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Structural elucidation of a polysaccharide from *Lonicera japonica* flowers, and its neuroprotective effect on cerebral ischemia-reperfusion injury in rat

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ABSTRACT

A water-soluble polysaccharide, LJPB2, was purified from *Lonicera japonica* flowers. The present study was aimed to illustrate its structural features and its neuroprotective effect via anti-oxidant activity on focal ischemia/reperfusion (I/R) injury in rat brain. Via chemical and spectral methods, LJPB2, a polysaccharide with molecular weight of 8.9×10^3 Da, was composed of arabinose, mannose, glucose, and galactose, in a molar ratio of 1.8: 1.0: 3.6: 3.7. In addition, the linkage analysis revealed that it mainly contained 1, 4, 6-linked mannose, 1, 4-linked glucose and 1, 4-linked galactose, with a highly branched structure of araban and terminal glucose. LJPB2 exhibited a strong capacity of scavenging DPPH free radical in vitro. Moreover, in vivo assay using a commonly used cerebral I/R model demonstrated that LJPB2 could significantly improve the neurological deficit scores and infract volume. In addition, LJPB2 remarkably reduced the MDA level and NO production, and elevated the SOD and GSH-Px enzyme activities in the rat brain tissues. These results certainly indicated that LJPB2 had a distinct neuroprotective effect related to its strong antioxidant capacity in the cerebral ischemia/reperfusion injury.

1. Introduction

Ischemia reperfusion (IR) injury is often referred to the parenchymal injury and dysfunction of organ where blood supply restores after a critical period of ischemia. It is often observed in organ transplants, and major organ resections [1]. Additionally, the IR injury often causes irreversible brain, heart, kidney, liver or other organs damage. Therein, the cerebral IR is the leading serious disability afflicting a large amount of the patients. The pathological events associated with cerebral IR injury causing neuronal injury and death in the brain failure include the interruption of cerebral blood flow, the production of oxygen free radicals, calcium overload, energy failure, cell apoptosis, and inflammatory reaction [2]. Accumulating evidence suggests that the damage to the neuronal cells may be correlated to the production of reactive oxygen species (ROS), free radicals and lipid peroxidation in the pathogenesis of cerebral IR injury [3]. Once the brain is injured, reactive oxygen and nitrogen species can be generated by several cellular pathways, including calcium activation of phospholipases, nitric oxide synthase, and xanthine oxidase. The reactive oxygen species (ROS) has recently been considered as secondary messengers to propagate pro-inflammatory or growth-stimulatory signals [4]. In the case that cellular defense systems have been impaired, the increasing generation of free radicals will result in oxidation of lipids, protein, and nucleic acids, which elicit significant alterations of cellular functions [5]. Antioxidants are known as free radical scavengers, which can protect the tissues from oxidative damage. Antioxidants, such as Vitamin E, Vitamin C and uric acid, have been studied for their roles in the neuroprotective effect in animal models or clinical studies [6,7]. These results indicate that ameliorating excessive ROS can reduce oxidative stress occurring in I/R injury. Therefore, supplementation of anti-oxidants can be a potent and feasible strategy to protect against the cerebral ischemia reperfusion injury.

In recent years, the studies of traditional Chinese medicines (TCMs) in the cure of I/R injury have gained more and more interest since substantial TCMs and their biological components are demonstrated to possess anti-oxidant and neuroprotective activities [8–10]. Given the low toxicity and small side effects, polysaccharides, the major constituents in herbal extracts, have attracted more scientists to explore their bioactive fragments. In

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addition, accumulating evidence indicates that polysaccharides are viewed as or demonstrated to be natural anti-oxidant constituents that are salutary to human health [11,12]. Therefore, it is worthy to explore the effective anti-oxidant polysaccharides for preventing and curing cerebral ischemia reperfusion injury.

Lonicera japonica flower, named as the Japanese honeysuckle, or known as "Jin Yin Hua" or "Ren Dong" in China, is native to eastern Asia [13,14]. In the past, its dry flower is usually used as a traditional Chinese medicine to treat various illnesses including allergy, fever, arthritis, and several diseases [15]. Previous reports have shown that extracts of its flower buds exhibit inhibition of nitric oxide (NO) generation, α-glucosidase and protein tyrosine phosphatase 1 B [15], attenuate the cytotoxicity induced by Aβ42 aggregation [16], and exert anti-oxidant [17], anti-tumor [18], anti-encystment and amoebicidal activities [13]. In addition, it is also consumed in health food and cosmetics. Given its traditional usage and current studies, this study is aimed to explore its neuroprotective effect of *Lonicera japonica* flower polysaccharide against oxidative stress in middle cerebral artery occlusion induced cerebral ischemia in rats.

2. Materials and methods

2.1. Materials

The dried *Lonicera japonica* flowers (4.0 kg) were purchased from Hu Qing Yu Tang Drugstore. DEAE-cellulose 52 was got from Whatman Co., and Sephacryl S-300 h was purchased from GE Healthcare Life Sciences. Bio Gel P-2 was purchased from Bio-Rad. All the other reagents of analytical grade were from Sinopharm Chemical Reagent Co. Ltd.

2.2. Extraction and purification of the polysaccharide from Lonicera japonica *flower*

The dried *Lonicera japonica* flowers (4 kg) were pre-treated with 95% ethanol (60 L) twice to remove liposoluble molecules at room temperature. After filtration and air drying, the residue was subjected to boiling water extraction for 4 times, and 4 h for each extraction. The extract was further concentrated and dialyzed against running water for 2 days. Subsequently, the retentate was concentrated to an appropriate volume and stirred with 15% (w/v) trichloroacetic acid (TCA) in ice bath for 2 h. The mixture was applied to centrifugation to remove the precipitate. Meanwhile, the supernatant was subjected to dialysis against running water for 2 days. Finally, the retentate was treated with 95% ethanol to yield the precipitate. The precipitate was pooled together and washed with absolute ethanol and acetone, then followed by vacuum dry. The dried precipitate was named as the crude polysaccharide LJP (218.7 g, yield 5.5%).

LJP (10 g) was dissolved in 100 mL distilled water and centrifuged to remove the extra insoluble constituents (1.3g). The supernatant was fractionated on a DEAE-cellulose 52 column (50 cm × 5 cm, Cl^- form), and eluted stepwise with water, 0.2, 0.4, and 0.6 M aqueous NaCl. The eluate was monitored by phenol-sulfuric acid method and appropriately pooled into four fractions, LJPA1 (4.2 g, yield 48.3% of LJP), LJPA2 (1.2 g, yield 13.8% of LJP), LJPA3 (0.8 g, yield 9.2% of LJP), LJPA4 (0.2 g, yield 2.3% of LJP). The major fraction, LJPA1 (2.0 g), was further fractionated by gel permeation chromatography on a Sephacryl S-300 column (100 cm × 2.6 cm), and eluted with 0.2 M NaCl to give LJPB1 (0.4 g) and LJPB2 (0.9 g). The schematic profile for the extraction and purification of the polysaccharide from *Lonicera japonica* flower was shown in Fig. 1.

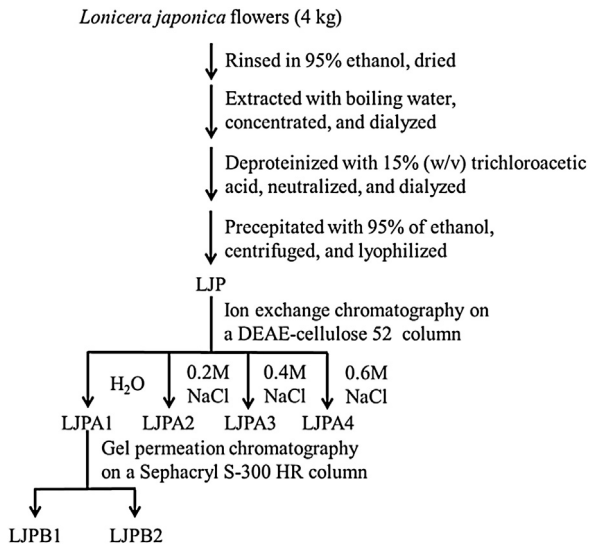

Fig. 1. Extraction, and purification profile of LJPB2 from flowers of Lonicera Japonica.

2.3. Physicochemical properties

The total neutral carbohydrate was determined by phenol-sulfuric acid method and the glucose was used as the reference [19]. The uronic acid content was measured by the *m*-hydroxyl biphenyl method, using glucuronic acid as the standard [20]. The protein content was determined by the Folin phenol method, using BSA as the standard [21]. The specific rotation was assayed using an Autopol VI instrument (Rudoph Research Analytical) at 25 °C at a wavelength of 589 nm.

2.4. Homogeneity and molecular weight determination

Homogeneity and molecular weight were determined by high performance gel permeation chromatography (HPGPC). The polysaccharides were dissolved in the elution buffer, applied to an Agilent 1260 HPLC system equipped with serial-connected KS-804 and KS-802 (8 mm × 300 mm, Shodex Co., Tokyo, Japan) columns, using 0.2 M NaCl as the mobile phase at a flow rate of 0.8 mL/min. The column temperature was kept at 40 °C. Other manipulation was referenced to the previous report [22].

2.5. Monosaccharide composition analyses

Monosaccharide compositions of polysaccharides were analyzed by gas chromatography (GC) [23]. Briefly, the polysaccharide sample (2 mg) was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 110 °C for 3 h. The hydrolyzates were subjected to reduction with sodium borohydride and subsequently derivatized into alditol acetates. The resulting alditol acetates were applied to GC analysis [24].

2.6. Methylation analysis

The vacuum dried polysaccharide (10 mg) was methylated by the modified Ciucanu and Kerek method [25]. Briefly, the polysaccharide was dissolved in DMSO (2 mL) at room temperature, and stirred vigorously with finely powered NaOH for 10 min. Iodomethane (0.5 mL) was added dropwise under ice bath in one hour, and stirred for 30 min at room temperature. Finally, the mixture was subjected to dialysis, and the retentate was freeze-dried to yield the methylated polysaccharide. The above procedure was performed for another 2–3 times. The completeness of methylation