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Development of chemical isotope labeling liquid chromatography mass spectrometry for silkworm hemolymph metabolomics

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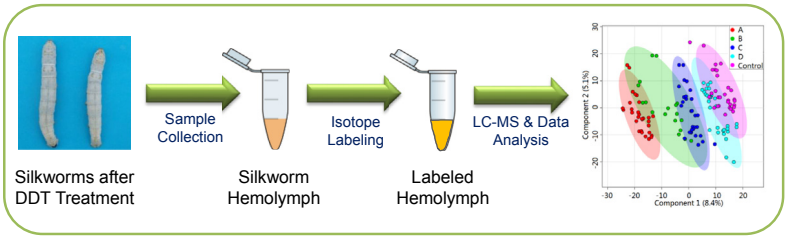
HIGHLIGHTS

- A method was developed for profiling silkworm hemolymph metabolome with high coverage.
- Dansylation isotope labeling LC-MS was used for amine/phenol sub-metabolome quantification.
- Metabolomic changes in silkworm after DDT exposure were studied.
- Among 2044 metabolites detected, 65 were positively identified and 1809 were putatively identified.
- 33 positively identified metabolites showed significant changes in five groups of silkworms.

GRAPHICAL ABSTRACT



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ABSTRACT

Silkworm (*Bombyx mori*) is a very useful target insect for evaluation of endocrine disruptor chemicals (EDCs) due to mature breeding techniques, complete endocrine system and broad basic knowledge on developmental biology. Comparative metabolomics of silkworms with and without EDC exposure offers another dimension of studying EDCs. In this work, we report a workflow on metabolomic profiling of silkworm hemolymph based on high-performance chemical isotope labeling (CIL) liquid chromatography mass spectrometry (LC-MS) and demonstrate its application in studying the metabolic changes associated with the pesticide dichlorodiphenyltrichloroethane (DDT) exposure in silkworm. Hemolymph samples were taken from mature silkworms after growing on diet that contained DDT at four different concentrations (1, 0.1, 0.01, 0.001 ppm) as well as on diet without DDT as controls. They were subjected to differential ^{12}C-/^{13}C-dansyl labeling of the amine/phenol submetabolome, LC-UV quantification of the total amount of labeled metabolites for sample normalization, and LC-MS detection and relative quantification of individual metabolites in comparative samples. The total concentration of labeled metabolites did not show any significant change between four DDT-treatment groups and one control group. Multivariate statistical analysis of the metabolome data set showed that there was a distinct metabolomic separation between the five groups. Out of the 2044 detected peak pairs, 338 and 1471 metabolites have been putatively identified against the HMDB database and the EML library, respectively. 65 metabolites were identified by the dansyl library searching based on the accurate mass and retention time. Among the 65 identified metabolites, 33 positive metabolites had changes of greater than 1.20-fold or less than 0.83-fold in one or more groups with p-value of smaller than 0.05. Several useful biomarkers

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including serine, methionine, tryptophan, asymmetric dimethylarginine, *N*-Methyl-D-aspartic and tyrosine were identified. The changes of these biomarkers were likely due to the disruption of the endocrine system of silkworm by DDT. This work illustrates that the method of CIL LC-MS is useful to generate quantitative submetabolome profiles from a small volume of silkworm hemolymph with much higher coverage than conventional LC-MS methods, thereby facilitating the discovery of potential metabolite biomarkers related to EDC or other chemical exposure.

1. Introduction

Silkworm, *Bombyx mori*, has been an economically very important insect for over 5000 years, mainly for silk production. With recent advances in genetic engineering technology, silkworm may be potentially used to produce other functional proteins and biomaterials [1]. Because silkworm is very sensitive to pollutants such as pesticide [2], heavy metals [3] and fluoride [4] as well as other chemicals such as pharmaceuticals [5], it has been used as a target species in environmental and health safety evaluation. It has also been traditionally used as a model system for lepidopteran study [6]. Since the completion of silkworm genome sequencing [7,8], functional genomic studies of silkworm on diverse areas of biological importance including developmental biology, reproduction and physiology have been extensively reported [6]. Many of these studies were focused on transcriptomic [9–11] or proteomic [12–14] investigation of silkworm. Very recently, research on using metabolomics to examine the metabolic changes induced by various stimulants or processes has been described [15–18]. Because metabolomics can provide complementary information to other Omics technologies, it is poised to play an increasingly important role in the future in large scale study of silkworm biology and related processes including developing genetically engineered silkworms [6].

Metabolome profiling is a critical part of metabolomics studies of silkworm. Traditional methods including NMR, GC-MS and LC-MS have been used for metabolome analysis of silkworm hemolymph [15–17,19] and larva brain [18], but with limited metabolic coverage. Because of a small size of samples available from each silkworm, generation of a metabolome profile with high coverage, which is often achieved by analyzing aliquots of the same sample using multiple techniques, is currently an analytical challenge. Mixing samples from a number of silkworms to form a pooled sample may increase the sample size for analysis. However, this is not ideal to account for intra-group biological variations in individual silkworms to reveal inter-group metabolic differences, particularly if the changes are small. In this work, we report a sensitive method based on high-performance chemical isotope labeling (CIL) LC-MS [20] to perform in-depth submetabolome profiling of silkworm hemolymph. To demonstrate the utility and analytical performance of this method for silkworm metabolomics, we applied this method to examine the metabolomic changes in hemolymph samples collected from individual silkworms with and without the exposure of dichlorodiphenyltrichloroethane (DDT).

DDT was a popular organochlorine pesticide several decades ago, but is now regarded as an endocrine disruptor [21]. It can modulate the endocrine system through mimicking endogenous hormone action and can cause adverse effects in wildlife and human [22,23]. Although DDT had been banned since 1970, the negative effects will still exist for a long time because of the presence of residues in the environment and ecosystem. Silkworm should be particularly suitable for the evaluation of endocrine disrupting effects of exogenous chemicals such as DDT. It is known that the complete endocrine system of silkworm consists of brain neurosecretory cells, suboesophageal ganglion, prothoracic glands, corpora allata, which can control the processes of growth, production, development and other aspects completely [24]. In addition, a wealth of background knowledge about genetics, physiology, biochemistry and genomics of silkworm [6] can provide us valuable information on endocrine disruption research. In our work, we applied CIL LC-MS metabolomics to generate metabolomic information in order to understand further how a simulant such as DDT affects silkworm growth as well as search for potential metabolite markers of DDT exposure. The latter is relevant to silk production in some part of the world where DDT residual levels in fields planted with mulberry trees could be still high [25–27].

2. Experimental

2.1. Chemicals and reagents

All the chemicals and reagents, unless otherwise stated, were purchased from Sigma-Aldrich Canada. The pesticide DDT was purchased from AccuStandard USA. For dansylation labeling, the ^{12}C-labeling reagent (dansyl chloride) was purchased from Sigma-Aldrich and the ^{13}C-labeling reagent was synthesized according to the method published previously [20]. These reagents are also available from the University of Alberta (mcid.chem.ualberta.ca).

2.2. Silkworm rearing and DDT treatment

The eggs of bivoltine hybrid “HuangKang 3” of silkworm were obtained from Sericultural Research Institute, Chinese Academy of Agricultural Sciences. The larvae were raised in incubator using a sterilized artificial diet developed at Zhejiang Academy of Agricultural Sciences, Hangzhou, China. The silkworms were raised intensively during the first instar with a condition of 29 °C and 90% humidity, followed with 1 °C temperature decrease and 5% humidity decrease in each instar. The door of incubator was kept open for 5 min to ventilate three times a day. Right after the first larval molted, size-matched larvae were selected and assigned randomly into the batches for the DDT treatment. Four concentrations of DDT (A = 1.0 ppm, B = 0.1 ppm, C = 0.01 ppm, D = 0.001 ppm) were used in this experiment. DDT was mixed with the diet. Meanwhile, the silkworms fed without DDT were considered as control. There were 3 replicate experiments for each DDT concentration as well as the control and 30 silkworms were used in each experiment. The diet was changed on alternate day from the first to the third instar, and every day in the fourth and fifth instar.

2.3. Hemolymph collection and preparation

From mid to late fifth instar, silkworm started to prepare for cocoon spinning. Because this period is very crucial to silkworm development, we analyzed the metabolome of silkworm after DDT exposure at this point. Five out of thirty larvae were randomly