Original paper

GC/MS-based metabolic profiling reveals important metabolic pathways in microsporidia, *Nosema antheraeae*

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Abstract

The microsporidian, *Nosema antheraeae* can infect Chinese oak silkworm, *Antheraea pernyi*, and cause pebrine disease. This disease could greatly reduced the income from tussah industry. For seeking a way to overcome this disease, more and more researchers were worked for exploring the infection mechanism of the spores. Here, in order to identify the metabolites of *N. antheraeae* for analyzing key metabolic pathways, Gas chromatography-mass spectrometry (GC/MS)-based metabolome was performed.

1975 metabolic characteristic peaks were assignment and finally fifty-two metabolites were identified, including amino acid, fatty acid, sugars, etc. Database searches showed that the monoisotopic mass of the obtained metabolites ranged from 57 to 539 Da. Metabolic pathway enrichment analysis revealed that many basic metabolism processes, including protein biosynthesis, amino acid metabolism, pentose phosphate pathway, etc were found. These results, for the first time, provided a tool to explore the metabolic profiling of the microspidia and may provide a new horizon for seaking pathogenic molecules during infection of spores.

Keywords

Metabolites; pathway analysis; *Antheraea pernyi*; pathway enrichment; energy metabolism.

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Introduction

The Chinese oak silkworm, *Antheraea pernyi*, which belongs to Lepidoptera: Saturniidae, is an important economic insect in North China for Tussar silk production, insect food and medicinal materials. In China, annual output of tussah cocoon is high to $7 \times 10^4$ t and approximately exports accounts for 90% of the world total of wild silk. Tussah production has become an important pillar industry of rural economy in most sericultural areas.

As a main pathogen, which can systematically infect *Antheraea pernyi*, *Nosema antheraeae* (Na), could lead to serious pebrine disease (XU & al [1]). This disease has greatly reduced the income from tussah industry and hindered the development of sericulture in China (WANG & al [2]). For seeking a way to overcome this disease, more and more studies were reported, which mostly focused on the structure of *N. antheraeae* spores, function of identified proteins and mechanism of the infection in recent years (WANG & al [3], TANG & al [4], GONG & al [5]). But no efficient method was obtained to prevent the pestilence, thus it was urgent to exploit a new research field. With the development of science and technology, metabolomics has been developed and it had become a new tool to analyze all the low molecular weight (less than 1000) metabolites in a cell at a certain time. It becomes a major component of systems biology. Up to now, it is a powerful tool not only for metabolite identification (VANYUSHKINA & al [6]), but also for the areas closely associated with human health and nursing, such as disease diagnosis (HUANG & al [7]), nutrition and food science, environmental science and so on. Most notably, metabolomics could not only play an important role in exploring the mechanisms of biological response (BARDING & al [8]), but also provide clues to mechanistic research (CHEN & al [9]). At present, a variety of analytical techniques were applied to metabolomics research. For the higher resolution and detection sensitivity, gas chromatography-mass spectrometry (GC-MS) was widely used for metabolite identification in the area of animal biology, plant physiology, etc. (CHEN & al [9], SALANŢĂ & al [10]).

So far, more and more studies were reported to explore the functional molecules and infection mechanism of microsporidia (YANG & al [11]). Host responses for seaking clues to reveal the immunization strategies or to control the spread of insect microsporidiosis, were also becoming a research hotspot (MA & al [12]). However, nearly all of the reported works were achieved using approaches describing changes on the genome level, proteome level or transcriptome level. Although metabolomics methods are efficient to reveal the correlation of genotypes and phenotypes and to understand the essential metabolic processes during the parasitic life, no studies analyzed the metabolome of microsporidia was reported. Here, this work aimed to identify as many intracellular metabolites as possible in microsporidia, *Nosema antheraeae*, for obtaining the metabolic profiling for revealing key metabolic pathways in mature spores.

Materials and Methods

Experimental spores and treatments

*Nosema antheraeae* was obtained from infected pupa of Chinese oak silkworm (*Antheraea pernyi*). Briefly, infected pupa was grinded in a mortar and suspended in sterile water. Spore purification was on the basis of the method reported for purifying microsporidia (*Nosema bombycis*) from silkworm (*Bombyx mori*) (MA & al [12]). Then, spores were purified with a percoll gradient centrifugation (10, 25, 50, 75 and 90% v/v) under aseptic conditions. The pellets of mature spores were rinsed twice with sterilized double distilled water and stored with antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) for later use (MA & al [12]).

Sample treatment for Gas chromatography-mass spectrometry (GC-MS) analysis

An improved sample preparation was refered based on the method for sample preparation in silkworm (CHEN & al [9]). In order to get rid of proteins, the *Ni* samples were diluted with acetonitrile (4×10⁷ spores: 400 μL), these mixtures were violently vortexed for 2 min with glass beads and three repeats were needed. Then placed on ice for 1 h, after that, all the mixtures were centrifuged to deproteinize under 4°C at 13,000 g for 15 min. The collected supernatants (320 μL) were transferred and lyophilized. Then, dissolving the lyophilized residues using 100 μL of methoxamine solution (20 mg/mL in pyridine) and followed by water bath at 37°C for 60 min and ultrasound for 20 min, successively. Nexty, 80 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was used for silylation under the same conditions for 60 min. To stop the reflection, 20 μL of N-heptane was employed and followed by centrifugation under 4°C at 14,000 g for 25 min. Finally, 120 μL of supernatants were obtained for GC-MS analysis. It is worth mentioning that instrument parameters for GC-MS analysis was referred to the reported work (CHEN & al [9]).

GC-MS data processing and analysis

First, CDF data format files were generated from the raw data files using Agilent ChemStation, and then the m/z features was matched using XCMS Online. Finally, the NIST 2011 (version 2.0) library and standard samples were used to analyze their mass spectra (LI & al [13]).

Results

MS spectra acquisition

Spores were purified from infected pupa of *Antheraea pernyi* using percoll gradient centrifugation. Glass beads were used to destroy cell walls to release metabolites and samples were harvested using acetonitrile for metabolite identification. Metabolite detection was accomplished by GC-MS. In order to obtain the most reliable data, the experimental parameters and the mass spectrometric peak recording, were optimised by standards.
Total ion current (TIC) was shown in Fig. S1. The NIST 2011 library was used for metabolites identification. There were three biological-independent repeats for metabolite detection. In order to reduce the repetitive error, two technological repeats were performed for each biological repeat. The two step chemical derivatization method was used to inhibit the multiple peaks of the reducing sugar (PASI KANTI & al [14]). In this study, 1975 metabolic characteristic peaks were assignment and finally fifty-two metabolites were identified.

**Metabolite content of Nosema antheraeae**

After identification using the NIST 2011 library, twenty-six metabolites were unknown and fifty-two metabolites were obtained (Table 1). According to certain features of the identified metabolites, the classification was as follows: carbohydrates, lipids, amino acid and others (shown in Fig. 1). In the data, fourteen amino acids were identified and took almost 27% of the identified metabolites, including Glycine, Alanine, Lysine etc. Fatty acid and carbohydrates were also identified which could provide energy and substrates during the process of *N. antheraeae* life cycle. Remarkably, trehalose, as an important production of trehalose synthetic pathway, was also found here and this suggested that similar to other microsoridian, such as *Nosema bombycis*, *Encephalitozoon cuniculi* and so on, trehalose metabolic pathway was also as the primarily-dependent pathway in *N. antheraeae* to produce glucose for causing gemination (UNDEEN & VANDERMEER [15]) and supporting energy (VIVARES & al [16]).

Database searches showed that the monoisotopic mass of the obtained metabolites ranged from 57 to 539 Da (Table 1). Based on monoisotopic mass, all the obtained metabolites were divided into five groups (Fig. 2). Notably, the proportion of metabolites with monoisotopic mass ranging from 100 to 400 Da was as high as 89.8%.
Table 1. Identification of metabolites and their pathways of Microsporidia, *Nosema antheraeae*, by GC-MS technique.

| Metabolite          | RT    | HMDB    | KEGG   | Metabolite          | RT    | Identity   | KEGG   |
|---------------------|-------|---------|--------|---------------------|-------|------------|--------|
| **Amino acid**      |       |         |        |                     |       |            |        |
| Glutamic acid       | 28.02 | HMDB00148 C00025 |        | Octadecanoic acid   | 40.73 | HMDB00827 C01530 |
| Glycine             | 18.61 | HMDB00123 C00037 |        | Oleic acid          | 40.18 | HMDB00207 C00712 |
| L-Alanine           | 12.19 | HMDB00161 C00041 |        | Palmitelaidic acid  | 36.25 | HMDB12328 -   |
| L-Aspartic acid     | 22.26 | HMDB00191 C00049 |        | Tetradecanoic acid  | 32.6  | HMDB00806 C06424 |
| L-Isoleucine        | 14.34 | HMDB00172 C00407 |        | α-Linolenic acid    | 41.93 | HMDB01388 C06427 |
| L-Leucine           | 17.63 | HMDB00687 C00123 |        | Decanedioic acid     | 22.34 | HMDB00792 C02678 |
| L-Lysine            | 34.46 | HMDB00182 C00047 |        | Nonanedioic acid     | 20.7  | HMDB00784 C08261 |
| L-Methionine        | 21.64 | HMDB00696 C00073 |        |                     |       |            |        |
| L-Proline           | 25.16 | HMDB00162 C00148 |        |                     |       |            |        |
| L-Threonine         | 18.28 | HMDB00167 C00188 |        |                     |       |            |        |
| N-α-Acetyl-L-Lysine | 32.83 | HMDB03759 C03681 |        |                     |       |            |        |
| L-Valine            | 11.51 | HMDB00883 C00183 |        |                     |       |            |        |
| Serine              | 17.09 | HMDB00187 C00065 |        |                     |       |            |        |
| Tyrosine            | 33.48 | HMDB00158 C00082 |        |                     |       |            |        |
| **Alcohol**         |       |         |        |                     |       |            |        |
| Adonitol            | 30.71 | HMDB00508 C00474 |        |                     |       |            |        |
| D-Glucitol          | 42.95 | HMDB00247 C00794 |        | D-α-Gluconic acid δ-lactone | 33.98 | HMDB01127 -   |
| Glycerol            | 17.92 | HMDB00131 C00116 |        | D-Gluconic acid      | 36.59 | HMDB00625 C00257 |
| Maltitol            | 53.07 | HMDB02928 G00275 |        | Gluconic acid        | 34.2  | HMDB00150 C00257 |
| Mannitol            | 44.2  | HMDB00765 C0392 |        | Sterol               |       |            |        |
| Glucitol            | 35.32 | HMDB00247 C00794 |        | Cholesterol          | 55.64 | HMDB00067 C00187 |
| Xylitol             | 30.62 | HMDB02917 C00379 |        | Sugars               |       |            |        |
| **Fatty acid**      |       |         |        |                     |       |            |        |
| (9Z,12Z)-Octadecadienoic acid | 40.06 | HMDB05047 C01595 |        | D-β-Talose           | 34.42 | - C06467 |
| Docosanoic acid     | 48.18 | HMDB00944 C08281 |        | D-β-Trehalose        | 51.03 | HMDB00975 C01083 |
| Eicosanoic acid     | 44.6  | HMDB02212 C06425 |        | D-β-Turanose         | 52.68 | HMDB11740 C19636 |
| Heptadecanoic acid  | 38.71 | HMDB02259 -     |        | D-Ribofuranose       | 51.77 | - C16639 |
| Hexadecanoic acid   | 36.71 | HMDB00220 C00249 |        | Galactose            | 37.9  | HMDB00143 C00124 |
| Heptadecanoic acid  | 38.71 | HMDB02259 -     |        | N-Acetyl-D-glucosamine | 38.19 | HMDB00215 C00140 |
| **Others**          |       |         |        | Palatinose           | 53.58 | - C01742 |
| **Vitamin**         |       |         |        | Sedoheptulose        | 38.92 | HMDB03219 C00447 |
| **Sugars**          |       |         |        | Vitamin              |       |            |        |
| Maltitol            | 44.2  | HMDB00765 C0392 |        |                     |       |            |        |
| Xylitol             | 30.62 | HMDB02917 C00379 |        |                     |       |            |        |

* The ID of HMDB and KEGG were listed in the 3rd and 4th column.
Pathway Analysis
Pathway analysis allows to locate metabolites on known biochemical pathways, which provides clues to explore their possible genetic roles. Pathway analysis tools can play a role in finding out biological functions of metabolites in biological systems. Here, MetPA program was employed to perform metabolic pathway enrichment analysis of identified metabolites, result was shown in Fig. 3. The results showed that total forty-two metabolic pathways were involved. These data suggested that a wide range of metabolic pathways were worked and basic metabolism processes, such as protein biosynthesis, galactose metabolism, ammonia recycling and so on, were maintain at relatively high levels. Metabolic pathways related to energy metabolism, including citric acid cycle (TCA), pentose phosphate pathway and mitochondrial electron transport chain were also found here. Insulin signalling pathway, which can play a key role in regulation of gene transcription, protein synthesis, cell growth and gene expression, was also existed with a low metabolic abundance.

![Figure 3. Metabolic pathway enrichment analysis result of *N. antheraeae* metabolites using MetPA program](image)

Discussion
Microsporidia can infect a variety of hosts, reductive evolutions on genome and metabolome levels were verified to exist. *Enterocytozoon bieneusi* was reported missing core carbon metabolism, including pentose phosphate and trehalose metabolism (KEELING & al [17]). Instead, metabolites related to metabolic pathways of energy metabolism, including citric acid cycle (TCA), pentose phosphate pathway and mitochondrial electron transport chain were found here. A further survey on the key catalyzing enzyme genes involved in pathways of TCA and pentose phosphate pathway in *N. antheraeae* genome data. Six pentose phosphate pathway-related genes, including two 6-phosphogluconate dehydrogenase genes, one 6-phosphogluconolactonase gene, one glucose-6-phosphate 1-dehydrogenase gene, one epimeraseepimerase gene and one isomerase encoding genes, which played key roles in pentose phosphate pathway, were obtained. This indicated that pentose phosphate pathway was relatively complete in *N. antheraeae*, likely in *Nosema bombycis* (PAN & al [18]). Dramatically different parasitic conditions were speculated as the most possible causes for the absence of pentose phosphate metabolism in *Enterocytozoon bieneusi*. 
Interestingly, no genes of TCA pathway were found in genome data of *N. antheraeae*. The acquisition of metabolites involved in TCA pathway might attribute to take in host-derived nutrients via membrane transporters. Further, many key enzymes related to glycolysis pathway, such as enolase, glucose-6-phosphate isomerase, 6-phosphofructokinase, fructose-bisphosphate aldolase, etc., were searched in *N. antheraeae* genome data. This indicated the existence of glycolysis, but the absence of TCA pathway implied that the amount of produced ATP might well below that of glucose completely oxidized. It would be interesting to uncover how about the spores to overcome this problem to obtain more ATP for meeting the demand of growth and reproduction. Fortunately, one ATP/ADP carrier protein was found in *N. antheraeae*, which was reported can obtain ATP or other energy molecules from host cells in obligate intracellular parasitic bacteria, including Rickettsia and Chlamydia (KRAUSE & al [19], TRENTMANN & al [20]). Thus, it was speculated that ATP/ADP carrier protein may involved in energy transportation to meet the need of *N. antheraeae* for energy.

Microsporidian mainly relied on trehalose metabolism to degrade trehalose to glucose, this can cause spore germination through enhancement of inside osmotic pressure (UNDEEN & VANDERMEER [15]). It can also play an important role in spore resistance to low temperature or dry conditions. In addition, the generated glucose by trehalose metabolism became one of the basic ways to provide energy for microsporidian metabolic activity (VIVARES & al [16]). At present, the main enzymes encoding genes involved in trehalose metabolism were identified in many microsporidian including *Nosema bombycis, Nosema locustae, Nosema ceranae, Encephalitozoon cuniculi*, etc., except *Enterocytozoon bieneusi*. All of trehalose-6-phosphate synthases (TPS), trehalose-6-phosphate phosphatases (TPP) and UDP-glucose pyrophosphorylases (UGPA) possessed conserved domains and alike gene locus. Interestingly, microsporidian parasitized in terrestrial or aquatic organisms showed distinct modes of trehalose metabolism (DOLGIKH & SEMENOV [21]). In this study, TPS, TPP and trehalase encoding genes were obtained in *N. antheraeae* genome database. Meanwhile, trehalase was found in GC-MS-based metabolomics data. This indicated that *N. antheraeae*, which was parasitic in Tussah, may exhibit the similar mode of trehalose metabolism to *Nosema bombycis* and showed no obvious changes concentration of reducing sugar in spores during polar tube extruded (DOLGIKH & SEMENOV [21]).

Here, fourteen amino acids were identified and took almost 27% of the identified metabolites, including Glycine, Alanine, Lysine etc., they were employed as resources for protein synthesis in the life cycle. However, genomic data showed that repertoire for the biosynthesis of amino acids in microsporidia was very restricted. For example, asparagine synthetase and serine hydroxymethyltransferase encoding genes were reported only in *Encephalitozoon cuniculi* genome (KEELING & al [17]). While, asparagine synthetase were identified here in *N. antheraeae* and serine hydroxymethyltransferase gene was also missing. The free amino detected in spores may be imported by parasite during their intracellular development. A further study to compare corresponding concentrations of major and minor metabolites during different developmental stages in microsporidia, can offer more details for confirming he presence of pathways in parasite metabolic system.

**Conclusion**

This work firstly analyzed important metabolic pathways in microsporidia, *Nosema antheraeae*, using GC-MS. The main metabolic pathways, which the detected metabolites participated in, included protein biosynthesis, ammonia recycling, urea cycle, galactose metabolism, alanine metabolism, arginine and proline metabolism, malate-aspartate shuttle, and so on. The results provided a new tool for understanding the metabolic pathways in spores. It may offer a new horizon for seeking pathogenic molecules during infection of spores on metabolonomic levels. Next work would continually work on comparing concentrations of metabolites in spores under different developmental stages.

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