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Extensive characterization and differential analysis of endogenous peptides from *Bombyx batryticatus* using mass spectrometric approach

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ABSTRACT

Bombyx batryticatus, the dried larva of *Bombyx mori* L. (4th–5th instars) infected with *Beauveria bassiana* Vuill, is an important animal-derived medicine effective against several diseases. The metamorphosis of silkworm can result insignificant changes in the levels of proteins and polypeptides in the 4th and 5th instar larvae. Here, we performed extensive characterization of *Bombyx batryticatus* peptides, including polypeptides containing cysteines, using an MS-based data mining strategy. A total of 779 peptides with various PTMs (post-translational modifications) were identified through database search and *de novo* sequencing. Some of these peptides might have important biological activities. Besides, the differential analysis of polypeptides between the head and body of *Bombyx batryticatus* was performed to provide a clinical basis for rational use of the drugs derived from it. This study illustrates the abundance and sequences of endogenous *Bombyx batryticatus* polypeptides, and thus, provides potential candidates for the screening of active compounds for future biological research and drug discovery studies.

1. Introduction

Medicines derived from animals are an important part of traditional Chinese medicine (TCM), characterized by high activities and unique and definite curative effects [1]. Compared to small molecule compounds in medicines derived from plants, most bioactive ingredients in medicines derived from animals are proteins or polypeptides, such as various animal toxins [2,3], antithrombotic peptides [4], and antibacterial peptides [5]. However, research on determining the ingredients of animal-derived medicines has lagged considerably with respect to that on plant-derived medicines, because of limited animal resources as well as because of the fact that during the heat process for sterilization and storage the polypeptide might endogenously produce new peptides or protein fragments [6]. Peptidomics is a highly efficient detection technology for comprehensive qualitative and quantitative analysis of all peptides in a biological sample, which relies on high sensitivity of mass spectrometry technology, rapid development of bioinformatics technology, and ever-improving protein database of all species [7,8]. Peptidomics can greatly improve the efficiency of finding new proteins and polypeptides in natural products by omitting the purification steps involved in the traditional extraction processes including plant bioactive peptides [9,10], which makes the conversion of one single active component into multiple components more synchronized [11]. It is pertinent to mention that different parts of animals used for medicine may have different effects and applications in actual clinical work because of the unbalanced distribution of active polypeptides in them; for example, the glands of *Scorpio* or *Scolopendra* contain high levels of toxin polypeptides [12]. Therefore, a differential analysis of polypeptides in the animal-derived medicines is necessary to find characteristic polypeptide markers.

Animal venoms are mainly composed of polypeptides and proteins. A combination of multiple “omics” techniques is used to discover novel proteins and peptide sequences in animal venoms and the activity of proteins is verified through protein recombination techniques [13] or solid-phase peptide synthesis technology [14], which play important roles in deciphering the mechanism of production of active proteins and peptides, the mechanism of drug activity, and in the designing of new drugs. Calvete [15] proposed a strategy for snake venomics, using reverse phase HPLC for fractionation of the crude venom and a combination of N-terminal sequencing, SDS-PAGE, and mass spectrometric determination of the molecular masses and cysteine content for characterization of each protein fraction, which can be used for BLAST analysis to establish a database of snake venom protein. Diego-García [16] studied

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the poison glands and venom of the spider, *Citharischius crawshayi*, using transcriptomics, peptidomics, and bioactive functional analysis, to construct a cDNA library and generate 236 expressed sequence tags (ESTs). Liang [17] studied the venom of the spider, *Ornithoctonus hainana*, and identified 192 mature sequences using a venomics strategy, which involved transcriptomics, peptidomics, and genomics approaches. A combination of such modern technologies can significantly improve the drug research, accelerate the research cycle, reduce the investment risk, and help in the sustenance of new drug research.

Bombyx batryticatus, a valuable traditional Chinese medicine, is the dried stiff larval stage of silkworm, *Bombyx mori* Linnaeus, infected with *Beauveria bassiana*. It was firstly recorded in 'Shen Nong's Herbal Classic' and has been widely used for clinical purpose either alone or with other compatible TCMs for thousands of years [18]. Because of unfavourable features of its unprocessed form, *Bombyx batryticatus* is often used as a processed product, mostly after stir-frying with bran, roasting with honey or ginger, and sauting with bran; the processing may, however, produce many new bioactive peptides [19]. Studies on *Bombyx batryticatus* and its application in traditional uses, origin, chemical constituents, pharmacology, and toxicity have recently been reviewed [20]. *Bombyx batryticatus* has been shown to contain various biological ingredients, including protein and peptides, fatty acids, flavonoids, nucleosides, steroids, coumarins, and polysaccharides, which play important roles in its multiple pharmacological effects, such as on nervous system [21,22], coagulation [23], cancer [24,25], and bacterial infection [26]. A BB octapeptide [4] was isolated from *Bombyx batryticatus* as a novel platelet aggregation inhibitory peptide with an amino acid sequence of Asp-Pro-Asp-Ala-Asp-Ile-Leu-Gln; cyclic peptides were also identified from *Bombyx batryticatus*, such as beauvericin [27], which is a cyclic three carboxylate peptide. The structure of BBPW-2 [24], an antioxidative polysaccharide, consists of β-D-(1→2,6)-glucopyranose and β-D-(1→2,6)-mannosyl units as backbones, α-D-(1→2)-galactopyranose and α-D-(1→3)-mannosyl units as branches, and α-D-Man and β-D-Glc as the terminals.

The presence of neuropeptides [28] and specific glands [29] in the head of *Bombyx batryticatus* suggests that the medical substances (especially polypeptides) in the head and the body might be different. Unfortunately, the sequences of most of these endogenous peptides remain unknown; especially, the differences in the endogenous polypeptides from silkworm head and body are not known. Therefore, a systematic approach should be taken for further research on the pharmacological activities of the natural peptides in *Bombyx batryticatus*. Our group had previously performed extensive studies on the analysis of a number of peptides in animal tissues and ginseng extracts [30–33], particularly for the discovery and identification of disulphide peptides [30]. In the present study, we identified the structures of *Bombyx batryticatus* polypeptides using nano-LC–MS and peptidomics technologies. Moreover, we have searched and characterized peptides containing cysteine residues through a derivative method, and have performed differential analysis to find out the different polypeptides present in the head and body of *Bombyx batryticatus*, which should provide a theoretical basis for further pharmacological research and clinic application.

2. Experimental

2.1. Chemicals and reagents

Three sources of *Bombyx batryticatus* were obtained from different Dalian Pharmacies, which were stored at 4 °C until use. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from Sigma Aldrich, formic acid was purchased from Tianjin Kemiou Chemical Reagent Co., Ltd (China), and pure water was prepared using a Milli Q system. Solid-phase extraction (SPE) cartridges were loaded on approximate 500 mg of C_{18} sorbent (Beijing Greenherbs Science and Technology Development Co., LTD. China) into the 2 mL polypropylene SPE tubes. DL-DTT (dithiothretol, 99% purity) and IAM (iodoacetamide, 98% purity) was obtained from Aladin (China).

2.2. Extraction, reduction and alkylation of Bombyx batryticatus peptides

Each source of *Bombyx batryticatus* herd was first grinded into powder using mortar and pestle, and 100 mg aliquot of *Bombyx batryticatus* powder was extracted using ultrasonic (Model: Ultrasonic Cleaner YQ-1000C with amplitude of 5026) with 1.5 mL of 50% methanol (in 2 mL EP tubes) in an ice-bath for 10 min. After the extract was subjected to 12,000 rpm/min centrifugation for 10 min successively, the supernatant was collected for nano-LC–MS analysis. Since the *Bombyx batryticatus* powder cannot be fully soluble in water, endogenous polypeptides with different polarity can be extensively extracted by ultrasonic-assisted method with 50% methanol due to its medium polarity, instead of direct water extraction like milk [34]. Besides, animal drugs often need to be processed at high temperature to facilitate storage and transportation, so ultrasonic extraction should not cause changes of endogenous peptides from *Bombyx batryticatus*.

The reduction and alkylation of peptide extracts from the *Bombyx batryticatus* was performed using the classic DTT/IAM reaction. First, the supernatant of crude extract by 50% methanol was transferred and dried by nitrogen gas, and was dissolved in 100 μL of 6 M urea in 100 mM Tris buffer (pH = 7.8). Then 20 μL of the reducing reagent (800 mM DTT in 100 mM Tris buffer, pH = 7.8) was added and the sample was mixed by gentle vortex and the reduction was carried out in the dark for 4 h at room temperature. A volume of 80 μL of the alkylating reagent (200 mM IAM in 100 mM Tris buffer, pH = 7.8) was then added to aklylate the sample for another 4 h in the dark at room temperature. After that, another 30 μL of the reducing reagent was added to consume any unreacted IAM. C18 SPE columns were then used to get the sample desalted. The SPE column was equilibrated with 0.1% formic acid before sample loading. The desalination and elution procedure were carried out using 0.1% formic acid and 80% methanol, respectively. The obtained desalted alkylated peptides were then concentrated by N_2 flow for subsequent nano-LC–MS analysis.

2.3. Nano-LC–MS analysis of Bombyx batryticatus peptides

A Thermo LTQ-Orbitrap Elite (Thermo Fisher Scientific, Bremen, Germany) system equipped was used to analyze *Bombyx batryticatus* extracts and their derivatives for peptide sequencing. An Ultimate 3000 nano-LC pump and a self-packed C18 column (75 μm ID, 25 cm length), of which the packing material was ReproSil-Pur C18 AQ particles (3 μm, 100 Å; Dr. Maisch GmbH) were coupled online to the mass spectrometer through a nanospray ion source. The elution direction of the trap column was reversed through a 6-port valve when it was switched to be coupled to the analysis column connected to the mass spectrometer *via* ESI interface. Conditions for LC–MS analysis were set as follows: a linear gradient elution of solvent A (0.1% formic acid) and solvent B (80% acetonitrile containing 0.1% formic acid) were used with the following stepped gradient program: 0–7 min, 5% B, 0.14 μL/min; 7–8 min, 5% B, 0.14-0.28 μL/min; 8–10 min, 5–25% B, 0.28 μL/min; 10–47 min, 25–80% B, 0.28 μL/min; 47–50 min, 80–95% B, 0.28 μL/min; 50–52 min, 95% B, 0.28 μL/min. The total running time was 52 min for each injection and the sample injec-