Original Article

Identification of the glutamine residue that may be involved in the transglutaminase-mediated intramolecular cross-linking of carp and walleye pollack myosin

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Abstract

In order to elucidate the molecular mechanism of transglutaminase-mediated myosin cross-linking, a fluorescent monodansylcadaverine (MDC) was incorporated into carp *Cyprinus carpio* myosin and the reactive Gln residues were analyzed by cyanogen bromide cleavage. The fluorescence was predominantly detected in 10.5 kDa BrCN-fragment, which is assumed to be located in subfragment 2 of myosin heavy chain. Furthermore, lysyl endopeptidase digestion of the 10.5 kDa fragment revealed that MDC was specifically incorporated into 520th Gln residue of the subfragment 2 domain. When meat paste prepared from walleye pollack *Theragra chalcogramma* frozen surimi was incubated with MDC, the fluorescence was mostly observed in 16 kDa BrCN-fragment and also slightly detected in other three bands. By the digestion of 16 kDa fragment with lysyl endopeptidase, it was elucidated that MDC was incorporated specifically into Gln-520 of myosin subfragment 2 as well as detected in carp. This domain around Gln-520 is likely to be a common critical region for dimer formation of myosin heavy chains for both fish species. In walleye pollack, other reactive Gln residues are presumed to be exist in the C-terminus of the light meromyosin. This slight difference may be significant in a capacity to form tetramers or even larger multimers.

Introduction

Transglutaminase (TGase, EC 2.3.2.13) is an enzyme that promotes protein polymerization through intermolecular or intramolecular ε-(γ-glutamyl) lysine bonding. In addition, it acts as a catalyst for acyl transfer reaction between the carboxamide groups of Gln residues and primary amines in proteins [1-3]. TGase is known to exist in fish muscle [4, 5]. It has been reported that this kind of endogenous TGase in muscle mediates the specific cross-linking of myosin heavy chain in actomyosin [6]. Myosin is a hexamer composed of two heavy chains and four light chains [7]. Structually, the myosin heavy chain is divided into subfragment 1 (S1) of the myosin head and a coiled-coil rod of the myosin tail. In addition, heavy meromyosin（HMM）refers to the part between the central region and the N-terminus, while light meromyosin（LMM）refers to the part between the central region and the C-terminus. HMM is further divided into S1 of the myosin head and a remaining region called myoin subfragment 2 (S2). These fragments of the myosin heavy chain have been identified through proteolytic degradation analysis [8, 9].

In a study by Kunioka and Ando [10], guinea pig liver TGase was used to bind the primary amine monodansylcadaverine (MDC) in rabbit HMM, which demonstrated that the myosin S2 has an incorporation site for MDC. Their study was conducted with physiological saline concentration in which myosin exits in the fibrillary form. Seki *et al.* used carp TGase under high salt concentrations to study the MDC incorporation site in carp myosin and found that TGase-mediated cross-linking initiates at the Gln residue of the S2 domain in the myosin heavy chain [11]. In another study by Maruyama *et al.* [12], it was proposed that 1-2 mol of fast reactive Gln residues existed in the carp myosin heavy chain. Araki and Seki showed that in cross-linking reactions with TGase under the condition similar to setting, heavy chain dimmers were formed and that even-numbered oligomers were dominant [6].

Based on previous findings, it has been suggested that TGase-mediated myosin cross-linking initially occurs at the S2 domain intramolecularly. Nevertheless, the reactive Gln residue in the S2 domain and the molecular mechanisms in cross-linking remain unclear. The study of cross-linking mechanisms is important to clarify the setting phenomenon in fish meat gelation. Furthermore, it is known that some species such as carp, salmon, and mackerel, have a weak setting whereas fish usually used in traditional *kamaboko* production (such as walleye pollack, golden thread, white

croaker, hoki, and lizardfish) are easy setting species [13, 14]. It is also important to clarify the similarities and differences in TGase-mediated cross-linking among fish species during setting. In this study, to obtain the basic information about the molecular mechanism of TGase-mediated intramolecular cross-linking of myosin at the initial stage of setting, we have examined the reactive Gln residue on the carp and walleye pollack myosin heavy chains involved in the TGase reaction using fluorescent primary amine MDC under the condition similar to setting.

Materials and Methods

Materials

Live cultured carp *Cyprinus carpio* were purchased from aquaculture farms near Hakodate. Frozen surimi (SA grade) of walleye pollack *Theragra chalcogramma* containing 4% sorbitol, 4% sucrose, and 0.2% polyphosphates was obtained from the Nippon Suisan Kaisha, Ltd. Monodansylcadaverine (MDC) was purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). Lysyl endopeptidase [EC. 3.4.21.50] was from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents were of analytical grade.

Preparation of carp myosin and muscle TGase

Carp myosin was prepared from the fresh dorsal muscle according to the method described by Nauss *et al.* [15]. Carp muscle TGase was partially purified by DEAE-cellulose and Sephacryl S-300 gel chromatography, according to the method described by Nozawa et al. [16]. TGase activity was determined at 25°C using the assay mixture containing 1.0 mg/ml succinylated casein, 1 mM MDC, 1 mM $CaCl_2$, 2 mM dithiothreitol (DTT), 0.1 M HEPES buffer (pH 7.5) and the enzyme. One unit of TGase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol MDC into succinylated casein for 1 min.

TGase-mediated MDC incorporation into myosin

For the TGase-mediated MDC incorporation into carp myosin, 5.0 units/ml of carp TGase was added to a reaction mixture containing 5.0 mg/ml myosin, 4 mM MDC, 0.5 M NaCl, and 1 mM $CaCl_2$ in 0.1 M HEPES buffer (pH 7.5), followed by incubation at 25°C for 2 h. For MDC incorporation using walleye pollack meat paste, the meat paste was prepared to give a protein concentration of 90 mg/g in 0.1 M Tris-HCl buffer (pH 7.0) with 4 mM MDC and 0.5 M NaCl. The meat paste was de-aerated for 5 min, packed into plastic tubes, and then incubated at 25°C for 2 h.

Cyanogen bromide cleavage

For direct BrCN-cleavage of carp myosin or walleye pollack meat paste, the proteins were first subjected to MDC incorporation and the reaction was terminated by 5% trichloroacetic acid (TCA). The precipitate was washed with ether/ethanol (1:1(w/w)) for 4 times and dissolved in 70% formic acid. One milliliter of the dissolved proteins was made to react with 10 mg of BrCN at 25°C for 1 h. The samples were then dried in a centrifugal vacuum evaporator. For SDS-PAGE, the dried samples were dissolved in 62.5 mM Tris-HCl buffer (pH 6.8)-2% SDS-10% glycerol-0.01% bromophenol blue and 0.1 M DTT.

SDS-polyacrylamide gel electrophoresis and transblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [17] using 15% slab gels. For semi-dry transblotting [18], proteins from the SDS-PAGE were transblotted onto a PVDF membrane (Immobilon ™ PSQ, Millipore Corporation) at 150 mA for 1-2 h with a semi-dry blotting system (Nihon Eido Co., Ltd.). Proteins incorporated with MDC were tested for fluorescence with UV Handy Light (MODEL UVGL-58, Fuankoshi Corporation). Membrane was stained with 0.25% CBB- 5% methanol-7.5% acetic acid and destained with methanol.

Lysyl endopeptidase digestion

PVDF membrane strips containing the transblotted target peptides were subjected to reducing and S-pyridylethylation. The membrane strips were then washed with 10% acetonitrile and digested with

lysyl endopeptidase for 15 h at 37ºC in 150 µl of 20 mM Tris- HCl buffer (pH 9.0) containing 10% acetonitrile at a molar ratio of 1/100 (mol/mol, E/S).

Purification of peptide by reversed phase HPLC

The digests treated with lysyl endopeptidase were applied to a Mightsil RP-18 (4.6 × 150 mm) HPLC column for isolation of the target peptides by a linear gradient of acetonitrile (2-70%) at a flow rate of 0.5 ml/min. Detection was performed using a HITACHI F-1050 Fluorescence Spectrophotometer (Ex 335 nm, Em 510 nm) coupled on to a HITACHI L-4000 UV Detector (220 nm). The fractions separated by Mightsil RP-18 were further purified using Inertsil ODS-3 column (2.1 × 150 mm) where necessary.

Amino acid sequencing of proteins

Protein samples transblotted onto a PVDF membrane were analyzed for their N-terminal amino acid sequence. The peptide samples isolated by reversed phase HPLC were spotted onto a PVDF membrane after removal of acetonitrile and applied to a protein sequencer (Procise^{TM} 492HT Protein Sequencing System, Applied Biosystems).

Results

Amine incorporation into carp myosin and analysis of BrCN-cleaved fragments

The reaction mixture was incubated with carp TGase for MDC incorporation into myosin at 25ºC for 2 h, which was subsequently recovered by precipitation with TCA and dissolved in 70% formic acid. This was directly added with BrCN to undergo cleavage at 25ºC for 1 h (Fig. 1). Since there was a mixture of products from the myosin heavy chain and light chain, it was anticipated that electrophoresis would give complex results patterns. However, except for low-molecular-weight peptides which produced some overlapping bands, fluorescence signals for a 10.5 kDa fragment were virtually specific in our observations.

Amino acid sequences of the fluorescent fragments (10.5 kDa, 21 kDa) and the non-fluorescent fragment (19 kDa) are shown in Table 1. These sequences were compared with that of known 10°C-type carp fast skeletal myosin heavy chain [19-21]. The 5 amino acids at the N-terminal end of the 10.5 kDa fragment matched with the amino acids located between positions 436 and 440 of the S2 domain. Therefore, this 10.5 kDa fragment derived from BrCN-cleavage of myosin is likely to be the region spanning positions 436 and 523 of the S2 domain, because both 435 and 523 residues are Met and there is no Met between positions 436 and 522. When carp myosin heavy chain or S2 was incubated with MDC and cleaved with BrCN, the fluorescence of MDC was also specifically detected in 10.5 kDa fragment of 436—523 of the S2 domain (data not shown).

Identification of glutamine residue as a carp myosin cross-linking site

The 10.5 kDa fragment (436—523 in S2) obtained from the BrCN-cleavage of myosin was transblotted onto a PVDF membrane and digested by lysyl endopeptidase. The digested peptides were separated by reversed phase HPLC using Mightsil RP-18 column. The UV absorbance at 220 nm (Fig. 2, upper solid line) and fluorescence intensity (Fig. 2, lower broken line) of the isolated peptides were monitored simultaneously. Although peptides K1—K16 were obtained, only K9 gave significantly sufficient fluorescence for detection.

The amino acid sequence of K9, which takes the form of AEL×RG×, could not produce signals for detection at the 4th and 7th residues. The sequence was identical to the region spanning 517 and 522 of the S2 domain (Fig. 3). The 4th amino acid of K9 corresponds to Gln of the known amino acid sequence. This Gln was unable to produce a signal probably due to the formation of covalent bonding with MDC. In other words, Gln-520 is likely to be the site of MDC incorporation. In addition, the 7th amino acid of K9 corresponds to Met-523 was undetectable probably because during BrCN-cleavage, Met was converted into homoserine lactone or homoserine. Therefore, it is revealed that the TGase-mediated amine incorporation site in carp myosin has very high specificity, with almost all incorporation occurring at Gln-520 of S2 domain. Since the TGase-mediated myosin cross-linking is inhibited in the presence of MDC, it is considered that Gln-520 specifically participate in myosin cross-linking, particularly in the dimerization of myosin heavy chain, suggesting that intramolecular cross-linking is initiated in the region between Gln-520 and the

nearby Lys residue, such as Lys-516 or Lys-525.

Analysis of carp myosin 19- and 21-kDa fragments

The amino acid sequence of the N-terminus of the 21 kDa fragment which exhibited some fluorescence was identical to that of 416—420 of LMM (LMM sequence), which means that this fragment is likely to be the region spanning 416—563 (C-terminus) of LMM (Fig. 3c). The non-fluorescent 19 kDa fragment yielded an amino acid sequence identical to that of 435—439 of LMM, suggesting that it is likely to be the region spanning 435—563 (C-terminus) of LMM. Based on these observations, we conclude that both the 19 kDa and 21 kDa fragments are part of the C-terminus. MDC in the 21 kDa fragment should be present in the part that is not overlapping with the 19 kDa fragment, which suggests that MDC incorporation occurs at Gln-423 of the LMM, which is the only Gln residue between Glu-416 and Met-434 (Fig. 3c). Although fluorescence from the 21 kDa fragment was very weak, the corresponding C-terminus region containing Gln-423 of LMM may also participate in cross-linking.

Amine incorporation into walleye pollack myosin and analysis of BrCN-cleaved fragments

Next, we studied the site of endogenous TGase-mediated cross-linking in fish proteins by preparing samples from frozen surimi of walleye pollack, which is an important raw material for *kamaboko* production.

The walleye pollack meat paste (90 mg/ml, 0.5 M NaCl, pH 7.0) was incubated with MDC and the paste was cleaved with BrCN (Fig. 4). Since many proteins other than the myosin heavy chain were present in the meat paste, it was anticipated that electrophoresis would produce complex result patterns, however, fluorescence was restricted to only a handful of the peptide fragments in the SDS-PAGE. These fluorescent peptide fragments (16 kDa, 17 kDa, 19 kDa, 26 kDa) thus obtained were analyzed for their amino acid sequence (Table 2). These sequences were compared with that of myosin heavy chain of walleye pollack fast skeletal muscle [22, 23].

Identification of glutamine residue as a major cross-linking site in walleye pollack myosin

Among the BrCN-cleaved products of MDC-incorporated walleye pollack myosin, the 16 kDa fragment exhibited the strongest fluorescence. The MDC-incorporated Gln residue in this fragment was further analyzed by lysyl endopeptidase digestion.

Lysyl endopeptidase digestion was performed as in carp myosin. The digests were then separated by reversed phase HPLC using Mightsil RP-18 column. For detection, UV absorbance at 220 nm (Fig. 5, upper solid line) and fluorescence intensity (Fig. 5, lower broken line) were monitored simultaneously. From the 16 kDa fragment, digestion peptides K1-K15 were obtained but only K7 produced fluorescence for detection. This K7 was subjected to amino acid sequence analysis.

The sequence of K7 was determined to be AEL×RG×, for which the 4th and 7th amino acid residues were not detectable. This sequence matched that of the region of 517—522 of S2 (Fig. 6). As in the case of carp myosin, the 4th amino acid of K7 corresponds to Gln of the known sequence. This Gln was unable to produce a signal probably due to the formation of covalent bonding with MDC, suggesting that Gln-520 is the MDC incorporation site. In addition, the 7th amino acid of K7 was undetectable probably because during BrCN-cleavage, Met was converted into homoserine lactone (or homoserine).

The 16 kDa BrCN-cleaved fragment of walleye pollack myosin contained two peptides (Table 2). However, when these were subjected to lysyl endopeptidase digestion, only one single fluorescence peak was produced as K7 (Fig. 5). It was considered that MDC incorporation only involves 16 kDa-1 which corresponds to 398—523 of S2, whereas 16 kDa-2 which corresponds to 229—388 of S2 does not participate in the reaction. Based on these results, it was revealed that the major site for endogenous TGase-mediated amine incorporation in walleye pollack was Gln-520 in the myosin heavy chain S2 domain, which was the same position as in the case of TGase-mediated cross-linking in carp myosin.

Analysis of walleye pollack myosin 17, 19 and 26 kDa fragments

The N-terminus 5 amino acid sequence of the 17 kDa fragment corresponded to 398—402 of the S2 domain, which was identical to those of 16 kDa fragment. However, the amount of fragment

available was not sufficient for further analysis. When the 26 kDa fragment was digested with lysyl endopeptidase, 14 peptides were obtained by reversed phase HPLC and only 2 peaks exhibited the fluorescence (data not shown). Although the MDC-incorporated Gln residues in 26 kDa fragment could not defined clearly, it is considered that the incorporation sites are restricted.

The amino acid sequence of the 19 kDa fragment, which produced a relatively weak fluorescence, was identical to 435—439 of the LMM region and thus may correspond to 435—564 of the LMM (Fig. 6). When this 19 kDa fragment was digested with lysyl endopeptidase and separated by HPLC, 14 digestion peptides were obtained (data not shown). The amount of peptide recovered was insufficient for protein sequencing. Nevertheless, it did produce a single fluorescence peak, suggesting that MDC incorporation takes place at other specific Gln residues within the region between 435 and 564 of LMM region in walleye pollack myosin heavy chain.

Discussion

TGase can serve as a catalyst for acyl transfer reaction between the γ-carboxamide groups of peptidyl Gln residues and primary amines. Primary amines have an antagonistic effect on the ε-amino acids of Lys residues in cross-linking of proteins. In view of this, this study was conducted to analyze the incorporation site for the fluorescent primary amine MDC and identify Gln residues participating in TGase-mediated myosin heavy chain cross-linking.

When MDC was incorporated into carp myosin and subjected to BrCN-cleavage, fluorescence was detected virtually only in the 10.5 kDa fragment which probably corresponds to 436—523 of the S2 domain, exhibiting very high specificity (Fig. 1). Upon lysyl endopeptidase digestion, only a major fluorescence peak was detected and it was estimated that MDC is specifically incorporated into Gln-520 of the S2 domain.

In contrast, when walleye pollack meat paste prepared from frozen surimi was used for MDC incorporation, three major fluorescence fragments were detected during BrCN cleavage (Fig. 4). The 16 kDa fragment, which was the strongest fluorescent signals of the three fragments, was subjected to lysyl endopeptidase digestion. The results were substantially identical to that of carp myosin, suggesting that MDC was specifically incorporated into Gln-520 of the walleye pollack myosin S2 domain. Based on the high reactivity exhibited by Gln-520, it is considered that Gln-520

is likely to participate in the dimerization of intramolecular myosin heavy chains.

It is known that the rod region of the myosin heavy chain contain a coiled-coil structure comprising two α-helices. In the amino acid sequence of one of these α-helices, a conserved seven-residue sequence repeat of a to g was observed. In this sequence repeat, hydrophobic amino acid residues occur at positions a and d, which stabilize the coiled-coil structure through hydrophobic interactions [24, 25]. The Gln-520 of the S2 domain, which is the putative site for TGase-mediated cross-linking, occur at position e (Fig. 7). In the vicinity of this Gln residue, there exists Lys-516, Lys-525, and Lys-3 in LMM at positions a, c, and g, respectively. The amino acid residue at position e lie comparatively adjacent to that at position a, c, or g of another helix chain; however, as the coiled-coil structure located near the C terminal end of S2 domain tends to be fragile, it is not certain whether Gln-520 occurs at position e, and its neighboring Lys residues lie at positions a, c, and g. However, it is required that both Gln and Lys substrates are physically located near each other for TGase-mediated cross-linking reaction. Therefore, it follows that the neighboring Lys residues may also participate in intramolecular cross-linking.

Since analysis of Lys residue as a substrate in cross-linking is not within the scope of this study, its role was not further characterized. For future studies, it should be required to analyze the dimmer of myosin heavy chains and to identify the Lys residues involving in TGase-mediated cross-linking. Nevertheless, the substrate specificity of TGase is generally dependent on Gln side chains, whereas Lys side chains exhibit relatively low specificity. In the case of myosin dimerization, there is a possibility that all of the Lys residues adjacent to Gln-520 may participate in a cross-linking reaction. The Gln-520 and these possible Lys residues are conserved between carp and walleye pollack myosin sequences. As mentioned, the coiled-coil structure near Gln-520 is a fragile structure with high reactivity. The molecular structure around Gln-520 of S2 domain may actually provide the optimal conformation as a TGase substrate in common.

We conclude that the Gln residue serving as a major amine incorporation site in both carp and walleye pollack myosins is the Gln residue in the 520th position of the S2 domain, and that it is a conserved part of the sequence critical for dimerization. Furthermore, based on analysis of lysyl endopeptidase digest of the fluorescent 19 kDa fragment from walleye pollack myosin, other reactive Gln residues in the region spanning 435―564 of the LMM may also exist (Fig. 6). For this particular region, no fluorescence was observed in carp myosin (Fig. 3). Although this constitutes

only a relatively small difference, it may well be involved in multiple cross-linking reactions, in particular, the formation of tetramers or even larger multimers. Further detailed investigation should be required to clarify the differences in the cross-linking mechanism among various fish species.

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Figure captions

Fig. 1 BrCN-cleavage of MDC-incorporated carp myosin. Carp myosin (5 mg/ml) was incubated at 25°C for 2 h with 5.0 units/ml carp TGase in 50 mM HEPES buffer (pH 7.5) containing 0.5 M NaCl, 1 mM $CaCl_2$, and 4 mM MDC. After incubation, the myosin was cleaved with BrCN in 70% formic acid at 25°C for 1 h. SDS-PAGE was performed using 15% polyacrylamide gel. The fluorescence of MDC-binding proteins was detected by UV-illumination (a) and then the proteins were stained with CBB (b)

Fig. 2 Reversed-phase HPLC chromatogram of lysyl endopeptidase digest of MDC-incorporated 10.5 kDa fragment. The MDC-incorporated 10.5 kDa fragment (100 pieces of PVDF membrane) was digested with lysyl endopeptidase in 20 mM Tris-HCl buffer (pH 9.0)-10% acetonitrile. The digest was applied to a reversed phase HPLC column Mightsil RP-18 and eluted with a linear gradient of 2—70% acetonitrile. Upper solid and lower broken lines indicate the absorbance at 220 nm and fluorescence intensity, respectively

Fig. 3 (a) Location of MDC-incorporated BrCN-fragments in carp myosin heavy chain. Black and shaded portions indicate the 10.5 kDa and 21 kDa fragments, respectively. (b) Amino acid sequence of subfragment-2 401—533 of carp 10°C type-myosin heavy chain [20, 21]. Thick line indicates K9 peptide derived from lysyl endopeptidase digestion of 10.5 kDa fragment which is enclosed with a broken line. The specific MDC-incorporated Gln residue (520) is boxed. (c) Amino acid sequence of light meromyosin 401—563 of carp 10°C type-myosin heavy chain [21]. Solid and broken lines indicate 21 kDa and 19 kDa fragments, respectively. Italic M, possible BrCN cleavage site

Fig. 4 BrCN-cleavage of MDC-incorporated walleye pollack paste. Walleye pollack paste (90 mg/ml, 0.5 M NaCl, 20 mM Tris-HCl buffer (pH 7.0)) was incubated at 25°C for up to 2 h with 4 mM MDC. After incubation, the paste was cleaved with BrCN in 70% formic acid at 25°C for 1 h. SDS-PAGE was performed using 15% polyacrylamide gel. The fluorescence of MDC-binding proteins was detected by UV illumination (a) and then the proteins were stained with CBB (b)

Fig. 5 Reversed-phase HPLC chromatogram of lysyl endopeptidase digest of MDC-incorporated 16 kDa fragment. The 16kDa fragment was digested with lysyl endopeptidase and separated by a reversed phase HPLC as described in the caption to Fig. 2. Upper solid and lower broken lines indicate the absorbance at 220 nm and fluorescence intensity, respectively

Fig. 6 (a) Location of MDC-incorporated BrCN-fragments in walleye pollack myosin heavy chain. Black and shaded portions indicate the 16 kDa-1 and 19 kDa fragments, respectively. (b) Amino acid sequence of subfragment-2 351—533 of walleye pollack myosin heavy chain [23]. Thick line indicates K7 peptide derived from lysyl endopeptidase digestion of 16 kDa fragment which is enclosed with a broken line. The specific MDC-incorporated Gln residue (520) is boxed. (c) Amino acid sequence of light meromyosin 401—563. Solid line indicates 19 kDa fragments. Italic M, possible BrCN cleavage site

Fig. 7 The amino acid sequence around Gln (520) of walleye pollack subfragment-2 and light meromyosin [23] in comparison with those of 10°C-type carp [20]. *a*, *b*, *c*, *d*, *e*, *f*, and *g* indicate positions in the seven-residue repeat for coiled-coil α-helices. S2, subfragment-2; LMM, light meromyosin. Bold Q and K indicate the possible cross-linking site

Table 1 Identification of BrCN-cleavage fragments of MDC-incorporated carp myosin heavy chain

BrCN-peptide [a]	Amino acid sequence	Presumed region in myosin heavy chain fragments (residue number)	Presumed numbers of amino acid	Calculated molecular mass
10.5 kDa	NAQRA	S2 (436—523)	88	10.2 kDa
21 kDa	ELTVK	LMM (416—563)	149	17.1 kDa
19 kDa	KGGKK	LMM (435—563)	130	15.0 kDa

[a] Each fragment corresponds to that in Fig. 1

Table 2 Identification of BrCN-cleavage fragments of MDC-incorporated walleye pollack paste

BrCN-peptide [a]	Amino acid sequence	Presumed region in myosin heavy chain fragments (residue number)	Presumed numbers of amino acid	Calculated molecular mass
16 kDa-1	EAVSK	S2 (398—523)	126	14.3 kDa
16 kDa-2	DLEND	S2 (230—388)	160	18.2 kDa
17 kDa	EAVSK	S2 (398— ?)		
19 kDa	KGGKK	LMM (435—564)	130	15.0 kDa
26 kDa-1	EAVSK	S2 (398)—LMM (58)	194	23.3 kDa
26 kDa-2	DLEND	S2 (230—397)	169	20.1 kDa

[a] Each fragment corresponds to that in Fig. 4

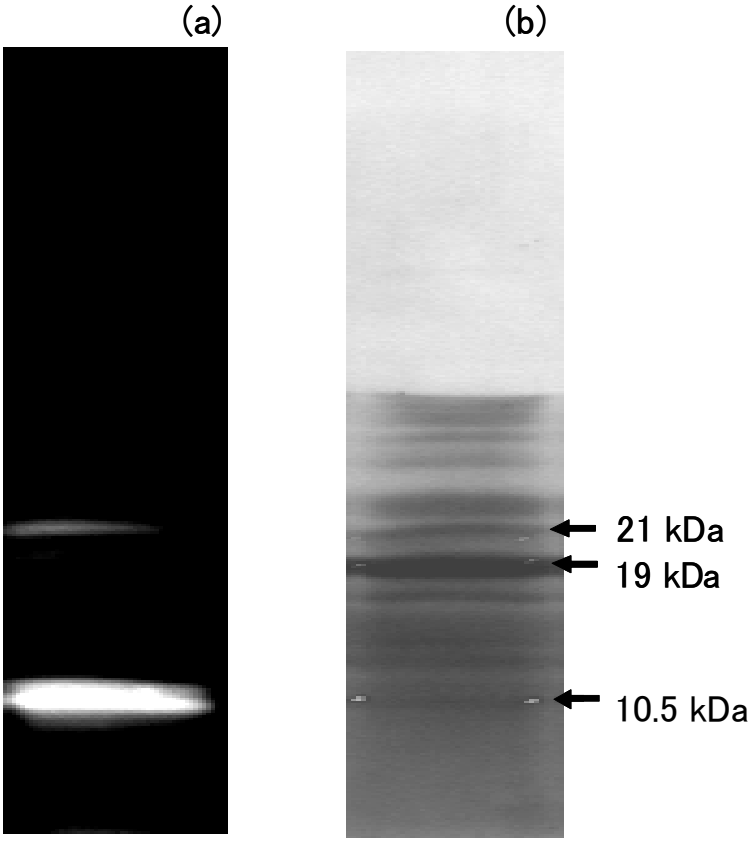

Fig. 1

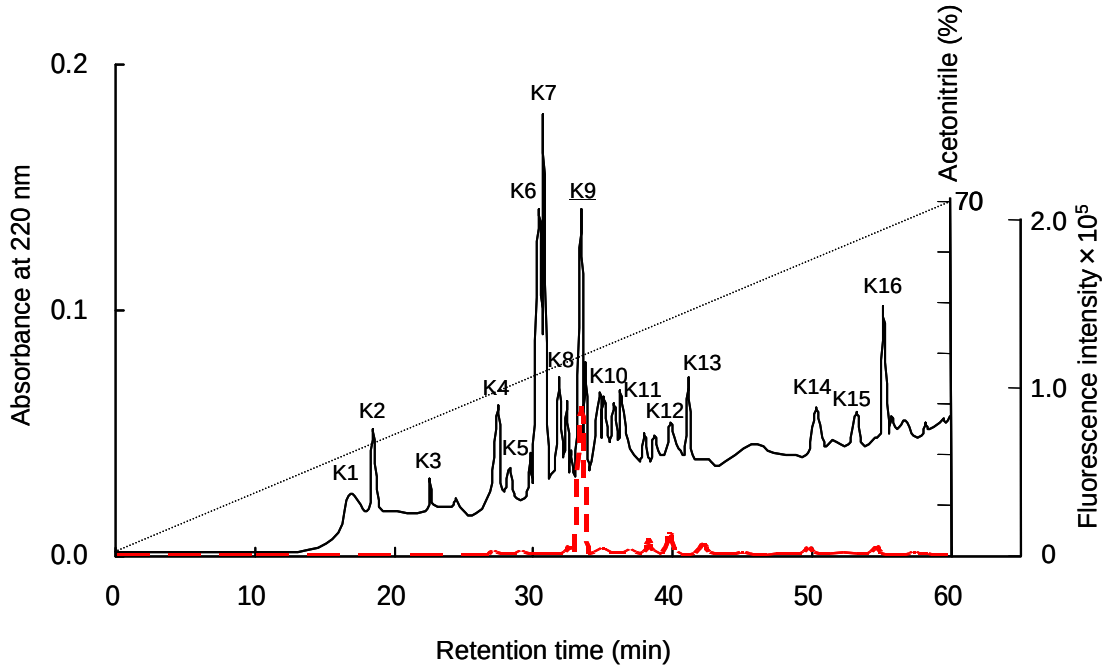
Absorbance at 220 nm
0.2
0.1
0.0
Acetonitrile (%)
70
Fluorescence intensity × 10⁵
2.0
1.0
0
K1
K2
K3
K4
K5
K6
K7
K8
K9
K10
K11
K12
K13
K14
K15
K16
0
10
20
30
40
50
60
Retention time (min)

Fig. 2

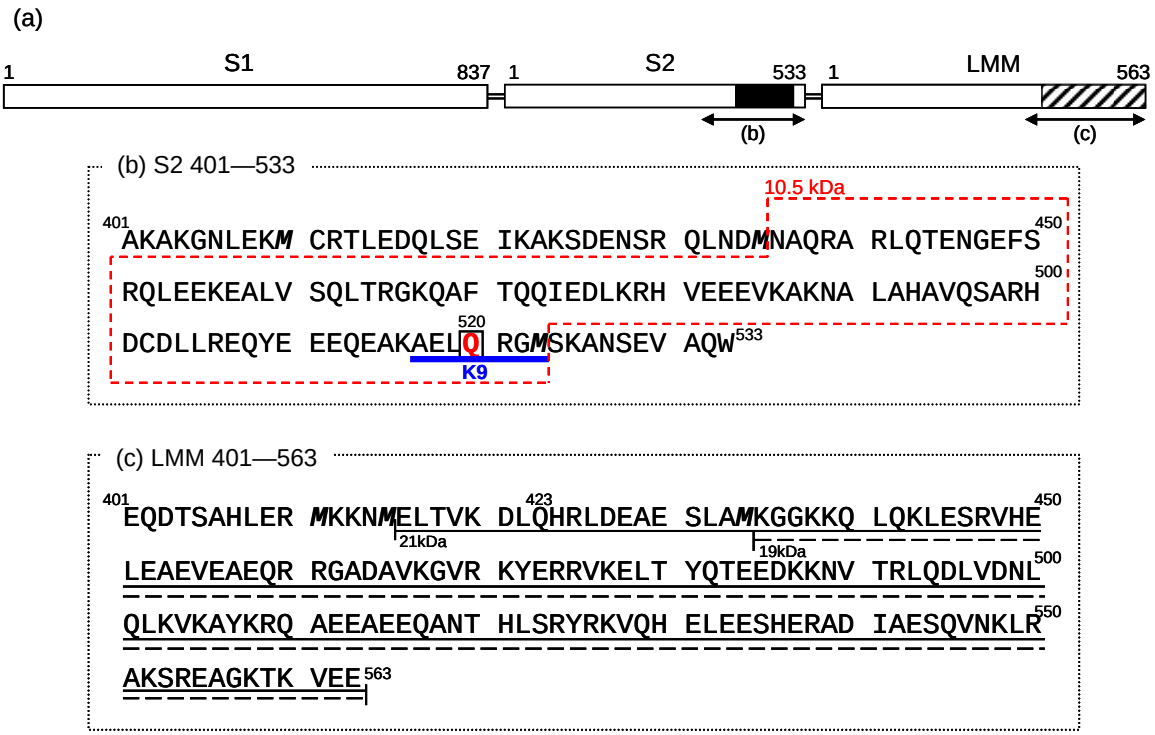
(a)
1
S1
837
1
S2
533
1
LMM
563
(b)
(c)
(b) S2 401—533
10.5 kDa
401
AKAKGNLEKM CRTLEDQLSE IKAKSDENSR QLNDMNAQRA RLQTENGEFS
450
RQLEEKEALV SQLTRGKQAF TQQIEDLKRH VEEEVKAKNA LAHAVQSARH
500
DCDLLREQYE EEQEAKAELQ RGMSKANSEV AQW
520
533
K9
(c) LMM 401—563
401
EQDTSAHLER MKKNMELTVK DLQHRLDEAE SLAMKGGKKQ LQKLESRVHE
423
450
21kDa
19kDa
LEAEVEAEQR RGADAVKGVR KYERRVKELT YQTEEDKKNV TRLQDLVDNL
500
QLKVKAYKRQ AEEAEEQANT HLSRYRKVQH ELEESHERAD IAESQVNKLR
550
AKSREAGKTK VEE
563

Fig. 3

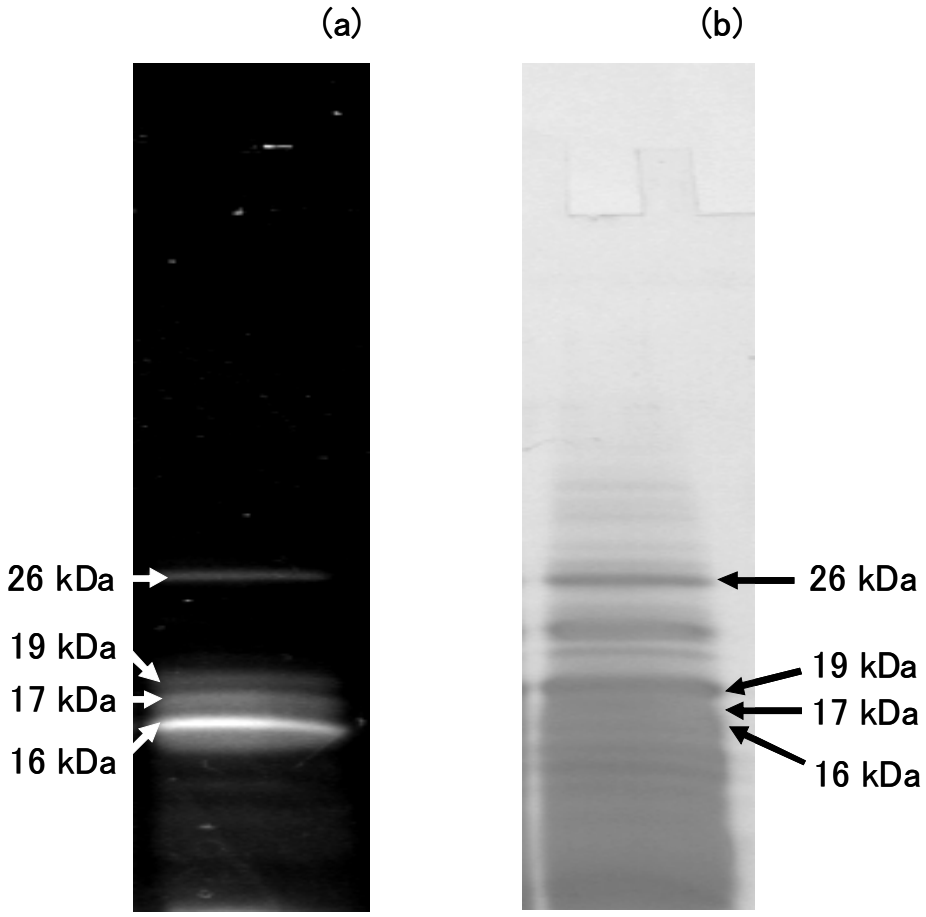

Fig. 4

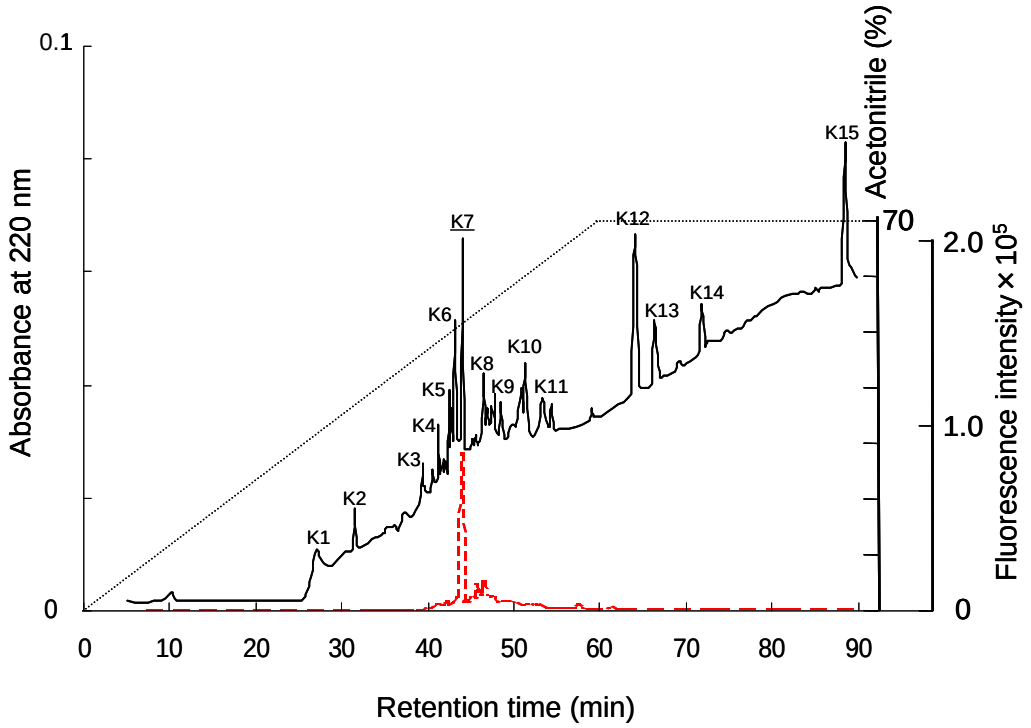
0.1
0
Absorbance at 220 nm
Acetonitrile (%)
70
2.0
1.0
0
Fluorescence intensity × 10⁵
K1
K2
K3
K4
K5
K6
K7
K8
K9
K10
K11
K12
K13
K14
K15
0
10
20
30
40
50
60
70
80
90
Retention time (min)

Fig. 5

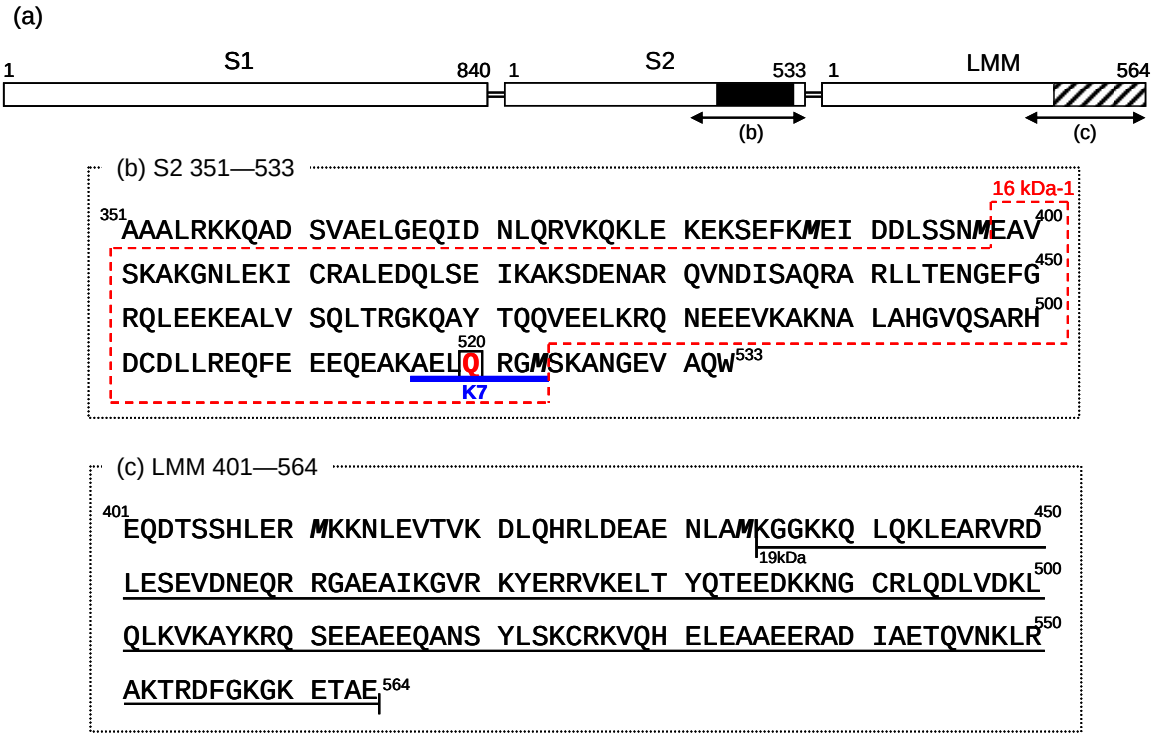
(a)
1
S1
840
1
S2
533
1
LMM
564
(b)
(c)
(b) S2 351—533
16 kDa-1
351
AAALRKKQAD SVAELGEQID NLQRVKQKLE KEKSEFKMEI DDLSSNMEAV
400
SKAKGNLEKI CRALEDQLSE IKAKSDENAR QVNDISAQRA RLLTENGEFG
450
RQLEEKEALV SQLTRGKQAY TQQVEELKRQ NEEEVKAKNA LAHGVQSARH
500
520
DCDLLREQFE EEQEAKAELQ RGMSKANGEV AQW
533
K7
(c) LMM 401—564
401
EQDTSSHLER MKKNLEVTVK DLQHRLDEAE NLAMKGGKKQ LQKLEARVRD
450
19kDa
LESEVDNEQR RGAEAIKGVR KYERRVKELT YQTEEDKKNG CRLQDLVDKL
500
QLKVKAYKRQ SEEAEEQANS YLSKCRKVQH ELEAAEERAD IAETQVNKLR
550
AKTRDFGKGK ETAE
564

Fig. 6

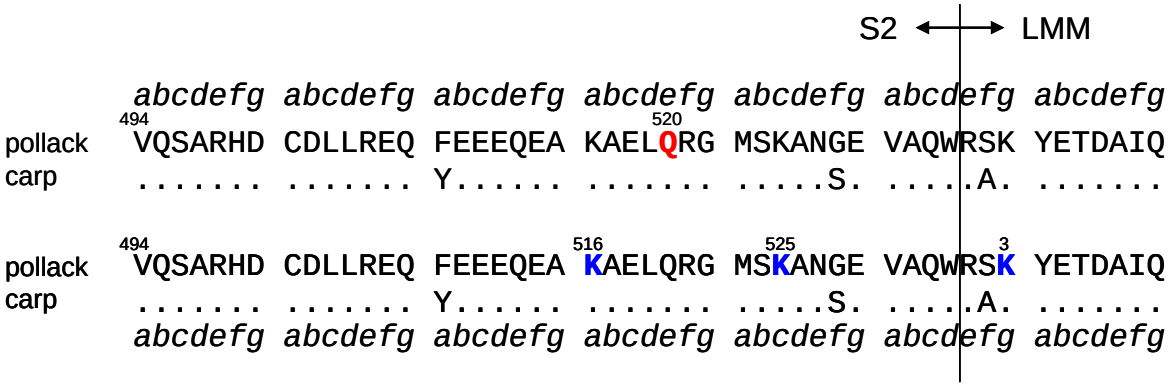
S2
LMM
abcdefg abcdefg abcdefg abcdefg abcdefg abcdefg abcdefg
494 520
pollack VQSARHD CDLLREQ FEEEQEA KAELQRG MSKANGE VAQWRSK YETDAIQ
carp .... Y..... ....S. .....A. .....
494 516 525 3
pollack VQSARHD CDLLREQ FEEEQEA KAELQRG MSKANGE VAQWRSK YETDAIQ
carp .... Y..... ....S. .....A. .....
abcdefg abcdefg abcdefg abcdefg abcdefg abcdefg abcdefg

Fig. 7