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Differential Scanning Calorimetry and Circular Dichroism Spectrometry of Walleye Pollack Myosin and Light Meromyosin

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The thermodynamic properties of myosin and its C-terminal fragment, light meromyosin (LMM), from walleye pollack, a typical cold-water fish efficiently utilized on an industrial scale, were analyzed by using differential scanning calorimetry (DSC) and circular dichroism (CD) spectrometry. Recombinant walleye pollack LMM expressed in *Escherichia coli* was also subjected to DSC and CD measurements for reference. The two proteins prepared from frozen surimi showed three endothermic peaks, the transition temperatures (T_m) of which were quite similar, although overall DSC patterns differed considerably from one another. Their α-helical contents determined by CD were low compared to values reported before for other species. On the other hand, recombinant LMM gave four endothermic peaks at 27.4, 30.8, 36.5, and 43.4 °C in DSC and showed an α-helical content of ∼80%. The peak at 27.4 °C could not be observed in walleye pollack LMM prepared from frozen surimi and thus was possibly attributed to its C terminus, because this extreme C-terminal region is supposedly truncated during preparation of LMM by tryptic digestion.

KEYWORDS: CD; DSC; light meromyosin; myosin; recombinant protein; thermodynamic properties; walleye pollack

INTRODUCTION

Myosin is of great importance in the contractile process in living muscle and constitutes >50% of myofibrillar proteins in vertebrate skeletal muscles. Its molecule consists of two heavy chains, each of ∼200 kDa, and four light chains, each of ∼20 kDa (*1*). The N-terminal half of each heavy chain is folded into a globular head or myosin subfragment 1 (S1), which contains the actin- and ATP-binding sites. The remainder participates in 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 occurs 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) and LMM (*2*). S2 can be produced by further digestion of HMM or rod (*3*).

Although fish myosins have structures similar to those of mammalian myosins, previous studies showed that the former myosins are very unstable in comparison with the latter (*4*). Moreover, species-specific differences in the thermal stability of actomyosin Mg^{2+}-ATPase (*5*, *6*) and myofibrillar Ca^{2+}-ATPase (*7*) strongly suggest that the stability of fish myosin is closely associated with the temperature under which the fish lives; that is, the colder the environmental temperature, the more labile myosin is. Therefore, the stability of fish myosin would also differ considerably among fish species.

In addition, myosin is the protein most responsible for the heat-induced gel-forming ability of meat (*8*, *9*). The formation of gels is correlated with the process of thermal unfolding in different portions of proteins, that is, the thermal denaturation process. Therefore, factors influencing protein stability or interactions may also affect gel properties and have considerable implications for fish processing and storage. Walleye pollack (*Theragra chalcogramma*) lives at very low environmental temperatures generally in the range of 2−5 °C (*10*). It is very important for the food industry to understand species-specific properties of walleye pollack myosin, because this fish is one of the major species used for the production of frozen "surimi", a raw material for "kamaboko". As such, walleye pollack seems to be a good target to evaluate how the environmental temperature influences the thermodynamic properties of fish myosin.

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For various studies of myosin fragments, proteolysis has been used as a tool to produce these fragments. However, purification of thermally unstable walleye pollack myosin fragments through proteolysis is difficult and limited by those proteolytic sites that occur naturally within the molecule. It also probably contains the products of several genes expressed in the same tissues (*11*), making it impossible to obtain truly homogeneous preparations of proteolytic tail fragments. Expression in *Escherichia coli* of different myosin heavy chain gene fragments has no such limitation because DNA fragments encoding desired protein segments can be easily engineered (*12*).

The objective of this study was to analyze differential scanning calorimetry (DSC) and circular dichroism (CD) spectrometry of walleye pollack myosin and LMM prepared from frozen surimi and, further, to compare these results with those obtained for full-length walleye pollack recombinant LMM (rLMM) expressed in *E. coli*.

MATERIALS AND METHODS

Materials. Frozen walleye pollack surimi (FA grade), containing 4% sorbitol and 0.2% polyphosphates, were kindly supplied by Nippon Suisan Kaisha Co., Ltd. (Tokyo, Japan), cut into 100 g blocks while frozen, packaged in polyethylene bags, and stored at −80 °C until use. The pAP-LMM plasmid previously established and containing a DNA insert that encodes walleye pollack LMM (*13*) was used for PCR amplification of LMM DNA to construct an expression vector. The expression vector plasmid pET-11a and *E. coli* strain BL21(DE3)pLysS were purchased from Novagen (Madison, WI). All other reagents were of analytical grade.

Preparation of Myosin and LMM. Myosin was prepared from thawed surimi according to the method of Hwang et al. (*14*).

A study on the time course of the digestion of myosin at concentrations of 10−15 mg/mL in 30 mM Tris-HCl (pH 8.2) containing 0.5 M KCl, 10 mM 2-mercaptoethanol, and 1 mM $CaCl_2$ with *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (type XIII, Sigma, St. Louis, MO) was carried out to determine suitable experimental conditions in which myosin would be digested to yield LMM. The digestion of myosin was started by the addition of trypsin in 0.01 N HCl at an enzyme-to-substrate weight ratio of 1:500 (w/w) at 10 °C. At certain time intervals, aliquots were taken from the reaction mixture and digestion was stopped by the addition of soybean trypsin inhibitor (type I-S, Sigma) at an enzyme-to-inhibitor weight ratio of 1:5. The fragmentation pattern of myosin was then analyzed by SDS-PAGE as described below.

LMM was purified essentially according to the method of Nakaya et al. (*15*). Briefly, the digests were centrifuged at 40000*g* for 1 h after dialysis against 10 mM sodium phosphate (pH 6.5) at 4 °C. The pellet containing LMM was dissolved in and dialyzed against 20 mM sodium pyrophosphate (pH 7.5) and further purified by ion-exchange chromatography on a DEAE-Toyopearl 650M column (1.4 × 26 cm) equilibrated with the same buffer. Proteins adsorbed were eluted with a linear gradient from 20 to 50 mM sodium pyrophosphate (pH 7.5). Fractions containing the purified LMM were concentrated by ultrafiltration with an Amicon PM-30 (Millipore, Bedford, MA) and a UP-20 ultrafilter membrane (Advantec, Tokyo, Japan) and dialyzed against 50 mM Tris-HCl (pH 8.0) containing 0.6 M KCl, 5 mM $MgCl_2$, and 1 mM DTT to be used for DSC and CD analyses.

Protein concentrations were determined according to the biuret method of Gornall et al. (*16*) using bovine serum albumin (BSA) as the standard.

Bacterial Expression and rLMM Purification. Expression vector pET-wpLMM containing a DNA fragment encoding walleye pollack LMM was constructed as follows. A DNA fragment for an insert encoding LMM was amplified by PCR using the pAP-LMM plasmid (*13*) as a template and a set of primers, LMM-6F (5′-dAGGAATC-CGCTAGCAGGAGCAAGTATGAAACTGATGCTATCC-3′) and LMM-7R (5′-dTATGGATCCT TATTCAGCAGTCTCCTTTCCTTTCC-3′), containing *Nhe*I and *Bam*HI recognition sites, respectively. PCR products were digested with *Nhe*I and *Bam*HI restriction endonucleases and ligated to an *Nhe*I−*Bam*HI site of pET-11a. The relevant plasmid pET-11a contains *Nhe*I and *Bam*HI sites in a multiple cloning site. After the sequence of the inserted DNA fragment was confirmed by DNA sequencing, expression vector pET-wpLMM was transfected into *E. coli* strain BL21(DE3)pLysS, in which the expression was controlled by a *lac*I repressor.

Walleye pollack rLMM was expressed in *E. coli* and purified essentially as described by Kakinuma et al. (*17*, *18*). Crude rLMM preparation obtained from cell lysate by treatments with low and high ionic strength buffers was further purified according to the same method as described for LMM from frozen surimi using DEAE ion-exchange chromatography except that a linear gradient from 20 to 50 mM sodium pyrophosphate (pH 7.5) was replaced by that from 0.03 to 0.6 M KCl in 20 mM sodium pyrophophate (pH 7.5).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Eletrophoresis (SDS-PAGE). SDS-PAGE was performed according to the method of Laemmli (*19*) using 7.5−20% polyacrylamide gradient gels containing 0.1% SDS. The gel was stained with a solution containing 0.05% Coomassie Brilliant Blue (CBB) R-250, 50% methanol, and 10% acetic acid and destained with a solution containing 25% methanol and 7% acetic acid.

N-Terminal Amino Acid Sequencing. The N-terminal amino acid sequence was determined by using the method of Matsudaira (*20*). Proteins separated on SDS-PAGE were electrically transferred onto an Immobilon PVDF membrane (Millipore) and stained with CBB R-250. The parts of the membrane carrying the blotted proteins were cut out with a clean razor and placed together onto a Blott Cartridge of an Applied Biosystems model 476A protein sequencer with an on-line system model 610A (Foster City, CA).

DSC and CD Spectrometry. DSC was performed with a differential microcalorimeter MC2 (MicroCal, Amherst, MA) equipped with a personal computer system, essentially according to the method of Nakaya et al. (*15*, *21*). The solvent used was 50 mM Tris-HCl (pH 8.0) containing 0.6 M KCl, 5 mM $MgCl_2$, and 1 mM DTT. Protein concentrations were in the range of 1.2−5.4 mg/mL. DSC scans were performed at a rate of 45 °C/h in the temperature range of 10−70 °C. DSC data were analyzed using a software package, Origin, developed by MicroCal. An approximate subunit molecular weight of LMM used for data analysis, 64000, was calculated from the amino acid sequence (*13*). The heat capacity data were fit by using nonlinear least-squares, initially assuming that $\Delta H_{cal}/\Delta H_{vh} = 1$, where ΔH_{cal} and ΔH_{vh} are calorimetric and van't Hoff enthalpy, respectively. When data were not fit satisfactorily, heat capacity curves were subsequently fit by allowing ΔH_{cal} and ΔH_{vh} to float. After minimization by appropriate computer programs, the values for the thermal transition, including the transition temperature (T_m), ΔH_{cal}, and ΔH_{vh}, and the molar excess heat capacity (ΔC_p) were obtained.

CD spectra were measured at various temperatures from 20 to 60 °C in the same buffer as in DSC measurement with a JASCO J-720W spectropolarimeter (Tokyo, Japan). While constant N_2 flux was employed, a jacketed cell of 0.2 cm optical path length was used, and the temperature was controlled by circulating thermoregulated water. Wavelength and protein concentrations for measurement were in the ranges of 240−195 nm and 1.2−5.4 mg/mL, respectively.

α-Helical content was estimated by means of the equation described below on the basis of the datum (*22*) that the mean residue ellipticity, $[\theta]_{222}$, of poly(L-glutamic acid) is −36000 deg cm^2/dmol when the substance is completely helical in structure:

$$\alpha\text{-helical content} = 100\% \times \{[\theta]_{222}/-36000\}$$

Fractional helical content (f_H) within the temperature range of 20−60 °C was calculated as

$$f_H = (\theta_T - \theta_{60})/(\theta_{20} - \theta_{60}) \times 100\%$$

where θ_{20}, θ_{60}, and θ_T are the experimental values of mean residue ellipticity (222 nm) at 20 and 60 °C and temperature *T*, respectively (*23*).

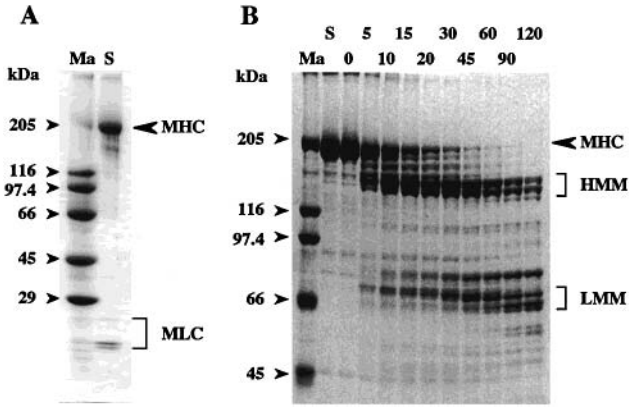

Figure 1. Ten micrograms of myosin was applied to 7.5–20% polyacrylamide slab gels containing 0.1% SDS. Myosin purified from frozen surimi (A) was digested with trypsin at an enzyme-to-protein weight ratio of 1:500 at 10 °C in 30 mM Tris-HCl (pH 8.2) containing 0.5 M KCl, 10 mM 2-mercaptoethanol, and 1 mM $CaCl_2$ (B). Numerals above the photograph in (B) indicate digestion time (min). Abbreviations: Ma, molecular weight markers; S, myosin prior to tryptic digestion; MHC, myosin heavy chain; MLC, myosin light chain; HMM, heavy meromyosin; LMM, light meromyosin.

All of the experiments described under Materials and Methods were repeated at least twice until good reproducibility was obtained.

RESULTS

Preparation of Myosin and LMM. Freshly killed walleye pollack muscle tissues were difficult to obtain for protein experiments, so myosin was extracted from frozen walleye pollack surimi. This surimi was prepared from fresh walleye pollack, which was harvested in the northern seas, processed on board fishing vessels with addition of cryoprotectants, and stored frozen at − 20 °C. Lane S in **Figure 1A** shows the SDS-PAGE pattern of myosin purified from frozen surimi. Although several minor components are seen in the photograph, the purity of myosin was >95%. Such purity has been demonstrated to be enough to be analyzed for DSC and CD measurements (*15*, *18*, *21*).

To determine the optimal conditions giving the highest yield of LMM, the time course of tryptic digestion was traced for myosin from frozen surimi of slightly less purity but with higher yield. Such impurities were easily removed during the purification process of LMM. **Figure 1B** shows SDS-PAGE patterns of the tryptic digests of walleye pollack myosin at 10 °C. Bands around 66 kDa were subjected to N-terminal amino acid sequence analysis and confirmed to be of LMM (data not shown). Apparently the digestion pattern was simple with parallel increases of HMM (bands around 140 kDa) and LMM (bands around 66 kDa) accompanying the disappearance of myosin heavy chain (205 kDa). It took 90 min for myosin heavy chain to be completely digested, giving distinct bands corresponding to HMM and LMM. Thus, the conditions of tryptic digestion for LMM preparation were set as 10 °C for 90 min.

By dialyzing the digests obtained against low ionic strength buffer followed by centrifugation, HMM remained in the supernatant and LMM precipitated (*24*). The LMM fraction was further purified using ion-exchange chromatography. **Figure 2A** shows the elution profile of walleye pollack tryptic digests from the DEAE-Toyopearl 650M column, which gave two main peaks, numbers 1 and 2, in the increasing order of elution volume, as monitored by UV absorption at 230 nm. Selected fractions indicated by numerals in **Figure 2A** were subjected

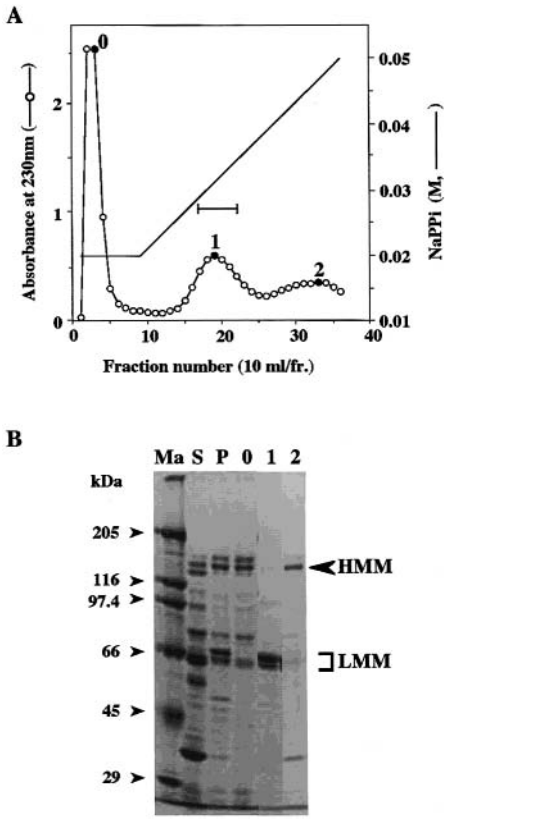

Figure 2. Elution profile in DEAE-Toyopearl ion-exchange chromatography (A) and SDS-PAGE patterns of selected fractions (B) for walleye pollack myosin tryptic digests. Myosin in 0.5 M KCl was digested at 10 °C for 90 min with trypsin at an enzyme-to-myosin weight ratio of 1:500. Numerals above photographs are fractions from a DEAE-Toyopearl 650M column (1.4 × 26 cm). Proteins were eluted with a linear gradient up to 50 mM sodium pyrophosphate (pH 7.5). Bars represent LMM fractions. Abbreviations: Ma, molecular weight markers; S and P, supernatant and precipitate, respectively, obtained when tryptic digests were dialyzed against a low ionic strength buffer; HMM, heavy meromyosin; LMM, light meromyosin; NaPPi, sodium pyrophosphate.

to SDS-PAGE (**Figure 2B**). Fraction 1 contained predominantly LMM and fraction 2 predominantly HMM. Fractions that were concentrated through ultrafiltration are shown by a horizontal bar in **Figure 2A**.

The LMM fractions thus purified and concentrated were dialyzed against 50 mM Tris-HCl (pH 8.0) containing 0.6 M KCl, 5 mM $MgCl_2$, and 1 mM DTT. **Figure 3A** shows the SDS-PAGE pattern of purified LMM with two bands of 66 and 63 kDa. As both bands presented the same N-terminal amino acid sequence as described below, the difference in molecular weights was attributed to the degree of digestion of the C-terminal region, assuming the sequence is unique.

Figure 3B shows the N-terminal amino acid sequences of walleye pollack LMM compared to that of rabbit myosin heavy chain (*25*) and to those of fast muscle myosin heavy chain isoforms predominantly expressed in carp *Cyprinus carpio* acclimated to 10 and 30 °C, respectively (*26*, *27*). Walleye pollack LMM was obtained by digestion with trypsin and other LMMs by digestion with chymotrypsin. Trypsin cleaves peptide chains by acting on the carboxyl sides of lysine and arginine, whereas chymotrypsin cleaves by acting on the carboxyl side of aromatic residues such as tyrosine, tryptophan, and phenylalanine and large hydrophobic residues such as leucine and isoleucine (*28*). Both enzymes seem to attack the same hinge region between S2 and LMM. This hinge region is flexible and

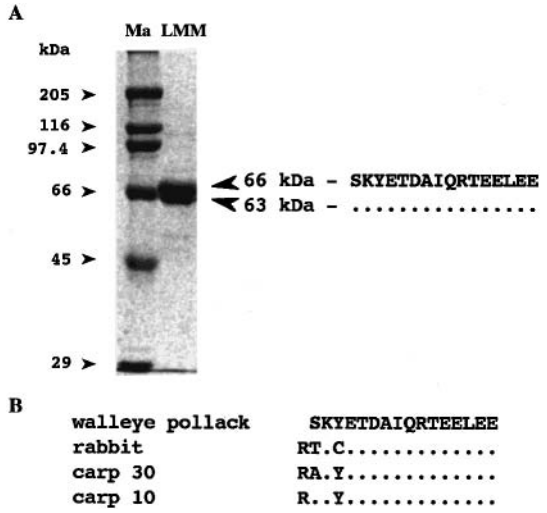

Figure 3. SDS-PAGE pattern of purified walleye pollack LMM and N-terminal amino acid sequences (A) in comparison with other LMM sequences (B): (A) Ma, molecular weight markers; (B) amino acids identical to those of walleye pollack are represented by dots. The sequences were cited for rabbit fast skeletal myosin heavy chain from Maeda et al. (*25*) and for those of carp predominantly expressed at 10 °C (carp 10) and 30 °C (carp 30) from Imai et al. (*26*).

known to be vulnerable to cleavage by proteolytic enzymes (*29*). Thus, as shown in **Figure 3B**, digestion of walleye pollack myosin with trypsin yielded LMMs starting with serine.

Preparation of rLMM. A 1692 bp DNA fragment encoding LMM of 546 amino acids was ligated into the expression vector pET-11a. The construct, pET-wpLMM, was then introduced into *E. coli* strain BL21(DE3)pLysS. We examined expression of the desired protein, rLMM, through preliminary expression and determined its N-terminal amino acid sequence. The addition of isopropyl β-D-thiogalactopyranoside (IPTG) into bacterial cultures induced the expression of an extra protein having ~66 kDa, which comprised ~30% of the total proteins, but did not affect the growth rate of the bacteria (data not shown). N-Terminal amino acid sequence analysis of this band confirmed that it was certainly LMM as described later. The expressed protein was completely soluble upon cell lysis, indicating that neither extensive aggregation of the expressed proteins nor partition into bacterial inclusion bodies had occurred.

The purification protocol for rLMM was based on the properties of its solubility in different ionic strength buffers. rLMM was precipitated and dissolved alternatively in low and high ionic strength buffers, respectively, and finally purified through DEAE-Toyopearl 650M ion-exchange column chromatography as described before for LMM. As shown in **Figure 4A**, the chromatography gave two main peaks, numbers 1 and 2, in the increasing order of elution volume, when the eluates were monitored by UV absorption at 230 nm. Selected fractions indicated by numerals in **Figure 4A** were subjected to SDS-PAGE (**Figure 4B**). Fraction 0 corresponds to the pass-through fraction and fraction 1 the purified rLMM free from minor bands. Although fraction 2 presented a high peak in absorption at 230 nm, proteins could not be detected in SDS-PAGE, suggesting that it probably belonged to DNA. The fractions containing rLMM shown by a horizontal bar (**Figure 4A**) were concentrated through ultrafiltration. The cDNA expression plasmid, pET-wpLMM, codes for a protein of 564 amino acids with a predicted molecular weight of 64826, which was in good agreement with its molecular mass of 66 kDa estimated from SDS-PAGE in the present study. The N-terminal amino acid

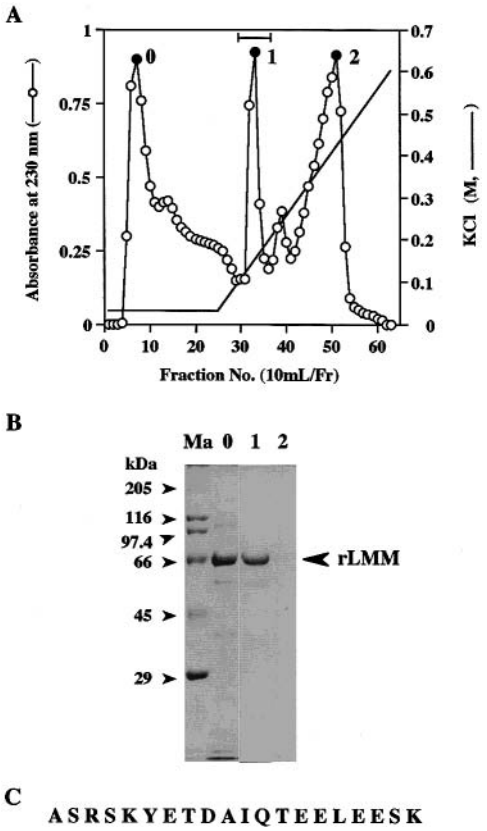

Figure 4. Elution profile in DEAE-Toyopearl ion-exchange chromatography (A) and SDS-PAGE patterns of selected fractions (B) for walleye pollack LMM expressed in *E. coli* (rLMM) and N-terminal amino acid sequence of purified rLMM (C). Numerals above photographs represent fractions from a DEAE-Toyopearl 650M column (1.4 × 26 cm). Proteins were eluted with a linear gradient up to 0.6 M KCl. Bars represent LMM fractions. Abbreviations: Ma, molecular weight markers.

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 (*30*, *31*).

DSC Analysis. Results of DSC at pH 8.0 in 0.6 M KCl for walleye pollack myosin, LMM, and rLMM are shown in **Figure 5**, whereas thermodynamic parameters derived from deconvolution of DSC data are shown in **Table 1**.

Two prominent endotherms were found for myosin and an asymmetrical shape of the first peak implied overlapping of two peaks (**Figure 5A**). Thus, the deconvolution analysis gave three endotherms, where the highest endothermic peak was observed at 33.7 °C, followed by those at 41.3 and at 28.2 °C. The thermal denaturation of walleye pollack myosin was partially reversible (data not shown). The rod portion of the myosin molecule may be responsible for this reversibility (*21*). Rabbit skeletal myosin undergoes thermal denaturation in at least three steps over a temperature change of 20 °C, probably due to the unfolding of different domains in the molecule (*32*, *33*).

Table 2 shows the T_m values obtained in this study for walleye pollack myosin together with those from carp acclimated to 10 and 30 °C (*21*), chicken (*34*), and rabbit (*35*). The lowest T_m of walleye pollack myosin was lower than those of any other myosins, suggesting a low thermal stability of the former myosin.

Three prominent endotherms were again found for walleye pollack LMM as in the case of its myosin counterpart. However, the T_m values for endotherm peaks of LMM were 29.9, 36.7,

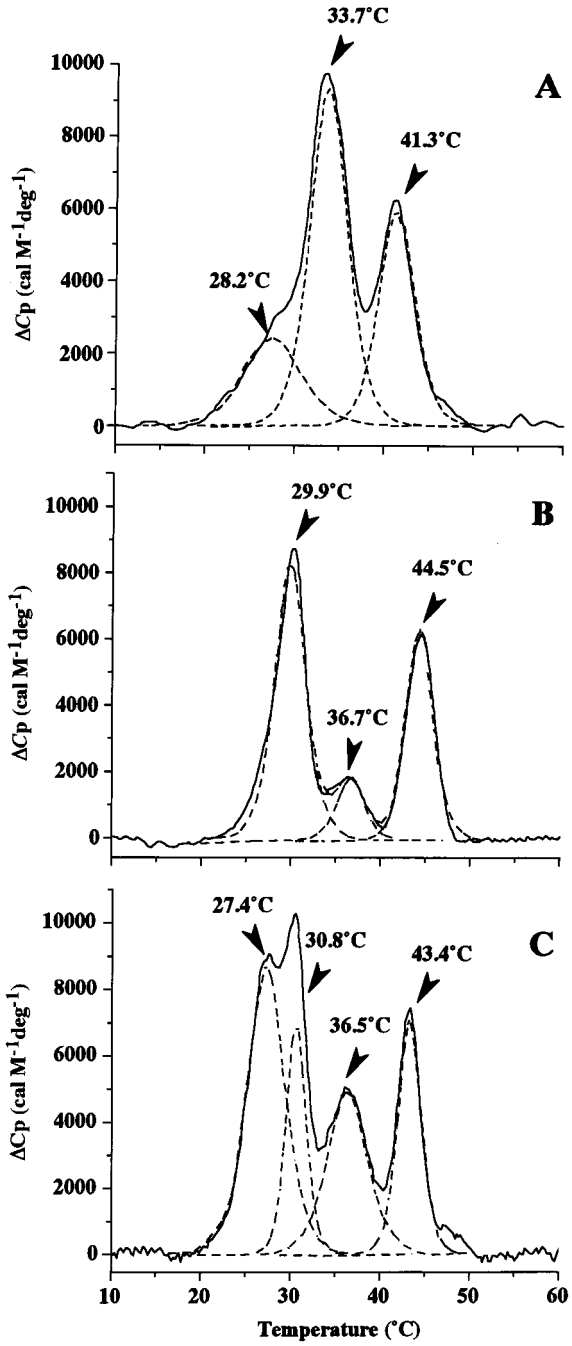

Figure 5. DSC scans of walleye pollack myosin (A), LMM (B), and LMM expressed in *E. coli* (rLMM) (C), together with their computer-calculated differential endotherms by the convolution analysis using three or four peaks (dashed lines). Protein concentrations were 1.2–5.4 mg/mL in 50 mM Tris-HCl (pH 8.0) containing 0.6 M KCl, 5 mM $MgCl_2$, and 1 mM DTT. The scan rate was 45 °C/h, and data were collected every 15 s. ΔC_p represents molar excess heat capacity.

and 44.5 °C with ΔH_{cal} values of 157, 193, and 204 kcal/mol, respectively, their sizes being quite different from those of myosin (**Figure 5B**; **Table 1**).

Habitat-temperature-related tendency as observed with myosin described before was also apparent with data for LMM shown in **Table 3**. **Table 3** shows the T_m values for walleye pollack LMM in comparison with those from carp acclimated to 10 and 30 °C (*15*), chicken (*36*), and rabbit (*37*). The lowest T_m of walleye pollack LMM was lower than any of those for other LMMs, suggesting a low thermal stability of walleye pollack LMM.

Table 1. Thermodynamic Parameters on the Thermal Unfolding of Walleye Pollack Myosin and LMM[a]

	T_m (°C)	ΔH_{cal} (kcal/mol)
myosin[b]	28.2	84 (4.1)[c]
	33.7	136 (2.6)
	41.3	150 (4.8)
LMM[b]	29.9	157 (4.0)
	36.7	193 (26.5)
	44.5	204 (8.1)
rLMM[b]	27.4	129 (2.7)
	30.8	253 (12.1)
	36.5	128 (4.3)
	43.4	233 (9.4)

[a] Abbreviations: LMM, walleye pollack light meromyosin purified from frozen surimi; rLMM, walleye pollack light meromyosin expressed in *E. coli*. [b] Values obtained from **Figure 5**. [c] Values in parentheses are ratios of ΔH_{vh} to ΔH_{cal}.

Table 2. Transition Temperatures of Endothermic Peaks Obtained from the Thermal Unfolding of Myosins from Different Species[a]

species	T_m (°C)			
walleye pollack	28.2	33.7	41.3	
10 °C carp	32.8	34.9	47.4	
30 °C carp	35.9	39.7	49.1	
chicken	35.3	48.1	49.9	67
rabbit	43	46	49	54

[a] Data were cited for myosins from carp acclimated to 10 °C (10 °C carp) and 30 °C (30 °C carp) (*21*), from chicken (*34*), and from rabbit (*35*).

Table 3. Transition Temperatures of Endothermic Peaks Obtained from the Thermal Unfolding of LMMs from Different Species[a]

species	T_m (°C)			
walleye pollack	29.9	36.7	44.5	
10 °C carp	32.5	39.5		
30 °C carp	39.2	47.3		
chicken	39.2	50.0		
rabbit	42.4	48.8	49.0	53.7

[a] Data were cited for LMMs from carp acclimated to 10 °C (10 °C carp) and 30 °C (30 °C carp) (*15*), from chicken (*36*), and from rabbit (*37*).

SDS-PAGE analysis on LMM gave two bands of 66 and 63 kDa, the former corresponding to the size of rLMM (see **Figure 3**). However, the two bands for LMM contained the same N-terminal amino acid sequence. Thus, the 63 kDa walleye pollack LMM could be the product of tryptic digestion lacking a C-terminal part of the whole LMM molecule, because molecular heterogeneity of walleye pollack myosin heavy chain is negligible (*38*). The α-helical content of LMM was very low compared to those of other species as described later. This may be partly due to tryptic digestion of an extremely C-terminal part of LMM during the purification process even in the 66 kDa component of LMM preparation. Then, the expression of the whole LMM in *E. coli* was carried out for subsequent study on its thermodynamic properties that are shown in **Figure 5C** and **Table 1**. Four prominent endotherms were found for rLMM and so were in deconvolution analysis. It was noted that the endothermic peak at 27.4 °C was specific to rLMM and not observed in LMM. Furthermore, there was a difference of ~1 °C for the peaks at the highest temperature at ~44 °C between LMM and rLMM. Such a difference between rLMM and LMM has been reported for carp (*15*, *17*). Sarcomeric myosin heavy chains contain a nonhelical tail piece in their C-terminal part (*39*−*42*). Therefore, this tail piece is possibly cleaved away

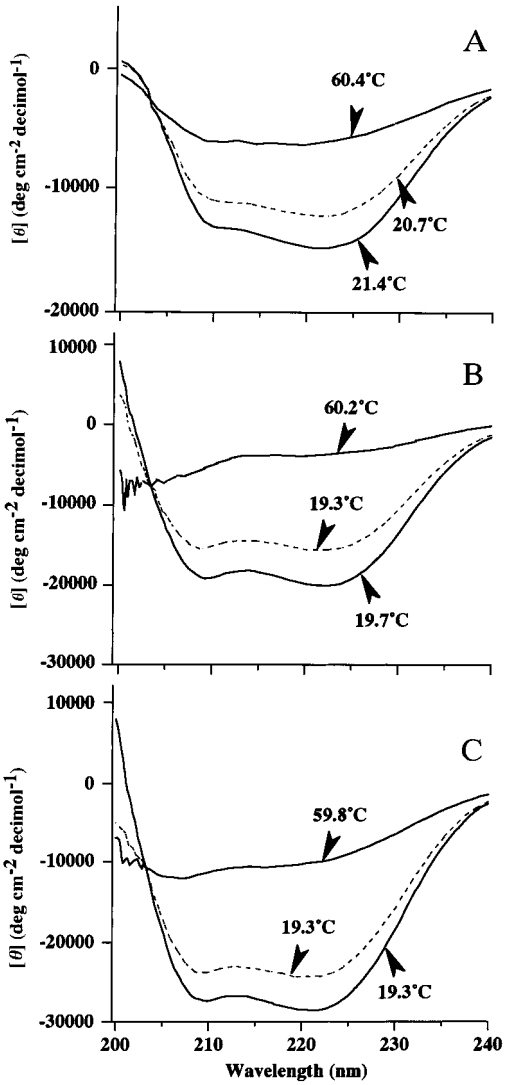

Figure 6. CD spectra of walleye pollack myosin, LMM, and rLMM. CD spectrometry was performed in 50 mM Tris-HCl (pH 8.0) containing 0.6 M KCl, 5 mM $MgCl_2$, and 1 mM DTT. Typical results at about 20 and 60 °C are shown for myosin (A), LMM (B), and rLMM (C). Reversibility was examined after heating at ~60 °C and subsequent measuring at ~20 °C (dashed line). [θ] represents the mean residue ellipticity. Protein concentrations were 1.2–5.4 mg/mL. Measuring temperatures are indicated.

during limited proteolysis to prepare LMM from myosin, showing thermodynamic properties of LMM different from those of full-length rLMM. Alternatively, LMM obtained from frozen surimi was partially denatured during preparation procedure or frozen storage.

CD Analysis. CD spectra at pH 8.0 for walleye pollack myosin, LMM, and rLMM are shown in **Figure 6** at representative low and high temperatures. All of these preparations showed CD spectra typical of α-helix at low measuring temperatures. Assuming that 100% α-helical content corresponds to a [θ] value of −36000 deg cm^2/dmol at 222 nm (*22*), walleye pollack myosin was calculated to be 42% α-helical at ~20 °C (**Figure 6A**). Ogawa et al. (*43*) reported 68, 65, 50, and 38% α-helices at 4 °C for myosins from rabbit, bigeye tuna *Thunnus obesus*, sea bream *Pagrus major*, and walleye pollack, respectively, assuming that 100% α-helical content corresponds to a [θ] value of −40000 deg cm^2/dmol at 222 nm. Recalculation of these values with the conversion factor used in this study gave 76, 72, 56, and 42% α-helices, respectively, in the same species order. Thus, the value of 42% for walleye pollack in this study agreed well with that reported by Ogawa et al. (*43*), emphasizing its low α-helical content. The low α-helical content of walleye pollack myosin may be due to preparations from fish stored on ice for 1 day reported by Ogawa et al. (*43*) and from frozen surimi in this study. Ogawa et al. (*43*) observed that cold-water fish such as walleye pollack underwent structural changes of myosin during preparation, gradually unfolding once extraction was started, by monitoring the α-helical content for different fish species through CD analysis.

The increasing minimum levels at 222 nm with increasing temperature at pH 8.0 in 0.6 M KCl indicated the decrease of the α-helical content for our myosin (**Figure 6A**). Finally, the CD spectra hardly changed over 50 °C, showing that the myosin molecule was completely unfolded. When the temperature was lowered to ~20 °C, the minimum at 222 nm was recovered to ~35% of its original value (**Figure 7**).

CD spectra of walleye pollack LMM and rLMM at pH 8.0 and ~20 °C also had a typical pattern of α-helix (**Figure 6B**,**C**). According to Yang et al. (*22*), with a [θ] value of −36000 deg cm^2/dmol at 222 nm, LMM and rLMM were calculated to have α-helical contents of 56 and 80%, respectively. The lower α-helical content of LMM is probably caused by denaturaion of LMM during myosin preparation or during frozen storage of walleye pollack surimi. The α-helical content of rLMM was comparable to 90% reported for LMM preparations from carp acclimated to 10 and 30 °C (*15*) and from rabbit fast skeletal muscle (*2*). When the measuring temperature was lowered to ~20 °C after heating at 60 °C, the α-helical content determined by the minimum at 222 nm was recovered to 44% for LMM and 67% for rLMM (**Figure 6B**,**C**).

Fractional α-Helical Content of Myosin, LMM, and rLMM. Figure 7 shows the fractional α-helical contents at pH 8.0 in 0.6 M KCl as a function of measuring temperature for walleye pollack myosin, LMM, and rLMM. Curves of their decreasing rate derivatives together with data for DSC analysis are superimposed in the same figure.

DSC endothermic peaks of LMM and rLMM agreed well with those of the derivative profile of α-helical content, implying that DSC endotherms reflect unfolding of α-helix as described before (**Figure 7B**,**C**). Two sharp decreases in the helicity of LMM, shown as the maxima in the derivative profile, occurred in the temperature range of the first and third endothermic peaks. The α-helical content of rLMM decreased rapidly during the endothermic process as in the case of LMM. The first decrease in helicity at ~30 °C was steeper than the LMM counterpart, reflecting the two consecutive endotherms in rLMM.

Apparently, the decrease in helicity of walleye pollack myosin occurred gradually in the temperature range examined (**Figure 7A**). Because myosin contains S1, S2, and LMM, the former two subfragments are considered to be responsible for the different patterns between myosin and LMM.

DISCUSSION

Species-specific differences in the thermal stability of actomyosin Mg^{2+}-ATPase (*5*, *6*) and myofibrillar Ca^{2+}-ATPase (*7*) suggest that the stability of fish myosin changes depending upon the habitat temperature under which the fish lives; that is, the colder the environmental temperature, the more labile myosin is. DSC runs in the present study for myosin and LMM prepared from walleye pollack, a typical cold-water fish, clearly demonstrated their low thermal stability (see **Tables 2** and **3**).

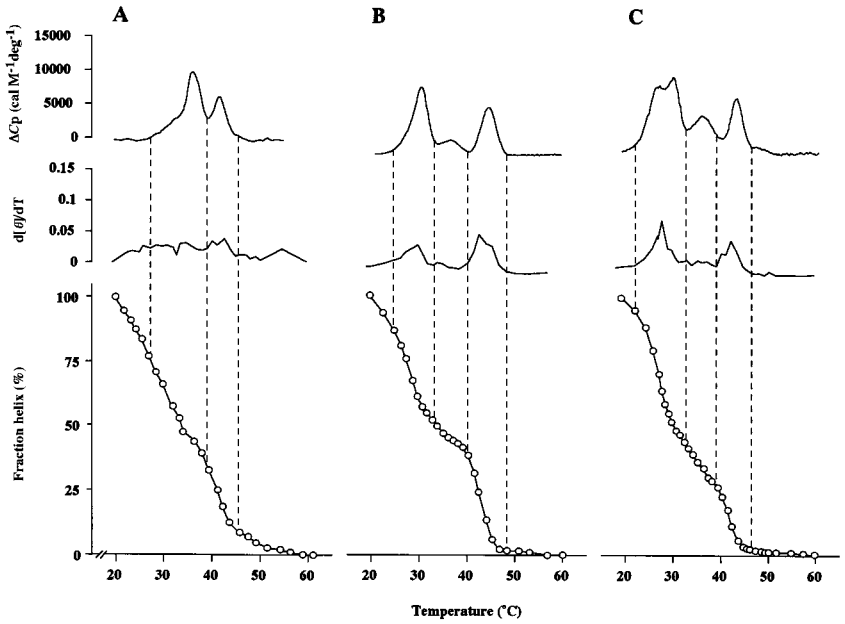

Figure 7. Effects of temperature on thermodynamic properties of walleye pollack myosin (A), LMM (B), and LMM expressed in *E. coli* (rLMM) (C) observed by DSC and CD measurements. The lower, middle, and upper panels show the profiles of fractional α-helical content, the rate derivatives of the mean residue ellipticity, and DSC curves, respectively. The decreasing rate derivatives were calculated from the increment of [θ] at 222 nm per unit change of temperature (°C). The temperature ranges between dashed lines show the main peaks.

It was uncertain whether the above evidence resulted from tryptic digestion of the C terminus of LMM during the preparation process. Moreover, SDS-PAGE analysis gave two bands of 66 and 63 kDa for walleye pollack LMM, the former corresponding to the size of rLMM (see **Figures 3** and **4**). However, the two bands for LMM contained the same N-terminal amino acid sequence. Therefore, the 63 kDa walleye pollack LMM could be the product of tryptic digestion lacking a C-terminal part of the whole LMM molecule. The α-helical content of LMM was very low compared to those of other species as described before. Thus, the expression of the whole LMM in *E. coli* was carried out for subsequent study on its thermodynamic properties (see **Figure 5** and **Table 1**).

Whereas rLMM exhibited four endothermic peaks, LMM prepared from frozen surimi exhibited only three; an endothermic peak at the lowest T_m, 27.4 °C, of rLMM could not be observed in LMM. This peak seems to be attributed to the C-terminal part of LMM, because it has been suggested that LMM irrespective of different origins has a nonhelical tail piece at the C terminus, which is susceptible to proteolytic cleavage (*39*−*42*). Moreover, it is noted that DSC measurement with chimeric carp LMM consisting of N- and C-terminal halves, from either the 10 or 30 °C type, demonstrated that the C-terminal half is largely responsible for thermodynamic properties of the entire LMM molecule (*18*). It has been demonstrated that eight amino acid substitutions located in the C-terminal region of the 69kDa fragment (residues 302−525) are major candidates for the residues responsible for the differences in thermostability between 10 and 30 °C type carp LMMs (*45*). Recent investigation on walleye pollack LMM also showed that a C-terminal one-third of this fragment was unstable and irreversibly unfolded by heat treatment (*46*).

Alternatively, the difference between walleye pollack LMM and rLMM in the present study might be caused by denaturation of LMM as mentioned before during the preparation process. The lower α-helical content of LMM from the surimi suggests such a possibility. Because the same chromatographic procedures were employed for purification of LMM and rLMM, the preparation procedure for myosin or the frozen storage of surimi might have damaged the proteins concerned. It is well-known that walleye pollack myosin is one of the most thermally unstable fish myosins (*4*−*7*). It is very difficult to obtain myosin preparation from walleye pollack as pure as rabbit myosin (data not shown). Thus, it is noted that our study has limitations in the correct interpretation of inherent thermodynamic properties in DSC and CD measurements for walleye pollack myosin and LMM. However, our results gave new insights into such markedly unstable, but industrially very important, proteins.

Although walleye pollack myosin presented the T_m values similar to those of LMM, the overall DSC patterns were markedly different between the two proteins. Such a difference may be derived from the domain structure of myosin, which consists of S1, S2, and LMM. Analyses of the deduced amino acid sequences of walleye pollack LMM and S2 showed substitutions that could strengthen and others that could weaken the coiled coil structure in comparison with sequences of other species (*13*). However, there was no predicted, specific substitution that could be responsible for the lower thermal stability of walleye pollack myosin.

In this study we expected to provide some basic information on the thermodynamic properties of proteins from fish living in different environmental temperatures, which is probably useful for future investigation. This work together with our previous studies (*16*, *38*) initiates an understanding at the molecular level of the major significance in the utilization of fish myosin as a functional ingredient in the manufacture of food products. It is noted that white croaker *Pennahia argentata*, the other important fish used as material for surimi production with higher gel-forming ability than walleye pollack, has also been determined for the primary structure of myosin heavy chain (*47*). It is interesting to compare the thermodynamic properties of myosin between these two fish species in association with amino acid variations, which is currently under investigation in our laboratory.

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