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Analysis of the neutral polysaccharide fraction of MCP and its inhibitory activity on galectin-3

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Abstract The pH-modified citrus pectin (MCP) has been demonstrated to inhibit galectin-3 in cancer progression. The components and structures of MCP related to this inhibition remained unknown. In this paper, we fractionated MCP on DEAE-cellulose column into a homogenous neutral fraction MCP-N (about 20 kDa) and a pectin mixture fraction MCP-A (wide molecular distribution on Sepharose CL-6B chromatography). Both MCP-N and MCP-A inhibited hemagglutination mediated by galectin-3 with minimum inhibition concentration (MIC) 625 and 0.5 μg/ml, respectively. MCP-N was identified to be a type I arabinogalactan (AG-I) with a main chain of β-1→4-galactan. MCP-N was digested by α-L-arabinofuranosidase to give its main chain structure fraction (M-galactan, around 18 kDa), which was more active than the original molecule, MIC 50 μg/ml. The acidic degradation of M-galactan increased the inhibitory activity, MIC about 5 times lower than M-galactan. These results above showed that the functional motif of the β-1→4-galactan fragment might lie in the terminal residues rather than in the internal region of the chain. Therefore, MCP-N and its degraded products might be developed to new potential galectin-3 inhibitors. This is the first report concerning the fractionation of MCP and its components on galectin-3 inhibition. The information provided in this paper is valuable for screening more active galectin-3 inhibitors from natural polysaccharides.

Keywords Citrus pectin · Galectin-3 inhibitor · Arabinogalactan · Hemagglutination assay

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Introduction

Galectin-3 is a member of the galectin family, which has a conserved carbohydrate-recognition domain and specifically recognizes β-galactoside moieties [1]. It is highly expressed in some metastatic cancer cells and overexpression of galectin-3 has been shown to protect cancer cells from apoptosis [2]. Galectin-3 interacts with its glycol-ligands on cell surface or in the extracellular matrix and regulates cell-cell and cell-matrix recognition and adhesion processes, which play an important role in cancer growth and progression [3]. Galectin-3 has been shown to promote the adhesion of tumor cells to endothelium through the interaction with tumor-associated Thomsen-Friedenreich glycoantigen, being expressed highly in tumor cells, and the embolization between homogenic tumor cells in circulation [4–7], which are the two important steps during early stages of cancer metastasis. As galectin-3 is a key player in cancer metastasis, it has become a target protein in anticancer drug research. Many research works have focused on producing specific galectin-3 inhibitors to inhibit or block the endogenous bioactivities of galectin-3 in cancer [8–10]. Several pectic polysaccharides from edible plants have been shown to possess anti-galectin-3 activities. As a type of natural products, pectin has rich sources and low toxicity. Therefore, among the galectin-3 inhibitors, pectin is of special value and attracted special attention [11–15].

Citrus pectin (CP) is an acidic polysaccharide extracted from citrus fruits. It consists predominantly of homogalacturonan, less amount of rhamnogalacturonan I and minor rhamnogalacturonan II structural domains [16]. CP has been modified by adjusting pH, degrading main chain *via* β-elimination at high pH followed by partial degradation of the neutral carbohydrates at low pH, to produce a mixture of low molecular weight polysaccharides, namely modified citrus pectin (MCP) [11, 17]. MCP has better anticancer activity. The experimental results demonstrated that MCP, *via* inhibiting galectin-3, effects on multiple steps of tumor metastasis, such as cancer cell arrest in target organs, extravasation invasion, clonogenic survival and growth, angiogenesis, and cancer cell resistance to chemotherapy [11, 17–21].

MCP is the degradation product of CP. It contains all of the structural elements present in the CP, including the homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II domains and the side chains. To date, there is no report concerning the fractionation of MCP. The active components of MCP remain unclear. Recently, Gunning *et al.* reported that β-1→4-galactan from potato could bind to galectin-3 and inhibit its function [22]. Thus, it has been considered that the β-1→4-linked galactan originating from the side chains of rhamnogalacturonan I structural domain in CP might be the active components of MCP [16], but it has never been isolated from MCP. In past 2 years, we started a research program to fractionate MCP, aiming to evaluate the activity of the components of MCP and the interaction among those components. In this paper, we report the initial results about the analysis of an arabinogalactan from MCP and its related polysaccharides as well as their inhibitory activities against galectin-3.

Materials and methods

Materials

Citrus pectin was purchased from Sigma (P9135). Potato galactan (referred to as P-galactan in this paper) and the enzymes endo-β-1→4-Polygalacturonase (EC 3.2.1.15), α-L-Arabinofuranosidase (E.C.3.2.1.55) and endo-β-1→4-D-Galactanase (E.C. 3.2.1.89) were from Megazyme (Bray, Ireland). Human recombinant galectin-3 (Q6IBA7) was purchased from R&D Systems, Inc. Monoclonal antibody against galactin-3 (A3A12) was from Santa Cruz Biotechnology, Inc. The chromatographic supports DEAE-cellulose and Sepharose CL-6B were from Sigma. Asialofetuin (ASF) was prepared by mild acid hydrolysis of fetuin (Sigma) in 0.05 M H_2SO_4 at 80 °C for 1 h. Other reagents and chemicals were of analytical grade.

General methods

Sugar contents were determined by phenol-sulphuric acid method according to the published methods [23]. Gel-permeation and ion-exchange chromatographies were monitored by assaying the total sugar contents. High performance liquid chromatography (HPLC) was carried out on a Shimadzu 10Avp HPLC system equipped with 10Avp HPLC Pump, SPD-10Avp UV–VIS Detector, and RID-10A Refractive Index Detector.

Monosaccharide composition analysis was performed by HPLC according to the methods described previously [23, 24]. Briefly, the sample (2.0 mg) was hydrolyzed using anhydrous methanol (1.0 ml) containing 2.0 M HCl at 80 °C for 16 h, then the products were hydrolyzed with 2 M TFA (0.5 ml) at 120 °C for 1 h. The released monosaccharides were derived by 1-phenyl-3-methyl-5-pyrazolone (PMP) and subsequently analyzed by HPLC.

Gel permeation chromatography was performed on Sepharose CL-6B column (1.5×90 cm), eluting with 0.15 M NaCl at a flow rate of 0.15 ml/min. The eluate was collected at 3 ml per tube and assayed for distribution of total sugar.

The homogeneity and molecular weight of polysaccharides were estimated by high performance gel-permeation chromatography (HPGPC) using a TSK-G3000 PW_{XL} column (7.8× 300 mm) as described previously [24]. The column was pre-calibrated with standard dextrans (50 KDa, 25 KDa, 12 KDa, 5 KDa and 1 KDa) using linear regression.

Preparation and fractionation of MCP

MCP was prepared from CP by pH modification according to the published methods [3, 17]. Briefly, CP was solubilized as a 1.5 % solution in distilled water, and its pH was adjusted and maintained at 10.0 with 3 M NaOH for 1 h at 55 °C. The solution was then cooled to room temperature and its pH was adjusted to 3.0 with 3 M HCl. After overnight incubation at room temperature, the pH of the solution was adjusted to 6.8 with 3 M NaOH. MCP was precipitated by adding 95 % ethanol to the solution up to 70 % and recovered by centrifugation, acetone washing and air-drying.

MCP (10.0 g) was dissolved in distilled water (1.0 l), applied to a DEAE–cellulose column (10×20 cm) and eluted first with distilled water and then with 0.5 M NaCl to give a neutral polysaccharide fraction (MCP-N) and an acidic polysaccharide fraction (MCP-A), respectively.

Degradation of MCP-N by enzymatic or acidic hydrolysis

MCP-N (100.0 mg), dissolved in 50 mM sodium acetate buffer (20.0 ml, pH 4.5), was treated with α-L-Arabinofuranosidase (0.5 U/ml) at 35 °C for 30 h. Then

the solution was boiled at 100 °C for 5 min to deactivate the enzyme, centrifuged, dialyzed (molecular weight cut off 1,000 Da) against distilled water and finally lyophilized to obtain the fraction M-galactan.

M-galactan (30 mg) was treated with 0.2 M trifluoroacetic acid (6.0 ml) at 80 °C for 4 h and then neutralized with 10 % aqueous ammonia to pH 6–7. The solution was desalted on Sephadex G-10 (3×20 cm) column and freeze-dried to give the fraction M-galactan-oligo.

Enzymatic hydrolysis of M-galactan and P-galactan

M-galactan or P-galactan (20.0 mg) was dissolved in 0.2 M sodium borate buffer (4.0 ml, pH 7.3), and incubated with endo-β-1→4-Galactanase (0.25 U/ml) at 30 °C for 24 h. The enzyme was deactivated by heating the solution at 100 °C for 5 min. The solution was centrifuged, and the hydrolyzed products were analyzed by HPGPC.

NMR spectroscopic analysis

The ^{13}C NMR spectrum was recorded using a Bruker 5 mm broadband observe probe at 20 °C with a Bruker Avance 600 MHz spectrometer (Germany), operating at 150 MHz [23]. The sample (20.0 mg) was dissolved in D_2O (99.8 %, 0.5 ml) with overnight stirring at room temperature. All the experimental data were recorded using standard Bruker software.

Hemagglutination assay

According to the method published previously [12], chicken erythrocytes prepared from fresh blood collected in Alsever's medium (2.05 % glucose, 0.8 % sodium citrate, 0.42 % sodium chloride, and 0.055 % citric acid) were washed four times with 0.15 M NaCl. The cells were suspended at 4 % (V/V) in 0.02 M PBS (pH 7.4) containing 1 mg/ml trypsin and incubated for 1 h at 37 °C. After washing with 0.15 M NaCl, the cells were fixed in 0.02 M PBS (pH 7.4) containing 1 % glutaraldehyde for 1 h at room temperature followed by termination with five volumes of 0.1 M glycine in PBS (pH 7.4). The fixed cells were washed and adjusted to 10 % (V/V) with PBS (pH 7.4). They were maintained at 4 °C until use. Hemagglutination assays were performed in microtiter V plates. Each well contained 1 % bovine serum albumin in 0.15 M NaCl (25.0 μl), 0.15 M NaCl (control, 25.0 μl) or the test samples in this solution (25.0 μl), 12.5 μg/ml galectin-3 (25.0 μl), and 4 % (V/V) chicken erythrocyte suspension (25.0 μl). The cells were added last, followed by vigorous shaking. Agglutination was allowed to proceed for 90 min at room temperature. Minimum Inhibitory Concentration (MIC) of the test samples was determined.

ASF-induced homotypic cell aggregation assay

The assay was performed according to the published method [10]. Briefly, MDA-MB-231 cells were harvested using 1 mM EDTA and re-suspended in PBS at 0.6×10^6 cells/ml. Single-cell suspensions (0.5 ml) were placed in 2 ml Eppendorf tubes, mixed with either buffer (control) or 15 μg/ml asialofetuin (ASF) in the presence or absence of test samples, and agitated at 100 rpm for 1 h at 37 °C. The aggregation was terminated by the addition of 8 % paraformaldehyde (170.0 μl) and examined under a microscope for the number of single cells. ASF-induced aggregation under each condition was calculated according to the following equation: $A = 1 - (N/N_c) \times 100$, where N and N_c represent the number of single cells in the presence of ASF and that in the control buffer, respectively. The inhibition of cell aggregation by test samples was calculated using the following equation: $1 - (A_t/A_0) \times 100$, where A_t and A_0 represent ASF-induced aggregation in the presence and absence of test samples, respectively.

Results

Fractionation of MCP

CP was modified to MCP by adjusting pH. Sugar composition analysis indicated that MCP was composed of 85 % galacturonic acid (GalA), 1.6 % rhamnose (Rha), 9.3 % galactose (Gal) and 4.0 % arabinose (Ara), which is similar to that of CP in literature (Table 1) [16]. MCP showed a broad molecular weight distribution on Sepharose CL-6B column, with an average molecular weight of 30 KDa (Fig. 1). MCP was separated on DEAE-Cellulose, eluted first with water to give an unbound fraction MCP-N (9.2 %, w/w) and then with 0.5 M NaCl to give a bound fraction MCP-A (62.4 %, w/w). As expected, MCP-A was a pectin mixture containing 89.3 % of GalA and showed a wide molecular distribution on Sepharose CL-6B (Fig. 1). MCP-N mainly consisted of 78.6 % Gal and 21.4 % Ara showed a narrow and symmetrical peak on Sepharose CL-6B column (Fig. 1), which indicated that MCP-N was a homogenous arabinogalactan. MCP-A will be studied further. This paper concerned MCP-N.

Structural analysis of MCP-N

MCP-N was an arabinogalactan. It showed a single, relatively narrow and symmetrical peak on HPGPC, indicating a relatively homogenous fraction. Its molecular weight was around 20 KDa based on the standard curve (Fig. 2a). In order to investigate the structure features of MCP-N, it was treated with α-L-arabinofuranosidase. Molecular weight

Table 1 The monosaccharide compositions (MCS), molecular weight (Mw) and minimum inhibitory concertration (MIC) of MCP and related polysaccharides. MCS and Mw were determined as described in materials and methods. MIC on galectin-3 was measured using the hemagglutination assay

Sample	MCS (mol %)					Mw (kD)	MIC (μg/ml)
	Rha	GalA	Gal	Ara	Glc		
CP	1.0	83.9	5.3	2.4	–	[a]nd	[a]nd
MCP	1.6	85	9.3	4.0	–	30	0.6
MCP-N	–	–	78.6	21.4	–	20	625.0
MCP-A	2.7	89.3	6.2	1.8	–	19–1000	0.5
M-galactan	–	–	96	4.0	–	18	50.0
M-galactan-oligo	–	–	97.3	2.7	–	1.8	12.5
P-galactan	6.1	11.3	70.0	10.0	3.6	431	10.5

[a]*nd* not determined

distribution analysis indicated that the major part of the molecules has not been digested. The remaining polysaccharide was a MCP deriving galactan (M-galactan), containing 96 % Gal and 4 % Ara (Table 1). M-galactan gave, on TSK-G3000 PW_{XL} column, a single, narrow and symmetrical peak. Its molecular weight was estimated to be 18 KDa, slightly lower than MCP-N (Fig. 2b). These data suggested that M-galactan constituted the core structure of MCP-N, while Ara residues located at the periphery. ^{13}C NMR spectroscopy of M-galactan (Fig. 3) displayed six signals assigned to C-1 (103.32 ppm), C-2 (70.78 ppm), C-3 (72.37 ppm), C-4 (76.62 ppm), C-5 (73.47 ppm) and C-6 (59.70 ppm) of β-1→4-linked galactose residues [25]. Therefore, M-galactan was a linear β-1→4-galactan. Several very weak signals in the NMR spectrum might be derived from terminal Gal and Ara residues, indicating also that MCP-N was a type I arabinogalactan [26].

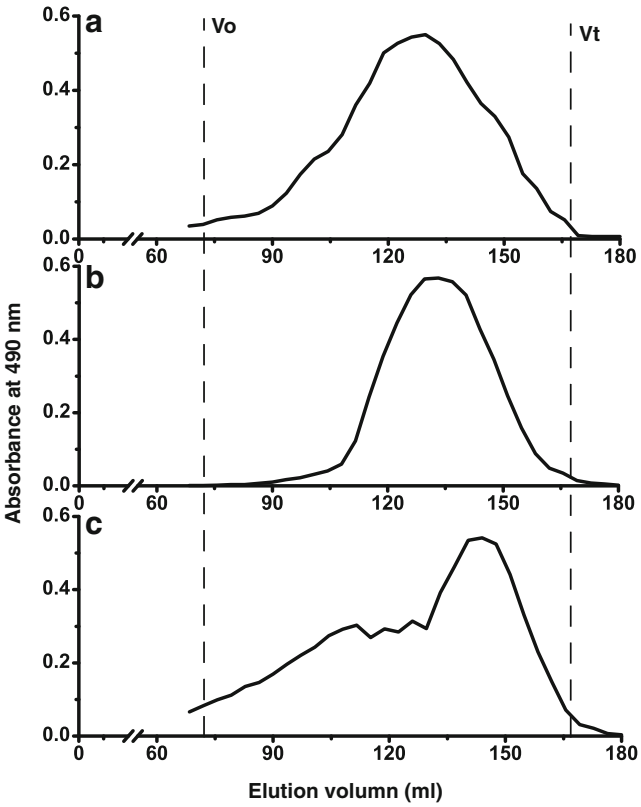


Fig. 1 The elution profiles of MCP (**a**), MCP-N (**b**) and MCP-A (**c**) on Sepharose CL-6B column. One milliliter sample at 5.0 mg/ml was applied onto a Sepharose CL-6B column (1.5×90 cm) and eluted with 0.15 M NaCl at a flow rate of 0.15 ml/min. The eluate was collected at 3 ml per tube and assayed for the distribution of total sugar by phenol–H_2SO_4 assay. Vo, void volume, Vt, total volume

To confirm the structure of M-galactan, it was subjected to treatment with the enzyme endo-β-1→4-D-galactanase, which hydrolyzes β-1→4-galactan [16, 27]. Under the conditions for complete digestion, nearly all M-galactan molecules were hydrolyzed into small oligosaccharides, which appeared near the total volume on TSK-G3000 PW_{XL} column (Fig. 2c). The sensitivity of M-galactan to this enzyme confirmed the conclusion drawn from the ^{13}C NMR data that M-galactan was β-1→4-galactan. Taken together, MCP-N was an arabinogalactan with molecular weight around 20 KDa, β-1→4-galactan composed its core structure and Ara residues are located at the periphery of the molecules.

The inhibitory activities of MCP-N and its related polysaccharide fractions on galectin-3

Galectin-3 mediated hemagglutination assay has been widely used for the evaluation of potential galectin-3 inhibitors [12]. The MIC required for completely inhibiting the agglutination was used to quantify the activity of each polysaccharide fraction. The assay was set up according to the literature and verified by the positive and negative controls as follows: lactose, a well known galectin-3 inhibitor, exhibited inhibition at 10.0 μg/ml, while sucrose, a non-competitive disaccharide, did not show inhibition up to the concentration of 8000.0 μg/ml. The inhibitory activities of MCP and its sub-fractions are listed in Table 1. As seen, the MIC of MCP was 0.6 μg/ml, being 17-fold lower than that of lactose, which was consistent with the results in literatures [11, 17]. For the components of MCP, the neutral fraction MCP-N, a type-I arabinogalactan, showed MIC

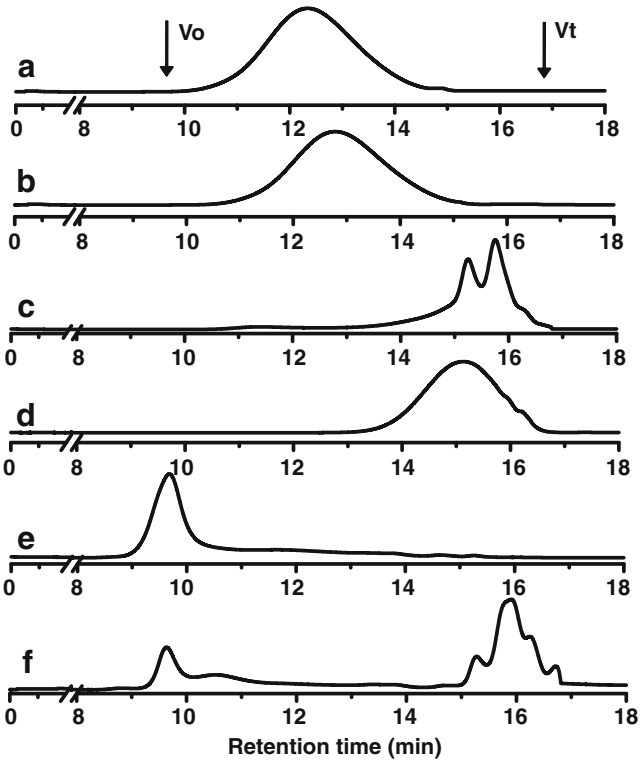


Fig. 2 HPGPC of MCP-N (**a**), P-galactan (**e**) and their derivatives (**b**, **c**, **d**, **f**) on TSK-G3000 PW_{XL} column. The samples (20.0 μl, 5.0 mg/ml) were injected into the column, eluted with 0.2 M NaCl at a flow rate of 0.5 ml/min and monitored using a refractive index detector. M-galactan-g (**c**) and P-galactan-g (**f**) represented the digests of M-galactan (**b**) and P-galactan (**e**), respectively, by endo-β1, 4-galactanase. (**c**) M-galactan-oligo; Vo, void volume; Vt, total volume

625.0 μg/ml; the acidic fraction MCP-A, a mixture of pectins, showed MIC 0.5 μg/ml, much lower than that of MCP-N, which suggested that the activity of MCP-A was better than that of MCP-N. After removing Ara residues of MCP-N by α-L-arabinofuranosidase, the remaining core region M-galactan showed MIC 50.0 μg/ml, 13-fold lower than MCP-N.

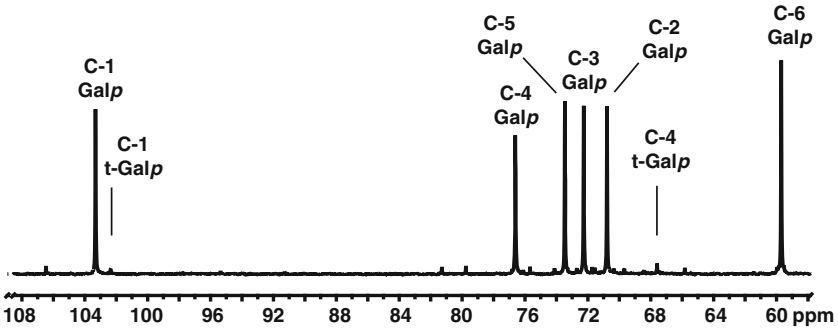
The inhibition of galectin-3 could be demonstrated in ASF-induced cancer cell aggregation assay, which involved the interaction between the sugar epitopes on ASF and the galectin-3 on cancer cells [28]. In literature report, MCP has been shown to inhibit the aggregation [17]. In this study, the assay was verified using a monoclonal antibody against galectin-3, which nearly completely inhibited the aggregation at 0.8 μg/ml (Fig. 4c). MCP exerted 51 % inhibition at 1.0 μg/ml (Fig. 4d), consistent with the observation in literature [17]. MCP-N and M-galactan did not show obvious inhibition at 1.0 μg/ml. They showed inhibition at higher concentrations. MCP-N exerted 15 % and M-galactan 32 % inhibition at 100.0 μg/ml (Fig. 4e, f). These results were similar to the observation in hemagglutination assay. Both hemagglutination and aggregation assay showed that MCP-N was a galectin-3 inhibitor. But its inhibitory activity was weaker than that of its mother sample MCP and its enzymatically hydrolyzed product M-galactan.

M-galactan, the core structure of MCP-N, possesses stronger inhibition on galectin-3 than the whole molecule. To investigate if the entire molecule was required for the activity and/or where the functional motif located within the molecule, we degraded M-galactan by partial acid hydrolysis yielding a series of oligosaccharides, referred to as M-galactan-oligo. M-galactan-oligo had an average molecular weight of 1.8 KDa (Fig. 2d). Its sugar composition was similar to that of its parental material M-galactan (Table 1). Hemagglutination assay showed that its MIC was 12.5 μg/ml, 4-fold lower than M-galactan. Thus, the degradation resulted in 4-fold increase of the activity. This result indicated that the inhibitory activity on galectin-3 did not require the participation of the whole molecule. The proportion of the terminal residues increased after degradation and internal residues decreased accordingly. Therefore, we speculate that the functional motif of M-galactan might lie in the terminal rather than the internal region of the molecule.

Comparison of the activities of M-galactan and P-galactan

P-galactan is a component of potato pectin, it is the only structurally characterized pectin component identified so far to interact with galectin-3 [22]. Here, we compared the activities of M-galactan with P-galactan using the hemagglutination assay. The MICs were 10.5 μg/ml for P-galactan and 50.0 μg/ml for M-galactan. Clearly, both galactans were effective at 10 μg/ml range. But P-galactan had slightly

Fig. 3 ^{13}C NMR spectrum of M-galactan



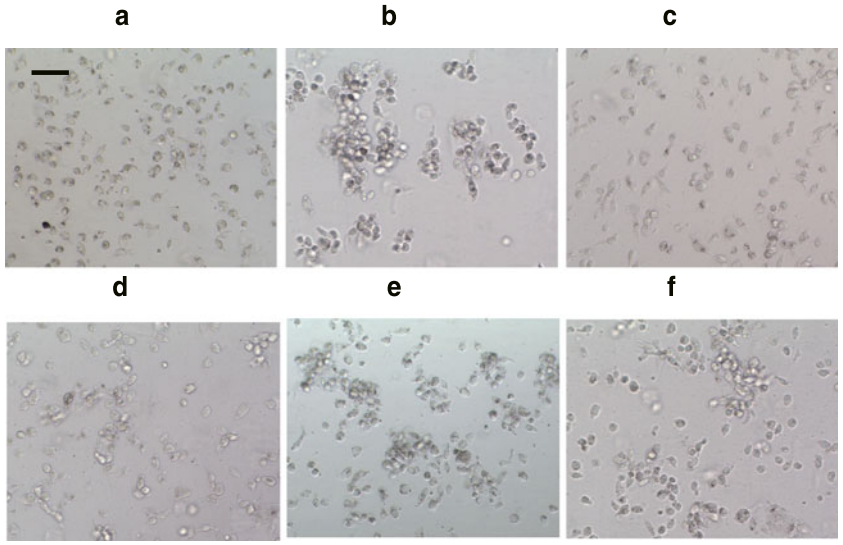


Fig. 4 The inhibition of ASF-induced cancer cell aggregation. MDA-MB-231 cells were agitated in the absence (**a**) or presence (**b**, **c**, **d**, **e**, **f**) of ASF supplemented with or without test samples. The cells under each condition were photographed using a phase-contrast microscope. The inhibition of the aggregation by the test samples (0.8 μg/ml galectin-3 antibody (**c**), 1.0 μg/ml MCP (**d**), 100.0 μg/ml MCP-N (**e**) and 100.0 μg/ml M-galactan (**f**)) was determined as described in material and methods. Scale bar was 10 μm

stronger activity than M-galactan. The reason for this difference is surely due to their structural differences. P-galactan had very high molecular weight. It was eluted at the void volume on the TSK-G3000 PW_{XL} column (Fig. 2e). According to literature, its molecular weight is around 272 kD [22], 15 times larger than that of M-galactan. M-galactan can completely be digested by endo-β-1→4-galactanase into small oligosaccharides, while under same conditions, two third of P-galactan was digested into small oligosaccharides and one third remained as large fragments (Fig. 2f). This indicated that P-galactan was not composed solely of galactan, which can be digested by endo-β-1→4-galactanase. Indeed, the sugar composition analysis showed P-galactan, besides 70 % Gal and 10 % Ara, contains 11.3 % GalA, 6.1 % Rha, and 3.6 % Glc (Table 1). Beased on the sugar compositions, P-galactan may have a rhamnogalacturonan I (RG-I) backbone to which the galactan chains attached, showing more complex structures than M-galactan.

Discussion

MCP was shown to be a potent galectin-3 inhibitor [11, 17]. The components of MCP related to their activity are currently unknown. To address this issue, we set out to separate MCP and examined the activity of individual components. In this paper, a neutral fraction MCP-N was purified and characterized. It was identified as an arabinogalactan with a β-1→4-galactan core and Ara residues in peripheries. Further, we prepared the core region, a β-1→4-galactan fragment of 18 KDa. Although the probable presence of galactan side chains has been reported before [29], they have never been prepared and isolated. It is for the first time that the arabinogalactan from MCP and its component fragment of β-1→4-galactan were isolated and analyzed.

MCP and its components for inhibitory activities on galectin-3 were investigated by using galectin-3 mediated hemagglutination and ASF-induced cancer cell aggregation assays. Both neutral and acidic fractions inhibited galectin-3. But the acidic fraction MCP-A showed even stronger inhibition than the neutral fraction MCP-N. This means that MCP-A contributes more than MCP-N to the inhibition of MCP on galectin-3. Currently, we are working on the fractionation and activity of MCP-A and the results will be reported later.

MCP-N is a type I arabinogalactan pectin. Its activities depend on its structures. When MCP-N was treated with α-L-arabinofuranosidase, the Ara residues in side chains were cleaved to produce the main chain β-1→4-galactans, which significantly increased the activity. We explained this phenomenon as such that the Ara substitution, occurring near the functional sites, hindered the access of galectin-3. The increase of the activity after Ara removal was also observed in the case of P-galactan [22]. The functional motif of M-galactan was probed by acidic hydrolysis. M-galactan was hydrolyzed into a mixture of oligosaccharides, namely M-galactan-oligo with molecular weight of 1.8 KDa (approximately 11 Gal residues), which increased the activity. It appeared that the functional motif resided at the chain terminal. The more Gal residues in the chain terminal, the more active the galactan might be.

Both M-galactan and P-galactan are composed of Gal residues. Their inhibitions on galectin-3 are in the same order of magnitude but some what different. The differences might result from different structures such as the number and type of substitution and the number and length of galactan chains in a molecule. P-galactan contains GalA and has more complex structures than M-galactan. The different inhibitory activities on galectin-3 were observed between MCP-N and MCP-A, two fractions from MCP.

MCP-A is an acidic fraction containing 89.3 % GalA, structurally different from the neutral fraction MCP-N without GalA. After removing Ara residues from MCP-N, the core structure M-galactan contains 96 % Gal. As structural differences, M-galactan inhibited galectin-3 much stronger than MCP-N, but about 100 times lower than MCP-A. The pectin structure-activity relationship on inhibition of galctin-3 is studed further in our research group and the results will be reported.

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