Purification, chemical analysis and inhibitory effects on galectin-3 of enzymatic pH-modified citrus pectin

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A R T I C L E  I N F O
Keywords:
Modified citrus pectin
Polygalacturonase hydrolysis
Purification
Chemical analysis
Galectin-3 inhibitor

A B S T R A C T
Modified citrus pectin (MCP), a commercially available dietary supplement prepared from citrus pectin, contains several different polysaccharide domains, but its primary chemical structure and the binding epitopes that antagonize galectin-3 function remain unclear. In this study, five fractions were isolated from MCP after endopolygalacturonase degradation (EMCP) and a combination of DEAE-cellulose and Sepharose CL-6B or Sephadex G-75 chromatography. Their primary structures, abilities to inhibit galectin-3-mediated hemagglutination, and antiproliferation activities on MCF-7 and A549 cell lines were studied. Results showed that EMCP-3p, one of the five fractions, was composed of Glc (89.8%), Gal (3.8%), Ara (3.1%), GalA (1.1%), Man (0.9%), and Rha (1.3%) with an average molecular weight of 88.4 KDa, which had the most substantial degree of galectin-3 inhibition with an MIC of 31.25 μg/mL, and it exhibited remarkable cytotoxicity against MCF-7 (36.7%) and A549 (57.4%) cell lines. These results provide new insight into the structure-function relationships of EMCP-derived polysaccharides.

Introduction

Pectin, a biomacromolecule present in plant cell walls, is a structural complex polysaccharide rich in galacturonic acid (GalA). Pectin usually contains four structural domains: homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA) (Maxwell, Belshaw, Waldron, & Morris, 2012). The HG domain, known as the “smooth region” of pectin, is a linear polymer composed of α-(1 → 4)-α-GalA residues, which are usually found to be partially methylated at the C-6 position and acetylated at the C-2 or C-3 positions. RG-I, known as the “hairy region” of pectin, consists of a main chain of alternating α-Rha and α-GalA repeat units [-4)α-GalA-(1 → 2)α-Rha-(1)α, which is highly branched with α-Ara- and β-Gal-rich side chains linked at C-4 of Rha in the backbone. RG-II, a highly complex branched structure pectin domain, consists of a 9 or 10 α-(1 → 4)-GalA unit main chain and four different branched saccharide β chains linked at C-2 or C-3 of GalA in the backbone (Maxwell et al., 2012; Mohnen, 2008; Perez, 2003; Ridley, O’Neill, & Mohnen, 2001; Willats, Knox, & Mikkelsen, 2006; Yapo, 2011).

The application of pectin in the food industry is usually limited due to its large size and cannot be absorbed by human, so some approaches have been applied to break it down into smaller fragments that can be fermented by gut microbiota. The most studied pH-modified pectin is obtained from citrus, named pH-modified citrus pectin (MCP). One of these pH modification steps is an alkaline treatment, which causes β-elimination reactions, resulting in depolymerization and deesterification of GaIA residues in the pectin backbone. The other step is acid treatment, which cleaves and releases neutral saccharide branches from RG domains in the pectin backbone (Eliaz & Raz, 2019; Glinsky & Raz, 2009).

Cancer is one of the major threats to human health, with an estimated 19.3 million new cancer cases (excluding nonmelanoma skin cancer) and 9.9 million cancer deaths in 2020 (Sung et al., 2021). Galectin-3 (Gal-3), a β-galactoside-binding lectin, has a C-terminal carbohydrate recognition domain (CRD) connected to a long N-terminal collagen-like proline- and glycine-rich tail (Argüeso & Panjwani, 2011; Sung et al., 2021).
fractions of EMCP were eluted, initially using dH₂O (EMCP-N), followed by NaCl(aq) at different concentrations of 0.05 M (EMCP-1), 0.1 M (EMCP-2), 0.2 M (EMCP-3), and 0.3 M (EMCP-4). (B) EMCP-1, EMCP-2, and EMCP-3 were applied to a Sephadex G-75 column, and EMCP-4 was applied to a Sepharose CL-6B column.

Barondes et al., 1994; Gao et al., 2012; R. Y. Yang, Rabinovich, 2005; Ruvolo, 2015; Song et al., 2014). In addition, tumor cell immune escape is also associated with the proliferation, adhesion, and metastasis of several types of cancers while also inhibiting cancer cell apoptosis (Blanchard, Yu, Collins, & Bum-Erdene, 2014; Liu & Rabinovich, 2005; Ruvolo, 2015; Song et al., 2014). In addition, tumor cell immune escape is also associated with Gal-3-induced immune cell apoptosis (Hsu, Chen, Bum-Erdene, 2014; Liu & Rabinovich, 2005; Ruvolo, 2015; Song et al., 2014). In addition, tumor cell immune escape is also associated with the proliferation, adhesion, and metastasis of several types of cancers while also inhibiting cancer cell apoptosis (Blanchard, Yu, Collins, & Bum-Erdene, 2014; Liu & Rabinovich, 2005; Ruvolo, 2015; Song et al., 2014).

Fig. 1. Elution profile of EMCP on DEAE-cellulose and gel-permeation chromatography. (A) EMCP was applied to a DEAE-cellulose column, and five fractions of EMCP were eluted, initially using dH₂O (EMCP-N), followed by NaCl(aq) at different concentrations of 0.05 M (EMCP-1), 0.1 M (EMCP-2), 0.2 M (EMCP-3), and 0.3 M (EMCP-4). (B) EMCP-1, EMCP-2, and EMCP-3 were applied to a Sephadex G-75 column, and EMCP-4 was applied to a Sepharose CL-6B column.

Barondes et al., 1994; Gao et al., 2012; R. Y. Yang, Rabinovich, & Liu, 2008). The biological functions of Gal-3 are due to the specific binding of glycoconjugates with its carbohydrate recognition domain (Laaf, Bojarova, Elling, & Kren, 2019). Gal-3 is associated with the development and malignancy of cancers, and it has been identified as a “culprit molecule” in different stages of cancer progression, acting to promote the proliferation, adhesion, and metastasis of several types of cancers while also inhibiting cancer cell apoptosis (Blanchard, Yu, Collins, & Bum-Erdene, 2014; Liu & Rabinovich, 2005; Ruvolo, 2015; Song et al., 2014). In addition, tumor cell immune escape is also associated with Gal-3-induced immune cell apoptosis (Hsu, Chen, & Liu, 2009). Thus, Gal-3 has become an important molecular target in the development of anticancer therapeutics.

It has been reported that MCP can bind to the CRD on Gal-3 and exhibit anticancer activity both in vitro and in vivo, such as metastatic cell cycle arrest in target organs, inhibiting angiogenesis and invasion, and enhancing cancer cell apoptosis in response to cytotoxic drugs (Blanchard et al., 2014; Eliaz & Raz, 2019; Glinsky & Raz, 2009; Zhang, Xu, & Zhang, 2015). However, compared with the large number of biological activity studies of MCP, its structure has been less studied. Yifa Zhou’s and Guhuai Tai’s laboratories tried to fractionate MCP into dozens of homogeneous subfractions and found that RG-I-rich subfractions with β-(1→4)-a-galactan side chains were more active than the other RG-I-rich and HG-rich subfractions, e.g., MCP-2a, a homogeneous subfraction, exhibited the greatest inhibitory effect with an MIC (minimum inhibitory concentration) of 12.5 ± 0.6 µg/mL (Zhang et al., 2016). However, all of these homogeneous subfractions contained notable amounts of GalA residues (MCP-2a contained 35.1% GalA), which might affect their binding to Gal-3. In this study, five subfractions were isolated from MCP after endo-polygalacturonase hydrolysis, and their primary structures, abilities to inhibit galectin-3-mediated hemagglutination, and antiproliferation activities against MCF-7 and A549 cell lines were studied.

Materials and methods

Citrus pectin (Reagent grade) was obtained from Kuerhuaxue Company (Beijing, China). Endo-polygalacturonase (≥5 units/mg), various dextrans (1 KDa–2000 KDa, analytical standard for GPC), and eight monosaccharides (glucuronic acid (GlcA, ≥95%), glucose (Glc, ≥98%), galacturonic acid (GalA, ≥98%), galactose (Gal, ≥99%), arabinose (Ara, ≥98%), fucose (Fuc, ≥98%), rhammose (Rha, ≥98%), and mannose (Man, ≥98%)) were purchased from Sigma. Sepharose CL-6B and Sephadex G-75 were purchased from Amersham Pharmacia Biotech Company. Recombinant human Gal-3 was prepared in our laboratory as previously reported (Zhou et al., 2020). All other reagents were of analytical grade and were purchased in China.

Preparation and purification of enzymatic MCP (EPCP)

MCP was obtained from CP using pH modification as previously described by our laboratory (Zhang et al., 2020). Briefly, the CP solution was treated with NaOH(aq) for 1 h at pH 10.0 followed by HCl(aq) for 12 h at pH 3.0, and the solution was neutralized by NaOH(aq). MCP was precipitated by adding ethanol to the solution up to 70%. MCP (5 g) was dissolved in 200 mL sodium acetate buffer (25 mmol/L, pH 4.2) and precipitated by adding ethanol to the solution up to 70%. MCP was treated with NaOH(aq) for 1 h at pH 10.0 followed by HCl(aq) for 12 h at pH 3.0, and the solution was neutralized by NaOH(aq). MCP was dissolved in 200 mL sodium acetate buffer (25 mmol/L, pH 4.2) and incubated with Endo-PG (50 U) at 50 °C for 2 h at static condition. This MCP solution was digested twice again by Endo-PG under the same conditions. The hydrolysates were dialyzed (MWCO 1 kDa) and freeze-dried to yield enzymatic MCP (EMCP).

EMCP (1 g) dissolved in 100 mL distilled water was applied to a DEAE-cellulose column (2.6 cm × 50 cm), eluted with distilled water to give the neutral fraction (EMCP-N) and then eluted with 0.05 mol/L, 0.1 mol/L, 0.2 mol/L and 0.3 mol/L NaCl(aq) to obtain four acidic fractions (EMCP-1, EMCP-2, EMCP-3, and EMCP-4). The acidic fractions (EMCP-1, EMCP-2, EMCP-3, and EMCP-4) were further purified on a Sepharose CL-6B (1.0 cm × 60 cm) column or a Sephadex G-75 (1.0 cm × 60 cm) column, which was eluted with 0.15 mol/L NaCl, and the flow rate was 0.1 mL/min. The total sugar content of each tube (1.2 mL) was detected by phenol–sulfuric acid assay, and the appropriate eluates were collected, dialyzed, and freeze-dried to obtain four fractions (EMCP-1p, EMCP-2p, EMCP-3p, and EMCP-4p).
**Homogeneity and molecular weight analysis**

The molecular weights were analyzed by gel-permeation chromatography on a Waters Ultrahydrogel 250 column (7.8 × 300 mm, Waters Corporation, Milford, Massachusetts, USA) coupled to a Shimadzu HPLC system and a Schambeck RI200A refractive index detector as described previously (Zhang et al., 2020). The columns were precalibrated by standard dextrans (1 KDa, 5 KDa, 10 KDa, 50 KDa, 2000 KDa), and the molecular weights of the EMCP fractions were calculated by linear regression analysis.

**Thiobarbituric acid method**

The thiobarbituric acid (TBA) method was performed according to a previously published protocol (Karkhanis, Zeltner, Jackson, & Carlo, 1978). In brief, each polysaccharide sample was treated with 0.1 mol/L H₂SO₄ at 100 °C for 0.5 h to release 3-deoxy-D-manno-oct-2-ulonic acid (Kdo). The hydrolysates were reacted with NaAsO₂, periodic acid, and thiobarbituric acid, and dimethylsulfoxide was added to the solution at 25 °C. If the polysaccharide sample contained Kdo, a red chromophore would be produced and could be detected.

**FT-IR analysis**

The polysaccharide sample was mixed with dried KBr powder, and this mixture was pressed into a 1-mm pellet. An FT-IR instrument (Bruker Tensor 27) was used to obtain the spectra ranging from 400 cm⁻¹ to 4000 cm⁻¹. The resolution was 4 cm⁻¹, and scan number was 32.

**NMR analysis**

A polysaccharide sample (20–30 mg) was dissolved in 1 mL D₂O (99.8%), and a Bruker AV600 spectrometer (Karlsruhe, Germany) was used to give the 13C NMR spectrum at 25 °C. Acetone was used as an internal standard.

**Gal-3-mediated hemagglutination method**

The Gal-3-mediated hemagglutination method was carried out as previously described (Zhou et al., 2020). In brief, a transparent microplate (V type) was used in this experiment, and each well of this microplate contained 25 µL 0.15 mol/L NaCl (control) or 25 µL polysaccharide sample solution, 25 µL 1% bovine serum albumin (BSA) dissolved in 0.15 mol/L NaCl, 25 µL 15 µg/mL Gal-3 solution (PBS, pH 7.4), and 25 µL 4% (V/V) chicken erythrocyte suspension (PBS, pH 7.4). After standing for 0.5 h at 25 °C, the concentrations of the polysaccharide samples that could completely inhibit the aggregation of the chicken erythrocytes were recorded, and the minimum inhibitory concentration (MIC) was used to describe its binding affinity to Gal-3.

![Fig. 2. Homogeneity and average molecular weight of the EMCP fractions.](image-url)
EMCP preparation and purification

MCP was obtained from CP using pH modification as previously described by our laboratory (Zhang et al., 2020), and EMCP (yield 42.0%) was obtained from MCP using Endo-PG hydrolysis. As shown in Fig. 1A, EMCP (1 g) dissolved in 100 mL dH2O was added to a DEAE-cellulose column (2.5 cm × 50 cm), which was eluted with dH2O to obtain one neutral fraction (EMCP-N, yield 2.9%), followed by elution with 0.05 mol/L, 0.1 mol/L, 0.2 mol/L and 0.3 mol/L NaCl to obtain four acidic fractions (EMCP-1, EMCP-2, EMCP-3, and EMCP-4). As shown in Fig. 1B, the acidic fractions (EMCP-1, EMCP-2, EMCP-3, and EMCP-4) were further purified on a Sepharose CL-6B (1.0 cm × 60 cm) column or a Sephadex G-75 (1.0 cm × 60 cm) column, which was eluted with 0.15 mol/L NaCl at 0.1 mL/min. Based on the elution profile, the appropriate eluates were collected, dialyzed, and freeze-dried to obtain four purified fractions: EMCP-1p (0.8%), EMCP-2p (2.5%), EMCP-3p (1.8%), and EMCP-4p (19.6%). In addition to the purification of MCP by chromatography, do Prado et al. (2019) fractionated MCP by sequential ultrafiltration using 30, 10, and 3 kDa MWCO Amicon Ultra-4 Centrifugal Filters based on different molecular weights to give four fractions: MCP30 (68%), MCP 30/10 (15%), MCP10/3 (10%), and MCP3 (7%).

Homogeneity and average molecular weight of the EMCP fractions

The homogeneity and average molecular weight of each fraction were determined by HPGPC on a Waters UltraHydrogel 250 column. As shown in Fig. 2, EMCP-N, EMCP-1p, EMCP-3p, and EMCP-4p each yielded a major single peak, suggesting that they were homogeneous, and their average molecular weights were estimated to be 3.1 kDa, 21.2 kDa, 88.4 kDa, and 117 kDa, respectively. EMCP-2p gave multiple peaks, indicating that it was heterogeneous, and we did not purify it further. The average molecular weight was estimated to be 19.3 kDa-90.8 kDa. Compared to the sequential ultrafiltration method, the average molecular weight of MCP30/10 (10–30 kDa), MCP30 (>30 kDa), and MCP10/3 (3–10 kDa) obtained by do Prado et al. (2019) were smaller than that of EMCP fractions.

Monosaccharide compositions of the EMCP fractions

The monosaccharide compositions of these fractions are provided in Fig. 3. EMCP-N was composed of Glc (39.9%), Gal (34.4%), Ara (12.5%), and Rha (13.2%); EMCP-1p was composed of Glc (77.4%), Gal (8.7%), Ara (4.3%), GaA (4.5%), Man (2.5%), and Rha (2.6%); EMCP-2p was composed of Glc (78.4%), Gal (7.2%), Ara (4.8%), GaA (4.9%), and Man (4.7%); EMCP-3p was composed of Glc (89.8%), Gal (3.8%), Ara (3.1%), GaA (1.1%), Man (0.9%), and Rha (1.3%; and EMCP-4p was composed of Glc (67.0%), Gal (10.2%), Ara (4.9%), GaA (13.8%), and Man (4.1%). The acidic fractions mainly consisted of neutral monosaccharide residues (such as Glc, Gal, Ara, Man), while their content of GaA was much lower, which showed that the HG domains of MCP were removed by Endo-PG hydrolysis. Compared to the sequential ultrafiltration method, the amount of GaA of MCP30/10 (10–30 kDa), MCP30 (>30 kDa), and MCP10/3 (3–10 kDa) obtained by do Prado et al. (2019) was much higher than that of EMCP fractions, which might be due to the limited hydrolysis using endo-polygalacturonase. Wu et al (2020) used endo-polygalacturonase hydrolysis to remove nearly half of the GaA residues from 49.8% in EP-1 to 26.47% in EP-2. Besides, after enzymatic hydrolysis, EP-2 showed much higher amount of Glc residues as compared to WSP.

FT-IR spectra of the EMCP fractions

FT-IR spectroscopy, one of the principal methods used to study the basic structure of complex polysaccharides, was utilized to analyze the
EMCP fractions. As shown in Fig. 4, the FT-IR spectra of the EMCP fractions were indistinguishable, with only slight differences in their intensity of absorption. The strong absorption at approximately 3415 cm⁻¹ and 3405 cm⁻¹ by the EMCP fractions suggests that these fractions contained hydroxy groups, and the absorption at approximately 2933 cm⁻¹ and 2923 cm⁻¹ were attributed to methyl or methylene groups, which are both structural features of polysaccharides. In addition, the absorption at approximately 1607 cm⁻¹ was attributed to the C–O bonds in the acidic polysaccharide fractions, and the absorption at approximately 1639 cm⁻¹ was attributed to the C–O group in EMCP-N (Ji et al., 2019; Liang et al., 2019); the absorption at approximately 1415 cm⁻¹ was attributed to the C–H group; and the absorption at 1023 cm⁻¹ and 1099 cm⁻¹ suggested that EMCP-1, EMCP-2, EMCP-3, and EMCP-4 all contained some uronic acid. The absorption at approximately 1025 cm⁻¹ indicated that EMCP-N contains some arabinogalactan–related domains (Capek et al., 2003), and absorption at 1153 cm⁻¹ was related to C–O stretching vibration of 6-OH owing to Glc or/and Gal residues (Coimbra, Barros, Barros, Rutledge, & Delgadillo, 1998; Zhang et al., 2020); the absorption at approximately 613 cm⁻¹ was due to the pyranose rings of polysaccharides (Zhang et al., 2019).

Inhibitory activities of EMCP fractions on Gal-3-mediated hemagglutination

The Gal-3-mediated hemagglutination method is usually performed to analyze the inhibitory activities of Gal-3 inhibitors. The MIC value is utilized to quantify the activity of the inhibitor toward complete inhibition of hemagglutination. This assay was carried out as described previously (Zhou et al., 2020). The inhibitory activities of the EMCP fractions are shown in Fig. 5A. EMCP-N, EMCP-1p, EMCP-2p, EMCP-3p and EMCP-4p exhibited different activities; in particular, EMCP-3p exhibited the strongest inhibition with a MIC of 31.25 µg/mL, followed by EMCP-2p with a MIC of 125 µg/mL. EMCP-N, EMCP-1p and EMCP-4p exhibited the weakest inhibition with the same MIC of 250 µg/mL. Compared to MCP-2a reported by Zhang et al., the MIC value of EMCP-3p was slightly higher than that of MCP-2a (MIC 12.5 µg/mL) (Zhang et al., 2016), which suggested that the HG domain could be important in MCP activity by maintaining the structural conformation of the entire molecule (Gao et al., 2013).

Anti-proliferation activities

Gal-3 can promote the proliferation and migration of some cancer cells, so the MTT method was utilized to analyze the inhibitory activities of EMCP fractions on Gal-3. The antiproliferative effects of the EMCP fractions were evaluated on MCF-7 and A549 cell lines in vitro. As shown in Fig. 5B, the viabilities of the MCF-7 and A549 cells were clearly inhibited by all EMCP fractions in a concentration-dependent manner. The five fractions exhibited cell viability in this order: EMCP-3p (36.7%) > EMCP-4p (45.9%) > EMCP-N (53.4%) > EMCP-2p (55.4%) > EMCP-1p (56.9%) against MCF-7 cells and EMCP-2p (53.4%) > EMCP-3p (57.4%) > EMCP-1p (64.8%) > EMCP-4p (82.8%) > EMCP-N (89.3%) against A549 cells. Especially at a concentration of 2.0 mg/mL EMCP-3p, the viability of MCF-7 cells (36.7%) was much lower than

Fig. 4. FT-IR spectra of the EMCP fractions.
that of A549 cells (57.4%), suggesting that A549 cells are less sensitive to Gal-3 inhibitors, which may be due to the lower expression level of Gal-3 in A549 cell lines than in MCF-7 cells (Wei et al., 2019; Wu et al., 2020).

These results suggest that the EMCP fractions could exert antitumor activity as Gal-3 antagonists against some cancer cells with high expression levels of Gal-3, indicating the potential of EMCP fractions for curing some cancers, especially EMCP-3p. Compared to pectin or polysaccharides prepared from other plants, the anti-proliferation effect of EMCP-3p against MCF-7 cell lines was stronger than that of *Dictyophora indusiata* polysaccharide (Liao et al., 2015), *Angelica sinensis* polysaccharide (Zhang et al., 2016), and pectin from citrus canning...
the work reported in this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 32060035), the Technology Research and Development Program of Guizhou (No. qiankehezicheng [2018]2803), the Program for Excellent Young Talents of Zunyi Medical University (No. 18zy-006).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fexch.2021.100169.

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