ANALYSIS OF OXIDIZED METHIONINE IN MYOSIN ISOFORMS OF PORCINE *LONGISSIMUS THORACIS* MUSCLE AT 24H POSTMORTEM

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Abstract – This study was conducted to analyze the methionine oxidized at 24h postmortem in the myosin heavy chain (myosin isoform) protein of porcine skeletal muscle. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify the oxidized methionine by detection of monoisotopic mass changes (15.9994amu). At 24h postmortem, 5.8%–19.2% of total methionine composed of myosin isoforms was modified. Myosin-7 showed the lowest level of methionine oxidation among the myosin isoforms, due to its low sequence coverage and content in porcine skeletal muscle. Therefore, these results indicate that methionine oxidation could be related to type and composition of myosin isoforms.

Key Words – Methionine oxidation, myosin, LC-MS/MS.

I. INTRODUCTION

Oxidation is inevitable in meat and meat products, and results in meat quality deterioration, such as rancidity and discoloration. The reactive species, including reactive oxygen species and reactive nitrogen species, various oxidative products, and transition ions, are considered major factors for oxidation in meat and meat products [1, 2]. Meat proteins undergo denaturation and proteolysis during postmortem, processing, and storage. Thus, control technologies to delay or prevent oxidation in meat and meat products are needed. From this point of view, to understand the various protein oxidation mechanisms in meat systems is important. Processes such as carbonylation, metal ion-catalyzed oxidation, cross-linking, hydroxylation, sulfoxidation, and nitrosylation, as well as products of modification, have been defined in previous reports [3, 4]. Among the products of modification, methionine sulfoxide and methionine sulfone result from methionine oxidation. Methionine is an amino acid that is very sensitive to reactive oxygen species [5]. Therefore, in the present study, oxidized methionine of myosin protein from pork loin at 24h postmortem was analyzed. Though various technologies could be applied to the analysis of methionine oxidation, LC-MS/MS analysis was used in this study.

II. MATERIALS AND METHODS

Sample preparation-Three muscles (*longissimus thoracis*) were taken from porcine carcasses (180 days old, castrated males) between the 4th and 5th *thoracic vertebrae* at 24h postmortem in a commercial slaughtering house. For exclusion of PSE (pale, soft, and exudative) and DFD (dark, firm, and dry), muscle pH (5.68±0.02), lightness (CIE L*) value (49.20±1.13), and drip loss (2.42±0.30) were checked using a pH-meter (MP230; Mettler-toledo, Greifensee, Switzerland), colorimeter (CR-300; Minolta Co., Tokyo, Japan), and plastic bag, respectively. Muscle samples were homogenized in lysis buffer consisting of 7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT, and 30 mM Tris with a protease inhibitor cocktail solution (GE Healthcare, Sweden) using a polytron homogenizer (IKA Labortechnik T25-B; Selangor, Malaysia) at 3,000 rpm for 20 s. The homogenates were centrifuged at 17,000 × g at 4°C for 50 min and the supernatants containing fat were removed. Eluted fractions were precipitated by TCA/acetone precipitation. Pellets were then mixed with buffer containing 10% v/v mercaptoethanol, 4% w/v SDS, 1 M Tris-HCl, 20% w/v glycerol, and 0.2% w/v bromophenol blue, and the protein concentration was adjusted to 1.0 mg/ml by using the Bradford [6] method with BSA as a standard.

Gel electrophoresis and in-gel digestion-Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was conducted in gels composed

of 0.1 M glycine, 0.4% (w/v) SDS, 0.2 M Tris-HCl (pH 8.80), 10% (w/v) acrylamide/Bis (50:1), and 45% (w/v) glycerol. The myosin heavy chain (myosin isoforms) bands were de-stained with 50 mM NH_4HCO_3 buffer (pH 7.8) containing 30% (v/v) acetonitrile, and dried completely in a SpeedVac (SPD1010; Thermo Fisher Scientific Inc., USA) for 10 min. The dried gel pieces were rehydrated in 10 µL (2.5 ng/µL) of trypsin solution (Promega, UK) in 50 mM NH_4HCO_3 buffer (pH 7.8) at 4°C for 2 h. After 10 µL of 50 mM NH_4HCO_3 was added, the gel slices were incubated at 37°C for 12 h.

LC-ESI/MS and data analysis-A nano-LC and LTQ mass spectrometer (Agilent 1100; Thermo Electron, USA) was used. Mass spectra were acquired using data-dependent acquisition with a full mass scan (400–1800 m/z) followed by MS/MS scans. Sequences of the MS/MS spectra were identified by an NCBInr database search using the MASCOT search engine (Matrix Science MASCOT software). The queries having >23 ion scores were identified (p<0.05).

III. RESULTS AND DISCUSSION

Four myosin isoforms (myosin-1, -2, -4, and -7) were identified by analysis of LC-ESI/MS as shown in Table 1.

Table 1. Myosin isoforms identified by LC-MS/MS

Myosin isoform	Accession number[a)]	MW (Da)[b)]	Size (aa)[c)]	Sequence coverage (%)	Queries matched	Queries with oxidized M[d)]
1	gi|5360750	2240841939	57	298	6	
2	gi|5360746	2240611939	48	243	6	
4	gi|5360748	2241461937	60	345	6	
7	gi|120604892	2240951935	38	150	2	

Data are means.
[a)] Accession numbers were taken from the NCBI database.
[b)] Theoretical molecular weight.
[c)] Peptide size (amino acids) recorded in NCBI database.
[d)] Methionine.

The matched queries with four individual sequences of myosin isoforms ranged from 150 to 345 and the sequence coverage (%) was 57, 48, 60, and 38 for myosin-1, -2, -4, and -7, respectively. Among the queries matched, 20 were identified as peptides with oxidized methionine (Met). The compositions of Met in total amino acid sequences of myosin isoform were 2.53% (49), 2.53% (49), 2.68% (52), and 2.69% (52) for myosin-1, -2, -4, and -7, respectively. 8, 8, 10, and 3 Mets were modified and they occupied 16.3%, 16.3%, 19.2%, and 5.8% of total Met in each myosin isoform (myosin-1, -2, -4, and -7, respectively). These results could be related to the composition of myosin isoforms in porcine muscle. The content of slow myosin isoform (-7) is lower than that of fast myosin isoform (-1, -2, and -4) in porcine muscle [7].

The MS/MS spectrum of ^{1098}IEDEQALAMQLQK^{1110} modified at Met-1106 is shown in Fig. 1. The fragment at m/z 767.25 (2+) was identified as ^{1098}IEDEQALAMQLQK^{1110} and this fragment corresponded to the fragment at m/z 759.05 (2+). The mass difference (15.9994amu) is observed in both b(9)- and y(5)-fragment ions. Therefore, Met sulfoxide was formed by Met oxidation.

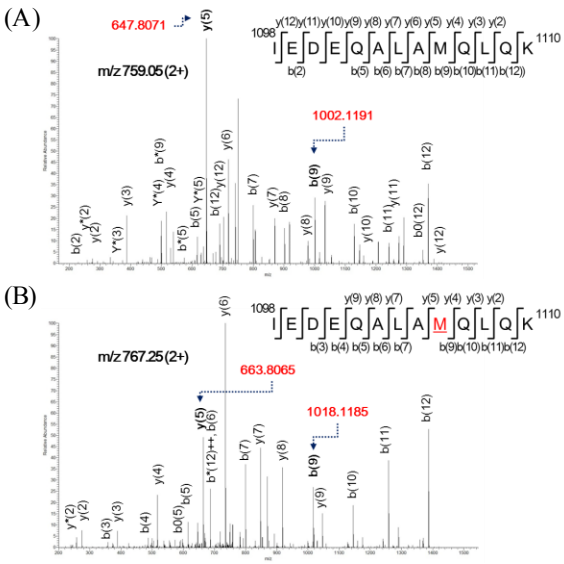

Fig. 1. LC-MS/MS results of methionine-oxidized myosin tryptic peptide (IEDEQALAMQLQK, residues 1098-1110 of myosin-1) from the porcine *longissimus thoracis* muscle. (A) spectrum of unmodified peptide; (B) spectrum of oxidized peptide at Met-1106 (M).

^{881}MVTLLKEK^{888}, ^{1582}DEEIDQMKRNHIR^{1594}, and ^{1832}RNAESVKGMR^{1841} were identified with modification at Met-881, -1589, and -1840 of myosin-2, -4, and -7, respectively (Table 2). These queries are unique peptides composed of three myosin amino acid sequences. The remaining

seven queries are common peptides existing in two or more myosin isoforms. Thus, just 10 queries were identified as peptides with oxidized Met. The neighboring amino acids can affect oxidation susceptibility. Met surrounded by acidic amino acids such as alanine, threonine, and serine is more susceptible to oxidation than Met surrounded by basic amino acids [5]. In the present study, three Mets were located at sites with nearby acidic amino acid (alanine).

Table 2. Methionine-oxidized peptides identified from the porcine myosin isoforms by LC-MS/MS

Sequences	Mr (expt)	Mr (calc)	Myosin isoform	Residues
IEDEQALAMQLQK	1532.87	1532.72	myosin-1	1098-1110
(*m/z* 767.44)			myosin-4	1096-1108
DEEIDQMKRNHR	1700.09	1699.84	myosin-4	1582-1594
(*m/z* 567.70)				
MFLWMVTR	1099.85	1099.37	myosin-1	438-445
(*m/z* 550.93)			myosin-2	438-445
			myosin-4	438-445
			myosin-7	435-442
MQGTLEDQIISAN	2476.90	2476.76	myosin-1	215-237
PLLEAFGNAK			myosin-2	215-237
(*m/z* 826.64)			myosin-4	215-237
MVTLLKEK	978.13	977.30	myosin-2	881-888
(*m/z* 490.07)				
NLTEEMAGLDET	1650.45	1650.80	myosin-1	981-995
IAK			myosin-2	981-995
(*m/z* 551.16)				
RNAESVKGMR	1163.34	1163.31	myosin-7	1832-1841
(*m/z* 388.79)				
SAETEKEMANM	2062.78	2063.22	myosin-1	846-862
KEEFEK			myosin-4	844-860
(*m/z* 688.60)				
ELEEISERLEEAG	2651.95	2650.82	myosin-1	1146-1169
GATSAQIEMNK			myosin-2	1146-1169
(*m/z* 884.99)			myosin-4	1144-1167
MKKNMEQTVK	1253.55	1252.51	myosin-2	1786-1795
(*m/z* 418.86)			myosin-4	1784-1793

M on the bar indicates oxidized methionine.

IV. CONCLUSION

Formation of Met sulfoxide (addition of an oxygen) was identified by confirmation of monoisotopic mass change (15.9994amu). Met oxidations ranging from 5.8% to 19.2% were found among the total Mets composed of each myosin isoform. For myosin-7, a relatively small quantity of queries was matched to amino acid sequences and the smallest number of oxidized Mets was detected among the myosin isoforms. These results indicate that Met oxidation could be related to type and composition of myosin isoforms.

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