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•Editorial•

Difficulties in research of Chinese medicine polysaccharides

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[ABSTRACT] Polysaccharides from Chinese medicines are attracting increasing attention to their wide range of valuable biological activities. As these polysaccharides are mostly from edible materials, their safety can be greatly ensured. Therefore, the Chinese medicine polysaccharides have been the focus of research and development of new drugs and health products. However, there are rarely successful cases. Here, based on the authors' own research experience, the difficulties and challenges in chemical analysis and mechanism study of Chinese medicine polysaccharides are discussed, in the hope of eliciting more innovative ideas and solutions.

[KEY WORDS] Polysaccharides; Chemical analysis; Mechanism study

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Introduction

The main dosage form of traditional Chinese medicine in clinical use is water decoction, in which polysaccharides are the dominant component. And most of the Chinese medicines which have tonic properties, especially Qi, Yin and blood enrichment, are rich in polysaccharides, e.g. Ginseng Radix, Astragalus Radix, Angelica Sinensis Radix, Ganoderma, and Dendrobii Caulis [1-4]. The polysaccharide content can even reach beyond half of the total dry weight in Dendrobium Caulis samples. In recent years, more and more studies have shown that these polysaccharides have a variety of biological activities, such as immune-enhancement, inhibition of tumor growth, anti-virus, regulation of blood sugar level, myocardial protection, etc. [5-12]. Therefore, polysaccharides are the main active ingredients of Chinese medicines. More importantly, the sources of these active polysaccharides are mostly edible plants so that the safety can be guaranteed. As a result, the active polysaccharides have always been a hot spot in the development of Chinese Medicine healthcare products and new drugs. However, the progress is not satisfactory. The main reason is that polysaccharides are highly complex polymer compounds with high polarity. The nature of polysaccharides is similar to proteins but have more diverse sugar linkages that makes them non-crystallizable, and difficult-to-detect and difficult-to-isolate [13]. This article will discuss the difficulties and solutions in the study of polysaccharides based on author's own research experience.

The confusing concept of purity

Polysaccharides naturally exist in form of carbohydrate mixture in Chinese medicines. Therefore, the concept of purity of small molecules which is based on a single molecule, is not suitable for the category of Chinese medicine polysaccharides. As a result, how should "pure polysaccharides" be defined? There is currently no consensus on this issue. Some studies use sugar content as the standard and the minimum requirement for "pure polysaccharide" is set at 90%. Some studies require not only a higher sugar content, but also a symmetric molecular weight distribution pattern in a narrow range. Some other studies further classified polysaccharides according to their charges (so-called acidic, neutral and alkaline polysaccharides). However, separation and purification based on molecular weight and charge are highly dependent on chromatographic materials, and different chromatographic materials can give out different results. In the end, the purification of polysaccharides (in other words, the separation of the polysaccharide mixture) would be an endless task, due to the lack of a uniform and clear concept of polysaccharide purity.

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Dedicated to Professor SUN Han-Dong on the Occasion of His 80th Birthday

The author believes that the natural properties of Chinese medicine polysaccharides should be fully respected, and different criteria should be adopted according to the individual research purpose. If the main purpose is quality control or activity evaluation, it is recommended not to pursue the so-called "single polysaccharide", because such a purification process is very complicated and difficult to be repeated. It will lose its comparability between different sample batches. It will also bring unsolvable problems to the quality control of polysaccharides. If the "pure polysaccharides" for research are uniformly defined on the principle of no small molecules, no protein and with high sugar content, the quality of extracted polysaccharides from different batches of the same Chinese medicine will then become more comparable and repeatable. As a result, when evaluating pharmacological activities, different studies can be compared. Due to the close or identical material basis, it is easier to compare extracted polysaccharides between different batches and different studies, and the repeatability of product quality will be greatly improved. However, the specific delineation of the requirements needs further discussion, and it needs the consensus of the official parties, enterprises, universities and institutes. In addition, if the research objective is mainly on the chemical structure characteristics, then under the premise of ensuring the sample size, it is possible to pursue a relatively single molecular feature as much as possible in order to obtain accurate structural information. Even so, the reproducibility of the sample should be taken into account so as not to reduce the reference value of the research outcomes.

Concerns with chemical analytical methods

The chemical analytical methods that are going to be discussed mainly refer to the traditional polysaccharide structure elucidation techniques, such as molecular weight determination, monosaccharide composition analysis and methylation analysis (sugar linkage), etc. [14-16]. These methods are classical methods with a long history, but they have shown considerable limitations in application. At present, the most commonly used molecular weight determination method is the gel permeation chromatography, which determines the relative molecular weight by comparing the retention time of the polysaccharide sample to a series of reference standards. This kind of method has the following three prominent limitations. First, it relies heavily on reference standards. The results given from different series of standards (Pullulan and Dextran series) can be very diverse. Furthermore, the natural polysaccharide structure is more complicated than the reference standards, so that the data obtained may not truly reflect the actual value. Second, it is heavily dependent on the columns. The results given by different but same type of columns are also variated. The main cause is that the molecular weight of the sample exceeds the applicable range of the column so that the retention time is shorter, resulting in inaccurate data. Third, it is the nature of the Chinese Medicine polysaccharides. In nature, the Chinese Medicine polysaccharides are a mixture of saccharides that are in different molecular weights. Therefore, different purification methods will produce different "purified polysaccharides". The author suggests that from the perspective of quality control for the specific Chinese Medicine polysaccharides, it should emphasize the molecular weight distribution patterns of the sample, with appropriate standard, column, mobile phase elution procedure and detection method instead of the detected molecular weight value. In this way, the stability and comparability on the molecular weight between different batches of samples can be greatly improved.

The proportional relationship provided by the monosaccharide composition analysis and the methylation analysis is only based on the relative values of the peak areas of the hydrolysates after acid hydrolysis of the original or methylated polysaccharides. However, this value is not stable because studies have found that different acid hydrolysis conditions can greatly affect the yield of hydrolysates [17-18]. Both abovementioned analytical methods require full hydrolysis, and the hydrolysis conditions are relatively harsh. Under normal circumstances, if only monosaccharide peaks are observed under a certain hydrolysis condition, it can be regarded that the polysaccharide has been completely hydrolyzed. However, such a criterion is not reliable. For example, *Dendrobium officinale* polysaccharide can be completely hydrolysed under three different acid hydrolysis conditions: 100 °C, 4 h, 110 °C, 4 h, 120 °C, 4 h hydrolysis. However, the monosaccharide composition ratio is quite different in different hydrolytic condition. The experimental results showed that the actual ratio of mannose to glucose decreased from 3.8 : 1 at 100 °C, 4 h to 2.8 : 1 at 110 °C, 4 h, and finally rose to even 3.1 : 1 at 120 °C, 4 h. When the acid hydrolysis conditions become more intense, the monosaccharides produced by the hydrolysis will also undergo significant degradation, which in turn causes a significant change in the proportion of the composition based on the peak area. In addition, from the experimental data, it can be observed that when the acid hydrolysis condition was 120 °C, 4 h, the actual content of mannose was decreased by 6.6% when compared that at 110 °C, 4 h. Because methylation analysis relies heavily on the proportion of monosaccharide composition so as to deduce the structure of the entire polysaccharide, it is often the case that even polysaccharides are extracted from the same Chinese medicines, different structures are reported. Moreover, the structural information of the backbone and side chains is always mixed together in methylation analysis, therefore, it is impossible to distinguish results from the main chain and side chain. More innovative analytical methods other than monosaccharide composition analysis and methylation analysis in structure elucidation are needed.

The authors' research team found a simple and effective method that not only accurately analyzes the backbone structure of polysaccharides, but also determines the content of

different polysaccharides in Chinese medicines formulae [19]. This method involves three parts. The first part is to partially hydrolyze the polysaccharide, and then the resulting oligosaccharide fragments are derivatized with *p*-aminobenzoic ethyl ester (ABEE) to form a glycoside compound. Finally, the oligosaccharides were separated by preparative HPLC. The advantage of this method is that derivatized oligosaccharides become glycosides which belong to the category of small molecules. In addition, the oligosaccharides have ultraviolet absorption after derivatization, so these labeled oligosaccharides can be easily separated by commonly-used columns. By mass spectrometry (MS) sequencing and nuclear magnetic resonance (NMR) spectroscopy of these labeled oligosaccharides, we were able to obtain their saccharide sequences and the entire linking structure at a higher confidence level. After analyzing the labeled oligosaccharides with different sugar unites, it is able to intergrade a series of sequence information of the fragments so as to obtain the backbone sequence of the polysaccharide. In addition to the structure elucidation of polysaccharides, we have also established the application of utilizing these oligosaccharide markers in the qualitative and quantitative analysis of polysaccharides. The content of the labeled oligosaccharides can be accurately determined by the extracted ion chromatogram (EIC) in liquid chromatography–mass spectrometry (HPLC-MS). Because different polysaccharides have different characteristic oligosaccharide fragments, qualitative analysis of polysaccharides in Chinese medicine formulae can be achieved by identifying the specific labeled oligosaccharides. At the same time, we found that the polysaccharide has a good linear relationship with its specific oligosaccharide markers. The method validation shows that the linear relationship can be applied to the accurate quantification of polysaccharides. Therefore, this reliable qualitative and quantitative method can be widely applied to the quality control of polysaccharides in single herb, Chinese Medicine formulae and related products.

Weakness of the spectral analytical method

The development of spectroscopic techniques, especially NMR spectroscopy, provides an effective means for the study of small molecules and even the structure elucidation study of macromolecules like proteins. The structure elucidation study of polysaccharides is also increasingly employing NMR spectroscopy, especially two-dimensional nuclear magnetic resonance (2D NMR) spectroscopy as an essential means. However, when people are relying more and more on NMR spectroscopy, they often overlook its limitations resulting in many judgement errors. First, NMR spectroscopy is highly dependent on the signal resolution in the determination of structural information. Once signals overlap, it will induce a huge challenge to the analysis of the 2D NMR spectrum. The situation is particularly serious in the analysis of polysaccharides, a multitude of signals are concentrated in an extremely narrow range of 3.5 to 5 ppm resulting in severely signal overlapping. Thus, one signal in the 2D NMR spectrum may have multiple interpretations, and it is easy to give erroneous results, whether it is determined from reference data or subjective judgment. Moreover, polysaccharides in nature are in the state of a mixture. Even if the partially hydrolyzed oligosaccharides are tested and analyzed, it is also in the form of mixture. Therefore, signals from various oligosaccharides are heavily overlapped so that it is impossible to accurately determine the saccharide linkage information. The limits of spectral analytical methods should be highlighted by the researchers.

Advances in mechanism study on polysaccharides' bioactivities

To better understand the mechanism of the biological activities of these molecules, the first thing is to determine whether the polysaccharide can be absorbed or not. The reported works [20-23] showed that on the one hand, some orally-dosed polysaccharides labeled with fluorescence can be absorbed and enter into circulative system and distributed to organs such as liver, lung and kidney. On the other hand, some polysaccharides were proved to be non-absorbable. All these works suggest that although the polysaccharides' absorption behavior is still problematic, low bioavailability is definitely confirmed, comparing to small molecules. Currently, many scientists have directly bypassed the existing problems of absorption and turned to open a new front of developing polysaccharide injections. However, with the increasingly strict regulation of Chinese medicines injections, this direction is also out the tantalizing prospect. Are there any commonly existing mechanisms of orally-dosed polysaccharides? Recently, taking the marker polysaccharide (DOP) of *Dendrobium officinale* as an example, the authors aimed to explore the dynamic distribution and degradation of orally-dosed DOP in mice and *in vitro* using near-infrared fluorescence imaging and kind of chromatographic analysis [24]. The results indicate that, 1) neither DOP nor fluorescence-labeled DOP (FDOP) was absorbed; 2) both DOP and FDOP were undigested and were quickly degraded to short-chain fatty acids in the large intestine; 3) DOP modulated gut microbiota, which could be associated with DOP's suppression of 4T1 tumor growth in mice. All these findings suggest that some (maybe not all) bioactive polysaccharides share the common destiny: indigestible and non-absorbing, end in modulating bioactivities-associated gut microbiota.

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