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Immunomodulatory effect of natural and modified Citrus pectin on cytokine levels in the spleen of BALB/c mice

Rihab Merheb, Roula M. Abdel-Massih *, Marc C. Karam

Department of Biology, University of Balamand, P.O. Box: 100, El-Koura, Lebanon

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ABSTRACT

Pectin is present in the cell wall of different vegetables and fruits. Beside its importance in the plant cell wall, pectin has enticed great attention for its beneficial effects on human health. It was shown to decrease cholesterol levels, to possess anti-oxidative, anti-bacterial and anti-cancer activity. The immunomodulatory activity of pectin and its mechanism of action is recently being investigated. In this study, the differential immunomodulatory activities of both CP (citrus pectin) and MCP (modified citrus pectin) were investigated. Females BALB/c mice (20–25 g) were randomly divided into 7 groups and different concentrations of CP and MCP (0%, 1.5%, 3% and 5%) were added to their drinking water for 21 days. Then, the splenic level of IL-1β, IL-4, IL-10, IL-17, IFN-γ and TNF-α were evaluated using ELISA. Both CP and MCP exhibited immunomodulatory activities by increasing the levels of the pro-inflammatory cytokines IL-17, IFN-γ and TNF-α levels. This tendency seems to be regulated by the up-regulation of IL-4 levels but with no major effect on those of IL-10. Therefore, CP and especially MCP have potential immunomodulatory effects which might be highly beneficial in immunotherapy.

1. Introduction

Pectin is a highly branched and complex polysaccharide present in the cell walls of a wide variety of fruits [1]. The concentration, the structure and the degree of methylation of pectin in the plant cell wall varies between different types of plant species and even within the same species at different maturation stages [2]. Pectin is involved in important cell wall functions including strengthening the cell wall [3] and regulating ion transport [4]. Pectin's major classes are homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II [2,5]. Homogalacturonan is a linear chain of galacturonic acid residues [6]. Rhamnogalacturonan I, consists of a repeating disaccharide unit galacturonic acid and rhamnose with side-chains mainly arabinogalactan, arabinan and galactan [7–9]. Rhamnogalacturonan-II is a highly branched and complex polysaccharide. RG-II plays a critical role in cell wall functions such as immunomodulatory functions [1]. It induces the production of interleukin-6 (IL-6) by macrophages *in vivo* [10].

Modified citrus pectin (MCP) is a treated form of citrus pectin [11] that has a low molecular weight [12]. MCP fragments are rich in galactans that, theoretically, are absorbed in the small intestine [11–15]. Courts suggested that the short chain galactan and arabinogalactan can be absorbed *via* the paracellular pores in the small intestine following oral consumption of MCP [16]. MCP showed strong anti-proliferative activity and anti-metastatic effect on cancer cells such as MAT-LyLu prostate cancer cells [17].

Pectasol-C, a form of MCP, is prepared by enzymatic treatment and has a molecular weight between 5 and 10 kD. Pectasol-C is marketed as a natural health supplement and several studies report its ability to inhibit cancer progression, inflammation and fibrosis [12]. Different studies investigated the immunomodulatory effect of different forms of pectin [18]. Pectic polysaccharides isolated from *Angelica gigas* were found to activate macrophages, natural killer cells and lymphocytes [18]. Pectin fragments increase the functions of many innate and adaptive immune effector cells such as macrophages, dendritic cells, natural killer cells, B lymphocytes and T lymphocytes [19–21] through the production of cytokines [20]. Lim et al. [22,23] demonstrated that pectin enhanced the concentration of immunoglobulin A (IgA) in the serum and in mesenteric lymph node (MLN) lymphocytes and decreased serum Immunoglobulin E (IgE) production in Sprague-Dawley rats. Through various studies, the alteration in Ig production by spleen and MLN lymphocytes was attributed to bile acids alteration [22,24,25] or due to the interaction of short-chain fatty acids (SCFAs) with lymphocytes in the intestinal mucosa [23,24,26].

Due to the their important potential clinical implications and since, to date, very few studies investigated the *in vivo* effects of natural and modified citrus pectin on the immune system, the aim of this study is to determine the immunomodulatory activity as to the levels of the secretion of the pro-inflammatory cytokines IL-1β, IL-17, TNFα and IFN-γ or anti-inflammatory cytokines IL-4 and IL-10 in the spleens of treated BALB/c mice.

* Corresponding author.
E-mail address: roula.abdelmassih@balamand.edu.lb (R.M. Abdel-Massih).

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2. Methods

2.1. Animals and diets

Female BALB/c mice were housed at 23 (±2 °C), 12:12 hour light cycle and were provided with solid food and water *ad libitum*. Different concentration of citrus pectin, modified citrus pectin or water were added to study their effect on cytokine production in the spleen. Handling was performed according to the Guide for Care and Use of Laboratory Animals of the National Institute of Health.

Eight-week-old female BALB/c mice (20–25 g) were randomly selected and divided into seven groups (n = 5). Each group was placed in a transparent plastic cage with 4–5 cm of sawdust.

Lyophilized citrus pectin was kindly provided by Megazyme. Modified citrus pectin, PectaSol-C was kindly provided by Eco-Nugenics (Santa Rosa CA, USA).

Group one or the control was given distilled water (0.0% of CP or MCP). The mice in the rest of the groups were given drinking water supplemented with low CP concentration (1.5%), intermediate CP concentration group (3%), high CP concentration group (5%), low PectaSol-C concentration group (1.5% MCP), intermediate PectaSol-C group (3% MCP) and high PectaSol-C concentration group (5% MCP) for three weeks. The pH of the CP and MCP solutions were pH 3 and pH 5.5 respectively. Mice were monitored for their uptake of food and liquid in addition to their survival. The amounts of the prepared solutions consumed by the different groups over the experimental period were documented (Table 1), and fresh solution was prepared every 3 days.

2.2. Immunoassay procedures

After 21 days of feeding, the mice were sacrificed by neck dislocation immediately before dissection and spleen extraction. The spleen of each mouse was weighed and placed in a clean Eppendorf containing 1 ml of RIPA buffer that contains 25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxy-cholate and 0.1% SDS. One tablet of protease inhibitors was added to 50 μl of RIPA buffer prior to use. The spleen was homogenized using a homogenizer and centrifuged for 10,000*g* at 4 °C for 30 min. The supernatants were then removed and aliquoted in Eppendorf endotoxin free tubes at −80 °C until ELISA was performed. Mini ELISA Development kits (Peprotech) were used for the quantitative measurement of the following cytokines: IL-1β (900-M47), IL-4 (900-M49), IL-10 (900-M53), IL-17 (900-392), IFN-γ (900-M98) and TNF-α (900-M54). All cytokine assays were performed in accordance to the manufacturers' instructions. Briefly, ELISA microplates were first coated by adding capture antibody that is specific for the protein of interest and incubated overnight at room temperature. Block buffer was then added for 1 h followed by the addition of the spleen supernatant samples or control groups. Samples were incubated for 2 h. Detection antibody was next added for another 2 h. Avidin-HRP conjugate was later added for 30 min and ABTS substrate was added for color development. The plates were washed after each step. Plates were read using the BioTek Epoch plate reader at 450 nm and results were obtained *via* the GEN5 software.

2.3. Gel filtration chromatography

Gel filtration chromatography is a method that separates molecules according to their molecular weight or sizes. Large molecules fractionate first whereas small molecules fractionate last. Sepharose CL-6B is a suitable column to separate and to compare the molecular sizes of pectin products.

Solutions of 1% citrus pectin and PectaSol-C were fractionated separately on a column of Sepharose CL-6B (22 cm in length). The column was washed with distilled water and the different polysaccharide fractions were eluted sequentially. The column was used over a period of 2 weeks. It was under a pressure of 5 cm of water resulting in a flow rate of 18 ml/h. A fraction collector (Bio Rad) collected samples of 700 μl of eluted solution. Blue dextran (high molecular weight) and Cobalt (II)-Chloride-Hexahydrate (low molecular weight) were used as molecular weight standards. A mixture of blue dextran and cobalt chloride (2% each) were dissolved in water and were run through the column.

2.4. Dubois method

Dubois method is a phenol sulfuric acid reaction that determines the total amount of carbohydrates present in a sample [27]. Phenol liquid 5% (300 μl) and sulfuric acid (1500 μl) were added to 300 μl from each fraction. The absorbance of each sample was read at 490 μm. D-Glucose anhydrous was used as a standard for this method. D-Glucose anhydrous (0.1 g) was dissolved in 10 ml of distilled water, and serial dilutions were performed to prepare the different concentrations of the standards. The eluted fractions *versus* absorbance at 490 nm wavelength of each solution were plotted on one graph to determine carbohydrate concentration.

2.5. Statistical analysis

The concentrations of each cytokine were analyzed by one-way analysis of variance (ANOVA) test. GrapPad Prism software 6 (GraphPad Software Inc., San Diego USA) was used to compare the significances among the groups. Error bars on graphs represent the standard error deviation for each sample. p values of <0.05, 0.01 and 0.001 were considered statistically significant.

3. Results

3.1. Comparison of molecular weight of pectin and MCP using gel filtration

According to literature, the molecular weight of pectin is a major parameter that influences its potency in biological activities [28]. In order to compare the molecular weight of CP and MCP, samples were run on Sepharose CL-6B columns and the Dubois Phenol-Sulfuric method was performed on the eluted polysaccharide fractions. As shown in Fig. 1, CP fractions gave a peak between fractions 8–11 whereas MCP fractions

Table 1
The amount of solutions with different concentrations of CP and MCP consumed by the different groups of mice.

1.5% pectin = 650 ml	1.5% MCP = 950 ml
3% pectin = 780 ml	3% MCP = 1120 ml
5% pectin = 850 ml	5% MCP = 1185 ml

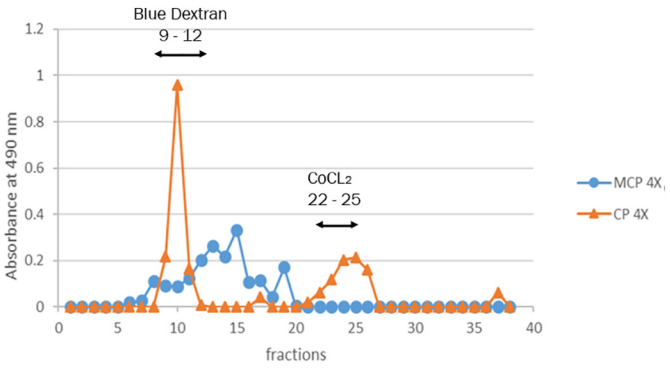

Fig. 1. Elution patterns of citrus pectin and modified citrus pectin on Sepharose CL-6B. Citrus pectin (CP) and modified citrus pectin (MCP) where run on Sepharose CL-6B. Fractions were collected, treated with the Dubois Phenol-Sulfuric method and their absorbance was determined. Blue dextran high molecular weight marker ran from fractions 9 to 12. $CoCl_2$ the low molecular weight marker eluted between fractions 22 to 25. CP and MCP were diluted (4×) before running on the column.