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Systematic identification of mitochondrial lysine succinylome in silkworm (*Bombyx mori*) midgut during the larval gluttonous stage

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ABSTRACT

Lysine succinylation is a newly identified protein post-translational modification (PTM) of lysine residues. Increasing evidences demonstrate that this modification is prevalent in mitochondria and regulates many vital cellular processes, especially metabolism. Here, we determined the succinylome of the silkworm (*Bombyx mori*) midgut mitochondria during the larval gluttonous stage (the fifth instar) using succinylated peptides enrichment coupled with nano HPLC/MS/MS. A total of 1884 lysine succinylation sites on 373 mitochondrial proteins were identified. The bioinformatic analysis reveal that succinylated proteins are significantly enriched in central metabolic processes and mitochondrial protein synthesis. Several apoptosis and detoxification related enzymes or proteins are succinylated. The findings suggest the crucial role of lysine succinylation in silkworm midgut metabolism and resistance. Our data provide a rich resource for further analysis of lysine succinylation in silkworm.

Significance: Insect midgut is the vital tissue for nutrient metabolism and also for xenobiotic metabolism. There is a growing body of knowledge on regulation of midgut function at the gene or protein levels in silkworm, however, the regulation at post-translation modification level remains largely unknown. We provide a first global analysis of the mitochondrial lysine succinylome in silkworm midgut. A total of 1884 lysine succinylation sites on 373 mitochondrial proteins were identified. Bioinformatics results suggest an important role of this modification in regulating metabolism and mitochondrial protein synthesis. Our data greatly expand the catalog of lysine succinylation substrates and sites in insects, and represents an important resource for understanding the physiological function of lysine succinylation in insect midgut.

1. Introduction

Silkworm (*Bombyx mori*) is one of the economically important insects for silk production and is also an ideal lepidopteran model playing roles in basic research and biotechnology [1]. Lepidoptera are among the most severe pests of agricultural importance. Researches on silkworm have contributed to the sericulture development and help to better understand of the biology of lepidopteran pests.

Insect midgut is a vital tissue with crucial role in feeding behavior. It is also a barrier to foreign substances including pathogens and toxins. The genome-wide microarray [2] and proteome [3,4] of silkworm midgut have been investigated. However, the post-translational modifications (PTMs) of this tissue have not been reported yet. Although cellular function is controlled through gene expression programs, PTMs often decorate proteins, providing more precise mechanisms for the modulation of protein properties and functions.

Lysine succinylation (Ksu) is a newly identified protein post-translational modification (PTM) of lysine residues [5]. In the past few years, studies of succinylation have been performed in plant, bacteria, yeast, parasite and mammals [6–19]. Compared with other lysine PTMs, such as lysine acetylation and malonylation, lysine succinylation is highly likely to have more profound or specific regulatory roles in cellular functions because protein characteristics are altered more dramatically with greater changes in charge and additions of larger structural moieties [5]. It produces a two-unit charge shift with a change in the charge status from $+1$ to -1. Increasing evidences show that lysine succinylation plays regulatory roles on enzymes and metabolism processes. However, the regulatory significance of lysine succinylation in insects remains largely unknown.

By analogy to acetylation using acetyl-CoA as a donor, succinylation

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uses succinyl-CoA as a donor [5]. Succinyl-CoA and acetyl-CoA are both particularly abundant metabolic intermediates in mitochondria. Increasing data show that mitochondrial proteins are highly succinylated in vivo [16–17]. Protein acylations are likely regulated via both enzymatic and non-enzymatic mechanisms. Emerging evidence suggests that lysine succinylation is likely predominantly mediated non-enzymatically in mitochondria [20], which is favored by the high concentrations of succinyl-CoA in mitochondria and by the alkaline microenvironment in the mitochondrial matrix [20–22]. Non-enzymatic regulation suggests a possible link between the metabolic status of mitochondria and the level of protein acylation. Mitochondria is the center of metabolism. Lysine succinylation is particularly prominent on mitochondrial proteins in metabolically active tissues [7,17–18]. In mouse liver, 70% of succinylation sites are located on mitochondrial proteins, and those succinylated proteins are enriched significantly in metabolic pathways [7].

The fifth instar of silkworm larvae is the very important stage in which the silkworm larva consumes the most food (approximately 85% of the total food of the entire larval stage) to accumulate sufficient nutrition and energy to meet remaining life requirements, because larva is the only feeding stage in the life cycle. The total nutrition demanded for silk protein synthesis, pupae, adults (moth) and eggs is dependent on the food intake of this stage. It is also the transition period from larvae to pupae (metamorphosis). Therefore, very active and specific metabolism in this stage is anticipated. We assumed that lysine succinylation PTM in silkworm midgut mitochondria would be abundant and most of enzymes will be succinylated in this stage.

Here, we report on the systematic identification of the lysine succinylome of silkworm midgut mitochondria in the fifth instar larvae using protein fractionation, affinity enrichment, and HPLC-MS/MS analysis to gain insights into lysine succinylation during the larval gluttonous stage. We finally identified 1884 succinylated sites from 373 mitochondrial proteins. Those succinylated proteins are involved in diverse biological functions and cellular processes, especially in central metabolism and protein synthesis. The data will contribute in understanding the regulatory role of lysine succinylation in silkworm midgut biological function, and furthermore, advancing the study of lysine succinylation in insects.

2. Materials and methods

2.1. Silkworm raising and mitochondria preparation

The silkworm strain P50 was maintained in the Sericultural Research Institute, Zhejiang Academy of Agricultural Sciences. Silkworms were raised on fresh mulberry leaves at 28 °C and 85% humidity for the first three instars and at 27 °C and 85% humidity for the last two instars. The midguts were dissected under a stereoscopic microscope on the third day of the fifth instar. Approximately 4.5 g of midgut from 45 silkworms was used in this experiment. The dissected midguts were rinsed with 0.7% saline solution three times after careful removal of Malpighian tubes and trachea from the midguts.

The midgut mitochondria were then isolated and purified using the differential centrifugation and density gradients method described by Anderson with slight modification [23]. The midguts were homogenized in 10 volumes ice-cold isolation buffer (0.25 M sucrose, 0.1 M Tris, 0.01 M EDTA, pH 7.5) supplemented with an EDTA-free protease inhibitor mixture (Roche Diagnostics GmbH, Mannheim, Germany) with a hand 15-mL glass dounce homogenizer (Knotes Glass, Vineland, NJ). Eight passes with “A” (loose-fitting) pestle and six passes with “B” (tight-fitting) pestle were performed. The homogenate was centrifuged at 1000 × *g* for 10 min to remove nuclei, cell debris and unbroken cells. Then, the supernatant was centrifuged at 10,000 × *g* for 10 min. The precipitate was resuspended with isolation buffer, and the centrifugation was repeated at 1000 × *g* and 10,000 × *g* for 10 min each. The resultant precipitate was the crude mitochondria. The crude mitochondria were resuspended in 15% Percoll and then loaded onto discontinuous gradients consisting of 23% Percoll layered on 40% Percoll. After centrifugation at 31,000 × *g* for 5 min, the purified mitochondria were collected at the interface of 23% and 40% Percoll layers and washed with isolation buffer three times. All the procedures were conducted at 4 °C.

To ensure completion and purity, purified mitochondria were visualized using transmission electron microscopy. Briefly, the purified mitochondria pellet was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) for 2 h at 4 °C, followed by 2.0% osmic acid for 2 h at 4 °C. The sections were post-stained with uranyl acetate and observed with an FEI Tecnai G2 Spirit transmission electron microscope.

2.2. Monoclonal antibody against mitochondrial protein cytochrome c

To investigate the purified mitochondria, monoclonal antibody against mitochondrial cytochrome *c* (CYC) protein was manufactured according to the reported method with a slight modification [24]. Firstly, the full length cDNA(NM_001309575.1) was synthetized and then cloned into the pET-28a expression vector (Novagen). This recombinant plasmid was transformed into *Escherichia coli* RosettaTM2(DE3). The produced protein was then purified by using Ni-NTA Sepharose chromatography with 250 mM imidazole.

Five BALB/c mice, 4 to 6 weeks old, were immunized by IP injection the purified mitochondrial CYC. Each mouse was injected four times with 100 μg of the purified protein once every 2 weeks. Three days after the final immunization, all mice were tail-bled, and the serum was assayed for antibody activity by indirect ELISA. Those two mice with the highest anti-CYC antibodies were selected for fusion. Splenocytes from administered mice were mixed with the Sp2/0 myeloma cells (American Type Culture Collection) at a ratio of 1:10 in presence of polyethylene glycol. Hybridoma cells were thoroughly cultured, and subsequently the reactivity of the culture supernatant was screened by ELISA. Lastly, positive hybridoma lines were isolated by the limiting dilution method [25]. Positive monoclonal hybridoma cells were then injected into the peritoneum of BALB/c mice to produce ascetic fluid. This ascetic fluid was drained, and purified using G-Sepharose column (Pierce) according to the manufacturer's instructions.

2.3. Western blotting

The extracted silkworm midgut mitochondrial proteins were subjected to gel electrophoresis on 10% SDS-PAGE, and transferred to PVDF membranes. The PVDF membrane was blocked, washed, and then incubated with 1:2000 dilutions of the monoclonal antibodies against CYC. The membranes were washed to remove unbound antibody, and then incubated with HRP-conjugated goat anti-mouse IgG polyclonal antibodies (1:5000). After washing, membrane was subjected to ECL regents (Millipore) before the exposure to X-ray and the development. The protein bands on the membrane were scanned and analyzed with a VersaDoc5000 imaging system (Bio-Rad).

2.4. Protein extraction, trypsin digestion, HPLC fractionation and affinity enrichment

Samples were ground in liquid nitrogen. The powder was lysed in lysis buffer (8 M urea, 1% Triton-100, 65 mM DTT) supplemented with 0.1% Protease Inhibitor Cocktail (Roche Diagnostics) on ice for 30 min, followed by sonication four times in an ice bath, 30 s each time. The supernatant was collected after centrifugation at 20,000 × *g* at 4 °C for 10 min. Finally, the protein was precipitated in pre-cooled 15% TCA for 2 h at − 20 °C. The supernatant was removed after centrifugation at 15,000 × *g* at 4 °C for 10 min. The obtained protein was washed with cold acetone three times and then redissolved in buffer (8 M urea, 100 mM NH_4CO_3, pH 8.0). The protein concentration was measured