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Short communication

Structural diversity requires individual optimization of ethanol concentration in polysaccharide precipitation

Jun Xu, Rui-Qi Yue, Jing Liu, Hing-Man Ho, Tao Yi, Hu-Biao Chen*, Quan-Bin Han*

School of Chinese Medicine, Hong Kong Baptist University, 7 Baptist University Road, Kowloon Tong, Hong Kong

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ABSTRACT

Ethanol precipitation is one of the most widely used methods for preparing natural polysaccharides, in which ethanol concentration significantly affects the precipitate yield, however, is usually set at 70–80%. Whether the standardization of ethanol concentration is appropriate has not been investigated. In the present study, the precipitation yields produced in varied ethanol concentrations (10–90%) were qualitatively and quantitatively evaluated by HPGPC (high-performance gel-permeation chromatography), using two series of standard glucans, namely dextrans and pullulans, as reference samples, and then eight natural samples. The results indicated that the response of a polysaccharide's chemical structure, with diversity in structural features and molecular sizes, to ethanol concentration is the decisive factor in precipitation of these glucans. Polysaccharides with different structural features, even though they have similar molecular weights, exhibit significantly different precipitation behaviors. For a specific glucan, the lower its molecular size, the higher the ethanol concentration needed for complete precipitation. The precipitate yield varied from 10% to 100% in 80% ethanol as the molecular size increased from 1 kDa to 270 kDa. This paper aims to draw scientists' attention to the fact that, in extracting natural polysaccharides by ethanol precipitation, the ethanol concentration must be individually optimized for each type of material.

1. Introduction

Natural polysaccharides are important biologically active components of many medicinal herbs, and have thus been attracting increasing multidisciplinary research interest [1]. Several challenges exist in this research field: crystal structure determination, quality evaluation, in vivo detection, and molecular target exploration [2–5]. However, before these challenges can be effectively tackled, a more fundamental problem must be addressed, namely, accurate, consistent sample preparation [6–8].

As the commonly-used sample pretreatment operation, ethanol precipitation is generally the first step in preparing crude polysaccharides from water extracts [9–11]. To some extent, previously, the methodology of ethanol precipitation for some specific samples, e.g. *Citrus* pectins [12], inulinases [13], water extracts of Danshen (*Salvia miltiorrhiza* Bge.) and Chuanxiong (*Ligusticum chuanxiong* Hort.) [14], was investigated in terms of ethanol concentration, supernatant pH value, and refrigeration temperature. The results indicated that the yield of total saccharides increased as ethanol concentration increased. The effect of supernatant pH value is not very significant. Although temperature decreased from 25–5 °C also led to an increase of saccharide yield, as the precipitation is usually performed at around 4 °C in a laboratory refrigerator, ethanol concentration seems to be the most important variable in ethanol precipitation.

We found and reviewed a total of 171 publications in *ScienceDirect* from Jan 1 to May 30, 2013 (Fig. 1) in which ethanol precipitation was used for the preparation of natural polysaccharide. In more than 70% of these publications – i.e. an overwhelming majority – the ethanol concentration used was 70–80%, which seems a standardized condition. Approximately 15% of these papers did not mention the ethanol concentration they used, suggesting that ethanol concentration was not considered important. And none of these publications include an optimization of the ethanol concentration. Some questions naturally arise: Will a fixed ethanol concentration (e.g. 70–80%) completely precipitate all polysaccharides in every type of natural product? Will varied ethanol concentrations extract different polysaccharides from the same sample? Will different polysaccharides within similar molecular sizes share the same optimal ethanol

* Corresponding author. Tel.: +852 34112906/+852 34112060; fax: +852 34112461.
E-mail addresses: hbchen@hkbu.edu.hk (H.-B. Chen), simonhan@hkbu.edu.hk (Q.-B. Han).

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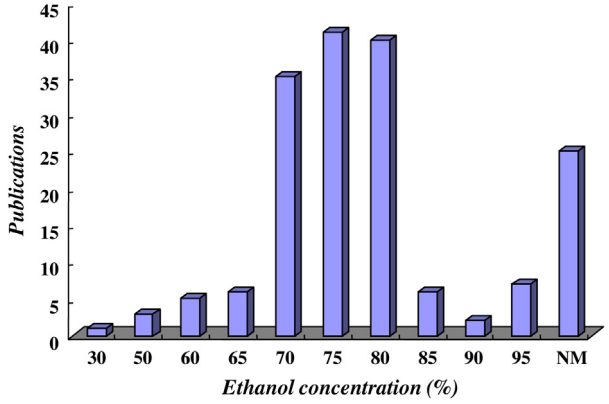

Fig. 1. The statistical results of the ethanol concentration used for polysaccharide precipitation from natural products in the published paper in *ScienceDirect* Database since Jan, 2013 (data was processed on May 30, 2013) (NM: not mentioned).

concentration? Should ethanol concentration be optimized for each natural product?

In order to answer these questions, in the current study we first used two series of reference glucans, branched dextrans and unbranched pullulans, to qualitatively and quantitatively evaluate the effect of ethanol concentration on the precipitation of polysaccharide by HPGPC (high performance gel permeation chromatography). Multiple parameters that could affect the ethanol precipitation results, such as structural features, molecular size, and ethanol concentration, were systematically investigated. Eight commonly-used polysaccharide-rich herbal/fungi materials were then used as natural samples to determine if and how variation in ethanol concentration affected natural polysaccharide precipitation.

2. Experimental

2.1. Materials and chemicals

Eight commonly used medicinal herbs/fungi, namely *Angelica sinensis*, *Codonopsis pilosula*, *Dendrobium officinale*, *Ligusticum wallichii*, *Panax ginseng*, *Panax notoginseng*, *Ganoderma lucidum* and *Ganoderma sinensis*, were selected as representative polysaccharide-rich natural samples. The herbal/fungi materials were purchased from herb markets in mainland China and were authenticated by Dr. Chen Hubiao. The voucher specimens were deposited at School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China.

Deionized water was prepared by Millipore Milli *Q*-Plus system (Millipore, Bedford, MA, USA) and ethanol was purchased from RCI Labscan Ltd. (Bangkok, Thailand). The reference glucan substances, dextrans and pullulans (Fig. 2) with known molecular sizes (1–270 kDa for dextrans, 6–805 kDa for pullulans), and glucose were bought from Sigma (St. Louis, MO, USA).

2.2. Preparation of water extracts

Herbal material was dried and powdered. For each sample, 10 g of powder was first ultrasonically extracted with 100 mL of acetone for 1 h to remove liposoluble substances, and then reflux-extracted with water at 100 °C (100 mL) for 1 h, twice. The decoctions were combined and centrifuged at 3500 rpm for 10 min. The total sugar content in the solution, calculated as glucose, was adjusted to about 2.0 mg/mL for further analysis [15].

Table 1
Calibration curves of the HPGPC quantitative assay of dextrans and pullulans.

	Mw (kDa)	Range (mg/mL)	Equation	R^2
Dextrans	1	0.13–4.23	$y = 1.8387x + 3.8191$[a]	0.9981
	5	0.23–3.68	$y = 2.0166x + 3.7503$	0.9975
	12	0.22–3.59	$y = 1.9444x + 3.7895$	0.9997
	25	0.11–3.51	$y = 1.749x + 3.7965$	0.9971
	50	0.12–3.72	$y = 1.8751x + 3.7307$	0.9989
	80	0.21–3.30	$y = 1.9263x + 3.6545$	0.9991
	270	0.23–3.67	$y = 1.9301x + 3.671$	0.9973
Pullulans	6	0.12–3.69	$y = 1.812x + 3.8354$	0.9991
	10	0.21–3.41	$y = 1.7843x + 3.8979$	0.9994
	21.7	0.15–4.89	$y = 1.797x + 3.7683$	0.9987
	48.8	0.11–3.48	$y = 1.8902x + 3.8747$	0.9974
	113	0.10–3.32	$y = 1.8518x + 3.7968$	0.9963
	210	0.13–4.14	$y = 1.81x + 3.7918$	0.9955
	366	0.12–3.84	$y = 1.7973x + 3.8851$	0.9955
	805	0.11–3.66	$y = 1.8666x + 3.7908$	0.9967

[a] x and y means the logarithms of corresponding saccharide concentration and HPGPC peak area.

2.3. Ethanol precipitation

Aqueous stock solutions of dextrans and pullulans with different molecular sizes (2 mg/mL, 5 mL) were precipitated by adding ethanol to make a final concentration of 10–90% (v/v), respectively, and left overnight (12 h) at 4 °C. After centrifugation (3500 rpm) for 10 min, the precipitate was collected, washed with ethanol, dried (water bath, 70 °C) to remove any residual ethanol, and then was completely re-dissolved in 5 mL hot water (60 °C) by drastic mechanical vibration for 2 h. Finally, each solution was filtered through a 0.22 μm syringe filter (Agilent Technologies, USA) for HPGPC analysis [16]. Solutions of the herbal samples were prepared using the same method.

2.4. HPGPC analysis

HPGPC analyses were performed on an Agilent 1100 series (Agilent Technologies, Palo Alto, CA) equipped with DAD and ELSD and two tandem TSK GMPW$_{XL}$ columns (300 mm × 7.8 mm i.d. 10 μm) at 40 °C. Ammonium acetate aqueous solution (20 mM) was used as mobile phase at a flow rate of 0.6 mL/min. DAD was set at 260 nm and 280 nm. The parameters of ELSD were set as: the drift tube temperature was 120 °C, and nebulizer nitrogen gas flow rate was at 3.2 L/min, impact-off mode. An aliquot of 20 μL solution was injected for analysis. Because polysaccharides have no UV absorption, UV detector was set at 260 nm and 280 nm in order to monitor the existence of nucleic acid and/or peptide in this study.

Aqueous stock solutions of dextrans and pullulans with different molecular weights were diluted to appropriate concentrations for the construction of calibration curves. At least five concentrations of each solution were analyzed in duplicate, and then the calibration curves were constructed by plotting the logarithm of the peak area versus concentration of each analyte.

3. Results and discussion

3.1. Impact of ethanol concentration, molecular size and structural features on precipitation

The reference standards dextrans and pullulans were precipitated at different concentrations of ethanol (10–90%). The obtained precipitates were quantitatively determined using the established HPGPC calibration curves (Table 1). The recovered yields of these glucans are shown in Fig. 3, and their individual HPGPC chromatogram can be found in Supplementary Figs. 1 and 2.