RESEARCH PAPER

Accepted 18 April 2001
Revised 12 April 2001
Received 17 July 2000
Indian J. Pharm. Sci. 2001, 63(4) 292-294

Immunostimulant Activity of Inulin Isolated from *Saussurea lappa* Roots

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Polyfructoscan, inulin was extracted from the dried roots of *Saussurea lappa*. The crude inulin obtained was purified by fractional recrystallization using ethanol. Inulin was characterized by IR, NMR, DSC and X-ray diffraction studies. The fructose content in inulin isolated from *Saussurea lappa* roots was quantitatively estimated to be 95.89% w/w. Inulin in the insoluble form was found to significantly potentiate the delayed type hypersensitivity response to SRBC and also increase the total WBC count.

Inulin is an unbranched chain of β-D-(2—>1) polyfructofuranosyl-α-D-glucose. The terminal glucose is linked to fructose as in sucrose[1]. Many plants belonging to the family Asteraceae, Campanulaceae and Roaceae, contain inulin as the reserve food. Inulin as a 10% sterile aqueous solution is used as a measure of the efficiency of glomerular filtration in kidneys[2].

It is reported that inulin from Dahlia tubers crystallizes in a series of polymorphic forms with different solubility rates[1]. The stable solubility polymorph, gamma inulin, is reported to be a potent activator of the Alternative pathway of complement (APC) and was also found to show anti-melanoma activity in B16/C57BL mice[3].

Saussurea lappa roots (Synonym-Kuth, Fam-Compositae) are reported to contain essential oil (1.5%), alkaloids saussurine (0.05%), resin (6%), inulin (18%), a fixed oil and other minor constituents like tannins and sugars[4]. The present investigation was aimed at isolation and characterization of inulin from the dried roots of *Saussurea lappa* and testing it for immunostimulant activity using *in vivo* models.

MATERIALS AND METHODS

Saussurea lappa root (powdered) was procured from the local market. The identity was verified by means of macroscopic and microscopic investigation. D-fructose was purchased from S.D. Fine Chemicals, Mumbai. Tryptamine hydrochloride was purchased from Hi-Media, Mumbai. All other chemicals and reagents used were of analytical grade.

Extraction:

The coarse powder (100 g) of the dried roots was extracted with water (300 ml) with stirring at 60-70° and filtered. The filtrate was kept overnight at room temperature, during which time inulin separated out. The precipitate was then filtered, washed with cold water and dried. The crude precipitate was purified by dissolving in hot water and subsequent reprecipitation from the aqueous solution using alcohol. The yield was approximately 10% w/w with respect to the crude drug.

Characterization:

The optical rotation of the isolated sample (2% w/v solution in water, 20°) was recorded on Polamet A, Karl Zeiss Jena polarimeter. The IR spectrum was recorded by KBr disc method on a Jasco FTIR 5300 spectrophotometer. The ^{13}C-NMR spectrum was recorded on a Bruker AMX-500 FT-NMR at 500 MHz, in deuterated DMSO. Powder XRD pattern was recorded using Phillips X-Ray diffractometer (PW1130/00) at a scanning rate of 2° per min.

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The isolated sample of inulin was hydrolyzed by boiling with 10% w/v oxalic acid solution[5]. The hydrolyzate was neutralized using calcium carbonate and concentrated at lower temperature. The constituent sugar in the hydrolyzate was identified by ascending paper chromatography in n-butanol: pyridine: water (7.5: 1.5: 1). For detection of the spots, the paper was sprayed with aniline hydrogen phthalate and heated for 10 min at 100-105°.

The fructose content in inulin was quantitatively estimated by a spectrophotometric method, which includes tryptamine hydrochloride reagent[6]. Standard fructose solution (1 mg/ml) was diluted with water to obtain a standard curve in the range of 0-40 µg/0.2 ml solution. Inulin solution (1 mg/ml) was prepared in water with the aid of heat and diluted to a concentration of 40 µg/0.2 ml solution. The absorbance of the solution was recorded on a Shimadzu 160A UV-visible spectrophotometer at 522 nm against water.

***In vivo* studies:**

The isolated inulin sample was passed through 400 mesh. This powder was suspended in normal saline solution and the suspension was used for animal experiments. Swiss albino mice of either sex (20-25 g) were divided into 6 groups of five animals each. One group served as control, while other groups served as treated groups. In all the animal experiments, doses of 5, 10, 20, 40 and 80 mg/kg were administered i.p. for a period of 5 days to treated groups. At the end of day 5, blood was withdrawn from the retroorbital plexuses and analyzed for total WBC count, RBC and haemoglobin using ERMA PC 607 cell counter.

Carbon clearance test:

The change in macrophage phagocytic activity was determined by carbon clearance test[7]. Inulin suspension was administered prior to injection of carbon particles. Mice were injected with 0.1 ml carbon suspension (Zeichentusche drawing ink, Germany) intravenously, through the tail vein. Blood samples were collected from the retro-orbital plexuses immediately before and at 3, 6, 9, and 12 min. after injection of carbon suspension. An aliquot of 25 µl of blood sample was lysed with 2 ml of 0.1% acetic acid and absorbance was measured spectrophotometrically at 675 nm.

Delayed type hypersensitivity reaction (DTH) using SRBC as an antigen:

Mice were primed with 0.1 ml SRBC (Sheep RBC) suspension containing $1x10^9$ cells intraperitoneally on day 0 and challenged on day 7 with $1x10^8$ cells in left hind footpad[8,9]. The suspension was administered i.p. on day 3 and continued till the day of challenge. The thickness of footpad was measured at 0 h and at 24 h after challenge using Mitutoyo Dial caliper (Mitutoyo Mfg. Company, Japan). The difference in the thickness of the left hind paw at 0 h and at 24 h was used as a measure of DTH reaction.

Statistical analysis:

The results were analyzed by Student's t-test. Statistical significance was considered at $P<0.05$.

RESULTS AND DISCUSSION

In the present investigation, inulin was purified by fractional recrystallization using ethanol. The specific optical rotation of the sample in water was found to be within the range specified in BP 1993 for inulin. The IR spectrum showed a sharp peak at 3389 cm^{-1} for hydroxyl group and at 1030 cm^{-1} for symmetric C-O-C stretch. The ^{13}C NMR spectrum showed signals due to anomeric carbon at δ 103.227 ppm. This was assigned to the anomeric carbon of β-D-fructofuranose residues[10]. The ^{13}C NMR spectrum shows that inulin has a linear chain (2—>1) linked β-D-fructofuranose chain. The XRD pattern of the isolated sample showed two peaks at 2θ values of 12.1° and 21.8°, which are relatively sharp and these are the analytical lines for inulin[11].

Paper chromatography of the hydrolyzed sample showed a spot corresponding to the spot of standard D-fructose (Rf-0.22). The standard curve for fructose was recorded at 522 nm. Correlation coefficient value close to 1 indicated that Beer Lambert law was obeyed over the concentration range of 40 µg/0.2 ml. Using this standard curve, the fructose content in inulin isolated from *S. lappa* roots was found to be 95.89% w/w.

The effect of inulin on the haematological parameters was studied. After 5 days administration of inulin suspension, i.p, significant increase in the total WBC count was observed at the doses of 40 mg/kg and 80 mg/kg (Table 1). No changes were observed in the RBC count and haemoglobin concentration.

TABLE 1: EFFECT OF INULIN ON BLOOD CELLS IN MICE

Dose mg/kg	WBC x 10^3 cells/µl
Control	11.68±1.052
5	13.28±2.708
10	14.66±3.562
20	14.72±3.993
40	15.4±2.884*
80	17.48±2.210*

Each value is expressed as mean±s.d. of five observations, *P<0.05 is considered significant as compared to control by Student's t-test.

In order to study the effect of inulin on the macrophage phagocytic activity, *in vivo* carbon clearance test was performed. Inulin did not significantly change the phagocytic index at any of the tested doses. This indicated that inulin isolated from *S. Lappa* roots probably does not directly affect the phagocytic activity of granulocytes and macrophages.

In order to study the effect of inulin on T cell function, inulin suspension (i.p.) was tested on sheep red blood cell induced DTH reaction in mice. In the control group the edema due to DTH was found to reach a peak value of 25.447% at 24 h after challenge. Inulin at 80 mg/kg significantly potentiated DTH response to SRBC (Table 2). This indicated that inulin possibly activates the T-lymphocytes. Probably these activated T-cells then release vasoactive amines and multiple hormonal substances called lymphokines. These substances then may function as mediators of the ensuing hypersensitivity response particularly by attracting and activating macrophages[12].

It is reported that the insoluble activators of APC such as lipopolysaccharide, inulin, zymosan, etc when incubated with the serum form an enzymatically active complex, capable of activating C3 and C5 components of the complement system[13]. Davis and Allison reported that the activated complement components participate in macrophage activation which inturn leads to release of lysosomal enzymes. So antitumor polysaccharides also may activate macrophages indirectly by the action of activated fragments of complement[13]. Thus it may be possible that inulin in the insoluble form causes activation of the complement through APC, which inturn causes activation of macrophages and lymphocytes. Hence the effect of inulin isolated from *S. lappa* roots on the complement system and B cell mediated immune response needs to be studied.

Currently, the studies on the different polymorphic forms of inulin are more or less empirical as they are based merely on their solubility properties. Hence, the crystal structure and conformation of these different forms needs to be established using techniques like 2D-NMR and fibre diffraction studies. Also further studies are required to derive the mechanism of immunostimulant activity of inulin isolated from *S. lappa* roots and also verify its implication in adjuvant cancer therapy.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. (Mrs.) Sudha Srivatsav, Tata Institute of Fundamental Research, Mumbai for NMR studies and Mr. Sekhar, CIRCOT for X-ray diffraction studies.

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