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Structural difference between myofibrillar protein, paratropomyosin, and tropomyosin as revealed by high-performance liquid chromatography

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Running Title: Difference between PTM and TM by HPLC.

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ABSTRACT

Paratropomyosin (PTM) which composes myofibrils functions to weaken the rigor linkages formed between actin and myosin during postmortem aging of muscles. PTM has the similar physico-chemical properties of tropomyosin (TM) that is a regulatory protein of myofibrils. So far, it is unclear whether PTM is definitely different from TM, because primary structure of PTM has not been determined yet. The aim of this study was to clarify structural difference of PTM from TM. PTM was prepared by column chromatography immediately after slaughter from broiler breast muscle, and purified by high-performance liquid chromatography (HPLC). Purified PTM was successfully separated from TM, and recovered PTM molecule was reduced with dithiothreitol to separate again by HPLC. Two subunits were obtained and peptides from each digested subunit by V8 protease were recovered by HPLC, and then amino acid sequences of the peptides were analyzed by protein sequencing. As the result, some amino acid residues were replaced from that of TMα1 isoform which is the major isoform of TM, and also different between two subunits. Therefore, it is concluded that PTM clearly differs from TM and suggested that functional difference in PTM from TM is attributed to amino acid replacements in subunits composing PTM.

Key Words: HPLC, meat tenderness, myofibrillar protein, paratropomyosin

INTRODUCTION

A myofibrillar protein, paratropomyosin (PTM) weakens the rigor linkages between actin and myosin, and contributes to meat tenderization during postmortem aging (Takahashi *et al*. 1985). PTM was found at the A-I junction of sarcomeres in living muscle and in muscle immediately postmortem, and translocated from its original position to thin filaments by an increase of calcium ion concentration to 0.1 mM during postmortem storage of muscles (Hattori & Takahashi 1988). Purified PTM binds to F-actin (Nakamura & Takahashi 1985) and strongly inhibits the Mg^{2+}-ATPase activity of reconstituted actomyosin and myofibrils (Takahashi *et al*. 1982; Takahashi *et al*. 1987). It weakens rigor tension generated in glycerinated muscle fibers and restores rigor-shortened sarcomeres (Yamanoue & Takahashi 1988).

On the other hand, skeletal muscle tropomyosin (TM) is a regulatory protein of myofibrils and a rod-shaped coiled-coil dimer assembled from highly α-helical monomers (Sodek *et al.* 1972). TM is an integral constituent of thin filament in muscle and actin cytoskeleton (Perry 2001) and regulates muscle contraction with troponin complexes. In multicellular animals, TMs are a family of actin filament binding proteins and they exhibit extensive cell type specific isoform diversity (Lees-Miller & Helfman 1991; Vindin & Gunning 2013). In skeletal muscles TM were separated into two bands, α and β by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE) (Cummins & Perry 1973) and TM dimers bind along the actin filament in a head-to-tail overlap domain with each molecule spanning seven actin monomers (Hitchcock-DeGregori & Varnell 1990; Xu *et al*. 1999), while PTM was electrophoresed as a single band at the same 34-kDa by SDS-PAGE (Takahashi *et al*. 1987).

Physico-chemical properties of PTM are similar to that of TM, for example, content of α- helix, amino acid composition and the same molecular weight by gel filtration chromatography (Takahashi *et al*. 1985; Takahashi *et al*. 1987). However, PTM function is different from TM, so we postulated that functional difference is attributed to structural difference between PTM and TM. The aim of this study was to strictly separate PTM from TM and to analyze the structural difference between PTM and TM, thereby contributing to determine primary structure of PTM and elucidating functional difference of PTM from TM.

MATERIALS AND METHODS

Preparation of Proteins

Proteins were prepared from broiler (*Gallus gallus*) breast muscle immediately after slaughter. Myofibrils were prepared by the method of Perry and Grey (1956). PTM was prepared according to procedure described previously (Takahashi *et al*. 1985). TM was extracted from myofibrils (Scellini *et al*. 2010) and prepared by the method of Bailey (1948).

Purification by high performance liquid chromatography (HPLC)

Prepared PTM and TM were purified by HPLC according to procedure of Lemon *et al*. (2011) with slight modifications. The HPLC system consisted of L-6200 Intelligent Pump and L-6000 Pump, an L-4000 UV Detector (Hitachi, Tokyo, Japan), and a Chromatocorder 12 (System Instruments, Tokyo, Japan) equipped with a Sepax Bio-C18 column (4.6 × 250 mm; Particle size, 3.0 μm; Sepax Technologies, Delaware, USA) or a Zorbax 300SB-C18 column (4.6 × 150 mm; Particle size, 3.5 μm; Agilent Technologies, Tokyo, Japan). Samples from breast muscles were dialyzed against a

92 solution containing of 20% acetonitrile, 0.1% trifluoroacetic acid (TFA), and 8 M urea
93 by Easy Sep (MWC 3,000; Tomy Seiko, Tokyo, Japan) for 3 h under room temperature.
94 The column was equilibrated with 0.1% TFA and 35% acetonitrile at 0.8 ml/min. After
95 loading 30-50 μl of dialyzed samples on the equilibrated columns, purified PTM and
96 TM were eluted by the linear gradient increase of 35-50% acetonitrile at flow rate of 1
97 ml/min during 25 minutes for the Sepax column or 15 minutes for the Zorbax column.
98 Eluted peaks were collected manually at the detector outlet monitoring the absorbance
99 at 215 nm and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis
100 (SDS-PAGE) and mass spectrometry.
101 *Reduction of sulfhydryl bonds and separation of PTM and TM subunits*
102 PTM and TM purified by HPLC were dialyzed against a solution containing 1 M
103 NaCl, 5 mM EDTA, 20 mM Tris-HCl (pH 7.5) for 20 hours at 4°C. After adding a final
104 concentration of 10 mM of dithiothreitol (DTT) powder, PTM and TM stand for 1 hour
105 at room temperature with occasional stirring, then were retained overnight at 4°C. An
106 equal volume of a solution of 1 M NaCl, 200 mM iodoacetamide, 5 mM EDTA, 20 mM
107 Tris-HCl (pH 7.5) was added to samples to prevent re-oxidation of the subunits, and
108 gently shaken at room temperature for 2 hours. After dialying to remove excess
109 iodoacetamide, samples were concentrated using Amicon® Ultra-4 (Merck Millipore,
110 Tokyo, Japan). Reduced and blocked PTM and TM subunits were separated and
111 recovered with HPLC system according to the same method described above.
112 *Mass spectrometry*
113 PTM and TM subunits obtained by HPLC separation were subjected to
114 SDS-PAGE and stained with CBB R-250 (Coomassie Brilliant Blue R-250). After the
115 gel pieces were excised at each band and dried completely, in-gel digestion was

116 performed using the In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific, Yokohama,
117 Japan) at 37°C for 30 minutes according to the product instructions. Alkylated and
118 digested peptides were analyzed by LC/MS system, which consisted of the Nanoflow
119 HPLC system (Paradigm MS2; AMR, Tokyo, Japan) equipped with a Zaplous column
120 αPep-C18 (0.1 x 150 mm; Particle size, 3 μm; AMR) and the LTQ Orbitrap Discovery
121 (Thermo Fisher Scientific, Yokohama, Japan). Proteins were identified in the Swiss-Prot
122 database (UniProt Consortium) using the MASCOT software (Matrix Science, Tokyo,
123 Japan).

124 *Internal amino acid sequence analysis*

125 The obtained PTM subunits were digested with V8 protease (Roche-Diagnostics,
126 Tokyo, Japan) at 37°C for 24 hours. The resulting peptide mixtures were concentrated
127 by a Micro Vac (MV-100; Tomy Seiko, Tokyo, Japan) and separated by HPLC with a
128 Zorbax 300SB-C18 column. Column was equilibrated with 0.1% TFA in MilliQ water.
129 Flow late was 0.6 ml/min. After loading 30-50 μl of sample, gradient elution was
130 performed by the linear increase in 0-80% acetonitrile with 0.1% TFA in 120 min.
131 Separated peptide fragments were collected and concentrated using a Micro Vac. The
132 peptide fragments were applied to a protein sequencer (Model 492; Applied Biosystems,
133 Foster City, CA, USA) to determine amino acid sequences by the method of Edman
134 (1950).

135 *SDS-PAGE*

136 SDS-PAGE was carried out by the method of Laemmli (1970) using 12%
137 polyacrylamide separating gel. Sample was prepared by adding a solution of 2%
138 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue, 4% SDS, 0.02% sodium
139 azide, 0.1 M Tris-HCl (pH 6.8) and heated for 1 minute at 97 °C. Twenty microliter of

each sample were applied to wells and electrophoresised at 30 mA in a separating gel. The gel was stained with CBB or silver staining (Yan *et al*. 2000).

Determination of protein concentration

Protein concentration of PTM and TM were determined by applying $E_{1\%}$ 277 = 5.56 and 3.17, respectively by ultraviolet absorption method (Takahashi *et al*. 1985). Protein concentration of purified PTM and TM subunits were determined by BCA protein assay (Smith *et al.* 1985) with Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan) using BSA as a standard.

RESULTS AND DISCUSSION

Preparation of Proteins

Figure 1 shows that both PTM fraction prepared according to the procedure described by Takahashi *et al*. (1985) and TM fraction prepared by the method of Bailey (1948) were mainly electrophoresed at the same 34-kDa by SDS-PAGE and these results are consistent with the results of previous reports (Cummins & Perry 1973; Takahashi *et al*. 1985). But, prepared PTM fraction (Fig. 1b, an arrow) seemed to slightly contain TM, because TMβ subunit (an arrowhead) with low mobility appeared as a upper band of PTM. On the other hand, prepared TM fraction (Fig. 1c) was mainly composed of TMα subunit and contained concomitantly TMβ subunit similarly to a study of Cummins and Perry (1973). PTM fraction prepared by the method of Takahashi *et al*. (1985) sometimes includes TM as an impurity, because physico-chemical properties of PTM are similar to that of TM. Therefore, the PTM fraction needs further purification to remove TM for subsequent analysis.

Purification of PTM by HPLC

In order to remove TM from prepared PTM fraction, we adapted HPLC method equipped with reversed-phase columns (Figs. 2 & 3). Separation of PTM fraction using a Sepax Bio-C18 column resulted in major four peaks and the most dominant peak (a) that eluted at 18.6 minutes of retention time (RT) was considered to be PTM (Fig. 2A). In the other peaks, elution of peaks (b), (c), and (d) from column were at 20.0, 23.0, and 23.5 minutes, respectively. The major four peaks separated by HPLC were collected and analyzed by SDS-PAGE (Fig. 2B). The bands of all peaks were electrophoresed at 34-kDa except for the bands of lane (b) from peak (b), which appeared in both upper and lower bands. By mass spectrometry of each band of lanes (b) and (d) from peak (d), it was revealed that similarities in amino acid sequences of upper and lower bands of lane (b) were 72% of TMβ (NCBI database: P19352) and 82% of TMα1 (NCBI database: P04268), respectively, and that of the band of lane (d) was 83% of TMα1 (data not shown). Trypsin cleaves carboxyl sides of lysine and arginine residues of proteins. There are several regions in rich of lysine and arginine residues in above TM subunits, so it is considered that LC-mass spectrometry was not able completely to identify the sequences of very short fragments produced from such regions by trypsin digestion. Thus, we concluded that peaks (b) and (d) separated by HPLC were TMαβ and TMαα isoforms concomitant with preparation of PTM, respectively.

The results described above suggest that PTM is different from TM. To confirm the difference between PTM and TM, purified PTM recovered from peak (a) shown in Figure 2A and TM prepared from breast muscle were separated by HPLC equipped with a Zorbax 300SB-C18 column (Fig. 3). The RT of peak (a) in Figure 3A was 12.0 minutes. In prepared TM fraction, the RTs of peaks (c) and (d) were 12.0 and 14.9 minutes, respectively (Fig. 3B). All Bands of separated peaks were migrated at 34-kDa

188 by SDS-PAGE (Fig. 3C, D). In this TM fraction, TMαα with migration of 34-kDa was
189 main isoform and two bands of TMαβ isoform were not appeared differently from the
190 result of Figure 1c, because this result is in agreement with the report of Roy *et al*.
191 (1976), but does not support the report of Cummins and Perry (1973) that both subunits
192 are present in TM prepared from chicken breast muscle. Since the peaks (a) and (c)
193 eluted at the same RT, it could be considered that small amounts of PTM (peak c) were
194 included in TM fraction during preparation. Thus, purified PTM was clearly separated
195 from TM by HPLC method, indicating that PTM is different molecule from TM.

196 ***Separation of PTM and TM to two subunits***

197 Considering the similarity of physico-chemical properties of both PTM and TM
198 molecules, TM has been reported as a strand structure of α-helical coiled-coile with two
199 subunits (Squire & Morris 1998) and PTM is also expected to consist of two subunits.
200 Therefore, PTM and TMαα molecules purified by HPLC were reduced with DTT and
201 separated by HPLC equipped with a Sepax Bio-C18 column (Fig. 4A, B). Separation of
202 reduced PTM by HPLC resulted in appearance of two peaks, namely subunits 1 (peak a)
203 and 2 (peak b). RTs of subunits 1 and 2 were 23.0 and 24.0 min, respectively. On the
204 other hand, reduced TM was separated to two peaks and those RTs were 23.5 (peak c)
205 and 24.9 (peak d) min, respectively (Fig. 4B). Peak (c) seemed to be a TMαα molecule
206 that was not reduced to monomer of TMα subunit, because the peak (c) and peak (d)
207 shown in Fig. 2A were eluted at the same RT of 23.5 minutes by HPLC separation with
208 a Sepax Bio-C18 column.

209 All bands of PTM and TM subunits were electrophoresed at 34-kDa by
210 SDS-PAGE (Fig. 4C, D). Those bands were digested by trypsin and then analyzed by
211 mass spectrometry. The similarities of PTM subunit 1 and 2 to TMα1 isoform were 45%

and 69%, respectively, but both TM bands of lanes (c) and (d) shown in Figure 4D were 80% and 77% similarities in amino acid sequence of TMα1 isoform, respectively (data not shown). The similarities of TM bands slightly decreased in comparison with the result of lane d in Figure 2B because of less amounts of applied TM samples to mass spectrometry. These results suggest that PTM consists of two subunits with some structural difference and both subunits seem to be distinct from TMα1 isoform.

Internal amino acid sequence analysis

After each PTM subunit was recovered by HPLC method and digested in solution by V8 protease, the produced peptides were separated again by HPLC. Then, amino acid sequences of peptides were analyzed by protein sequencing. As the result, we observed different elution patterns on reversed-phase HPLC between digested subunits 1 and 2 (Fig. 5A, B), for example, three peaks (arrows, a, b, d) were appeared at different RTs of 30.5, 47.0, 29.0 minutes, respectively. Amino acid sequences of these peaks are shown in Figure 5C. Both peaks (c) and (e) were eluted at the same RT of 49 minutes, indicating that both peptides were identical (Fig. 5C) and produced from the same sequences in PTM subunits 1 and 2, corresponding to amino acid residues 43-52 of TMα1. Peptide of peak (d) eluted at RT 29.0 minutes was 80% similarity to amino acid residues 73-82 of TMα1 isoform. Peak (b) was detected only in subunit 1 (Fig. 5A). In subunit 1, amino acid replacement occurred from Met to Lys at residue 281 of TMα1 isoform (Fig. 5C-b). Since the replacement did not match any parts of sequences in TMα1 isoform, this amino acid residue is possible to compose inherent sequence of PTM. From the results described above, it was clarified that PTM molecule was composed of two different subunits with the same 34-kDa masses and that both subunits strictly differed from TMα1 isoform.

In recent study, the Tpm1.1 (α) and Tpm2.2 (β) isoforms of TM have been compared in terms of their end-to-end association, interaction with troponin, and thin filament activation of myosin (Lohmeier-Vogel & Heeley 2016). The results suggested that all of the issues were different between each TM isoform and the differences were derived from replacements of amino acids at the C-terminal end including His276Asn and Met281Ile. In particular, amino acid residue 281 is important to the interaction between N-terminal and C terminal for overlap in TM (Greenfield *et al*. 2006). Thus, the replacement of amino acid residue occurred in subunit 1 is highly probable to effect on PTM function different from that of TM.

CONCLUSION

In this study, we succeeded in remove TM from prepared PTM fraction by HPLC method. Also two subunits of PTM named subunits 1 and 2 were successfully separated and recovered. Both of two subunits were migrated at 34-kDa by SDS-PAGE, having different RTs on separation by HPLC. The results of mass spectrometry by trypsin digestion and protein sequencing by V8 protease digestion of subunits 1 and 2 clarified that PTM was strictly differed from TM and that both subunits 1 and 2 seemed to be distinct from TMα1 isoform, which is the major constituent with a mass of 34-kDa of TM molecule. These findings are very useful to determine primary structure of PTM molecule and then to elucidate the reason for the functional difference between PTM and TM.

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FIGURE LEGENDS

Figure 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns stained by CBB R-250 of myofibrils, PTM, and TM prepared from chicken breast muscle. Electrophoresis was performed at constant-current of 30 mA using a 12% polyacrylamide gel. Lanes MW, marker; a, myofibrils; b, PTM; c, TM.

Figure 2 Elution profile of prepared PTM fraction by HPLC using a Sepax Bio-C18 column and SDS-PAGE patterns of each separated peak. (A), an elution profile of peaks (a), (b), (c), and (d) at retention times (RTs) of 18.6, 20.0, 23.0, and 23.5 minutes, respectively; (B), SDS-PAGE patterns of peaks (a), (b), (c), and (d) recovered from the separation by HPLC.

Figure 3 Elution profiles of separation of purified PTM and prepared TM fraction by HPLC using a Zorbax 300SB-C18 column and SDS-PAGE patterns of each separated peak. (A), an elution profile of PTM separated to peaks (a) and (b) at RT 12.0 and 14.9 minutes, respectively; (B), an elution profile of TM separated to peaks (c) and (d) at RT 12.0 and 14.9 minutes, respectively. Each peak fraction was collected and analyzed by SDS-PAGE. (C), SDS-PAGE patterns of peaks (a) and (b) recovered from the re-separation of PTM by HPLC; (D), SDS-PAGE patterns of peaks (c) and (d) recovered from the separation of TM by HPLC.

Figure 4 Elution profiles of reduced PTM and TM subunits by HPLC using a Sepax Bio-C18 column and SDS-PAGE patterns of each separated peak. (A), an elution profile of PTM subunits separated to peaks (a) and (b) at RT 23.0 and 24.0 minutes, respectively; (B), an elution profile of TM subunits separated to peaks (c) and (d) at RT 23.5 and 24.9 minutes, respectively. Each peak fraction was collected and analyzed by SDS-PAGE. (C), SDS-PAGE patterns of peaks (a) and (b) recovered from the separation of PTM subunits by HPLC; (D), SDS-PAGE patterns of peaks (c) and (d) recovered from the separation of TM subunits by HPLC.

Figure 5 Elution profiles of digested PTM subunits by HPLC using a Zorbax

300SB-C18 column and amino acid sequences of recovered five peaks. After digestion of PTM subunits 1 and 2 by V8 protease, each subunit was separated by HPLC and analyzed by protein sequencing. (A), an elution profile of digested subunits 1 and recovered peaks a-c (arrows); (B), an elution profile of digested subunits 2 and recovered peaks d and e (arrows); (C), amino acid sequences of recovered five peaks a-e shown in Fig. 5A and 5B.

Figure 1

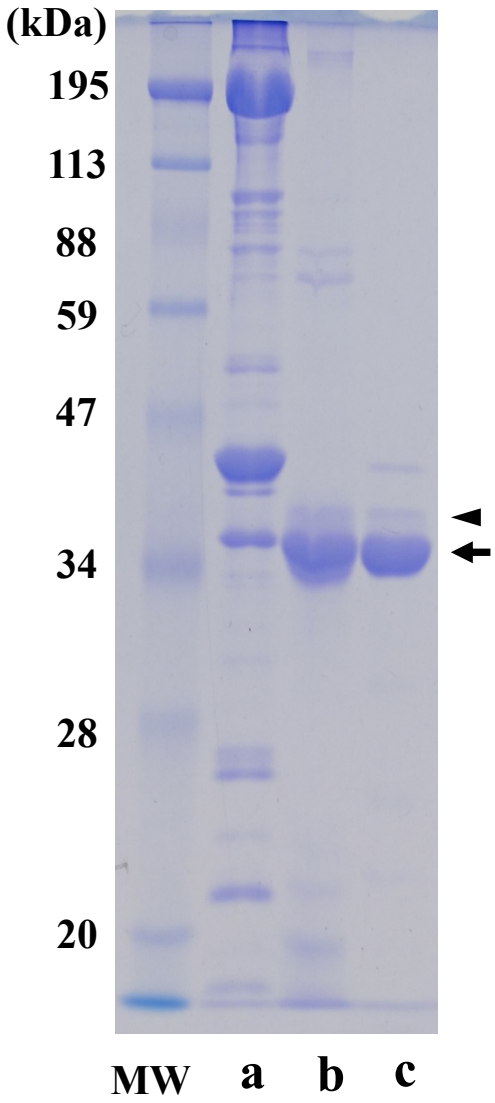


Figure 2

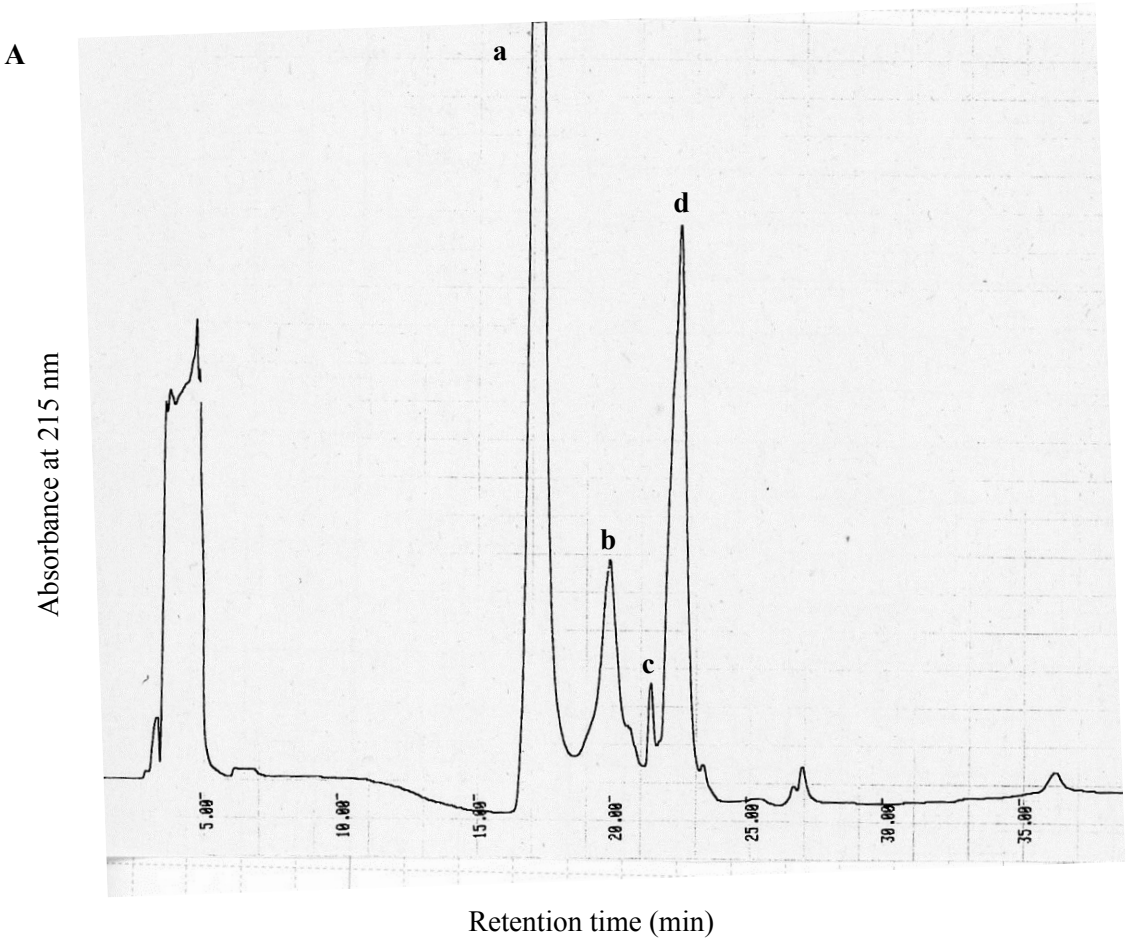

A
a
b
c
d
Absorbance at 215 nm
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Retention time (min)
B
MW
a
b
c
d
34-kDa

Figure 3

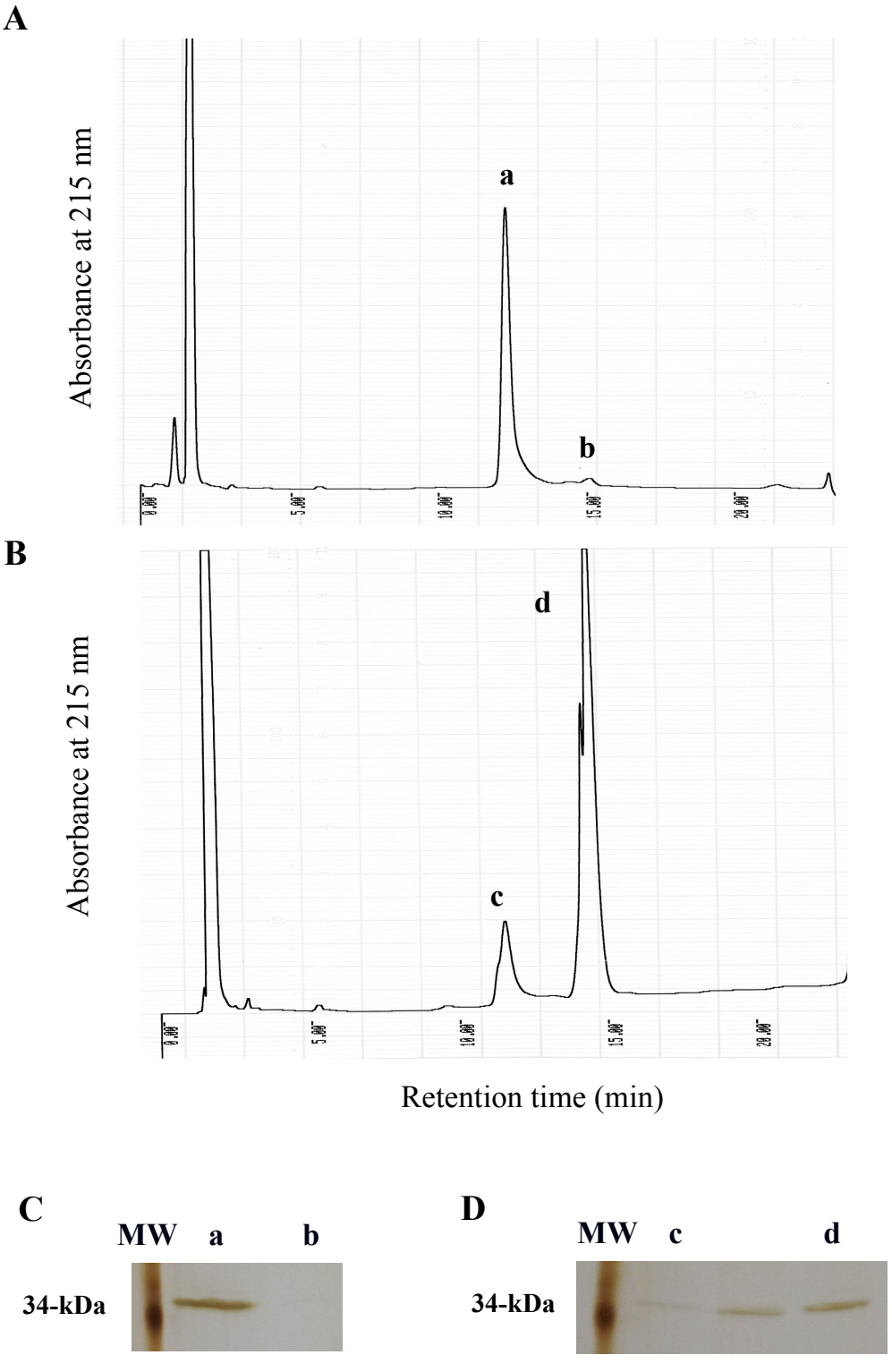

A
Absorbance at 215 nm
a
b
B
Absorbance at 215 nm
d
c
Retention time (min)
C
MW
a
b
34-kDa
D
MW
c
d
34-kDa

Figure 4

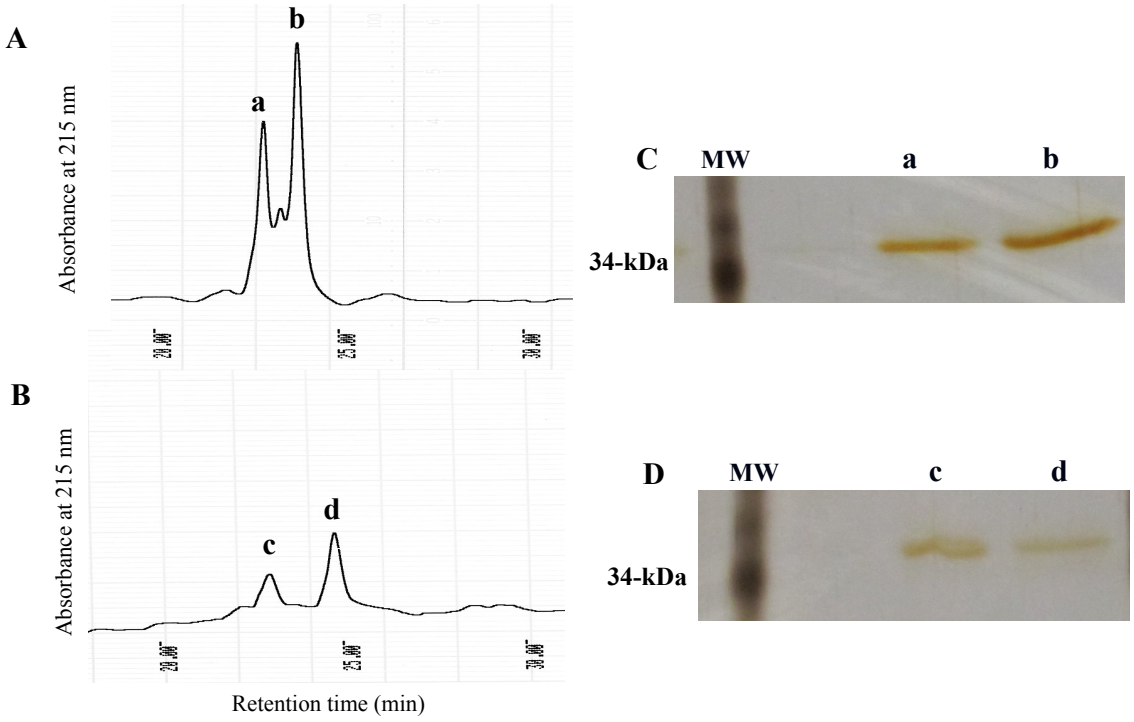
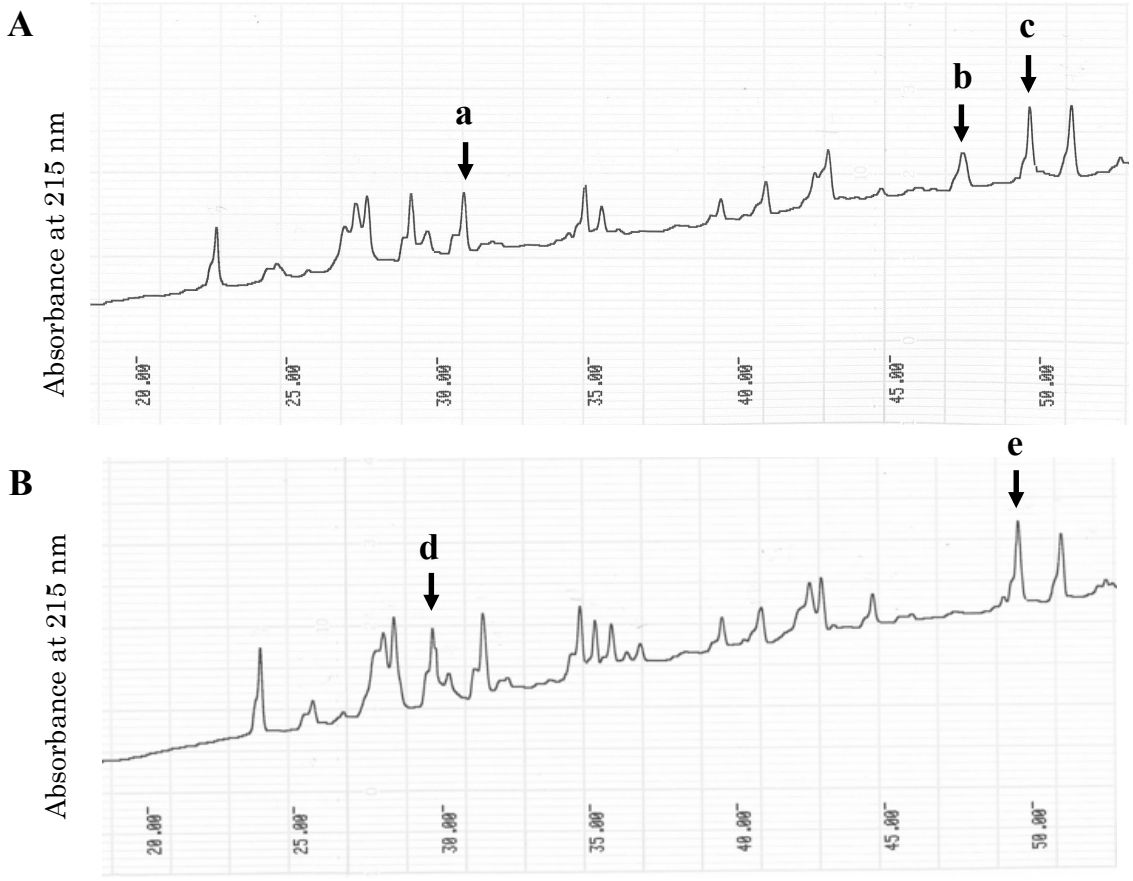

A
a
b
Absorbance at 215 nm
B
c
d
Absorbance at 215 nm
Retention time (min)
C
MW
a
b
34-kDa
D
MW
c
d
34-kDa

Figure 5



C

peak	amino acid sequence
a	LAKKKATDAE
b	LDHALNDKTS
c	LVALQKKLKG
d	LADQLATDAE
e	LVALQKKLKG