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Carbohydrate Polymers 59 (2005) 281–288
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

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

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Received 20 October 2003; revised 24 June 2004; accepted 7 July 2004
Available online 8 December 2004

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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doi:10.1016/j.carbpol.2004.07.005

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

15 min, centrifuged and dialyzed. The retentate was lyophilized to give HBN-E.

2.9. Immunological assays in vitro

Inbred ICR male mice, 6–8-weeks-old, weighing 20±2 g, were obtained from the Shanghai Experimental Animal Laboratory, Chinese Academy of Sciences (certificate No. ZhongKeHuDongGuan99-003). Different dilutions of the polysaccharide samples were incubated with mouse splenocytes in the presence of mitogen ConA (5.0 μg/ml) or LPS (20 μg/ml) at 37 °C in humidified 5% CO_2 atmosphere. T- and B-lymphocyte proliferations were assayed by MTT and ^{3}H-TdR method (Heeg, Reimann, Kabelitz, Hardt, & Wagner, 1985; Kang, Li, Meng, & Wang, 1996). All experiments were performed three times independently.

IL-2, IL-10, IL-12 and IFN-γ were assayed by the ELISA method. Mouse splenocytes (5×10^6/ml, 100 μl/well) and samples (different dilution, 50 μl/well) were incubated in 37 °C and CO_2 for 24 h in presence of SAC (1:2500) or ConA (20 μg/ml). The absorbance was measured at 450 and 570 nm.

2.10. Immunological assays in vivo

Animals and preparation of spleen cells. ICR female mice, 3-months-old, weight 20±2 g, were obtained from the Shanghai Experimental Animal Laboratory, Chinese Academy of Sciences (certificate No. Zhongke005). They were divided randomly into seven groups: normal saline (control group), 0.1, 1.0 or 10 mg/kg (positive groups). The mice were challenged by intraperitoneal injection (i.p.) of 0.2 ml of 5% SRBC on day 0. The polysaccharides were injected i.p. into the mice from day 1 to day 4, respectively. Mice were sacrificed on day 5 and their spleens were removed, minced and passed through a sterilized ion mesh (200 meshes) to obtain single cell suspensions. Erythrocytes in the cell mixture were destroyed by the rapid addition of H_2O. Finally, the cells were suspended to 5×10^6 cells/ml in the RPMI-1640 medium.

Proliferative response to T- and B-mitogen. Spleen cells (100 μl/well) were seeded into a 96-well plate in the presence of mitogen ConA (5.0 μg/ml) or LPS (10 μg/ml). After incubation for 44 h at 37 °C in a humidified 5% CO_2 incubator, lymphocyte proliferation was assayed by the MTT method (Li et al., 1990). The plate was incubated for another 4 h and then the resolver (100 μl/well) was added. The absorbance was measured by DG-3022 ELISA at 570 nm.

Antibody production and serum IgG level in vivo. The effect on antibody production was by using QHS assay (Xiang & Li, 1993). After the mice were killed on day 5, the spleen cells and serum were obtained for the antibody production assay and IgG determination, respectively. Fresh SRBC was washed twice with PBS (pH 7.2) and diluted to 1:20. A suspension of spleen cells (2×10^7 cells/ml) was prepared. Cell suspensions 1.0 ml, SRBC 1.0 ml and serum of pooled guinea-pig 1.0 ml were mixed and incubated at 37 °C for 1.5 h and then centrifuged. Absorbance at 520 nm of the supernatants was measured. Serum IgG level was measured by single immunodiffusion method (Xiang & Li, 1993). The diameters of samples in a rabbit anti-mouse plate diffusion ring were measured.

3. Results and discussion

3.1. Structural features

HBN, extracted from *C. asiatica* with hot-water, had specific rotation $[\alpha]_D^{20}: -10.4°$ (ca. 0.70, H_2O). It was repeatedly subjected to Sephacryl S-300 chromatography and Sephadex G-200 gel-permeation chromatography, and assayed using a refractive-index detector and also the phenol–H_2SO_4 method. The results showing HBN was homogeneous. In HPGPC, it showed only one symmetrical peak, and its molecular mass was estimated to be 5.4×10^5 Da (Fig. 1). No absorption at 280 nm and a negative response to the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) confirmed HBN did not contain protein. TLC analysis showed the original polysaccharide (HBN) contained Ara, Gal, mainly, and trace amount of Rha, GalA, as well as Xyl residues. The *m*-hydrobiphenyl method determined it to contain 8.9% GalA. With further quantitative determination and composition analysis of HBN and its carboxyl-reduced derivative (HBNR) by GC, Ara, Gal, Rha, GalA and Xyl residues were in molar ratio of 1.00:1.90:0.26:0.30:0.15 (Table 1). The ratio of GalA was established by the increase of Gal content in HBNR. The signal at δ 2.1 ppm in ^{1}H NMR indicated the presence of *O*-acetyl groups, and the content of *O*-acetyl groups were calculated to be 2.5% according to ^{1}H

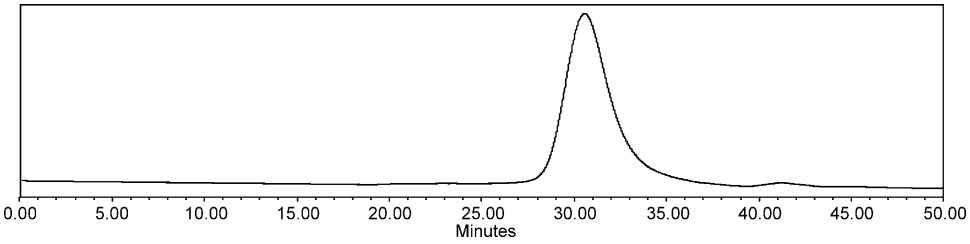


Fig. 1. Profile of HBN in HPGPC.

Table 1
Compositional analysis of HBN and its degraded polymers and oligomers (molar%)

	HBN	HBN-P	HBN-E	IB	IC	ID	IE
L-Ara	27.7	23.7	46.2	79.4	15.1	13.7	13.3
D-Gal	52.6	62.1	53.7	n.d.	61.1	63.5	37.9
L-Rha	7.3	5.9	Trace	20.6	14.2	15.4	35.3
D-GalA	8.9	6.0	Trace	n.d.	9.6	7.4	13.5
D-Xyl	4.5	1.3	Trace	n.d.	n.d.	n.d.	n.d.

n.d., not detected.

NMR and the Tamada method. The similar molar ratio of Rha and GalA residues suggested that Rha and GalA probably formed pectic rhamno-galacturonic acid chain structure.

The original polysaccharide, HBN, was treated with pectolyase to remove pectin structures. The resultant product, HBN-E, was analyzed with TLC and GC. The results (Table 1) showed that most of Rha and GalA residues were removed by pectolyase, and in HPGPC, the molecular mass of HBN-E was lowered to 4.1×10^5. All the results above indicated the pectic component of Rha and GalA was part of the original polysaccharide. It is probably the core since cleavage by pectolyase-treatment caused a substantial loss of molecular mass. When considering the effects of the removal of Xyl in HBN-E, the Xyl residues in HBN should form short chains and be attached to the RG core tightly. The loss of Xyl could be explained by the cleavage of the RG core by pectolyase-treatment and the loss in dialysis (molecular mass of xylosyl chains <3500). With respect to the linkage of Xyl in methylation analysis, the Xyl residues probably formed (1→2)-linked Xyl*p* chains.

Methylation of HBN (Table 2) directly showed it did not contain 2,3,6-Me_3-Gal, while methylation of HBNR (carboxyl-reduced derivative of HBN) showed it contained 2,3,6-Me_3-Gal. Thus, 2,3,6-Me_3-Gal should be derived from 1,4-linked Gal*p*A. Other linkages were analyzed and listed in Table 2.

The partial acid hydrolysis product of HBN, HBN-P was per-methylated and analyzed on GC-MS. The results (Table 2) showed partial Ara residues were removed and *O*-6 substituted Gal residues were lost, while the proportion of terminal and *O*-3 substituted Gal residues increased, compared with methylation analysis of HBN. It suggested that Ara*f* was attached to the *O*-6 of Gal*p*. Because of the presence of the high molar ratio of terminal Ara*f* in the original polysaccharide, most of *t*-Ara*f* should be linked to *O*-6 of Gal*p* directly and other *t*-Ara*f* possibly formed some short chains with 1,5- and 1,3,5-linked Ara*f*. Further methylation analysis HBN-M (oligomer product in hydrolysis) showed it contained a high ratio of 1,2,4-linked Rha*p* and 1,4-linked Gal*p*A, indicating that the RG core had a large proportion of side chains that formed tight hairy region.

HBN was oxidized with $NaIO_4$ to give HBN-I, and HBN-I was subjected to mild acid hydrolysis to give HBN-S. HBN-S was analyzed using HPGPC and gave a main peak with a molecular mass of 1.1×10^5 Da. Methylation analysis showed HBN-S mainly contained 1,3-linked Gal*p*, as well as small amount of 1,3,6-linked Gal*p* and Ara*f* residues (Table 2). The existence of 1,3,6-linked Gal*p* in HBN-S indicated the original polysaccharide contained →3)-Gal-(1→3)-Gal-(6←1)-Gal-(3← moiety. With the consideration that 1,6-linked Gal*p* in HBN-S derived from 1,3,6-linked Gal*p* in HBN, the structure of →6)/*t*-Gal-(1→3)-Gal-(6←1)-Gal-(3← existed in HBN. Thus, the galactosyl chains in HBN were very complex.

Oligomers in Smith degradation had been isolated on Sephadex G-10 to give IA, IB, IC, ID and IE (58/19/11/5/7, w/w), as shown in Fig. 2. Compositional analysis results are shown in Table 1. IB–IE did not contain Xyl residues. IA was a mixture of disaccharide and a small amount of monosaccharide. In ESI-MS, ion fragments *m/z* 248.9, 277.2 corresponded to $[\text{Ara-Glycerol}+2+\text{Na}]^+$ and $[\text{Gal-Glycerol}+\text{Na}]^+$, anion fragments *m/z* 339.3 corresponded to $[\text{Rha-GalA-1}]^-$. IB was the mixture of tri- and tetra-saccharides, and in ESI-MS, ion fragments *m/z* 452.5,

Table 2
Linkage analysis of HBN and its derivatives (molar%)

	Linkage	HBN	HBN-P	HBN-M	HBN-S
2,3,5-Me_3-Ara	*t*-Ara*f*	21.3	14.3	n.d.[a]	2.6
2,3-Me_2-Ara	1,5-Ara*f*	3.2	4.7	25.6	5.8
2-Me_1-Ara	1,3,5-Ara*f*	3.6	5.9	n.d.[a]	n.d.[a]
2,3,4,6-Me_4-Gal	*t*-Gal*p*	6.9	15.1	19.6	24.5
2,3,6-Me_3-Gal	1,3-Gal*p*	11.9	13.6	n.d.[a]	40.0
2,3,4-Me_3-Gal	1,6-Gal*p*	18.4	16.1	3.7	11.4
2,4-Me_2-Gal	1,3,6-Gal*p*	16.2	18.1	14.5	15.7
2,3,5-Me_3-Xyl	*t*-Xyl*p*	0.5	0.4	n.d.	n.d.[a]
3,5-Me_2-Xyl	1,2-Xyl*p*	3.6	1.1	n.d.	n.d.[a]
3,4-Me_2-Rha	1,2-Rha*p*	n.d.[a]	n.d.[a]	0.5	n.d.[a]
3-Me-Rha	1,2,4-Rha*p*	7.5	6.2	20.4	n.d.[a]
2,3,6-Me_3-Gal	1,4-Gal*p*A[b]	6.9	4.5	15.7	n.d.[a]

[a] n.d., not detected.
[b] Carboxyl-reduction data.

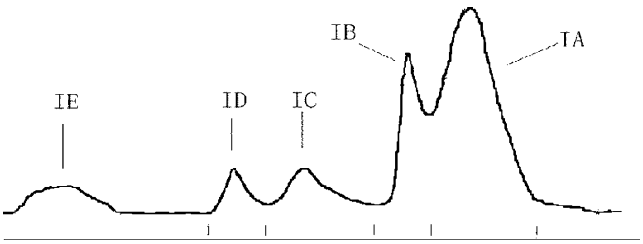


Fig. 2. Profile of the isolation of IA, IB, IC, ID and IE on G-10 column (detected with RI detector).

456.5, 512.7, 568.6 and anion fragment *m/z* 644.7 corresponded to $[Ara_2\text{-}Rha+2+Na]^+$, $[Ara_3+H_2O+2+Na]^+$, $[Ara_3\text{-}Gly+2+Na]^+$, $[Ara_4+Na]^+$ and $[Gal_2\text{-}Rha\text{-}GalA\text{-}H_2O]^-$ or $[GalA_2\text{-}Rha_2\text{-}H_2O\text{-}1]^-$. ID was analyzed by ESI-MS, and ion fragments *m/z* 696.7, 712.6 and anion fragments *m/z* 514.7, 644.7 corresponded to $[Ara_4\text{-}Rha\text{-}H_2O+Na]^+$, $[Ara_4\text{-}Gal\text{-}H_2O+Na]^+$, $[GalA_2\text{-}Rha\text{-}1]^-$ and $[Gal_2\text{-}Rha\text{-}GalA\text{-}H_2O]^-$ or $[GalA_2\text{-}Rha_2\text{-}H_2O\text{-}1]^-$, respectively. These results confirmed the existence of RG core and arabinogalactosyl chains were linked to RG core. With the consideration of the mechanism of $NaIO_4$-oxidation, the Gal residues which were attached to the core were 1,3-linked Gal. And, a small amount of Ara residues formed arabinosyl chains and attached to *O*-4 of Rha directly.

In ^{13}C NMR spectra (Fig. 3), the signals at δ 110.4 and 108.5 ppm were assigned to the anomeric carbons of α-Ara*f* and β-Xyl*p*, respectively (Dong et al., 1999; Karacsonyi, Koracik, Alfoldi, & Kubackova, 1984). The signals at δ 104.6–104.4 ppm arise from C1 of β-Gal*p* (Sims, Craik, & Bacic, 1997). The weak signals at δ 102.0 and 100.6 ppm were assigned to C1 of α-Rha*p* and α-Gal*p*A, respectively (Cui, Eskin, Biladeris, & Marat, 1996; Shimazu & Tomada, 1985). As 76% Ara*f* residues were located at termini, the main signals of Ara*f* came from *t*-Ara*f*. Other signals were assigned and listed (Table 3 and Fig. 3) according to 2D NMR and references (Bao, Wang, Fang, & Li, 2002; Karacsonyi et al., 1984; Shimazu, Tomoda, Kanari, & Gonda, 1991; Sims et al., 1997). In ^1H NMR spectrum, the signals at δ 5.2 and 4.4–4.5 ppm were corresponded to H1 of α-Ara*f* and β-Gal*p* residues. H1 of α-Rha*p*, α-Gal*p*A and β-Xyl*p* had signals at δ 5.1, 4.9 and 4.7 ppm, respectively.

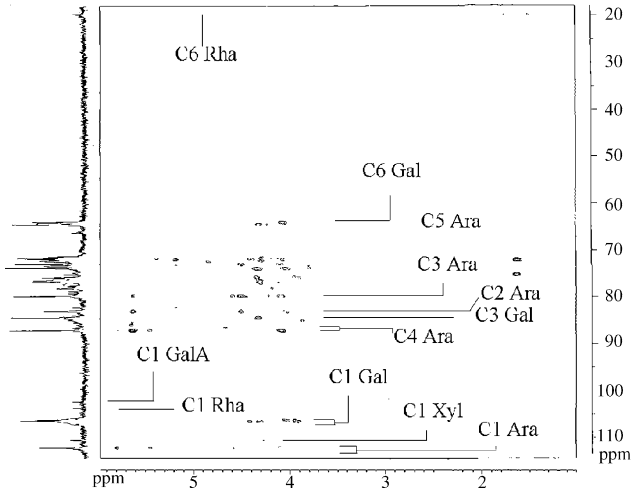


Fig. 3. HMBC of HBN.

Table 3
^{13}C NMR of HBN (ppm, r.t., D_2O, referring to NaOAc at δ 24.5 and 182.7 ppm)

	C1	C2	C3	C4	C5	C6
t-Ara*f*	110.4	81.3	77.8	85.1	62.5	
1,5-Ara*f*	110.4	81.3	77.8	85.1	70.6	
1,3,5-Ara*f*	110.4	81.3	84.3	85.1	70.6	
t-Gal*p*	104.4	71.1	73.8	69.6	74.6	62.0
1,6-Gal*p*	104.4	71.9	73.8	69.8	74.9	70.6
1,3-Gal*p*	104.6	71.9	82.5	69.8	74.9	62.0
1,3,6-Gal*p*	104.6	71.9	82.5	69.8	74.9	70.6
1,2-Xyl*p*	108.5	81.0	76.3	71.4	64.4	
1,4-Gal*p*A	100.6	71.3	71.3	80.2	73.2	178.3
1,2,4-Rha*p*	102.0	79.1	71.3	81.0	70.2	18.9

3.2. Immunological activity

HBN was tested by an immunological assay in vitro. As shown in Tables 4 and 5, HBN had significant immunoenhancing effect on T- and B-lymphocytes proliferation, and the effect on B cell was larger than that on T cell. To further study the immunological mechanism of HBN, Th1 and Th2 were assayed. Th1 concluded cell factors IFN-γ, IL-2, TNF-α and IL-12, and Th2 contained cell factors IL-4, IL-5, IL-6 and IL-10, etc. With the stimulation of SAC on APC, HBN enhanced the secretion of IFN-γ, IL-12 and IL-6, and inhibited IL-10. Among the results, HBN had obvious secretion-enhancing activity on IL-12. In the test of T cell induced with ConA, HBN increased the secretion of IL-2 and ILF-γ. HBN was prepared in different concentration (0.1–10 mg/kg) and tested in vivo. The results (Table 6) showed the original polysaccharide had obvious effect on the spleen index, but limited effect on thymus. In the concentration of 10 mg/kg, HBN inhibited the level of IgG. With ConA or LPS induction, T or B lymphocyte proliferation increased substantially.

3.3. Activity and structure

The derivatives of HBN were assayed in immunological tests (Table 4). From the results, HBN-E, HBN-I and HBN-S had obvious effects on T and B cells. The activities of HBN-I and HBN-S were similar, indicating that the arabino β-3,6-galactan moiety was the major contributor to immunoenhancing activity (Yamada, 2000). In comparison to the contents of *O*-3-substituted Gal and *O*-6-substituted Gal in HBN and HBN-S, HBN-S contained more *O*-3-substituted Gal, while HBN contained more *O*-6-substituted Gal. Thus, not only 1,6-linked Gal residues in arabinogalactan played an important role in

Table 4
Immunological assay in vitro

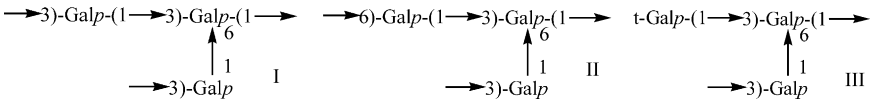
Groups	Concentration (μg/ml)	Cell-toxic assay[a]		T-cell[b]		B-cell[b]		Effects on cell factors[c] (mean±SD)	
		Mean±SD (OD)	*P*	Mean±SD (CPM)	Prolif. (%)[d]	Mean±SD (CPM)	Prolif. (%)[d]	IL-2 (pg/ml)	IFN-γ (pg/ml)
Control	Negative	0.100±0.015		3604±401		3604±401		666±12	6797±811
	Positive			58570±34455		16371±1309			
HBN	100	0.317±0.044	0.007	102854±15761	76	79047±16266	383	799±17	7956±892
	10	0.131±0.004	0.188	90925±5116	55	36062±4683	120	754±0	7437±95
	1	0.098±0.098	0.911	79823±14561	36	23322±1324	42	617±20	5597±43

[a] MTT method.
[b] ^{3}[H] TDR method.
[c] ELISA method, induced with ConA.
[d] ≥15 showed the sample was effective.

Table 5
Cell factors effect with Sac stimulated

Groups	Concentration (μg/ml)	Mean±SD				
		IL-10	IL-12	IFN-γ	TNF-α	IL-6
Control		2638±80	472±20	287±14	207±0	398±16
HBN	100	2467±63	332±3	499±12	186±4.2	504±8
	10	2204±102	422±8	460±56	236±23	471±8
	1	1948±0	731±72	629±76	242±23	540±8

immunoactivity, but also 1,3-linked Gal did so. With pectolyase treatment, the immunological activity of HBN-E became much weaker than that of the original polysaccharide, indicating that the RG core of arabinogalactan contributed the immunological activity, which had seldom been reported before. The loss of RG core decreased the immunological activity. Thus, the immunological activity of HBN was influenced by the distribution and cross-linking of different moieties (Table 7).



4. Conclusion

HBN, firstly obtained from *C. asiatica*, is an acidic arabinogalactan. Rha and GalA residues constitute the core, and, arabinogalactosyl chains and Xylosyl chains are attached to *O*-4 of Rha. Ara residues are linked to *O*-6 of Gal mostly. Galactosyl chains are very complex and contain the following moieties:

Among the three moieties, the proportion of **I** is more than the other two (**II** and **III**). According to the analysis of the arabinogalactosyl region, which is the main component in HBN, it possesses the structure of arabinogalactan (type **II**). Many arabinogalactans have been reported before and some polysaccharides are similar to HBN, such as AGP from *Nicotiana alata* (Gane et al., 1995), AGP4 from red wine (Pellerin et al., 1995), SSa3 from *Sophora subprostrata* (Dong, Yao, & Fang, 2003), DL-3Bb from *Diospyros Kaki* (Duan et al., 2003) and so on. But the molar ratio of residues

Table 6
Effects on spleen and thymus of mice in vivo

Groups	Weight (g)	Spleen (mg/10 g)	Thymus (mg/10 g)	QHS (OD value)	IgG (μg/10 μl)	^{3}[H]-TDR (CPM×10^{-3})	
						ConA	LPS
Control	23.6±0.7	89.5±16.4	42.4±5.1	0.89±0.01	179±28	9.7±1.8	21.1±1.5
0.1 mg/kg	23.4±1.0	104.4±11.7	49.0±7.2	0.89±0.01	156±14	9.9±1.1	21.5±2.0
1.0 mg/kg	23.3±1.2	120.4±17.2**	40.9±7.2	0.89±0.02	156±18	14.4±0.5**	39.2±0.9**
10 mg/kg	23.3±0.9	154.9±19.6***	42.5±8.4	0.90±0.004	142±15*	o.e.	o.e.

* $P<0.05$, ** $P<0.01$, *** $P<0.001$; o.e., over-estimated.

Table 7
Effect on T- and B-lymphocyte of HBN's derivatives

Sample	Conc. (μg/ml)	MTT test (mean ± SD ($OD_{570\ nm}$))	^{3}H TDR test			
			ConA		LPS	
			Mean ± SD (CPM)	Prolif. (%)[a]	Mean ± SD (CPM)	Prolif.(%)[a]
Control	Negative		4092 ± 677		4404 ± 352	
	Positive	0.168 ± 0.005	36105 ± 2566		22694 ± 1043	
HBN-S	1	0.277 ± 0.014[b]	44165 ± 1767	22	28112 ± 3296	24
	10	0408 ± 0.018[c]	50820 ± 1784	41	34697 ± 1473	53
	100	0.680 ± 0.010[c]	67764 ± 1455	88	55356 ± 2233	144
HBN-I	1	0.261 ± 0.004[c]	40898 ± 927	13	25508 ± 291	12
	10	0.415 ± 0.005[c]	56708 ± 1007	57	36299 ± 2756	60
	100	0.681 ± 0.018[c]	69540 ± 2653	93	54429 ± 4835	140
HBN-E	1	0.267 ± 0.004[c]	44544 ± 2611	23	29903 ± 1727	32
	10	0.426 ± 0.013[c]	49984 ± 898	38	35863 ± 2240	58
	100	0.672 ± 0.013[c]	63484 ± 590	76	48735 ± 3476	115

[a] ≥15 showed the samples were effective.
[b] $P < 0.01$.
[c] $P < 0.001$.

and linkages are different to some extent. With immunological assays in vitro and vivo, HBN has significant immunoenhancing activity on the immune system. The study of this arabinogalactan (HBN) is helpful to the use of it in pharmacology and biotechnology.

Acknowledgements

We are grateful to Mr Yu Zhou and YongXin Ren for their help with the immunological assays and for valuable suggestions.

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