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Comprehensive relative quantitative metabolomics analysis of lycopodium alkaloids in different tissues of *Huperzia serrata*

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ABSTRACT

Qian ceng Ta, the whole plant of *Huperzia serrata*, is an important landscape and medicinal herbs and contains abundant bioactive lycopodium alkaloids. Although the structures of more than 100 lycopodium alkaloids in *Huperzia serrata* have been isolated and identified, the content and distribution of these alkaloids in different tissues are still unclear. In current study, an ultra-performance liquid chromatography-mass spectrometry based comprehensive metabolomics strategy was developed, including the extraction, separation, identification, and statistical analysis. The results showed that different types lycopodium alkaloids could be separated at different time-windows, which was helpful for further metabolite identification. Peak4388 and peak3954 were metabolite biomarkers for the different tissues according to the principle component analysis and partial least squares-discriminant analysis model. A computational tool based in-house database was also built up and used for putative identification. Of the 2354 true peaks after four-step filtration, 118 peaks were putatively identified as lycopodium alkaloids by using in-house database, and four of which was identified by authentic standards. Alternatively, another computational software was used to predict the fragmentation pattern, to dereplicate the structure of identified peaks, and identified the peak3585 to N-methylhuperzine A. The integration of both computational tools could be used for more metabolites identification.

1. Introduction

Qian Ceng Ta, the whole plant of *Huperzia serrata* (*H. serrata*), was an important Traditional Chinese Herbs (TCH) for the treatment of contusions, strains, swellings, schizophrenia, myasthenia gavis, and organophosphate poisoning since Tang Dynasty [1,2]. Huperzine A (HupA), a representative lycodine-type lycopodium alkaloid, was first isolated and identified by Chinese scientist in 1986 [2] and was found as a powerful, highly specific, and reversible acetylcholinesterase inhibitors [3,4]. This natural product has attracted widespread attention because of its unique pharmacological activities and low toxicity. Owing to the low content of HupA, slow growth of the plant, and no plant culture method for this plant, the supply for this compound is not sustainable [5]. Producing the lycopodium alkaloids in heterologous host by using synthetic biology is an alternative method, because this method has been successful to produce ginsenoside in yeast [6] and artemisinin in tobacco [7].

The biosynthetic pathway of HupA has been proposed based on labeled precursors feeding experiment by Ma and Gang [4]. Lysine/Ornithine decarboxylase (L/ODC) was proposed as the first enzyme

List of abbreviation: UPLC-MS, ultra-performance liquid chromatography-mass spectrometry; HPLC-MS, high-performance liquid chromatography-mass spectrometry; IS, internal standard; HupA, huperzine A; HupB, huperzine B; Lycop C, lycoposerramine C; Lycop D, lycoposerramine D; PCA, principle component analysis; PLS-DA, partial least squares-discriminant analysis; L/ODC, Lysine/Ornithine decarboxylase; CAO, copper amine oxidase; t_R, retention time; *m/z*, mass over charge.

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for HupA biosynthesis. Indeed, L/ODC could catalyze the first-step in the biosynthesis pathway of lysine-derived alkaloids in different lycopodium plants [8–10]. After the decarboxylation of lysine to produce cadaverine, it would be catalyzed by copper amine oxidase (CAO) to yield 5-aminopentanal, which is spontaneously cyclized to the first intermediate for lysine-derived alkaloids production, Δ1-piperideine [11]. However, the genes involved in the skeleton formation and modification are still unclear. Although some candidate genes for HupA biosynthesis were proposed based on the EST data and the global RNA-Seq data from *H. serrata*, none was characterized because of the lack knowledge of substrate and intermediates involved in HupA biosynthesis.

Metabolomics, focusing on the high-throughput qualitative and quantitative profiling of small molecular metabolites, has been used for the candidate genes mining and characterization by integrating with other omics [12]. In the past years, targeted metabolomics has been used for the detection of HupA in different lycopodium plants base on high-performance liquid chromatography (HPLC) to find the better sources than *H. serrata* [13]. To improve the efficiency of extraction and detection, an high performance liquid chromatography coupled with mass spectrometry (HPLC-MS) based method was developed for HupA detection, identification and quantification [14]. However, these works only focused on the content of HupA. Subsequently, another ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) based metabolomics method was developed and 27 lycopodium alkaloids were identified, of which only six metabolites were identified based on comparison of accurate MS and MS/MS data compared to the authentic standards [5]. Currently, more than 200 lycopodium alkaloids have been isolated and identified by NMR [4]. Given the limited authentic standards and recruited lycopodium alkaloids in the public databases, and limited reference MS/MS spectra, only few could be identified by commonly used tools. In addition, many lycopodium alkaloids had the same formula and skeleton, thus the MS and MS/MS spectra are similar, making it more difficult to identify. Combination of the computational tools will be more useful for the identification because the identification based on these tools were independent on the public databases.

In this study, a comprehensive relative quantitative metabolomics method for the extraction, detection and identification of lycopodium alkaloids in different tissues of *H. serrata* was firstly developed. For identification, a computational tool based lycopodium alkaloids specific in-house database was developed and identified118 putative chemicals. In addition, other computational software was also employed to dereplicate the structure of lycopodium alkaloids and identified the peak3585 to N-methyl-huperzine A based on the in-silico fragmentation pattern.

2. Materials and methods

2.1. Plant materials

The plants were collected from Xiangxi, Hunan, China, in January 2017. The plants were carefully rinsed in running tap water, and soil was removed by hand. Root, stem, and leaf were separated from the plant and ground to fine power in liquid nitrogen and ice-dried, and then stored at-80 °C until further using.

2.2. Authentic standards

The authentic standards of huperzine A, huperzine B (HupB), Lycoposerramine C (Lycop C) and lycoposerramine D (Lycop D) were separated from the crude alkaloids extracted from *H. serrata* and identified by NMR (Supplementary method) according to previous reports [2,15]. All purified authentic standards (>95% purity, Supplementary Figs. S1, S2) were dissolved in methanol with 100 ng/mL, filtered through 0.22 μM PTEE filters and stored at −20 °C until for using.

2.3. Lycopodium alkaloids extraction

The extraction of lycopodium alkaloids was carried out according to previous report with minor modification [16]. Around twenty mg dry power was weighted and extracted by 1 mL methanol including 0.1% (v/v) formic acid. After adding 400 ng caffeine as internal standard, the sample was sonicated for 15 min at room temperature. Centrifugation at 17000*g* for 10 min, the supernatants were filtered through 0.22 μM PTEE filters and then analyzed by UPLC-MS. Meanwhile, the blank samples were also treated with the same procedure. All samples including the blank were repeated three times.

2.4. Analytical platform

A waters Ultra performance liquid chromatography system (Waters Corporation, Milford, MA, USA) was used. That system was coupled to a Thermo Scientific Q-Exactive mass spectrometer equipped with a HESI interface (Thermo Scientific, Hemel Hempstead, U.K.). Thermo Xcalibur Tune software (version 3.0) was used for instrument control and data acquisition.

The separation was performed with a Waters Acquity HSS T3 C18 column (150 mm × 2.1 mm, i.d. 1.8 μm) in positive mode ESI. A linear biphasic LC gradient was conducted from 2% to 60% B over 12 min, and then kept at this phase for 2 min, followed by 3 min wash with linear biphasic gradient from 60% B to 2% B over 1 min, and 3 min re-equilibration with 2% B, where solvent A was 0.1% formic acid in water and solvent B was acetonitrile. The flow rate was 0.25 mL/min, column temperature was maintained at 30 °C, injection volume was 2 μL, and samples were maintained at 4 °C in the autosampler.

2.5. Data processing

The raw data files were firstly converted to mzXML format files and mzML format files using MSConvert [17]. After peak deconvolution and alignment processing by XCMS [18,19] and MS-DIAL [20], the given peak alignment table was further used to choose the true peaks before putative identification based on four-step filtration (Fig. 1) according to the previous method [21]. The filtered peaks were used for putative identification in different databases by using precursor ions (*m/z*) and/or MS/MS spectrum based on different software, xMSannotator [22], Metabosearch [23], and MS-DIAL [20]. Here, an in-house database for lycopodium alkaloids in *H. serrata* was also developed based on the computational tool, plantMAT [24]. Furthermore, MS-FINDER [25] and CSI:FingerID [26], were used for the prediction of in-silico fragmentations.

After processing by MS-DIAL [20], a plot view was shown and each plot was a detected peak. The information of spectrum, retention time, *m/z* value, and so on could be shown by left click. The interested peak (plot) could be linked to MS-FINDER [25] for exact identification by in-silico fragmentation pattern by right click. More details could refer to the manual tutorial (http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/MS-DIAL%20tutorial_vs9.pdf).

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