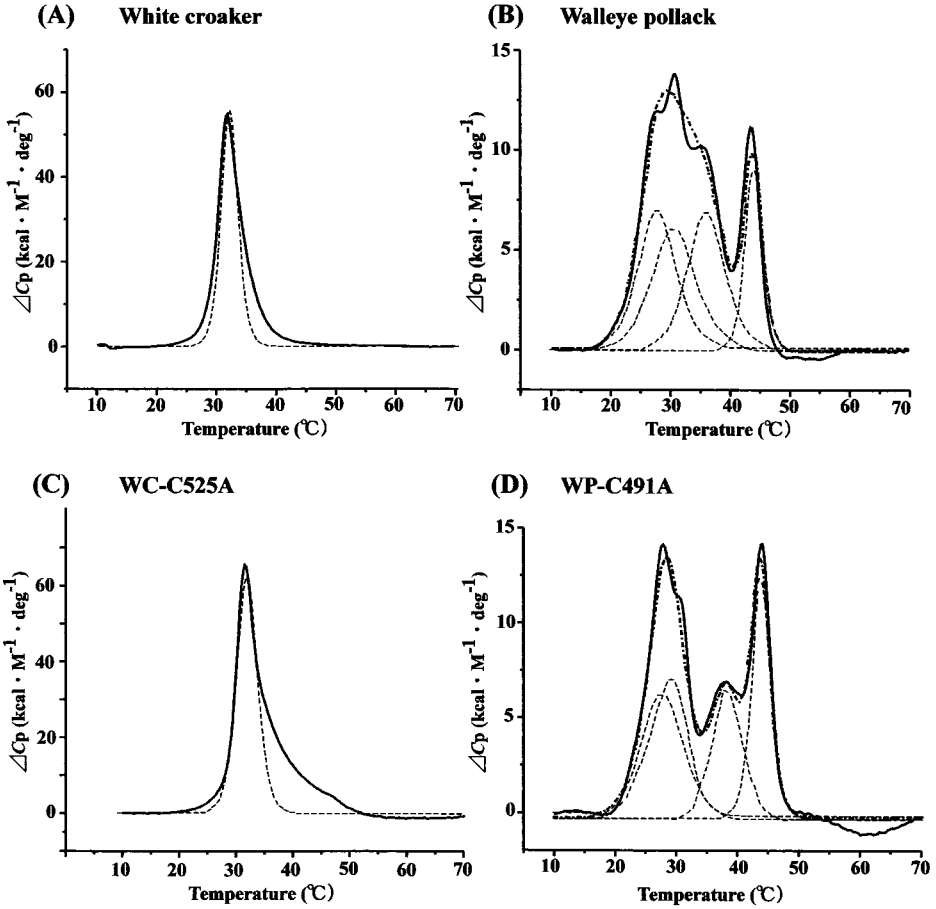

Figure 4. DSC scans of white croaker LMM (**A**), walleye pollack LMM (**B**), white croaker C525A LMM (**C**), and walleye pollack C491A LMM (**D**). WC-C525A LMM represents white croaker point-mutated LMM where Cys525 was replaced by alanine. WP-C491A LMM represents walleye pollack point-mutated LMM where Cys491 was replaced by alanine. The observed DSC patterns (solid lines) were subjected to smoothing treatment (bold-faced dashed lines), together with their computer-calculated differential endotherms by the convolution analysis (dotted lines).

by heating, its point-mutated counterpart, WC-C525A, was only slightly polymerized (**Figure 2C**). These results suggest that Cys525 plays a key role in polymerization of white croaker LMM based on disulfide bonding. On the other hand, walleye pollack point-mutated LMM, WP-C491A, hardly formed polymer as in the case of the native LMM. Therefore, it was thought that not only the existence of Cys525 but also the certain conformational changes of LMM upon heating would play an important role in polymer formation through disulfide bonding. Accordingly, we tempted to investigate the difference between white croaker and walleye pollack in thermodynamic properties of LMM, which are described below.

DSC Analysis. The thermodynamic properties of white croaker, walleye pollack, and their point-mutated LMMs were analyzed by using DSC. DSC runs for the LMMs are shown in **Figure 4** and **Table 1**. DSC scan patterns were different between white croaker and walleye pollack LMMs, where walleye pollack LMM showed essentially the same pattern as that in our previous paper (*30*). White croaker LMM exhibited a single endothermic peak at 32.1 °C, whereas walleye pollack LMM showed four peaks at 27.7, 30.5, 35.8, and 43.9 °C. Therefore, it is apparent that white croaker LMM unfolds in one step upon heating and walleye pollack LMM unfolds in several steps. These results raised the possibility that thermodynamic properties affect their polymer formation upon heating besides the presence of Cys525.

It is noted that white croaker WC-C525A LMM showed no change in thermodynamic properties in comparison to the native LMM before point mutation. This result agreed with the coiled coil prediction by algorithm Coils that such substitution does not change the tertiary structure (see **Figure 3A**). On the other hand, walleye pollack WP-C491A LMM markedly shifted the middle endothermic peak from 35.8 to 38.4 °C, with no change for other peaks. Therefore, it is suggested that walleye pollack LMM has four cooperative structural units and one containing Cys491 is associated with the middle endothermic peak. It has been reported that the endothermic peak at the lowest temperature peak reflects thermodynamic properties of the C-terminal

Table 1. Thermodynamic Parameters on Thermal Unfolding of LMMs from White Croaker LMM and Its Point-Mutated LMM of WC-C525A as Well as Walleye Pollack LMM and Its Point-Mutated LMM of WP-C491A

LMM[a]	T_m (°C)[b]	ΔH_{cal} (kcal/mol)
white croaker	32.1	217
walleye pollack	27.7	58.4 (1.5)[c]
	30.5	58.4 (1.3)
	35.8	61.4 (1.4)
	43.9	35.6 (5.7)
WC-C525A	32.1	203
WP-C491A	27.9	56.5 (1.4)
	29.1	51.2 (2.1)
	38.4	44.6 (2.6)
	43.9	49.2 (4.2)

[a] Values obtained from **Figure 3**. [b] T_m represents transition temperature. [c] The values in parentheses are a ratio of ΔH_{vh} to ΔH_{cal}. ΔH_{vh} and ΔH_{cal} are calorimetric and van't Hoff enthalpies, respectively.

part of LMM (*30*). The differences in polymer-forming ability upon heating between white croaker and walleye pollack LMMs were possibly caused by the differences in their thermodynamic properties, where the latter LMM was composed of four cooperative units for thermal unfolding. The circular dichroism spectra for four LMM preparations from white croaker and walleye pollack (data not shown) reflected their DSC patterns, suggesting a close relationship of endothermic peaks in DSC with unfolding of α-helical structure as reported before for carp LMM isoforms (*22*−*24*) and walleye pollack LMM (*30*). It seems that the rapid conformational change upon heating leading thermal unfolding of white croaker LMM in one step is necessary to form polymers of LMM in addition to Cys525. Alternatively, the interaction of Cys525 with another undefined region in LMM would be a prerequisite to polymer formation.

In this study, we investigated differences in polymer-forming ability and thermodynamic properties among white croaker and walleye pollack LMMs and their point-mutated LMMs. As a result, it was found that white croaker LMM formed polymers through disulfide bonding at Cys525. On the other hand, walleye pollack hardly formed polymers despite the presence of Cys525. These differences were caused by the species specific thermodynamic properties, which may be related to the presence of cooperative units showing four endothermic peaks in DSC for walleye pollack LMM, but not in white croaker LMM. We conclude that such differences in polymer-forming ability in association with altered thermodynamic properties result in different thermal gel-forming abilities between white croaker and walleye pollack myosins. Further investigations are required to address the question whether the disulfide bonding is formed at the intermolecular or intramolecular level.

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