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Proteomic analysis of individual fruit fly hemolymph

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ABSTRACT

Analysis of blood proteins holds critical promise for in depth understanding of physiological states. Protein content of hemolymph from *Drosophila melanogaster* is of particular analytical interest because the insect open circulatory system involves chemical signaling through the hemolymph. The challenge of working with this sample, however, is the nanoliter volumes of solution available for analysis. In this study, we developed a novel hyphenated Agilent nano-HPLC chip column-MS method to obtain proteomic information from individual fruit fly hemolymph, using a low-volume sample collection technique established previously. The total amount of individual *Drosophila* hemolymph protein is determined around 0.798 ± 0.251 μg/100 nL based upon a Bradford assay with BSA. Hemolymph samples around 50 nL were collected from single flies and digested using a customized micro-scale digestion protocol. Mass spectral analysis shows a total of 19 proteins were identified from the hemolymph of individual flies. Of these findings, 6 novel proteins have been identified for the first time with evidence at the translation level. Detection of 13 proteins well-known in the literature speaks to the method's validity and demonstrates the ability to reproducibly analyze volume-limited samples from individual fruit flies for protein content. This nano-scale analysis method will facilitate future study of *Drosophila* and lead to a more complete understanding of the physiology of the fly model system.

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1. Introduction

Drosophila melanogaster (commonly known as "fruit fly") has been an important animal model for studying molecular mechanisms underlying intracellular processes, cell–cell interaction, or the pathology of clinical diseases for decades. It affords many biological advantages such as an easily manipulated genome, a relatively short lifecycle, and a large body of genome data that would be a powerful resource for proteomic methodologies. However, the exceedingly small-size of fruit fly yields biological volumes in the low nanoliter level, which makes sample collection, handling and preparation quite a challenge. Methods to perform proteomic analysis on nL volumes of fluids from the fly would be an enabling tool for fly studies.

The physiology of the fly is relatively simple. It has a short lifecycle that makes maintenance in the laboratory straightforward and economical. With only four chromosomes, its genome system is significantly simpler compared to human [1]. This simplicity contributes to its power as a model and makes manipulation of the fly genome relatively easy. The fly genome is also homologous to almost three-quarters of the human genome [2,3]. Importantly, a good number of *Drosophila* genes were found to have human analogs relevant to clinical diseases. This great similarity and relevance to the human genome has prompted close studies of gene expression, metabolites digestion pathways, and proteomic functions inside the *D. melanogaster* biological system.

Unlike mammals, the fly has an open circulatory system in which hemolymph circulates through the whole body. Hemolymph, similar to blood, transports and exchanges nutrients, metabolites, and antibodies between organs and tissues. There is ongoing active chemical signaling between internal tissues and organ cells by proteins, peptides, and hormones in hemolymph [4]. Many of the secreted proteins in hemolymph serve as the innate immune system for the fly and protect against infections and external invasion [5]. Thus, hemolymph is an ideal choice for proteomic analysis in order to better understand the effects of particular protein post translation modifications and, more generally, the physiology of disease development in the fly.

Abbreviations: HPLC, high performance liquid chromatography; LTQ-FT Ultra, Finigan LTQ FT hybrid linear ion trap – Fourier Transform ICR mass spectrometer; BSA, bovine serum albumin; DTT, dithiothreitol; MALDI-TOF-MS, matrix assisted laser desorption/ionization-time of flight-tandem mass spectrometer.

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A diversity of approaches have been proposed to isolate specific cell populations, tissues and body fluid from small anatomically defined regions, for functional physiology studies. Recently, laser capture microdissection (LCM) has become a popular technique due to the ability to excise, ablate and isolate individual tissues and cells from morphologically defined spot sizes of less than 1 μm in diameter without mechanical contact or cross-contamination [6]. LCM has been widely employed on fixed cell preparation, for instance, cell smears and histological tissue section and in the clinical study of oncology [6–8]. However, LCM is critically sensitive to tissue processing and specimen preservation methods. Significant chemical alteration and molecule variability should be taken into consideration for subsequent proteomic analysis [9]. A proteomic profiling of *Drosophila* larval hemolymph clotting has been established by the Theopold group quantitatively and qualitatively, using a group of 10 animals [10]. The study of hemolymph coagulation is inspiring, but requires tedious and precise sample preparation, in order to harvest and pool microliters of hemolymph solution for SDS-PAGE separation prior to proteomic analysis. To obtain soluble proteomic information of *Drosophila* antenna from a population of flies, Anholt and Williams described an osmotic lysis method of hundreds of antennae in distilled water, followed with homogenization and centrifugation, which required several hours of processing [11].

In this study, we developed a novel LC–MS analysis method to collect proteomic information from individual fruit fly hemolymph which utilizes a unique small-volume sample collection technique developed in our lab previously [2,12]. A microscale in-solution digestion procedure has been established for efficient low volume hemolymph digestion. Peptides resulting from a tryptic digest of hemolymph were separated using an Agilent nano-HPLC chip system and then assayed via LTQ-FT MS/MS. From the study, a number of proteins were positively identified including six proteins that have only been predicted by evidence at the translation level.

2. Experimental

2.1. Chemicals and materials

2.1.1. Chemicals

Deionized water was obtained from a US Filter Purelab Plus purification system (Lowell, MA). Trypsin, Tris hydrochloride, dithiothreitol and iodoacetimide were all purchased from Sigma–Aldrich (St. Louis, MO). Glacial Acetic acid was purchased from Fisher Scientific (Itasca, IL). Bradford reagent Coomassie blue G-250 solution was from Thermo Scientific (Itasca, IL).

2.1.2. Materials

Fused-silica capillary (360/50-μm o.d./i.d.) was purchased from Polymicro Technologies (Phoenix, AZ). Tygon tubing (250-μm/20.6-mm o.d./i.d.) was purchased from Cole-Parmer (Vernon Hills, IL). Insulin injection syringes with a 1-mL volume were from Becton Dickinson Co. (Frankin, NJ). Centrifuge tubes were purchased from USA Scientific (Ocala, FL). The 0.400-mL Spectrosil Far UV quartz window cuvette cell is from Starna Cells, (Atascadero, CA).

2.2. Fruit fly sample collection

Hemolymph was collected from the Oregon-R strain of *D. melanogaster*, reared on standard cornmeal-agar medium [2] and maintained in UIC Biological Science Department. Only female flies were selected and sampled for hemolymph, in order to avoid the gender differences and to access larger hemolymph sample volumes. The sample collection technique was adapted from a protocol described previously [2]. Hemolymph samples were collected from individual fruit fly after 5-min cold-anesthetization. A single fruit fly was placed on a micro-dissection plate with the head taped to the dish, the wings cut, and the dorsal surface of the abdomen under view with a Leica MZ 6 dissection microscope (Leica Microsystem, Germany). A small incision was made with a microdissection scissors at the second abdominal tergite. The released hemolymph was immediately collected using a probe consisting of a 4 cm-long fused-silica capillary. This sampling probe was held by an electrode holder positioned in a three-dimensional micropositioner to adjust the probe position while collecting hemolymph. Collected hemolymph samples were then transferred to a 250 μL centrifuge tube and left to air-dry with the tube cap open and then stored at −20 °C before analysis. Three individual female adult flies were used for hemolymph collection as triplicate trials.

2.3. Proteomic sample preparation

2.3.1. Development of micro-scale digestion protocol

A volume of 50 nL (1 μM) BSA was thermally denaturized at 90 °C for 20 min in 2 μL of 0.4 M pH 7.8 stock Tris buffer before adding 2 μL (200 mM) of reducing agent (DTT) and alkylating reagent (iodoacetamide). Next, an aliquot of 0.2 μg/μL trypsin in 0.4 M stock Tris buffer solution was added to the BSA solution, giving a 1:50 protease-to-substrate ratio. The digestion was carried out at 37 °C overnight. Trypsin-digested BSA peptides were detected in MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) to confirm digestion efficiency. As evidence of the digestion, a list of BSA peptides that were identified was provided as a table in supplemental material.

2.3.2. Digestion and analysis of fruit fly hemolymph sample

With confirmation of digestion efficiency in BSA, the above microliter-scale digestion protocol was applied to each 50 nL fly hemolymph sample. Digested hemolymph samples were frozen at −20 °C until analysis. In order to estimate total protein amount in hemolymph sample regarding instrumental loading restriction, a Bradford assay absorption calibration curve of digested BSA was generated. A series of concentrations (0–30 μg/mL) of BSA tagged with Coomassie blue G-250 were measured at 595 nm by a Cary 300 Bio UV-Visible Spectrophotometer (Agilent Technologies, Santa Clara, CA).

2.4. Separation and MS identification

The digested hemolymph samples were reconstituted to be 10 μL for injection and then separated using an Agilent large capacity nano-HPLC chip system. This nano-HPLC chip is essentially a microfluidic device, with laser-ablated channels designed on a polyimide film [13,14]. The 150 mm-long chip integrates columns, connections, and valves together with an ESI-emitter mounted at the end, guiding separated analyte ions into a mass spectrometer for characterization. It has a 160-nL enrichment column for analyte enrichment and desalting. The HPLC column is composed of a 75-μm × 150-mm analytical column packed with ZORBAX C-18 stationary phase resins for highly complicated tryptic digest mixtures.

The analysis was performed with a 0.4 μL/min flow rate and a loading rate of 4 μL/min. The gradient began with 6% B (A is 95% H_2O, 5% acetonitrile with 0.1% formic acid, B is 5% H_2O, 95% acetonitrile with 0.1% formic acid) which was held for 5 min, then a 30 min gradient was run from 8% B to 45% B. The column was then washed at high organic content (80% B) for 2 min, before equilibrated back to initial starting conditions.

Hemolymph proteins were characterized using a Thermo LTQ-FT Ulltra (Waltham, MA). A survey full scan (m/z = 400–2000) at 50,000 resolution was acquired in which the five most intense ions

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