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Original article

Preparation and anticoagulant activity of the phosphosulfomannan PI-88

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Abstract

A yeast-derived phosphomannan mixture was chemically sulfonated and the composition and structure of the product mixture was studied. This phosphosulfomannan mixture, PI-88, is currently under clinical evaluation as an anti-cancer agent. Analysis using capillary electrophoresis demonstrated that PI-88 was a multi-component mixture. Gel permeation chromatography provided four fractions of PI-88 that contained components which differed in size from disaccharide to hexasaccharide, and by degree of sulfation. These fractions were characterised by spectroscopic and chromatographic methods and the structure of PI-88 is that expected based on the structure of the phosphomannan starting material. The anticoagulant activity of these fractions was evaluated and the structural requirements for activity are described.

Keywords: phosphomannan; phosphosulfomannan; anticoagulant; capillary electrophoresis; PI-88

1. Introduction

The exopolysaccharide of the yeast, *Pichia (Hansenula) holstii* NRRL Y-2448, consists of a phosphomannan core to which oligosaccharides are attached through a phosphodiester linkage [1,2]. These phosphomannans have been valuable as tools to study Man-6P receptors [3–6]. Spectroscopic characterisation of the major repeating side chain demonstrated that it was a pentasaccharide of the structure: 6-phospho-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p*, where Man is mannose, and *p* is pyranosyl [2]. In addition to the major pentasaccharide, substantial tetrasaccharide and minor amounts of di-, tri- and hexasaccharides were also shown to be present through NMR analyses of the mixture of side chains following their dephosphorylation [2,7]. Large-scale preparation of these phosphomannan oligosaccharides afforded multi-gram quantities for chemical modification (sulfonation) and further structural studies [8]. Analysis by capillary electrophoresis (CE) showed that the phosphomannan mixture was composed of Man-6-phosphate (3%), monophosphorylated disaccharide (3%), monophosphorylated tetrasaccharide (28.5%), monophosphorylated pentasaccharide (59%) and monophosphorylated hexasaccharide (1%). In addition, two other components were present (5.5% combined), one of which corresponded to monophosphorylated trisaccharide. There was evidence to suggest that the remaining component was monophosphorylated tetrasaccharylamine [8].

This phosphomannan oligosaccharide mixture is a precursor for a novel sulfonated oligosaccharide agent known as PI-88 [9]. PI-88 has recently been identified as

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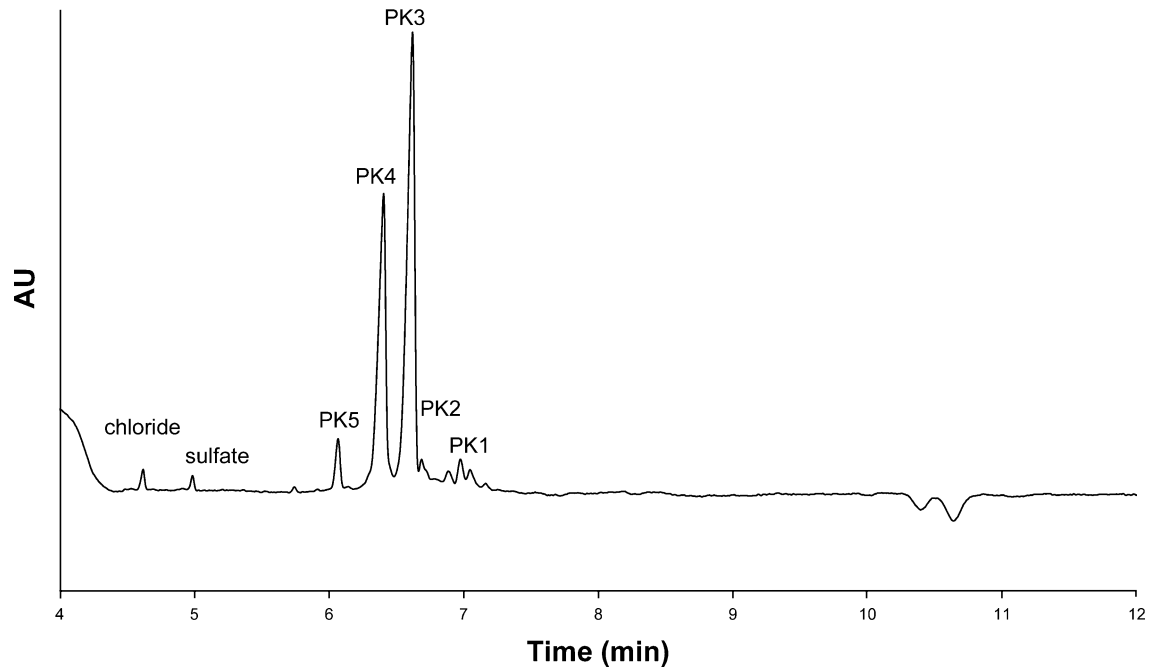

Fig. 1. Reversed polarity CE analysis of PI-88. The PK designations refer to the fractions afforded using LPLC–GPC (shown in Fig. 3). Absorbance units (AU) are shown as a function of migration time in minutes.

a promising inhibitor of tumour growth and metastasis and is currently undergoing Phase II clinical trials for this indication. PI-88 appears to block tumour growth by preventing the interaction of heparan sulphate with fibroblast growth factor and its receptor [9,10]. PI-88 is believed to block metastasis by inhibiting heparanase [11,12], blocking the breakdown of the extracellular matrix, thus preventing the spread of tumour cells.

The current study examines the components present in PI-88 using CE. PI-88 was fractionated by gel permeation chromatography to obtain individual phosphosulfomannans for structural characterisation. The anticoagulant activity of these phosphosulfomannans is evaluated and a structure–activity relationship established.

2. Results

Phosphomannans were prepared from *Pichia (Hansenula) holstii* NRRL Y-2448 in multi-gram scale as previously described [7]. This phosphomannan oligosaccharide mixture (phosphomannan precursor) was chemically sulfonated using sulphur–trioxide/pyridine in DMF for 3 days at 25 °C to afford phosphosulfomannans. Conversion to the sodium salt, removal of pyridine, decolorisation and diafiltration (NMWCO 1,000) afforded PI-88. Microanalysis for PI-88 was C12.5%, H2.4%, P1.2%, S17.7% and the $[\alpha]_D$ was 32 ± 2.

To assess its complexity, PI-88 was analysed by CE using an indirect detection method [13,14]. The resulting electropherogram showed 5–6 components in amounts ranging from $<1\%$ to $>60\%$ of the mixture (Fig. 1). PI-88, analysed by PAGE in a 22% acrylamide gel, showed the presence of multiple components having from two to six saccharide units based on structurally defined heparin oligosaccharide standards (Fig. 2, lane e). Similar analysis by HPLC–GPC confirmed PI-88 was a mixture of M_W 2100 with a polydispersity of 1.102.

LPLC–GPC was next used to separate PI-88 into fractions and the elution of carbohydrate was monitored using the phenol-sulphuric acid assay. Four fractions

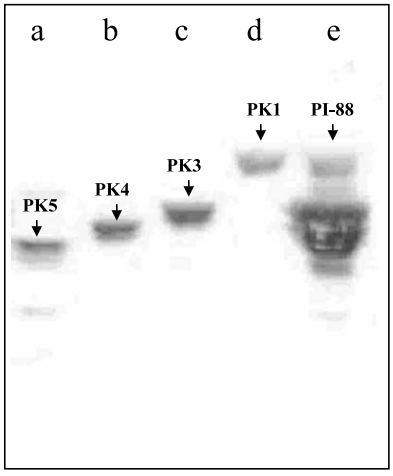

Fig. 2. PAGE analysis of PI-88 and PK fractions. The fractions, prepared using LPLC–GPC (shown in Fig. 3), are analysed in lanes: a, PK5; b, PK4; c, PK3; and d, PK1. Lane e contains the PI-88 mixture. The gel is visualised with Alcian blue staining.

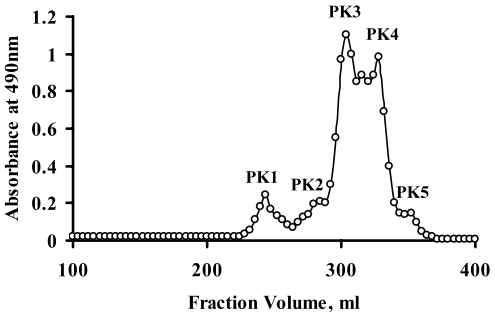

Fig. 3. LPLC–GPC fractionation of PI-88 into PK fractions 1–5 on a Bio-Gel P6 column. The peaks were determined using anthrone assay at an absorbance of 490 nm and plotted as a function of elution volume in millilitres.

(PK1, 3, 4 and 5) were obtained (Fig. 3) and after concentration each was desalted, freeze-dried and subjected to analysis. Fraction PK2 was obtained in quantities insufficient for analysis.

PI-88 fractions PK1, PK3, PK4 and PK5 analysed by PAGE showed each fraction contained a major component or components present within the original PI-88 mixture (Fig. 2). PK1 corresponded to the highest molecular weight component in PI-88, while PK5 contained the smallest molecular weight components based on both LPLC–GPC and PAGE analysis (Figs. 2 and 3). Analytical HPLC–GPC showed that these fractions ranged in M_w 1488 for PK5 to 3138 for PK1 and had significantly reduced polydispersity (1.025–1.045) compared to PI-88 (M_W 21 000, polydispersity 1.102), consistent with LPLC–GPC and PAGE analysis (Figs. 2 and 3). The co-injection of PK1, PK3, PK4 and PK5 with PI-88 on CE was used as a high-resolution method to establish the identity of each peak in the PI-88 mixture (Fig. 1). FABMS analysis of each fraction demonstrated that all the fractions prepared were still mixtures (Table 1). The fraction corresponding to PK4 was the purest, by FABMS, containing only tetrasaccharide(s). It is important to note that FABMS cannot be used as an unambiguous determination of purity or identity in the analysis of these fractions. The purity of PK4 is difficult to assess because the multiple ions might be associated with a mixture or arise from fragmentation of a single tetrasaccharide [15]. Since it is not possible to distinguish between isobaric phospho and sulfo groups, the identity of PK4 (and indeed all of the PK fractions) is difficult to establish by FABMS.

Ion chromatography was next used to assess whether each mole of PI-88 contained a single mole of phosphate ester as did its phosphomannan precursor (not shown). Ion chromatography demonstrated that the phosphomannan precursor contained 3.0–7.4% phosphate and PI-88 contained 4.4–5.2% ($n = 3$) phosphate, consistent with a single phosphate group in each phosphosulfomannan molecule. The microanalysis results also showed the presence of phosphorus (1.2%) as well as the presence of 2.9–3.1 sulfo groups per mannose. To further confirm the retention of the phosphate monoester after chemical sulfonation, the ^{31}P-NMR spectrum of PI-88 was compared to the ^{31}P-NMR spectrum of the

Table 1
Negative ion fast atom bombardment (FAB) spectra of PI-88 fractions

Sample	PK1	PK3	PK4	PK5
Hexa	2919.2 [M-16H+15Na]- 2853.5 [M-13H+12Na]- 2789.6 [M-21H+23Na-3NaSO$_3$]- 2642.9 [M-18H+21Na-4NaSO$_3$]-			
Penta	1998.9 [M-7H+10Na-4NaSO$_3$]- 1910.2 [M-3H+6Na-4NaSO$_3$]-	2335.8 [M-15H+16Na-2NaSO$_3$]- 2232.3 [M-14H+16Na-3NaSO$_3$]-		
Tetra		1969.4 [M-13H+13Na-NaSO$_3$]- 1866.7 [M-12H+13Na-2NaSO$_3$]-	2072.1[M-14H+13Na]- 1969.4[M-13H+13Na-NaSO$_3$]- 1867.3[M-12H+13Na-2NaSO$_3$]- 1765.3[M-11H+13Na-3NaSO$_3$]- 1663.3[M-10H+13Na-4NaSO$_3$]-	
Tri				1604.1 [M-11H+10Na]- 1501.1 [M-10H+10Na-NaSO$_3$]- 1399.6 [M-9H+10Na-2NaSO$_3$]- 1296.9 [M-8H+10Na-3NaSO$_3$]-
Di				1134.7 [M-8H+7Na]- 1032.8 [M-7H+7Na-NaSO$_3$]- 930.7 [M-6H+7Na-2NaSO$_3$]- 828.5 [M-5H+7Na-3NaSO$_3$]- 850.6 [M-6H+8Na-3NaSO$_3$]-

M represents the fully sulphated, protonated acid forms of fractions.

Table 2
Chemical shifts of oligosaccharides from PI-88

Mannose	Non-reducing end	Chemical shift (ppm)	
		Internal	Reducing end
H-1α	5.41–5.43	5.43–5.45	5.04–5.94 [a]
H-1β	–	–	5.10–5.76 [a]
H-2	n.d.	4.4	4.5
H-3	4.3–4.5	4.3–4.5	4.3–4.4
H-4	4.4–4.5	4.4–4.5	n.d.
H-5	4.20	4.23	4.11(α)
H-6 [b]	4.2–4.4	4.36	4.40

[a] *O*-sulfo.
[b] *O*-sulfo or *O*-phospho.

phosphomannan precursor (data not shown). Both spectra showed a peak at 3.9 ppm consistent with the presence of a phosphate monoester.

One-dimensional ^{1}H-NMR spectroscopy was performed on all of the PK fractions, the PI-88 mixture, and the phosphomannan precursor. The ^{1}H-COSY spectrum (not shown) was used to confirm the assignment of the chemical shifts of the mannose units in PI-88 (Table 2). The ^{1}H-NMR spectrum of the PI-88 mixture (not shown) and of PK4 (Fig. 4a) demonstrated that all the saccharide residues were α-linked. The mannose residue at the reducing-end, while appearing to be predominantly in the β-configuration, based on the signal at 5.76 ppm, also showed a minor signal at 5.93 ppm assigned to an α-anomeric proton. The signals at 5.76 and 5.93 ppm also indicate that the reducing-end C1 hydroxyl group had been sulfonated. The phosphate ester at the C6 of one of the mannose residues was clearly established by the presence of a signal at 4.38 ppm. The small multiple signals near 4.1 ppm corresponded to the presence of unsubstituted hydroxyl groups. The ^{13}C-NMR spectrum of PI-88 (not shown) and the HMQC spectrum of PK4 (Fig. 4b) suggest the presence of an *O*-sulfo group at the reducing end, based on the downfield shift of the C1 signal from ~87 to 99 ppm. The observation of an upfield shift in the C5 and C6 in the ^{13}C-NMR spectrum supports the presence of a phosphate group at C-6. PK4, which contained only tetrasaccharide as determined by FABMS analysis (Table 1), was subjected to ^{31}P-NMR analysis (Fig. 4c), which confirmed the presence of a single 6-phospho group in its structure. Based on these data a general structure can be proposed for the PK fractions comprising PI-88 (Fig. 5).

The anticoagulant activity of PI-88 and its fractions were next evaluated using clotting-based and amidolytic assays (Fig. 6). In human plasma, PI-88 [16,17] and its fractions produced concentration-dependent anticoagulant effects in the APTT and Heptest assays (Fig. 6b, c), although the effect on the prothrombin time was very weak (Fig. 6a). It is clear from the APTT responses that PI-88, PK3, and PK1 show a much greater activity than PK4 and PK5 (Fig. 6b). This result is consistent with the recent observation that the anticoagulant activity of sulphated oligosaccharides is dependent on chain length. Sulphated oligosaccharides smaller than pentasaccharide only show minimal APTT prolonging activity, while pentasaccharide and larger oligosaccharides display marked increases in activity [17]. The level of the effect on the APTT was comparable to that which has been observed for low molecular weight heparins (LMWHs), such as Enoxaparin [18]. The Heptest is a clot-based assay that measures both anti-Xa and anti-IIa activity of compounds. Historical data has shown that at low concentrations (< 2 μg mL^{-1}), this test reflects

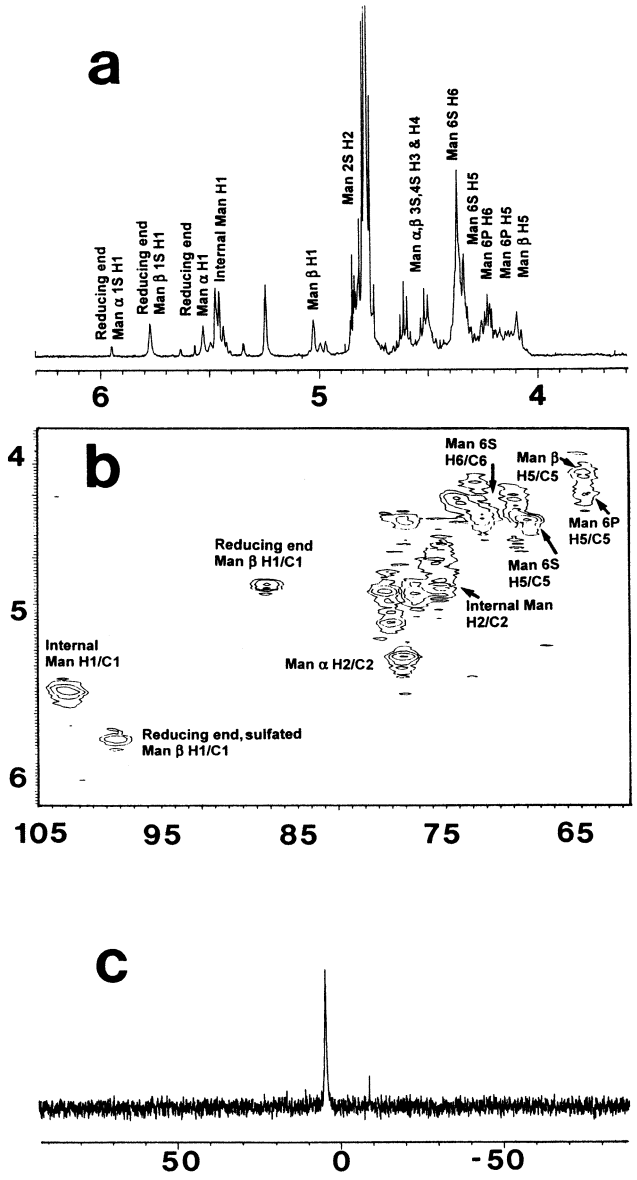

Fig. 4. NMR analysis of the PK4 fraction. Panel a shows the 1D 500 MHz ^{1}H-NMR spectrum. Panel b shows the partial 2D HMQC spectrum. Panel c shows the ^{31}P-NMR spectrum. All axes are labelled in ppm and important peaks are identified.

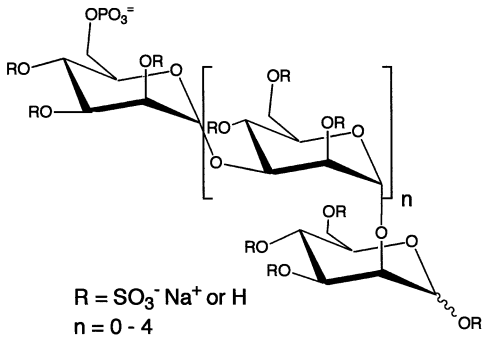

Fig. 5. Generalised structure of the oligophosphosulfomannan components of PI-88, where $n = 0$–4 and R is primarily $SO_3^- Na^+$ and occasionally H. The PK4 fraction is a tetrasaccharide(s), where $n = 2$.

predominantly the anti-Xa activity. PK1, PK4, and PK5 were shown to have a mild effect on Heptest®, which increased at the high concentrations in a dose-dependent manner (Fig. 6c). It is interesting to note that PK3 was more active than the parent compound PI-88.The Heptest results indicate that the parent and fractions exhibit anti-IIa activity. Therefore, each was assayed for anti-IIa amidolytic activity (Fig. 6d). In this case, PK3 and PK1 showed higher anti-IIa activity than PK4 and PK5, which is consistent with their higher activity as determined by the APTT and (for PK3) the Heptest® assays. Previous studies have indicated that PI-88 has no anti-Xa activity [16]. No fractions were found to have measurable anti-Xa activity (results not shown). It is surprising that the anti-IIa activity of the most active fractions are less than the parent compound.

3. Discussion

The phosphomannan mixture obtained from *Pichia holstii* could be chemically sulfonated to obtain a product PI-88 with a high level of *O*-sulfo group substitution. Complete chemical sulfonation of carbohydrates is often difficult to accomplish [19,20]. The PI-88 product retained a phosphate monoester at C6 of its non-reducing terminal mannose under these sulfonation conditions. Detailed analysis of PI-88 demonstrates that it is a mixture of phosphosulfomannans ranging in size from disaccharide to hexasaccharide. Size fractionation of PI-88 afforded four fractions having improved purity and each consisting of a major component having a different number of mannose residues. FABMS analysis of PK1 is consistent with a mixture comprised primarily of monophosphorylated hexasaccharide substituted with from 15 to 19 *O*-sulfo groups and containing a minor pentasaccharide component. PK3 was comprised of primarily a monophosphorylated pentasaccharide substituted with 14–16 *O*-sulfo groups and containing a minor tetrasaccharide component. PK4 was comprised only of monophosphorylated tetrasaccharide substituted with 9–13 *O*-sulfo groups. PK5 consisted of an equal mixture of monophosphorylated trisaccharide and disaccharides with a wide range of *O*-sulfo group substitution. The presence of a faint band running near the bottom of the gel in PAGE analysis (Fig. 2) also hints at the presence of a very minor amount of monosaccharide (possibly the tetra-*O*-sulfo-mannose-6-phosphate). One-dimensional ^{1}H-, ^{13}C- and ^{31}P-NMR spectroscopies were used to confirm the backbone structure of these phosphosulfomannans contained in

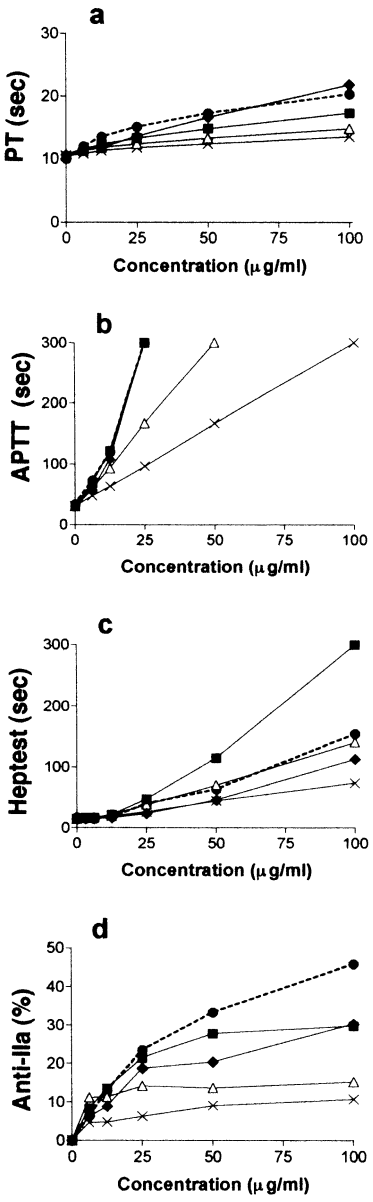

Fig. 6. Anticoagulant activity of the PK fractions of PI-88. Panels a, b, c and d show the respective responses of the PT, APTT, Heptest®, and anti-IIa assays to the PI-88 fractions. The symbols correspond to: PK1 (filled diamond); PK3 (filled square); PK4 (open triangle); and PK5 (X); PI-88 (filled circle–dashed line). The final data points shown for PI-88, PK1 and PK3 in the APTT assay (Fig. 6b) and for PK3 in the Heptest® (Fig. 6c) are off-scale as they are > 300 s.

PI-88. 2D-^{1}H-COSY and HMQC of PK fractions permitted the assignment of many of the signals and confirmed the generalised structure for PI-88 that is shown in Fig. 5.

The evaluation of the anticoagulant activity of PI-88 and its fractions afforded data suggesting that a precise oligosaccharide size is critical for high activity. PK3, consisting primarily of a pentasaccharide and corresponding to the major component in PI-88, afforded the highest anticoagulant activity of all the fractions in the APTT, Heptest® and anti-IIa amidolytic assays. The most surprising result in this study is the observation that the pentasaccharide component of PI-88, found principally in PK3, and not the larger hexasaccharide in PK1, demonstrates the greatest anticoagulant activity. These results suggest some degree of specificity for this activity, reminiscent of that observed for a specific pentasaccharide sized sequence found in heparin [21–23].

The anticoagulant activity of heparin has been principally ascribed to the presence of a structurally unique pentasaccharide sequence that interacts with antithrombin III (ATIII) [18]. The specificity of the pentasaccharide–ATIII interaction is well established [24] and was the driving force behind the successful introduction of LMWHs [18,25] and the recent clinical evaluation of a synthetic heparin pentasaccharide [26]. The binding of heparin pentasaccharide to ATIII causes a conformational change in this molecule, which enhances the serine protease inhibitor (serpin) activity of ATIII [25,27]. The serine protease Factor Xa can be inhibited by the ATIII–pentasaccharide complex, while the serine protease Factor IIa (thrombin) can only be inhibited by ATIII bound to this pentasaccharide sequence in the context of a larger (containing ≥ 18 saccharide units) oligosaccharide chain [25,27]. While heparin exhibits an anti-Xa/anti-IIa activity ratio of 1, the smaller LMWHs exhibit anti-Xa/anti-IIa ratios of >1. Some of the anti-IIa activity associated with heparin and LMWHs are due to a second serpin, heparin cofactor II (HCII), which does not bind heparin at its unique pentasaccharide, but rather interacts at highly sulphated domains common to the heparin polymer [28].

While unfractionated heparin has both anti-IIa and anti-Xa activity, LMWHs have predominantly anti-Xa activity [11,25]. In contrast to LMWHs, PI-88 and its PK fractions do not show any anti-Xa activity, which suggests that ATIII-mediated anticoagulant activity is not associated with these compounds. However, PI-88 and its PK fractions produced a concentration-dependent anti-IIa activity which has been attributed to HCII-mediated inhibition of thrombin [16,17]. This observation is consistent with the highly charged nature of PI-88.

The response to PK3 in the Heptest is greater than the parent compound, PI-88. Since approximately 59% of the PI-88 mixture consists of PK3, the greater response could be attributed to the fact that about twice the concentration of material produced the response in the pure compound compared to parent. The response to the parent compound is greater than any of the fractions in the anti-IIa assay. This observation suggests that there might be a synergistic interaction between the components to yield the higher activity seen with the parent compound.

It is important to emphasise that PI-88 is a multi-component mixture that is a promising inhibitor of tumour growth and metastasis and is currently undergoing Phase II clinical trials for this indication. This paper has investigated the anticoagulant activity of PI-88 and several of its subfractions. While most therapeutic agents are homogenous species, anticoagulant agents, such as heparin and PI-88, are important exceptions to this rule, as they are prepared from complex mixtures of carbohydrates. Efforts to prepare homogenous agents for detailed pharmacological investigation are required. Future work will also be directed at determining the precise mechanism of anticoagulant action of PI-88. Additional work will also be required to assess the in vivo efficacy of PI-88 as an anticoagulant/antithrombotic agent.

4. Experimental protocols

4.1. Materials

PI-88 was obtained from Progen Industries Limited, Brisbane, Australia. Bio-Gel P-6 (45–90 μm) and Bio-Gel P-2 (45–90 μm) were purchased from BioRad (Hercules, CA). Acrylamide (ultrapure) and Tris (ultrapure) were purchased from Life Technologies Inc. (MD, USA). Alcian blue dye, bromophenol blue dye and ammonium persulfate were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Glycine, disodium ethylene diaminetetraacetic acid (EDTA), boric acid, sucrose, *N*,*N*′-methylene bisacrylamide and *N*,*N*,*N*′*N*′-tetramethylenediamine (TEMED) were purchased from Fisher Chemical Company (Fair Lawn, NJ). Reagents used in the preparation and CE analysis of PI-88 were analytical grade. Diafiltration of PI-88 was performed with a Millipore (Bedford, MA) Pellicon ultrafiltration unit employing seven Pellicon 1K (0.46 m^2, NMWCO 1,000) Cassettes. Operating parameters were: inlet pressure 200 kPa, outlet pressure 150 kPa, retentate cross flow rate 27.6 L min^{-1}, permeate flux rate 10–11 L h^{-1} m^{-2} (18–30 °C). Reverse Osmosis was performed on a Millipore Proscale system equipped with two Helicon RO4 cartridges (0.37 m^2, Nanomax 50 membrane). Operating parameters were: temperature

15–22 °C, feed pressure 1200 kPa, inlet flow rate 8 L min^{-1}.

4.2. Synthesis and chemical characterisation of phosphosulfomannans

4.2.1. Chemical sulfonation of phosphomannan mixture

The oligosaccharide phosphate fraction from *P. holstii* NRRL Y-2448 phosphomannan [7] (478 g, 0.50 mol) was suspended in DMF (10 L). Sulphur trioxide pyridine complex (3.78 kg, 23.7 mol) was then added and the mixture stirred at 25 °C for 3 days, by which time the product had separated out as a thick oil. The DMF was siphoned off and the residue was washed with ethanol (3 × 1 L) and then dissolved in water (~3 L). The solution was then adjusted to pH 9.5 by addition of 1 M NaOH solution (~5 L). The liberated pyridine was then removed by extraction with dichloromethane (3 × 3 L) and the aqueous phase was diluted with water and decolourised by passage through a charcoal filter (Cuno R53S). The solution was diluted to 20 L with water and diafiltered firstly against eight volumes of 1 M NaCl solution, and then against purified water until the conductivity of the permeate was <0.2 mS cm^{-1}. The solution was then concentrated to 6 L by reverse osmosis, filtered (0.2 μm) and lyophilised to give PI-88 as a white, hygroscopic, amorphous solid (760 g); $[\alpha]_D$ +32 (*c* 1.0, H_2O).

4.2.2. Capillary electrophoresis (CE) of PI-88

Capillary electrophoresis was performed in reverse polarity mode on a Beckman P/ACE 5000 System equipped with a P/ACE UV absorbance detector. Beckman eCAP™ fused silica capillaries (50 μm i.d., 375 μm o.d., 57 cm long, 50 cm to detector) were used in these analyses. Indirect detection was used relying on 10 mM 5-sulfosalicylic acid, pH 3 (adjusted with NaOH) as the background electrolyte. The capillary temperature was 20 °C and the operating voltage was −20 kV. Samples were injected by pressure injection for 10 s and detected at 214 nm.

4.2.3. Low pressure liquid chromatography–gel permeation chromatography (LPLC–GPC)

The PI-88 mixture (200 mg mL^{-1}, in 0.2 M sodium chloride, pH 7.0) was loaded onto a Bio-Gel P-6 column (i.d. 2.6 × 96 cm), eluted with 200 mM sodium chloride, and fractions of 3 mL were collected (LBK 2211 SuperRac, Bromma, Sweden) at a flow rate of 16 mL h^{-1}. Four fractions corresponding to each prominent peak were pooled based on total carbohydrate analysis and the volume was reduced to about 2 mL using a rotary evaporator (Büchi Rotavapor R-114, Westbury, NY).

4.2.4. Total carbohydrate analysis

Total carbohydrate was determined by the phenol–sulfuric acid method. A 10 mL glass test tube containing 100 μL sample solution (either PI-88 or PK fractions, 1–10 μg) and 100 μL phenol solution (5%, w/v) were mixed for 5 s and 500 μL sulphuric acid (98%) was added and mixed for an additional 5 s. After remaining at room temperature for 30 min the absorbance at 490 nm was determined (Shimadzu UV-160 Spectrophotometer, Kyoto, Japan).

4.2.5. Desalting

Desalting was performed on a Bio-Gel P-2 column (i.d. 1.5 × 36 cm) and eluted with water at a flow rate of 1 mL min^{-1}. Fractions were collected and assayed for total carbohydrate. Fractions containing oligosaccharides were pooled and freeze-dried (Virtis lyophiliser, Gardiner, NY).

4.2.6. Polyacrylamide gel electrophoresis (PAGE) analysis

Buffer solutions were prepared as follows: Resolving gel buffer and lower chamber buffer contain 0.1 M boric acid, 0.1 M Tris, 0.01 M disodium EDTA, pH 8.3. Stacking gel buffer, 0.1 M boric acid, 0.01 M disodium EDTA, 0.1 M Tris–HCl, pH 6.3. Upper chamber buffer, 1.24 M glycine, 0.2 M Tris. Acrylamide solution (22%), 20.02% (w/v) acrylamide containing 2% (w/v) *N*,*N*′-methylenebisacrylamide and 15% (w/v) sucrose in resolving gel buffer. Stacking gel solution (5%), 4.75% (w/v) acrylamide and 0.25% (w/v) *N*,*N*′-methylenebisacrylamide in stacking gel buffer. Alcian blue (0.5%) in 2% acetic acid was used to stain the gel.

Oligosaccharide standards from bovine lung heparin, including a disaccharide, tetrasaccharide and hexasaccharide were prepared as previously described [29]. PAGE analysis was performed using a BioRad (Richmond, CA) Mini-Protean II slab cell and a BioRad Power Pac 1000 (Richmond, CA) power source. Analytical gels (7 × 8 cm, separated by 1 mm spacers) containing 22% total acrylamide were prepared. Electrophoresis was performed at 180 V for 100 min, and the bands visualised by Alcian blue staining [29].

4.2.7. Ion-chromatography

PI-88 and its phosphomannan precursor were each hydrolysed with trifluoroacetic acid (2.5 M at 100 °C, 6 h under nitrogen). After centrifugation at 1800 × g for 10 min, the sample was dried at 50 °C under a stream of nitrogen gas, dissolved in water and inorganic phosphate and sulphate anions were analysed by ion-chromatography using a TSK gel IC-anion-PW (4.6 mm i.d. × 50 mm) separation column and a Dowex 50W-X (H^+) (5.0 mm i.d. × 200 mm) suppressor column. The temperature of the separation was controlled at 30 °C and the mobile phase was 1.42 mM

sodium bicarbonate and 1.5 mM sodium carbonate (pH 7.8) at a flow rate of 1.0 mL min^{-1}. Detection was by suppressed conductivity.

4.2.8. Spectral characterisation

Optical rotation was measured using a Polax polarimeter (Atago, Tokyo). Negative-ion FABMS was performed on a VG analytical 7070E double focusing sector field mass spectrometer at the University of Minnesota, MS Service Laboratory by methods previously described in our laboratory [15]. Samples for NMR analysis were dissolved in D_2O (99.96% of atom) filtered through a 0.45 μm syringe filter and freeze-dried to remove exchangeable protons. After exchanging the sample three times, the sample was dissolved in D_2O (99.96% of atom). One-dimensional (1D) ^{1}H-NMR and two-dimensional (2D) COSY, HMQC and HMBC experiments were performed on a Varian VXR-500 spectrometer equipped with 5 mm triple resonance tunable probe with standard Varian software at 298 K. ^{31}P-NMR experiments were performed at 298 K on a Bruker Avance DRX-400 with a 5 mm BBO "X" nucleus tunable probe using 85% H_3PO_4 as external standard (0.00 ppm).

4.2.9. High performance liquid chromatography–gel permeation chromatography (HPLC–GPC)

PI-88 and its fractions were analysed on TSK G3000SW and TSK G2000SW columns (Tosoh Corp, Japan) run in series on a Waters Chromatography System (Lexington, MA) with detection by refractive index. Samples (20 μL at 10 mg mL^{-1}) were injected and separated in 0.3 M sodium sulphate at pH 5 at a flow rate of 0.5 mL min^{-1}.

4.3. Pharmacological evaluation of phosphosulfomannans

4.3.1. Anticoagulant activity in normal pooled human plasma

To determine the in vitro anticoagulant profile of PI-88 and its fractions, samples were supplemented to pooled normal human plasma in a 1:10 dilution and then serially diluted with plasma from 100 μg mL^{-1} and assayed immediately for all of the following tests. Prothrombin time (PT; Dade Thromboplastin C-Plus), activated partial thromboplastin time (APTT; Platelin L; Organon Teknika Corporation, NC), and Heptest® (Haemachem Inc., St. Louis) were used. PT assay [30] was performed using a fibrometer. Citrated plasma (100 μL) was incubated at 37 °C for 3 min after which pre-warmed (at 37 °C) Thromboplastin-C-Plus® (200 μL) was added and the time required for clot formation was measured. APTT assay was performed using a fibrometer. Citrated plasma (100 μL) was mixed with automated APTT reagent® (100 μL) and incubated at 37 °C for 5 min. Pre-warmed (at 37 °C) calcium chloride (100 μL of 0.025 M) was then added and the time to clot formation was measured. Heptest® [31] was performed using a fibrometer according to the manufacturer's instructions. Undiluted plasma (100 μL) was incubated with bovine Factor Xa (100 μL) for exactly 120 s at which time Recalmix® (reagent containing a bovine plasma fraction, brain cephalin and calcium) was added to the mixture and the time to clot formation was determined.

4.3.2. Anti-protease activity assays

Anti-Factor IIa (anti-IIa) activities [32] were determined by amidolytic assay (ACL 300 plus, Instrumentation Laboratory). Purified human thrombin (Ortho Diagnostic Systems) and Spectrozyme® Thrombin (American Diagnostica Inc.) as chromogenic substrate were used in the anti-IIa activity assay system. In a cuvette, antithrombotic agent supplemented platelet poor citrated plasma (25 μL) was pre-incubated with buffer (400 μL) at 37 °C for 2 min. Human Factor IIa (25 μL at 10.0 NIH units mL^{-1}) was added to the mixture held at 37 °C. After exactly 1 min, synthetic substrate (1.0 mM) specific for Factor IIa was added. The change in absorbance at 405 nm was monitored for 1 min (A_{405}/min) at 37 °C. Activity levels were calculated as a percentage inhibition of anti-IIa activity (%I). Anti-Factor Xa assays were performed in an identical manner using Spectrozyme® FXa (American Diagnostica Inc.), a specific chromogenic substrate for FXa, and bovine FXa (Enzyme Research Lab., South Bend, IN).

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