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Structure of polysaccharide from *Polygonatum cyrtonema* Hua and the antiherpetic activity of its hydrolyzed fragments

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Abstract

A neutral polysaccharide named PD was isolated from the traditional Chinese medicinal herb, *Polygonatum cyrtonema* Hua. Five fragments were isolated by Bio-Gel P4 chromatography from hydrolysates of PD. Using assays of cytopathic effect inhibition, neutral red dye uptake and plaque forming inhibition, it was proved that the fragments with degree of polymerization (DP) of 4 and 5 were the shortest ones which retained the activity against herpes simplex virus type 2 (HSV-2) in vero cell culture. The structures of PD and one of its activity-retaining fragments, B3, were determined by permethylation followed with reductive cleavage, mass spectrometry and nuclear magnetic resonance spectrometry. It was shown that PD was a branched fructan with average DP of 28. There was one two-residue side chain composed of (2 → 6)-linked β-D-fructofuranosyl (Fru*f*) residues every three (2 → 1)-linked β-D-Fru*f* residues in the backbone of PD, whereas B3 was a mixture containing 1-kestose and neokestose series of oligosaccharides of DP 3–5 without branches.

Keywords: *Polygonatum cyrtonema* Hua; Polysaccharide; Antiviral activity; Herpes simplex virus; Activity-retaining fragment; Structure

1. Introduction

Herpes simplex virus 1 and herpes simplex virus 2 (HSV-1 and HSV-2) are two kinds of ubiquitous pathogens that may cause serious morbidity in humans. In addition to producing mild oral and genital lesions, these viruses can cause more severe cutaneous, ocular and disseminated infections, particularly in those with immune deficiencies. HSV-1 is normally associated with oral-facial infections, ocular infections and encephalitis. HSV-2 usually causes genital infections, neonatal herpes and is also known as oncogenic virus which has the ability to convert cells into tumor cells (Lapucci et al., 1993; Liesegang, 2001; Souza et al., 2003; Kavita and Sanjay, 2002; Whitley et al., 1998). Both types of viruses establish latent infections in sensory neurons, moreover, upon reactivation, cause recurrent infections (Hook et al., 1992).

At present, antiviral chemotherapeutic agents such as acyclovir, valacyclovir and penciclovir are the standard drugs for treating various forms of HSV infections. They are modified nucleoside analogs that work as inhibitors of viral DNA polymerase. However, to be effective, they must be taken within hours of the onset of symptoms. Moreover, they all have undesirable complications more or less after prolonged high dose and may induce the emergence of drug-resistant strains (Collins and Darby, 1991). Because of the drawbacks of the nucleoside-based anti-HSV therapeutics and the increase of HSV infections with the growth of immunocompromised patients and HIV infections over the past decade (Xu et al., 1999), there is an urgent need to develop new antiherpetic drugs, especially those with different mechanisms of action than foregoing drugs.

In recent years, several classes of polysaccharides, including mannans, glucans, galactans, fucans, carrageenans, and their derivatives, especially their sulfated derivatives have been reported to possess antiviral activity against HSV (Carlucci et al., 1999). Our group also isolated a polysaccharide component named PD from *Polygonatum cyrtonema* Hua, a traditional Chinese medicinal herb. Previous studies revealed that PD could inhibit both HSV-1 and HSV-2 in vitro in vero cell culture, with a 50% effective concentration (EC_{50}) against stoker strain (HSV-1), 333 strain (HSV-2) and sav strain (HSV-2) of 3.95, 8 and 7.72 mg/ml, respectively (Gu et al., 2003). Furthermore, Zen reported that eye drops containing PD were markedly efficacious in the

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treatment of experimental rabbit herpes simplex keratitis (HSK) (Zen et al., 1998). The rabbits were scarified on their corneas and drops of viral solution were applied to the eyes. The treatment began after the confirmation of the successful establishment of rabbit HSK model by indirect immunofluorescence, and lasted for 18 days. In the observed group, the keratitis was alleviated by an average of six days of treatment, while nine days were required in the acyclovir eye drop group. Presently, a phase three clinical research trial of the eye drops containing PD is underway.

In this paper, we furthered our research of this antiherpetic polysaccharide, PD, by tracing its activity-retaining fragment and investigating the structure of PD and this activity-retaining fragment. To our best knowledge, it is the first time that hydrolysates of polysaccharide from *P. cyrtonema* Hua which retain antiherpetic activity have been reported.

2. Materials and methods

2.1. Materials and reagents

The roots of *P. cyrtonema* Hua were collected from Xingwen County of Sichuan Province, P.R. China, at springtime and authenticated by Professor Zuocheng Zhao at Chengdu Institute of Biology, Chinese Academy of Sciences. Specimen was deposited at the Herbarium of the same institute. RPMI-1640 medium and trypsin were purchased from GIBCO (USA). Et_3SiH and $Me_3SiO_3SCF_3$ were purchased from SIGMA (USA). All other reagents with analytical grade were obtained from manufacturers in China.

2.2. Preparation of PD

The roots of *P. cyrtonema* Hua were washed and cut into thin pieces, dried at 60 °C and pulverized to yield brown powder. After homogenized and refluxed in 85% ethanol, the powder was firstly extracted with 50% ethanol and subsequently precipitated with 100% ethanol to yield the crude extract, as described in detail elsewhere (Meng, 1996). Then the crude extract was purified by two-step chromatography. Firstly, anion exchange chromatography was carried out on LKB Chromatography System with a column (60 cm × 7.5 cm) filled with 685 alkaline resins. The column was equilibrated with 20 mmol/L Tris–HCl of pH 8.0, and eluted with the same medium followed by a 0.5 mol/L NaCl solution at a flow-rate of 500 ml/h. Secondly, the collected sugar fractions were subjected to Bio-Gel P4 column (40 cm × 2.6 cm) eluted with distilled water at a flow-rate of 50 ml/h. The saccharide was detected by ultraviolet detection at 206 nm combined with α-naphthol-H_2SO_4 reaction (Zhang, 1994).

Finally, the purified sugar fractions were collected and lyophilized to yield white powder, which was named PD. The saccharide and protein contents of PD were estimated by anthrone-H_2SO_4 method and Lowry–Folin test, respectively.

2.3. Hydrolysis of PD and separation of hydrolyzed fragments

6% (w/v) PD was hydrolyzed with 0.025 mol/L H_2SO_4 solution for 20 min at 65 °C. The hydrolysates were applied to chromatographic column (160 cm × 2.6 cm) filled with Bio-Gel P4 and eluted respectively with two eluent systems, distilled water and 50 mmol/L $(NH_4)_2CO_3$ solution, both at a flow-rate of 70 ml/h. The degree of polymerization (DP) of each fraction was analyzed by thin layer chromatography (TLC) on silica plate (10 cm × 25 cm) with ascending spread mode developed with 1-butaol: glacial acetic acid: water (2:1:1) and stained by acetone–diphenylamine, acetone–phenylamine, trichloracetic system (Zhang, 1994).

2.4. Cell and virus

Vero cells, provided by West China University of Medical Sciences, were used for virus stock production and antiviral activity evaluation. The cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and RPMI-1640 medium supplemented with 10% newborn calf serum, 100 U/ml penicillin and streptomycin.

HSV-2 (strain 333) was obtained from Wuhan Institute of Virology, Chinese Academy of Sciences. The virus stock was prepared in vero cell culture and stored at −70 °C. Virus infectivity was determined by cytopathic effect (CPE) assay (Mahmoud et al., 2002) and 50% tissue culture infectious dose ($TCID_{50}$) was measured before use.

2.5. Cytopathic effect (CPE) inhibition assay

Vero cells were seeded in 96-well flat-bottomed microplates and grown to near-confluence using the conditions described above. Test samples of different concentrations were added in quadruplicate, immediately followed by the addition of 100 $TCID_{50}$ of virus. Then the plates were incubated at 37 °C for 72 h. The cytopathic effect was detected by light microscopy and graded. The 50% effective concentration (EC_{50}) was calculated by Reed–Muench method (Zhang, 1998).

For each sample, 4 wells remained uninfected to observe any morphological changes due to the cytotoxicity of test samples. The 50% cell-inhibitory concentration (IC_{50}) was calculated by Reed–Muench method also.

2.6. Neutral red (NR) dye uptake assay

The antiviral activity was also evaluated by neutral red dye uptake assay. 200 μl of neutral red dye (0.04%, w/v in culture medium) was added to each well of the plates at the end of CPE inhibition assay. After incubation for 1 h, the NR solution was aspirated and wells were washed twice with PBS (pH 7.4). Then 200 μl of 70% ethanol solution was added per well to extract the dye. The plates were shaken for 5 min and the absorbance at 540 nm of each well was

read with a microplate reader. Percent protection or EC_{50} was calculated to evaluate the anti-HSV activity of samples (Zhang, 1998). Also, IC_{50} was determined by regression analysis between sample concentrations and the percentages of the absorbance values of the uninfected sample wells versus that of the uninfected cell controls.

2.7. Plaque formation inhibition assay

Vero cells were seeded in 24-well culture plates, and the monolayers were infected with 50 $TCID_{50}$ of HSV-2 (strain 333). After incubation of 1 h, the inoculum was removed and the cultures were overlaid with RPMI-1640 medium supplemented with 2% newborn calf serum, 0.6% (w/v) agarose and test samples with different concentrations. After incubation for 72 h at 37 °C and the removal of the agarose overlays, the plates were fixed with formalin and stained with crystal violet. The percentage of inhibition of plaque forming was calculated as [(mean number of plaques in untreated control − mean number of plaques in test)/mean number of plaques in untreated control] (Xu et al., 1999), and EC_{50} was deduced by Reed–Muench method.

2.8. Structure determination of PD and B3

Methylation and reductive cleavage were performed as described (Ciucanu and Kerek, 1984; Rolf et al., 1985). The permethylated PD was subjected to reductive cleavage to yield partially methylated alditol acetates and the products were directly applied to gas chromatographic–mass spectrometry (GC–MS) analysis. The peak areas were corrected by effective carbon response theory (Addison and Ackman, 1968; Sweet et al., 1975). Gas chromatographic separation was performed using a SUP-PTE-S capillary column of 30 m × 0.25 mm (inner diameter) × 0.25 μm (film thickness) with flame ionization detector (FID). The carrier gas was N_2 and temperature program was 152 → 182 °C at 3 °C/min and to 250 °C at 12 °C/min. Gas chromatographic–mass spectrometry was recorded on a HP 6890 GC–MS with temperature program of 100 → 250 °C at 4 °C/min and ionization voltage of 70 eV.

The sample from Peak B3 was dissolved in 10 mmol/L sodium acetate to determine the molecular weight by atmospheric pressure ionization mass spectrometry (API/MS), and in-source collision-induced dissociation (CID) measurement was carried out to observe fragment ions. The nuclear magnetic resonance (NMR) spectrum was recorded on Bruker Advance 500 spectrometer at 20 °C in D_2O.

3. Results

3.1. Determination of the smallest activity-retaining fragment of PD

To determine the smallest activity-retaining fragment, PD was hydrolyzed and the hydrolysates were applied to

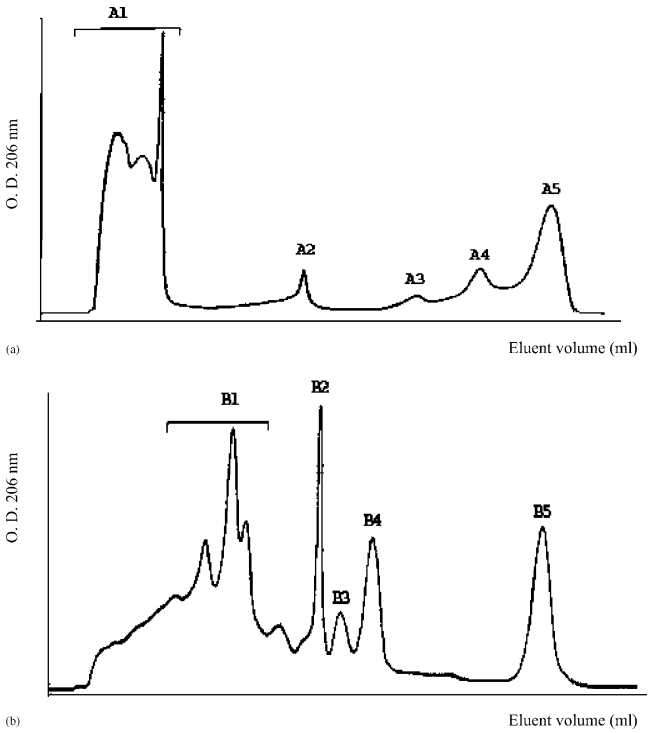

Fig. 1. Chromatograms of hydrolysates of PD separated with Bio-Gel P4. (A) Eluted with distilled water; (B) eluted with 50 mmol/L $(NH_4)_2CO_3$ solution.

Bio-Gel P4 gel filtration column eluted with distilled water to separate different fragments. The chromatogram is shown in Fig. 1A. Using inulin, melezitose and fructose as standard sugars in TLC analysis to determine the size of each fragment (data not shown), peak A5 was identified to be monosaccharide, peak A4 was a mixture of oligosaccharides ranging from mono- to tetra-saccharides and peak A3 contained tetra-, penta- and some larger oligosaccharides (Table 1).

We have previously determined that the EC_{50} of PD against HSV-2 (333 strain) was 8 mg/ml. Therefore, in the preliminary screening of activity-retaining fragments, the antiherpetic activities of fraction A1–A5 at the concentration of 8 mg/ml were tested by CPE inhibition and neutral red dye uptake assays. The percent inhibition was expressed by dividing the difference in absorption between test wells and virus control wells by the difference between cell controls and virus controls. As shown in Table 1, except A5, other fractions retained the activity similar to that of PD. So A4, which was a mixture of oligosaccharides from mono- to tetra-saccharides, was thought to be the shortest active fragment.

To investigate the antiherpetic activity of A4 further, the IC_{50} and EC_{50} of this fragment were determined by CPE inhibition, neutral red dye uptake and plaque formation inhibition assays (result shown in Table 2). A4 gave a dose-dependent response in inhibition of CPE and plaque formation of HSV-2 333 strain, and EC_{50}s were

Table 1
Antiherpetic activities of hydrolysates A_n evaluated by neutral red dye uptake assay

	PD (%)	Hydrolysates A_n (8.0 mg/ml)				
		A1 (%)	A2 (%)	A3 (%)	A4 (%)	A5 (%)
Percent inhibition	68	46	72	75	47	4
Oligosaccharide composition (degree of polymerization)				4, 5 and >5	1–4	1

Table 2
Antiherpetic activity of peak A4 evaluated by different assays

Assay used	IC_{50} (mg/ml)	EC_{50} (mg/ml)
CPE inhibition	>8	2
Neutral red uptake	>8	1.8
Plaque forming inhibition		3.3

estimated as 2 and 3.3 mg/ml by these two methods, respectively.

To further reduce the range of the degree of polymerization of the shortest activity-retaining fragment, the PD hydrolysates were again applied to Bio-Gel P4 column and eluted with 50 mmol/L $(NH_4)_2CO_3$ solution (chromatogram shown in Fig. 1B). As demonstrated in TLC analysis (data not shown), peak B5 primarily contained mono- and di-saccharides, B4 was mainly composed of tri-saccharides together with a small quantity of tetra- and penta-saccharides and peak B3 contained penta-, tetra- and a spot of tri-saccharides. Then the antiherpetic activities of B3 and B4, which were composed of oligosaccharides having degree of polymerization values overlapping with that of A4, were determined by CPE inhibition assays (Table 3). The result showed that B4, mainly tri-saccharides, did not hold the activity, while B3, mainly tetra- and penta-saccharides, retained the antiherpetic activity and was identified to be the shortest activity-retaining fragment.

3.2. Structure of PD

Former monosaccharide determination has shown that PD was exclusively composed of glucose and fructose (Meng, 1996). In order to further elucidate the structure of PD, complete methylation followed by reductive cleavage was carried out. Different methylated alditol acetates derived from PD were analyzed by GC–MS. Identification of all significant peaks was carried out according to previously obtained GC–MS data (Rolf and Gray, 1984; Thomas et al., 1992). The reductive cleavage of PD gave peaks 1–9 (Fig. 2), indicating that PD contained branched residues. The formation of peak 6 suggested the presence of (2 → 6)-linked residues and peak 7 indicated the presence of (2 → 1)-linked residues. Peak 4 was identified as 6-*O*-acetyl-1,5-anhydro-2,3,4-tri-*O*-methyl-D-glucitol according to GC–MS, suggesting the α-D-Glc*p* residue linked with a β-D-Fru*f* residue was also attached at position 6 as in neokestose (Thomas et al., 1992; Sabine et al., 2000; Sims et al., 2001).

Furthermore, the integrated area of each peak was corrected using effective carbon response theory (Addison and Ackman, 1968; Sweet et al., 1975) to obtain quantitative information, which gave the relative ratios of each kind of linkages existing in PD (Table 4). The presence of peak 1 implied that trace of glucose existed at the non-reductive end of the whole polysaccharide, which was consistent with the result that the difference between terminal and branched fructofuranosyl residues was less than 2 as should be the case for neokestose series.

Thus, on the average, the possible structure of PD was deduced as a branched fructan that had a DP of 28 on the assumption of a single D-glucosyl group per molecule (Sabine et al., 2000; Thomas et al., 1992), which was in agreement with the average molecular weight of 2000–5000 (Meng, 1996). There was one two-residue side chain composed of (2 → 6)-linked β-D-fructofuranosyl (Fru*f*) residues every three (2 → 1)-linked β-D-Fru*f* residues in the backbone of PD. The glucose mainly existed inside the molecule with neokestose form (Fig. 3).

3.3. Structure of B3

API/MS analysis of B3 (figure not shown) indicated that it was a mixture containing tri-, tetra- and penta-saccharides. The molecular ionic peaks at *m/z* 851 ($M + Na^+$), 689 ($M + Na^+$) and 527 ($M + Na^+$) corresponded to penta-,

Table 3
Antiherpetic activities of peak A4, B3 and B4 evaluated by CPE inhibition assay

	A4	B3	B4
IC_{50} (mg/ml)	>8	>8	>8
EC_{50} (mg/ml)	2	<2	>8
Oligosaccharide composition (degree of polymerization)	1–4	Mainly 4, 5	Mainly 3

Table 4
Peak assignment and molar ratios of residues per molecule of PD after GC–MS analysis

Residues	Peak number in Fig. 2	Molar ratio
Terminal α-D-Glcp	1	0.2
6-Linked α-D-Glcp	4	0.8
Terminal β-D-Fruf	2, 3	6.1
6-Linked β-D-Fruf	5, 6	5.6
1-Linked β-D-Fruf	5, 7	11.1
1,6-Linked β D-Fruf	8, 9	5.0

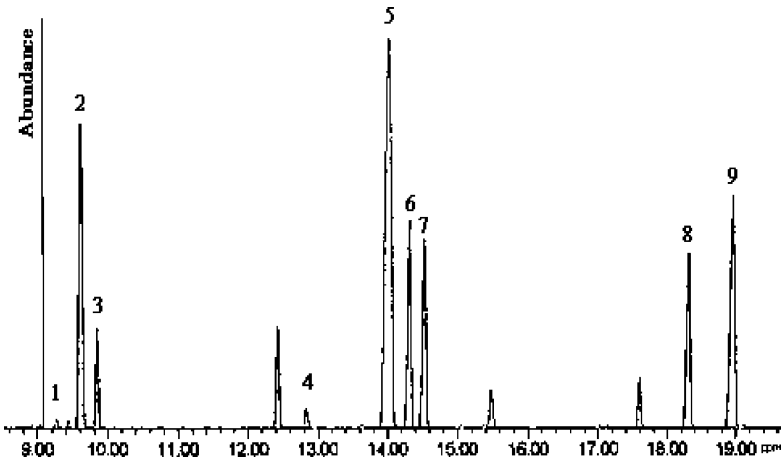

Fig. 2. GC chromatogram of the reductive cleavage products of PD (1–9 is the same as in Table 4).

tetra- and tri-saccharide, respectively. The results validated again that the molecular weight of all monosaccharides in PD was 180.

There were several series of visible degressive peaks of 162 fraction in CID API/MS analysis of B3 (figure not shown), such as the series of $[M + Na^+]$: 851 → 689 → 527 → 365 → 203 and the series of $[M + Na^+ - H_2O]$: 671 → 509 → 347 → 185. These regularly degressive peaks represented that the backbone of oligosaccharide released one residue sequentially, indicating no side chains existed in B3 (Anne, 1990; Beth and Burlingame, 1990).

There are many reports dealing with the characterization of ^{13}C-signals for fructan (Carpita et al., 1991; Bancal et al., 1991; Hammer and Morgenile, 1990; Heyer et al., 1998). According to former assignments, C-1 signal of glucose ranges between 93 and 94 ppm, around which we found two different signals in our own experiment (figure not shown), 93.980 and 94.481 ppm. The difference of about 0.5 ppm between the two signals showed that 1-kestose (94.481 ppm) and neokestose (93.980 ppm) series of oligosaccharides existed in B3 simultaneously. Neokestose series were consistent with the structure of PD, whereas 1-kestose series maybe came from the hydrolysis at C-6 of glucose in PD. Thus, based on the structure of PD, B3 was deduced to be a mixture containing neokestose and 1-kestose series of oligosaccharides of DP 3–5 without branches.

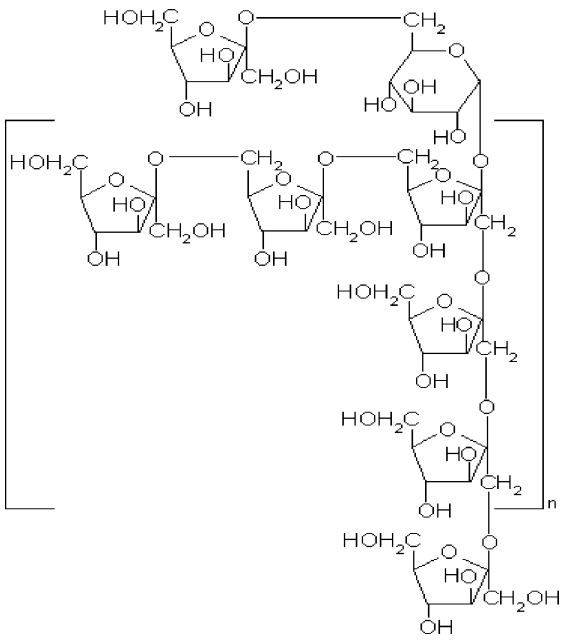

Fig. 3. Suggested structure of PD, $n = 5$, with a DP of 28.

4. Discussion

During the past decades, a wide variety of phytochemicals extracted from different plants and herb species have been investigated for their potential antiviral activities, including different kinds of polysaccharides (Baba et al., 1988; Gonzalez et al., 1987; Damonte et al., 1996; Eo et al., 2000; Tabba et al., 1989; Xu et al., 1999), most of which are sulphated saccharides. Mechanisms of the antiviral activities of these anionic polysaccharides are postulated to inhibit a step in virus replication subsequent to viral internalization (Gonzalez et al., 1987) or to inhibit the virus attachment to the host cells (Damonte et al., 1996; Yao et al., 1992).

However, as a fructan, PD and its fragments contain no ionic groups and thus may represent a different class of anti-HSV agents. Our group has isolated another fructan from *Polygonatum* sp., named PP, which can not inhibit the CPE development caused by HSV infection in vero cell culture, but possesses an obvious anti-aging activity (Zhao et al., 1995; Huang et al., 1999). As PD and PP are fructans with different structures, it is no doubt that the arrangement of different residues in fructans is essential to their functions. Moreover, the sulphated derivatives of PD and PP both can inhibit HSV infections in vitro, with an activity ten times higher than that of the original PD (Yan et al., 2000). Altogether, it seems to indicate that PD and the sulphated

derivatives have different molecular mechanisms of antiviral action.

It is reported that some polysaccharides will retain bioactivities when degraded, such as heparin, which retains anticoagulant activity with a penta-saccharide fragment (Choay et al., 1983; Grant et al., 1984). This seems to predict the existence of active centers in polysaccharides as there are in proteins. The study on active centers of polysaccharides should be noteworthy. It will not only reduce the difficulties of polysaccharide research, but will also benefit the structure-activity exploration, and may even make chemical synthesis of active polysaccharides possible (Zhang, 1999).

In this study, the hydrolyzed fragments of PD were separated by Bio-Gel P4 chromatography and a fraction corresponding to a mixture of tetra- and penta-saccharides was determined to be the shortest fragment that retained the antiviral activity against HSV-2 in vitro. The structure of this fragment, B3, was measured, which was proved to be consistent with the structure of PD. Elucidation of the structure of active B3 is important to understand its structure-function relationship and the molecular mechanism of its function. As noted in the results, B3 is still a mixture, due to the difficulties in separating oligosaccharide homologies and the restriction of techniques on hand. Therefore, only some preliminary structural information was gained in the present study. The definitive structure of the minimum fragment required for biological activity is still unavailable. Future studies may focus on the further separation of B3, its mechanism of action against HSV and its preventive or therapeutic effect on viral infections in vivo.

Acknowledgements

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