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Identification of ubiquitin conjugated Protein Phosphatase Inhibitor 1 from postmortem bovine muscle

Purevdorj Nyam-Osor, Barana Jayawardana, Juan Marcos Aro Aro, Ken-ichiro Shimada, Michihiro Fukushima, Mitsuo Sekikawa *

Graduate School of Animal and Food Hygiene, Department of Agriculture and Life Science, Obihiro University of Agriculture and Veterinary Medicine Inada-Machi, Obihiro, Hokkaido 080-8555, Japan

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ABSTRACT

Previously, we characterised the presence of ubiquitin and some ubiquitinated proteins (conjugates), which were considered as ubiquitin conjugates in the sarcoplasmic fraction of postmortem skeletal and cardiac muscles using SDS–PAGE and Western blot. In this study, an ubiquitin conjugate around 27 kDa was subjected to identification. The N-terminal amino acid sequence analysis generated by Edman degradation resulted in a 13 amino acid sequence (KIQFTVPLLEPHL), which is similar to that of Protein Phosphatase Inhibitor 1. Based on this result, Western blot analysis was performed by using a polyclonal antibody for Protein Phosphatase Inhibitor 1 and the conjugate was recognised and reacted clearly. Thus, the ubiquitinated protein around 27 kDa is identified as Protein Phosphatase Inhibitor 1.

1. Introduction

Skeletal muscle is believed to contain at least five proteolytic pathways (i.e. the lysosomal, the Ca^{2+}-activated and the ubiquitin–proteasome-dependent systems, caspases and metalloproteinases). Unfortunately, the precise substrates of these systems are poorly characterised (Taillandier et al., 2004). The ubiquitin–proteasome system, consisting of ATP, proteasomes and ubiquitin, plays an important role in the degradation of muscle proteins under various catabolic conditions (Taillandier et al., 1996). Studies to identify specific proteolytic pathways that are stimulated in experimental animal models of muscle atrophy have repeatedly demonstrated involvement of the ATP-dependent, ubiquitin–proteasome pathway (Price, 2003). In this pathway, ubiquitin, containing 76 amino acids, is conjugated to proteins destined for degradation (Hershko & Ciechanover, 1998). Ubiquitin conjugates are recognised by a 26S proteasome complex composed of two subproteasome complexes, a 19S regulatory particle, and a 20S catalytic particle. Ultimately, the ubiquitinated proteins (conjugates) are rapidly degraded by proteasome in an ATP-dependent manner, and the released peptides are further degraded into amino acids in the cytoplasm (Lin, Chen, Lee, Huang, & Sheu, 2005). In brief, the involvement of ubiquitin–proteasome pathway in muscle protein breakdown in many catabolic conditions is well documented, but the substrate or conjugate identification under those conditions remains obscure.

In our previous studies (Sekikawa, Seno, & Mikami, 1998; Sekikawa et al., 2000; Sekikawa, Yamamoto, Fukushima, Shimada, & Mikami, 2001), it was clearly characterised that the proteins of postmortem skeletal and cardiac muscle sarcoplasmic fractions contain ubiquitin (8.6 kDa) as an important component and some small amounts of other higher-molecular-mass proteins, which were considered to be ubiquitin conjugates. Identification and characterisation of ubiquitin conjugate from muscles can be vital to meat science, implicating antemortem muscle physiological status and other molecular biological studies. In some preliminary studies, a protein around 27 kDa, which is considered as an ubiquitin conjugate was prominently and frequently observed (Sekikawa et al., 1998; Sekikawa et al., 2001). Therefore, this study aimed to identify this conjugate protein, which is found in postmortem muscle.

2. Materials and methods

2.1. Sampling

Muscle samples (*M. quadriceps femoris*) were obtained from five Holstein Friesian cows. Early postmortem muscle tissues were ground, boiled for 5 min with thrice their weight of distilled water and allowed to cool to room temperature. Homogenisation was performed by an ultra high speed homogenizer (Physcotron NS-50, Niti-On, Tokyo, Japan). The homogenates were centrifuged

* Corresponding author. Tel.: +81 155 49 5770; fax: +81 155 49 5775.
E-mail address: sekikawa@obihiro.ac.jp (M. Sekikawa).

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under refrigeration (0 °C, 6000×*g*, 30 min) to obtain a pellet and a clear supernatant. The supernatant was dialysed against distilled water for 48 h and freeze dried.

2.2. N-terminal amino acid sequencing

Proteins of the sarcoplasmic fraction were separated by preparative 2-D gel electrophoresis, and blotted to polyvinylidene difluoride (PVDF) membranes by using 10% CAPS buffer. The PVDF membrane was stained with Ponceau S as described by Aebersold, Leavitt, Saavedra, Hood, and Kent (1987). Protein spot of interest was estimated from Western blots with ubiquitin antibody and excised. Amino acid sequence analysis for N-terminal residues was performed by Edman degradation using a protein sequencer (AB 470, Applied Biosystems, Foster city, CA, USA).

2.3. Western blotting

Based on amino acid sequence analysis, the Western blot was performed by using a polyclonal antibody of Protein Phosphatase Inhibitor 1 for the demonstration of the conjugate identification. An antibody for Protein Phosphatase Inhibitor 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which was developed in goat, was used as primary antibody and biotinylated anti-goat antibody (VECTORSTAIN® ABC kit, Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody.

3. Results and discussion

3.1. N-terminal amino acid sequencing

A 13 amino acid sequence (KIQFTVPLLEPHL) resulted from the N-terminal amino acid sequence generated by Edman degradation. When this sequence was compared with the ubiquitin reported previously (Schlesinger, Goldstein, & Niall, 1975) there was no correlation indicating that it is an ubiquitin conjugate. The obtained sequence was matched against the SWISS-PROT database. The output of the predicted N-terminal sequence was identical to that of Protein Phosphatase Inhibitor 1 (I-1). The sequence identity of the obtained 13 amino acid residue was exactly matched to amino acid residues from 9 to 22 of the I-1 of rat, mouse, human, dog and rabbit (Fig. 1). This result strongly suggests that the ubiquitin conjugate around 27 kDa in postmortem muscle is I-1. Therefore, to demonstrate the identification of this conjugate, the Western blot analysis was performed, subsequently.

3.2. Western blotting

The proteins of sarcoplasmic fraction were resolved on 15% SDS–PAGE and transferred to PVDF membrane, and the same membrane strips were incubated with ubiquitin and I-1 antibody, separately. The protein band, which was considered as ubiquitin conjugated I-1, was reacted with both antibodies. The ubiquitin conjugate was clearly recognised by the I-1 antibody and was recognised by the ubiquitin antibody (Fig. 2). On the peptide mass database, SWISS-PROT, the molecular weights of rat, mouse, human, dog, and rabbit I-1 are given as 18.73, 18.03, 18.71, 18.39 and 18.99, respectively. On average, ubiquitin and I-1 were around 8.6 and 18.4 kDa, respectively, according to their molecular weights. Therefore, it is logical to assume that when ubiquitin was attached to I-1, the conjugate was around 27 kDa. Together, these results confirmed that the ubiquitin conjugate around 27 kDa was Protein Phosphatase Inhibitor 1.

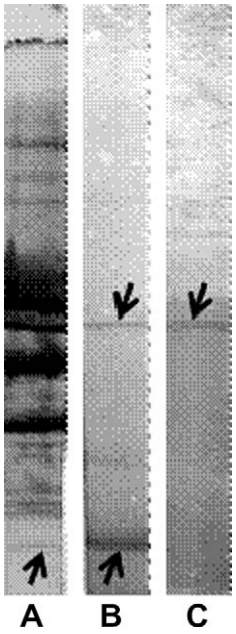

Fig. 2. Detection of ubiquitin conjugated I-1. (A) Protein staining of sarcoplasmic fraction. (B) Western blot with ubiquitin antibody. (C) Western blot with I-1 antibody. Arrows directing up are point ubiquitin band and down are point ubiquitin conjugated I-1 band.

I-1 was first identified from rabbit skeletal muscle in 1976 as an inhibitor of Protein Phosphatase 1 (PP1) and as a regulator of glycogen metabolism (Huang & Glinsmann, 1976). I-1 specifically inhibits PP1 when it phosphorylated by cAMP-dependent protein kinase (PKA) (Aitken, Bilham, & Cohen, 1982; Foulkes, Strada, Henderson, & Cohen, 1983; Huang & Glinsmann, 1976; Sikes & Shenolikar, 2005). The role of I-1 in regulating PP1 function has been investigated in many different physiological settings. These include the hormonal control of glycogen metabolism, synaptic plasticity controlled by neurotransmitters, growth of pituitary tumour cells and the control of muscle contraction (Oliver & Shenolikar, 1998). However, it is unknown yet what is the physiological meaning of this conjugate and the change in the function of I-1 which triggers the ubiquitin attachment to this protein labelling for targeted degradation through the ubiquitin proteasome system in muscle.

In conclusion, the ubiquitin conjugate around 27 kDa, which is found in the sarcoplasmic fraction of postmortem muscle, was identified as Protein Phosphatase Inhibitor 1 based on the amino sequence and Western bolt analysis. Its importance as an ubiquitin conjugate found in muscles remains unknown. Therefore, further studies on the characterisation of this conjugate's expression are needed. Currently, we are studying the relationship between the conjugate expression and muscle carbohydrate metabolism, considering the preslaughter stress which influences the amount of muscle glycogen stores at the time of slaughter, since I-1 is one of the important regulators of glycogen metabolism.

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        1       9           22                              50
Rat     MEPDNSPRKIQFTVPLLEPHLDPEAAEQIRRRRPTPATLVLTSDQSSPEV....
Mouse   MEPDNSPRKIQFTVPLLEPHLDPEAAEQIRRRRPTPATLVLTSDQSSPEI....
Human   MEQDNSPRKIQFTVPLLEPHLDPEAAEQIRRRRPTPATLVLTSDQSSPEI....
Dog     MEQDNSPRKIQFTVPLLEPHLDPEAAEQIRRRRPTPATLVLTSDQSSPEI....
Rabbit MEQDNSPRKIQFTVPLLEPHLDPEAAEQIRRRRPTPATLVLTSDQSSPEV....
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Fig. 1. Sequence alignment of rat, mouse, human dog, and rabbit I-1 (first 50 amino acid residues are shown). The 13 amino acid sequence obtained in this study is highlighted.

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