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Research paper

Global proteomic analysis of protein acetylation affecting metabolic regulation in *Daphnia pulex*

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ABSTRACT

Daphnia (*Daphnia pulex*) is a small planktonic crustacean and a key constituent of aquatic ecosystems. It is generally used as a model organism to study environmental toxic problems. In the past decade, genomic and proteomic datasets of Daphnia have been developed. The proteomic dataset allows for the investigation of toxicological effects in the context of "Daphnia proteomics," resulting in greater insights for toxicological research. To exploit Daphnia for ecotoxicological research, information on the post-translational modification (PTM) of proteins is necessary, as this is a critical regulator of biological processes. Acetylation of lysine (Kac) is a reversible and highly regulated PTM that is associated with diverse biological functions. However, a comprehensive description of Kac in Daphnia is not yet available. To understand the cellular distribution of lysine acetylation in Daphnia, we identified 98 acetylation sites in 65 proteins by immunoprecipitation using an anti-acetyllysine antibody and a liquid chromatography system supported by mass spectroscopy. We identified 28 acetylated sites related to metabolic proteins and six acetylated enzymes associated with the TCA cycle in Daphnia. From GO and KEGG enrichment analyses, we showed that Kac in *D. pulex* is highly enriched in proteins associated with metabolic processes. Our data provide the first global analysis of Kac in *D. pulex* and is an important resource for the functional analysis of Kac in this organism.

1. Introduction

Daphnia (*Daphnia pulex*) is a small planktonic crustacean and a key constituent in aquatic ecosystems. Along with other planktonic species such as those of the *Ceriodaphnia* and *Moina* genera, Daphnia is often used as a model organism for ecotoxicology studies by the USEPA, OECD, and NIH [1–4]. Genomic information on *D. pulex* has been investigated by the Daphnia Genomics Consortium and offers the opportunity for mass spectrometry-based proteomic research [2]. Proteomic analysis provides an insight into the cellular effects of toxicants on Daphnia and the changes in protein behavior in response to environmental stresses. The first proteomics dataset for Daphnia was compiled in 2009 and included 701,274 peptides of 531 proteins, based on predicted genomic coding regions [3].

The integration of proteomic knowledge enables the quantification of proteins and their identification in biofluids and tissues; this integration, which we term as "Daphnia proteomics," has the potential to provide new insights for toxicological research. The responses of the *D. pulex* proteome to environmental agents, temperature, and redox changes have been investigated [4,5]. For example, changes in protein expression profiles after 48 h of deltamethrin exposure have been described [6]. Likewise, redox proteomics has been used to assess the potential toxicity of silver nanoparticles, paraquat, and tamoxifen [7–9]. When *D. pulex* is exposed to acute and medium levels of the anticancer drug tamoxifen, proteins involved in protein degradation and carbohydrate and lipid metabolism are affected [8]. Recently, kairomone-mediated and light-dependent life-history changes in Daphnia were investigated using differential peptide labeling methods in LC-MS/MS-based proteomics [10]. In our previous study, 103

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phosphorylation sites in 91 Daphnia proteins were identified by titanium dioxide isolation technology for phosphopeptide enrichment in combination with online two-dimensional liquid chromatography and mass spectrometry (2D-LC-MS/MS) [11].

Although proteomics approaches have been used in Daphnia for ecotoxicological research, there is no comprehensive dataset of the diverse post-translational modifications (PTMs) of Daphnia proteins. One of the most important modifications is lysine acetylation (Kac), which is reversible and highly regulated. Since its initial discovery in histones about 40 years ago [12], Kac has been shown to have diverse biological functions, including the regulation of DNA-protein interactions, subcellular protein localization, regulation of transcriptional activity, and in protein stability [13]. In 2006, Kim et al. reported the first systematic description of acetylated residues in the genome (the "acetylome") of HeLa cells and mouse liver mitochondria using nano-HPLC/MS/MS analysis to identify the proteins involved [14]. During the past decade, developments in proteomic technology have enabled the wide-scale identification of acetylated proteins. In 2009, over 3600 lysine acetylation sites on 1750 proteins were identified in three human cell lines [15], and, subsequently, 1300 acetylated peptides were matched to 1047 human proteins [14]. Additionally, 15,474 acetylation sites have been identified in 16 different rat tissues [16,17]. Global acetylome data and the identification of the biological function of acetylated proteins have increased our understanding of the roles of lysine acetylation in various cellular processes.

In eco-toxicological studies, the responses of particular metabolic pathways allows for the assessment of potentially toxic reagents in Daphnia. Profiling of the global acetylome in Daphnia would greatly assist such studies. Here, we analyzed acetyllysine in *D. pulex* by immunoprecipitation (IP), using a pan-anti-acetyllysine antibody, and an LC-MS/MS system. Using this approach, we successfully identified 98 acetylation sites in 65 proteins in *D. pulex*. Furthermore, we demonstrated that Kac in *D. pulex* is highly enriched in proteins associated with metabolic processes.

2. Materials and methods

2.1. Daphnia culture

The *D. pulex* were cultured from ephippia purchased from MicroBioTest Inc. (Belgium; batch number *DP070508*). The ephippia were induced to hatch according to the supplier's instructions. In brief, the ephippia were washed in deionized water and then placed in aerated Elendt M4 medium in a petri dish [18]. The eggs were cultured at 20 °C for 96 h under continuous illumination (6000–10,000 lux). Neonates were transferred to fresh Elendt M4 medium at 20 °C and 16 h light/8 h dark light cycle in a plant culture chamber [19]. Daphnia were fed daily with *Chlorella* sp. and the yeast (*Saccharomyces cerevisiae*). The medium was renewed 3 times per week; cetyl alcohol (~1 mg/L) was added to the culture surface to prevent trapping of the Daphnia in the surface film [20]. The Daphnia were acclimated for a month under these conditions.

2.2. Daphnia protein extraction

For protein extraction, about 550 adult *D. pulex* were collected and the medium was removed. The Daphnia were rinsed in deionized-water and then frozen immediately in liquid nitrogen. Prior to sample preparation, the Daphnia were starved for 12 h in fresh media [5]. The collected Daphnia were lysed in Radio-Immunoprecipitation Assay buffer (RIPA buffer, 25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40 and 0.1% SDS) containing protease inhibitors (10 μM sodium pyrophosphate, 1 mM sodium fluoride, 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate) with 50 mM sodium butyrate and 30 mM nicotinamide as histone deacetylase (HDAC) inhibitors. The lysed sample was sonicated four times at 20% for 10 s on ice using a high intensity ultrasonic processor (Scientz, CT, USA). The sonicated sample was incubated at 4 °C for 1 h and centrifuged at 13,000 *g* for 10 min at 4 °C. The supernatant was transferred to a fresh Eppendorf-tube and the crude proteins were quantified using a BCA protein assay kit (Thermo Scientific Pierce, IL, USA). For protein precipitation, the proteins were included to a final concentration of 10% trichloroacetic acid (TCA) and incubated overnight at 4 °C and centrifuged for 10 min at 14,000 *g* at 4 °C. The pellet was washed twice in ice-cold acetone. Protein pellets were dissolved in 50 mM ammonium bicarbonate buffer.

2.3. Protein digestion by trypsin

Trypsin (Promega, WI, USA) was added to the protein solution at a ratio of 1:50 (w/w) of trypsin to protein and incubated at 37 °C for 16 h. Digested peptides were reduced with 5 mM dithiothreitol (DTT) at 56 °C for 30 min and alkylated with 15 mM iodoacetamide (IAA) at RT for 30 min in the dark. The peptide samples were then added to 15 mM cysteine at RT for 30 min in the dark. For additional digestion, trypsin was added to the peptide sample in a ratio of 1:100 (w/w) of trypsin to peptide and incubated at 37 °C for 4 h. Digestion was stopped by adding 50% acetonitrile containing 5% trifluoroacetic acid (final concentration of 1% TFA) to the peptide sample. Peptides were dried using a centrifugal vacuum concentrator.

2.4. Immunoblotting

To separate proteins, 40 μg of protein lysate was placed in 1× SDS sample buffer (50 mM Tris–HCl pH 6.8, 2% Sodium dodecyl sulfate, 10% glycerol, 0.1 M dithiothreitol, 12.5 mM EDTA, 0.02% bromophenol blue) and separated by SDS-PAGE using an 8% gel. The separated proteins were transferred to a PVDF membrane (Roche, Germany) using wet transfer at 300 mA for 1 h on ice. Protein-transferred membranes were blocked with 5% bovine serum albumin in Tris-buffered saline plus 0.1% Tween-20 (TBST). The membranes were then probed with an anti-acetylated lysine antibody (Cell Signaling, MA, USA, 1:1000) overnight at 4 °C with rotation. The membranes were washed in TBST and incubated with an HRP-linked secondary antibody (Cell Signaling, MA, USA, 1:2000) at RT for 2 h with rotation. The membranes were then washed in TBST and developed using ECL reagent (Amersham ECL Prime Western blotting analysis system, GE Healthcare, UK). Images of the membranes were obtained using an ImageQuant LAS 4000 mini (GE Healthcare, UK).

2.5. Affinity enrichment of Kac peptides

To enrich Kac peptides, the tryptic peptides were dissolved in NETN buffer (100 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid, 50 mM Tris–HCl, 0.5% NP-40, pH 8.0) and incubated with pre-washed antibody agarose beads (PTM Biolabs, IL, USA) at 4 °C overnight with moderate shaking. The agarose beads (20 μL) were washed by NETN buffer four times and twice with water. Enriched peptides were eluted from the beads using 0.1% TFA. Eluted fractions were combined, vacuum-dried, and analyzed by LC-MS/MS.