PERGAMON

Phytochemistry 52 (1999) 1089–1093

PHYTOCHEMISTRY

An immunologically active arabinogalactan from *Tinospora cordifolia*

Gajanan Chintalwar[a,*], Anjali Jain[a], Arjun Sipahimalani[a], Asoke Banerji[a], Percy Sumariwalla[b], Rupal Ramakrishnan[b], Krishna Sainis[b]

[a]*Bio-Organic Division, Bhabha Atomic Research Centre, Modular Laboratories, Trombay, Mumbai 400085, India*
[b]*Cell Biology Division, Bhabha Atomic Research Centre, Modular Laboratories, Trombay, Mumbai 400085, India*

Received 4 May 1999; received in revised form 30 June 1999; accepted 30 June 1999

Abstract

An arabinogalactan of mean M_r 2.2×10^6 has been isolated from the dried stems of *Tinospora cordifolia* and examined by methylation analysis, partial hydrolysis and carboxyl reduction. Purified polysaccharide showed polyclonal mitogenic activity against B-cells, their proliferation did not require macrophages.

Keywords: Tinospora cordifolia; Menispermaceae; Acidic arabinogalactan; Isolation; Structure; Immunological activity

1. Introduction

Tinospora cordifolia Miers (Menispermaceae), is an important medicinal plant cultivated throughout the Indian subcontinent. Through centuries, it has been extensively used in various Ayurvedic preparations for the treatment of various ailments (Kirtikar & Basu, 1933; Thatte, Chhabria & Karandikar, 1987). Our previous studies, of the methanolic extract of the plant, led to the isolation and characterisation of phenylpropanoids (Sipahimalani, Norr & Wagner, 1994), norditerpene furan glycosides (Gangan, Pradhan, Sipahimalani & Banerji, 1994, 1995), diterpene furan glycosides (Gangan, Pradhan, Sipahimalani & Banerji, 1996) and phytoecdysones (Gangan, Pradhan, Sipahimalani & Banerji, 1997). Recently, Thatte and Dahanukar (1989) have shown that the aqueous extracts of the plant stimulated the phagocytic and bactericidal activity of the neutrophils and macrophages. During the present studies a high molecular weight polysaccharide was isolated. It accounted for most of the immunostimulant activity.

2. Results and discussion

The dried and powdered stems of *T. cordifolia* were extracted with boiling H_2O. Addition of excess Me_2CO to the cooled aqueous extract gave a precipitate P which gave positive Molisch test, compatible with the presence of carbohydrate derived from polysaccharide. It was found to possess the immunogenic activity when tested on mouse spleen cells (stimulation index 11.0). Progressive purification resulted in the enhancement of mitogenic activity. Removal of proteinous matter from P resulted in the isolation of fraction T with enhanced mitogenic activity (stimulation index 17.4). Further purification by GPC gave a product T1 with considerably higher stimulation index of 66.8 (Table 1). The mitogenic activity was directed against B cells only; T cells were not affected (Sainis et al., 1997). The mean M_r of T1, as determined by GPC, was found to be around 2.2×10^6. Complete acid hydrolysis of T1 gave a mixture of monosaccharide derivatives which were identified and estimated by GC. The major components were galactose (32%), arabinose (31%) and galacturonic acid (35%) estimated by carbazole–H_2SO_4 method (Bitter & Muir, 1962). There was 1.4% rhamnose (Table 2). After partial hydrolysis,

* Corresponding author.

0031-9422/99/$ - see front matter
PII: S0031-9422(99)00386-6

Table 1
Mitogenic effect of polysaccharide fractions of *Tinospora cordifolia* on murine spleen cells

Treatment	Optimum concentration (μg/ml/10^6 cells)	^{3}H Thymidine incorporation (mean cpm ± s.e.m)	Stimulation index[a]
None	–	1040 ± 194	–
P	800	11,400 ± 900	11.0
T	600	18,090 ± 2300	17.4
T1	200	69,450 ± 1801	66.8

[a] Stimulation index = (^{3}H thymidine incorporation in cells treated with immunomodulator/^{3}H thymidine incorporation in untreated cells only).

Table 2
Monosaccharide composition of T1, its partially hydrolysed product T4 and monosaccharide T5 liberated due to partial hydrolysis (relative %)

	T1	T4[a]	T5
Galactose	32	53.8	13.2
Arabinose	31	16.7	57.1
Rhamnose	01.4	–	–
Galacturonic acid	35	13.6	25.4

[a] Unidentified portion is 15.9 %.

the M_r remained in the range of 10^6 indicating that most of the arabinose was not in the main chain. This was also indicated by the fact that most of the arabinose residues were liberated as monosaccharides (Table 2). Almost 65% of galacturonic acid was liberated after partial hydrolysis, indicating that major portion of the acid was present in the side chains, linked to the more stable linear part of T1, through arabinose units. Almost 80% of galactose remained unaffected by the partial hydrolysis, indicating it to be in the linear chain. The GC–MS analysis, of the partially methylated alditol acetates obtained by Hakomori methylation (Hakomori, 1964) of T1, complete hydrolysis, $NaBH_4$ reduction and acetylation showed the presence of terminal arabinose, 1,5-linked arabinose, terminal galactose, 1,4-linked galactose, 1,6-linked galactose and 1,3,6-linked galactose (Table 3). No derivative of rhamnose was detected. The presence of terminal arabinose (2,3,5-OMe_3 ara) was indicative of the easily hydrolysable furanoid form of arabinose (Wolfrom, Thompson & Timberlake, 1963). To determine the linkage of galacturonic acid, T1 was first reduced with 3-ethyl 3(dimethylaminopropyl)carbodiimide hydrochloride/$NaBH_4$ (Taylor & Conrad, 1972) and then subjected to Hakomori methylation, complete hydrolysis, $NaBH_4$ reduction and acetylation. The GC–MS analysis of the corresponding alditol acetates showed predominant enhancement of the 2,3,6-OMe_3-galactose, indicating that galacturonic acid is linked only at its 4- position. After partial hydrolysis the product T4 lacked terminal arabinose and 1,5 arabinose residues.There was an increase in the proportion of 1,6-linked galactose residues. The observations are compatible with the main chain being of 1,4 linked galactose residues with some 1,3,6-linked galactose units interposed in between and carrying side-chains or units on their 3-position. About one fifth of the galactose residues may be in the side-chains and one third of galacturonic acid remained glycosidically linked after partial hydrolysis.

These data thus show that the aqueous extract of *T. cordifolia* contains polyclonal B-cell activator which may be attributed to an acidic arabinogalactan polysaccharide. Similar immunomodulatory polysaccharides have been reported in other species of medicinal plants (Wagner, Stuppner, Schafer & Zenk 1988; Wagner & Jordan, 1988).

3. Experimental

3.1. Analytical methods

GC of alditol acetates (FID detector) was carried out using glass column (2 m × 3.5 mm i.d.) packed

Table 3
Methylation analysis of polysaccharide T1 and its partially hydrolysed polysaccharide T4 (Relative Molar %)

Polysaccharide partially methylated alditol acetate			T1	T4	Deduced glycoside linkage	
2,3,5	OMe_3	ara	7.8	-tr	term	araf
2,3	OMe_2	ara	6.4	–	1,5	araf
2,3,4,6	OMe_4	gal	0.6	2.9	term	galp
2,3.6	OMe_3	gal	6.2	7.4	1,4	galp
2,3,4	OMe_3	gal	0.3	4.8	1,6	galp
2,4	OMe_2	gal	2.0	3.6	1,3,6	galp

with 3% OV-225, temp. 180–240°, 4° min^{-1}, N_2 40 ml min^{-1}. GC–MS of partially methylated alditol acetates (70 eV) was carried out using fused silica DB- 9 column (50 m × 0.22 mm i.d), temp. 180–240°, 4° min^{-1}. Preparative GPC was carried out on Sephacryl S-400 column (350 mm × 25 mm i.d.; eluent, H_2O 0.4 ml min^{-1}.) Analytical HP-GPC was carried out on Polysep GFC-D 5000 column (Phenomenex, H_2O 0.7 ml min^{-1}, RI detector). TLC of the monosaccharides was carried out on SiO_2 F254 (E Merck) precoated plates, (BuOH:AcOH: H_2O:Et_2O, 9:6:1:3). Detection was carried out with silver nitrate.

3.2. Plant material

Stems of *Tinospora cordifolia*, growing within the Trombay campus of BARC, were harvested in the month of March, 1996. These were sun-dried, powdered and used for the isolation of the polysaccharide. The plant was identified and authenticated by Dr. V. Abraham, Scientific officer (retd.), Nuclear Agriculture and Biotechnology Division, B.A.R.C. as *T. cordifolia* (Willd) Miers. [Ref. Herbarium No. 1391].

3.3. Immunological test system

The mitogenic activities of P and its purified compounds were estimated as described earlier (Sainis et al., 1997). The crude and purified polysaccharide fractions were evaluated by culturing murine spleen cells (SC) with them in vitro, for 48 h in 5% CO_2 containing incubator. Proliferation was estimated by pulsing

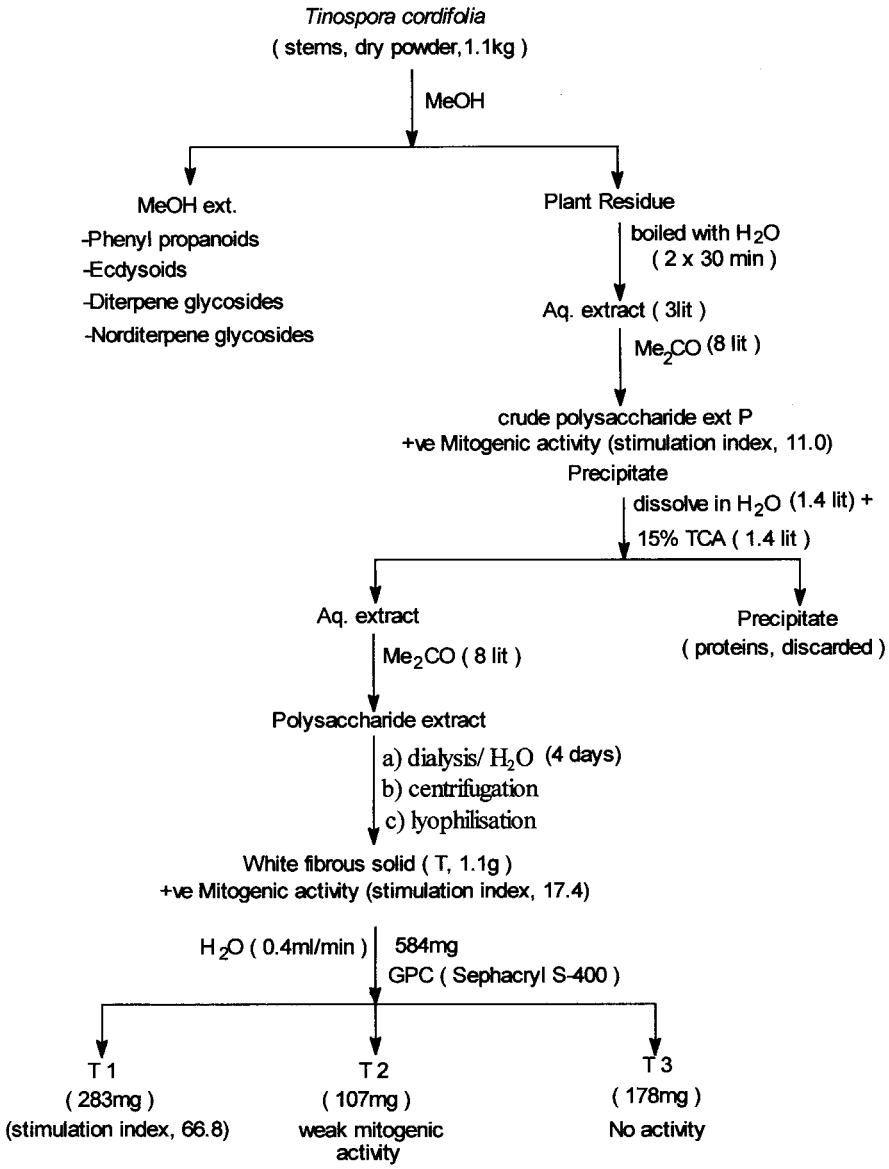

Fig. 1. Isolation and purification of bio-active polysaccharide fraction from *T. cordifolia*.

each well (2×10^5 cells) with 1 μCi ^{3}H-thymidine for 16 h and the incorporation of ^{3}H-thymidine was measured by liquid scintillation counting (Sainis et al., 1997).

The active fraction T1 was tested for any contaminations by bacterial lipopolysaccharide (endotoxin) using Sigma E-Toxate reagent (Amebocyte lysate of *Limulus polyphemus,* Sigma catalogue No. 210-8) following Sigma procedure No. 210. LPS from *Salmonella typhimurium* (Difco cat No. 3998-25) was used as positive control. The T1 preparation did not have LPS activity (test was negative).

3.4. Bioassay directed isolation and purification

Figure 1 outlines the steps involved in the bioassay directed isolation and purification of the bioactive polysaccharide.

Air dried stems of *Tinospora cordifolia* (1.1 kg) were powdered and extracted with MeOH followed by boiling H_2O. The cold aqueous extract was treated with Me_2CO to precipitate crude polysaccharide fraction P. It showed positive mitogenic activity against murine spleen cells (Table 1). It was freed from proteins and dialysed to remove any low molecular weight compound. The undialysable aqueous fraction was slightly turbid. It was centrifuged and freeze-dried to get white fluffy fibrous polysaccharide T (1.17 g), having enhanced immunostimulant activity (Table 1).

3.5. Gel permeation chromatography

Further purification of T was carried out on a Sephacryl S-400 column using H_2O as an eluent. Seventy fractions were collected. These were monitored by HP-GPC. Fractions, with similar retention behaviour, were pooled to give three fractions viz T1, T2 and T3 (Fig. 2). T1 was moderately soluble in water, showed a positive Molisch test and absence of N by sodium fusion test and Kjeldhal method. Fraction T1 was found to have further enrichment of activity (Table 1) as compared to T.

3.6. Mean molecular range (M_r) of T1 and T4

It was determined by comparing the HP-GPC retention-times of T1 (10.6 min) and T4 (10.4 min) with those of dextrans samples of known molecular weight viz 2.02×10^6 (10.9 min), 5.8×10^5 (14.8 min), 1.89×10^5 (15.8 min), 8.00×10^4 (16.5 min), 1.95×10^4 (17.3 min) and 1.04×10^4 (17.5 min). The retention-time of both T1 and T4 compared with those of a known dextran standard of 2.02×10^6.

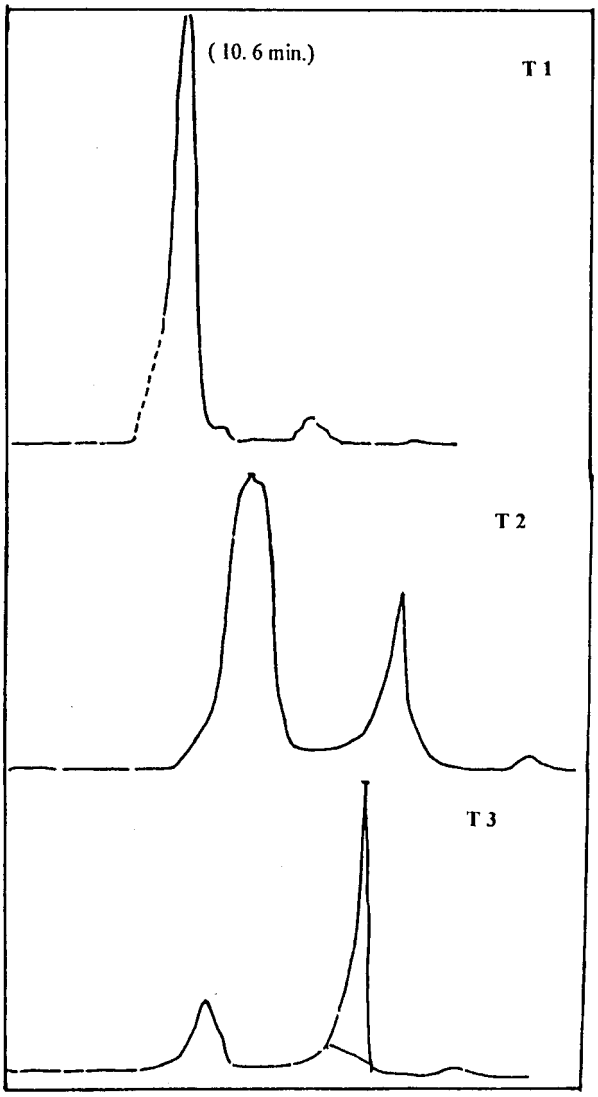

Fig. 2. HPGPC fractions, T1, T2 and T3.

3.7. Neutral monosaccharide composition

T1 (5 mg) was hydrolysed with 2 M TFA (130°, 3.5 h). After the hydrolysis, the solution was evaporated under reduced pressure and codistilled a number of times with MeOH. The alditol acetates were prepared by $NaBH_4$ reduction followed by acetylation with Ac_2O/pyridine. GC analysis indicated the relative percentages of galactose, arabinose and rhamnose (Table 2).

3.8. Identification and quantification of galacturonic acid

The presence of uronic acid was detected by carbazole-H_2SO_4 method (Bitter & Muir, 1962). T1 was reduced with 3-ethyl-3(dimethylaminopropyl)carbodiimide hydrochloride/2M $NaBH_4$ (Taylor & Conrad, 1972), hydrolysed with 2M TFA (130°, 3.5 hr) and the

corresponding alditol acetate prepared as above. GC analysis did not show any peak either corresponding to glucose or any other new monosaccharide. There was an increase in the proporation of galactose compared to that in T1. The absence of glucose indicated the absence of glucoronic acid residues. Galacturonic acid was quantitatively estimated as 35%.

3.9. Methylation analysis

T1 (20 mg) was dissolved in dry DMSO and methylated with methylsulphinyl carbanion, according the method of Hakomori (Hakomori, 1964). The complete hydrolysis was done with 2M TFA (130°, 3.5 h) and the methylated sugars were converted into their corresponding partially methylated alditol acetate by reduction with $NaBH_4$ followed by acetylation with Ac_2O/pyridine. The mixture thus obtained was subjected to GC–MS analysis (Table 3).

3.10. Partial hydrolysis

T1 (20 mg) was hydrolysed with TFA (0.02 M, steam bath, 1 h). Solvent was evaporated under reduced pressure and the product subjected to GPC on Sephacryl S-400 (H_2O, 0.4 ml/min) for separation of partially hydrolysed polysaccharide (T4, 6.7 mg) and liberated monosaccharides (T5, 6.0 mg). The neutral monosaccharide composition of the two fractions was determined as their alditol acetates. Galacturonic acid was quantitatively determined by carbazole-H_2SO_4 method (Table 2).

References

Kirtikar, K. R., & Basu, B. D. (1933). In E. Blatter, J. R. Cauis, & K. S. Mhaskar, (p. 77). In *Indian medicinal plants*, *Vol.1*. Basu, India: L.M. Allahabad.

Thatte, U. M., Chhabria, S. S., & Karandikar, S. M. (1987). *Indian Drugs*, *75*, 95.

Sipahimalani, A. T., Norr, H., & Wagner, H. (1994). *Planta Med.*, *60*, 596.

Gangan, V. D., Pradhan, P., Sipahimalani, A. T., & Banerji, A. (1994). *Phytochemistry*, *37*, 781.

Gangan, V. D., Pradhan, P., Sipahimalani, A. T., & Banerji, A. (1995). *Phytochemistry*, *39*, 1139.

Gangan, V. D., Pradhan, P., Sipahimalani, A. T., & Banerji, A. (1996). *Ind. J. Chem.*, *35B*, 630.

Gangan, V. D., Pradhan, P., Sipahimalani, A. T., & Banerji, A. (1997). *Ind. J. Chem.*, *36B*, 787.

Thatte, U. M., & Dahanukar, S. A. (1989). *Phytother. Res.*, *3*, 43.

Sainis, K. B., Sumariwalla, P. F., Goel, A., Chintalwar, G. J., Sipahimalani, A. T., & Banerji, A. (1997). In S. N. Upadhyay, *Immunomodulation* (p. 155). New Delhi, India: Narosa Publishing House.

Bitter, T., & Muir, H. M. (1962). *Anal. Biochem.*, *4*, 330.

Wolfrom, M. L., Thompson, M., & Timberlake, C. E. (1963). *Cereal Chem.*, *40*, 82.

Hakomori, S. I. J. (1964). *Biochemistry (Tokyo)*, *55*, 205.

Taylor, R. L., & Conrad, H. E. (1972). *Biochemistry*, *11*, 1383.

Wagner, H., Stuppner, H., Schafer, W., & Zenk, M. (1988). *Phytochemistry*, *27*, 119.

Wagner, H., & Jordan, E. (1988). *Phytochemistry*, *27*, 2511.