Comparative proteomics of cold-treated tardigrades reveals shifts in key biological processes and identifies putative ice-binding proteins

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Little is known about the molecular mechanisms that allow tardigrades to survive subzero temperatures, especially in terms of biological processes and the utilization of tardigrade-specific proteins related to cold tolerance. Here we explore shifts in proteins found in a model tardigrade species (*Hypsibius exemplaris*) in response to a mild, ecologically-feasible cold exposure, using comparative proteomics methods and ice-affinity purification to identify putative ice-binding proteins (IBPs). Thousands of adult tardigrades were exposed to cold (-10°C) or ambient temperatures (20°C) and either whole-body protein extractions or the final elution fractions of ice-affinity purification were analyzed using DIA spectrometry, from each thermal treatment. First, we identified differentially abundant proteins between the whole-body lysate of cold-treated and room temperature cultured tardigrades (N=3, per thermal treatment). We found a total of 2776 proteins identified among all samples, with 28 proteins being significantly more abundant after cold treatment and 61 proteins being significantly less abundant after cold treatment (q-value < 0.1, between cold-treated and room-temperature treated samples). Of these 89 differentially abundant proteins, 41 (46%) were unannotated in the tardigrade genome, with 58 (65%) having no significant pBLAST homologs among other taxa (E-value 0.05 or less). GO enrichment analysis illustrated several terms which were significantly enriched in proteins differentially abundant in response to cold. As our second experimental goal, we conducted ice-affinity purification on pools of thousands of cold-treated and room temperature cultured tardigrades (N=3, per thermal treatment), with the aim of enriching for proteins with a physical affinity to bind to ice (predicted to act as protective antifreeze proteins). PCA analysis suggested high variation in the proteomic profiles of these ice-affinity purified samples compared to whole-body samples, illustrating the impact of the selective protein enrichment. We ranked proteins found in the final ice-affinity elution fractions according to protein abundance, size, and known characteristics of ice-binding proteins (ice-binding motifs found in proteins of other organisms, predictions based on previously published machine learning methods, *de novo* protein structure modeling). We subsequently identified a list of top candidate ice-binding proteins (IBP) that we predict to help prevent damage by ice-formation in *H. exemplaris*, and which are otherwise currently uncharacterized. Four of these proteins were heterologously expressed and purified for future *in vitro* functional verification via thermal hysteresis assays. In summary, this study represents the first-ever high-sensitivity comparative proteomics study in response to cold stress in tardigrades, or any eukaryotic animal. The candidate ice-binding proteins identified here are novel and promising candidates for playing a functional role in tardigrade freezing tolerance, and may shed light on the evolution of ice-binding proteins in this unique phylum.

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