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Structural features of an immunoactive acidic arabinogalactan from *Centella asiatica*

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Abstract

A water-soluble acidic arabinogalactan, named HBN with a molecular mass of 5.4×10^5 Da determined by HPGPC, was obtained from *Centella asiatica*. HBN contained Ara, Gal, Rha, GalA and Xyl in molar ratios of 1.0:1.9:0.26:0.30:0.15. The acetyl content was estimated to be 2.5%. Using methylation analysis, partial acid hydrolysis, $NaIO_4$ oxidation–Smith degradation, RI, NMR, ESI-MS, HPGPC, pectolyase-treatment methods, the structure of HBN was elucidated. HBN had a core composed of Rha and GalA, with arabinogalactosyl and xylosyl chains were attached to this core. Seventy-six percent Ara residues were located at termini and linked to *O*-6 of Gal residues. HBN had remarkable immunoenhancing activities on T- and B-lymphocytes in vitro and vivo tests. It increased spleen index and inhibited the level of IgG. With the stimulation of SAC, it enhanced the secretion of IFN-γ, IL-12 and IL-6, and inhibited IL-10. Its derivatives by $NaIO_4$ oxidation–Smith degradation and enzyme-treatment possessed immunological activities in vitro.

Keywords: *Centella asiatica*; Immunological activity; Polysaccharide; Arabinogalactan; Pectolyase; Modification

1. Introduction

Arabinogalactans are widely distributed in the plant kingdom (Clarke, Anderson, & Stone, 1979). Usually, arabinogalactan contain a high proportion of β-D-Galp and α-L-Araf residues, and smaller and variable levels of a range of other neutral and acidic monosaccharides, including α-L-Ara*p*, α-L-Rha*p*, β-D-Glc*p* and β-D-Glc*p*A (Fincher, Stone, & Clarke, 1983). Arabinogalactans can be grouped into three main structural types. Arabino-1,4/6-β-galactans (type I) have backbone of 1,4-linked Gal*p*. Arabino-1,3/6-β-galactan (type II) have β-1,3-linked galactosyl backbone. Type III is formed by the cell wall glycoproteins containing Ara and Gal (Aspinall, 1980; Clarke et al.; Timell, 1969). *Centella asiatica*, predominantly growing in the Southern hemisphere, has been used as a remedy for sedation and against leprosy, ulcers (Chopra, Nayar, & Chopra, 1956; Maquart, Bellon, Gillery, Wegrowski, & Borel, 1990; Yoshinori, Reiko, & Tsumematsu, 1982) in oriental countries. An arabinogalactan (HBN) isolated and purified from *C. asiatica* was tested in immunological assays and it was found to exhibit significant immunoenhancing activity in comparison with that of other polysaccharides from this plant (Wang, Dong, Zuo, & Fang, 2003). In the present paper, we elucidate the structural features and pharmacological activity of HBN, as well as its structure–activity relationship.

Abbreviations: TCA, trichloroacetic acid; LPS, Lipopolysaccharide; ConA, Concanavalin; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium brimoide; CMC, 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene-sulphonate; TFA, trifluoroacetic acid; QHS, quantitative hemolysin spectrophotometer; APC, Antigen presenting cells; SAC, *Staphylococcus aureus* cowan strain 1; HPGPC, high-performance gel-permeation chromatography; HMBC, (1H-detected) heteronuclear multi-bond correlation; PBS, Phosphate buffered saline; SRBC, Sheep red blood cell.

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2. Experimental

2.1. Materials

The dried plant of *C. asiatica* was purchased from the Shanghai Medicinal Materials Cooperation, P.R. China (code: 000201), and stored as a voucher specimen in the Shanghai Institute of Materia Medica, Shanghai, P.R. China. Pectolyase was obtained from *Aspergillus japonicus* (P-3026, Sigma). MTT, ConA and LPS (from *E. coli.,* Serotype 055: B5) were products of Sigma. ^{3}H-TdR was obtained from Shanghai Institute of Nuclear Research. Medium RPMI 1640 was purchased from Gibco Laboratories. IFN-γ, IL-2, IL-10, IL-12, TNF-α and IL-6 were purchased from Beckton Dickinson Company. All other reagents were of the highest available quality.

2.2. General methods

NMR spectra were recorded on a Varian Mercury 400 NMR spectrometer. A polarization transfer pulse of 135° technology was used in DEPT experiment. HPGPC was performed with a Waters system instrument, including GPC software (Millennium^{32}), 515 HPLC pump, 2410 RI detector and 2487 dual λ absorbance detector. GC was done with a Shimazu-9A apparatus equipped with a 5% OV 225/AW-DMC-Chromosorb W column (2.5×3 mm). GC-MS was performed with a Shimadzu QP-5050A apparatus equipped with a db-1 capillary column (0.25 mm×30 cm). ESI-MS spectra were obtained with a VG Quattro MS/MS spectrometer. ^{3}H-TdR data were counted using a liquid scintillation counter (MicroBeta Trilux, Perkin–Elmer Life Science).

2.3. Isolation and purification

The dried *C. asiatica* (4000 g), previously defatted with 95% alcohol, was extracted with hot water for 4 h. The aqueous solution was treated with TCA to remove protein, extensively dialyzed (molecular weight cut of 3500–5000 Da). The retentate was concentrated, precipitated with EtOH and vacuum-dried at 40 °C, yielding the crude polysaccharide (yield: 2.1%, of the original dried *C. asiatica*). A portion (7 g) of the crude polysaccharide was fractionated on DEAE-cellulose (Cl^- form) column (50×10 cm), eluted with water and stepwise by 0.1, 0.3 and 0.5 M NaCl solutions to give five sub-fractions. The fraction eluted with H_2O (413 mg, yield: 5.9%, of the crude polysaccharide) was further separated on DEAE-cellulose (AcO^- form) column (50×10 cm) eluted with H_2O, 0.05 and 0.2 M NaOAc to give three fractions. The fraction eluted with 0.2 M NaOAc was further purified on Sephadex G-200 (100×2.6 cm) to give HBN (yield: 15%, of the crude polysaccharide).

2.4. Homogeneity and molecular mass

Determination was done by HPGPC on a linked column of Ultrahydrogel™ 2000 and 500 column, eluted with 0.003 M NaOAc at a flow rate of 0.5 ml/min. The column was kept at 30.0±0.1 °C, and was pre-calibrated by standard Dextran (T-700, 580, 300, 110, 80, 70, 40, 9.3 and 4, Pharmacia). All samples were prepared as 0.2% (w/v) solutions and 20 μl of solution analyzed in each run.

2.5. Chemical analyses

The neutral sugars were analyzed by GC after conversion of the hydrolysate into alditol acetates, as described before (Dong, Ding, Yang, & Fang, 1999). The samples were methylated four times according to the modified NaOH–DMSO method (Needs & Selvendran, 1993). The hexuronic acid content was determined by a modification of the *m*-hydroxylbiphenyl method (Kimberley & Jock, 1992), and was reduced before GC analysis. Reduction was carried out with CMC and $NaBH_4$ as previously described (Taylor & Conrad, 1972; Tomada, Gonda, Kasahara, & Hikino, 1986). The acetyl groups were determined using the Tomada method (Tomada, Shimizu, Shimada, & Suga, 1985). D,L-configurations were determined by the Gerwig method (Gerwig, Kamerling, & Vliegenthart, 1978)

2.6. Partial acid hydrolysis

One hundred milligrams of HBN was dissolved in 25 ml 0.016 M TFA, and was hydrolyzed at 60 °C for 1 h. After cooling, the solution was concentrated and dialyzed with distilled water, and the retentate was lyophilized to give a polymer (HBN-P, 18.6 mg). The dialysate was fractionated on Sephadex G-10 column to give a main fraction (HBN-M) and a series of oligosaccharides.

2.7. Periodate oxidation and Smith degradation

HBN (20.8 mg) was oxidized with 0.02 M $NaIO_4$ (20 ml) at 4 °C in the dark (Tomada et al., 1986; Vaishnav, Bacon, O'Neill, & Cherniak, 1998). The reaction was quenched with glycol (0.6 ml) after 7 d, and the solution was reduced, neutralized, dialyzed and lyophilized to give a degraded product (HBN-I). HBN-I was further hydrolyzed with 0.2 M TFA at 40 °C for 24 h, and then dialyzed. The retentate was lyophilized to give a sub-fraction (HBN-S), and the dialysate was further fractionated on a Sephadex G-10 column to give IE, ID, IC, IB and IA, according to elution times.

2.8. Pectolyase treatment

HBN (13.2 mg) which was dissolved in 3 ml distilled water was added with 150 μl 5 mg/ml pectolyase solution at 30 °C for 48 h. The solution was heated at 100 °C for