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Immunomodulating pectic polysaccharides from waste rose petals of *Rosa damascena* Mill

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ABSTRACT

A water-soluble polysaccharide (RP-1) was obtained from distilled rose petals of *Rosa damascena* Mill. as an attempt for valorization of the waste. RP-1 showed *in vitro* intestinal immune system modulating activity through Peyer's patch cells and IL-6 producing activity from macrophages. RP-1 lost most of its immunomodulating activity by degradation of the carbohydrate moiety with periodate. RP-1 was fractionated by anion-exchange and gel filtration chromatography and some of the fractions showed significant intestinal immune system modulating activity. The active fractions were suggested to be pectic polysaccharides and type II arabino-3,6-galactan from the component sugar analyses and the reactivity with Yariv antigen. When some active fractions were digested with endo α-D-(1→4)-polygalacturonase, highest molecular weight fragments which were considered as rhamnogalacturonan I, showed potent immunomodulating activities.

To our knowledge, this is a first report which explores the possibility for utilization of waste rose petals as a source of immunomodulating pectic polysaccharides.

1. Introduction

Bulgaria is one of the biggest world producers of rose oil. Almost 100% of the oil-bearing roses grown in Bulgaria are *Rosa damascena* Mill. Data from local authorities in Kazanlak, Bulgaria showed that for 2008 the rose oil produced is about 2000 kg [1]. For every one kg rose oil, approximately 4000 kg rose petals are needed. Roughly for 2008 was generated $8–10 \times 10^6$ kg waste. The waste material after distillation was used as a source of aroma substances, vitamin C, animal feed, fertilizer or just discarded [2,3].

Historically there are data that roses were cultivated and used for medical purposes by the ancient Chinese and Egyptians about 5000 years ago and lately in the ancient Greece and the Roman Empire [4,5]. During the Medieval the roses were used as moist conserve (as astringent, anti-infective medicine), syrup of roses (general tonic, anti-infective), honey of roses (antiseptic, mouth-wash), rose water (general tonic, anti-inflammatory eyewash), decoction of roses (analgesic), rose ointment and oil (antipyretic, antiseptic) [6]. Flowers of *R. damascena* also were used as traditional medicine in some area, and are known as cardiac tonic and bile secretion stimulants. Rose water internally was used to relieve weakness and anxiety [3,7]. Aqueous extracts of the rose flowers were reported to extend the life span of *Drosophila* flies [8]. Water and methanol extracts of *R. damascena* were found to exhibit moderate anti-HIV activity [9]. Recently a possibility for utilization of polyphenols from the waste rose petals was investigated [10,11], and it was shown that the polyphenolic compounds can be used as antioxidant supplements and for strawberry beverage stabilization. Another unexplored possibility for utilization of the waste rose petals is extraction of polysaccharides. Although there are scarce data about polysaccharide molecules in rose flowers, it has been reported on isolation of polysaccharide-peptide complex with anti-oxidant properties [12,13]. The suspension culture of roses has been also reported to produce xyloglucans [14,15], pectic polysaccharides [15] and arabino-3,6-galactans [16], therefore it will be expected that the waste rose petals are good source for these macromolecular compounds. Numerous reports suggested that these type macromolecular polysaccharides possess pharmacological and immunopharmacological activities against digestive system, immunological system, etc. [17]. Since arabino-3,6-galactans with a certain structural feature have been found to express important immunopharmacological activities [18,19], waste rose petals is predicted to contain immunostimulating polysaccharides. Although in the literature there are descriptions that the rose hips and petals contain significant amount pectins [4], to our knowledge till now there are no attempts to use waste rose petals as a source of bioactive macromolecular polysaccharides.

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The present paper describes isolation, chemical characterization and immunomodulating activities of pectic polysaccharide fractions from distilled rose petals of *R. damascena* Mill. for novel effective utilization of the waste material.

2. Materials and methods

2.1. Materials

The waste rose petals material (*R. damascena* Mill.) was obtained from distillery Zlatna roza, (Zlatosel, region of Plovdiv, Bulgaria – 2008 crop). The material was kept frozen (−18 °C) before further treated. Endo-α-D-(1→4)-polygalacturonase (EC.3.2.1.15) was prepared from Pectinase (Sigma) according to the procedure of Thibault and Mercier [20]. Sepharose FF and Sepharose CL-6B were purchased from GE Healthcare Bioscience (USA). β-D-Glucosyl-Yariv antigen and Acacia arabinogalactan standard were obtained from Megazyme (Australia). Phosphate-buffered saline (PBS), RPMI 1640 medium and Hank's balanced medium were purchased from Sigma (St. Louis, MO, USA).

2.2. General methods

The carbohydrate, uronic acid and protein contents of the polysaccharide fractions were determined by phenol–H_2SO_4 [21], *m*-hydroxybiphenyl [22] and Bradford [23] methods, respectively, using galactose, galacturonic acid and bovine gammaglobulin as respective standards.

Component sugars of polysaccharide fractions were analyzed as TMS methylglycoside derivatives (unmodified neutral sugars and uronic acids) after methanolysis with 1 M HCl in anhydrous MeOH for 15 h at 80 °C according to York et al. [24] by GC on a Hewlett–Packard Model 5890 series II gas chromatograph, and neutral sugars of the crude polysaccharide (RP-1) were measured as alditol acetates after hydrolysis with trifluoroacetic acid for 3 h at 120 °C according to Blakeney et al. [25] by GC on a Hewlett Packard Model 6890 series gas chromatograph.

The Mr. values of polysaccharide fractions were determined by high performance size exclusion chromatography (HPSEC) using a JASCO model 980 HPLC system equipped with combined columns of Asahi-pak GS-710 + GS-620 (Asahi Chemical Industry Co. Ltd., Tokyo) using 0.2 M NaCl as eluant. Relative Mr. values of polysaccharides were estimated from the calibration curve obtained by using standard pullulans (Showa Denko Co. Ltd., Tokyo).

The content of β-D-(1→3,6)-arabinogalactan in the polysaccharides was analyzed using a single radial gel diffusion using β-D-glucosyl-Yariv antigen according to the procedure of Holst and Clarke [26]. Ten microliters of tested polysaccharides and standard Acacia arabinogalactan (1 mg/ml) was applied to the gel. The amount of arabino-3,6-galactan content in a given test sample was determined by a calibration curve based on the square value of the halo formed using Acacia arabinogalactan (10 μg) as a standard.

2.3. Preparation of crude polysaccharide fraction (RP-1)

The alcohol-insoluble residue (AIR) was obtained from the waste rose petals of *R. damascena* Mill. according to Kratchanova et al. [27]. RP-1 was isolated from AIR as follows. AIR (70 g) was extracted with hot-water (1.4 L distilled water) at 80 °C for 1 hr with constant stirring, the mass was filtered and the residue was subjected to a second extraction with hot water (1 L) at 80 °C for 40 min with constant stirring. Then the extract was filtered again, and both filtrates were combined and evaporated using rotary evaporator (temperature 40 °C) to 1/3 of its initial volume. The concentrated filtrate was precipitated with 4 volumes of 96% ethanol under stirring for 24 h at 4 °C, and then the precipitate was filtered and dried. The obtained precipitate was dissolved in 250 ml distilled water and dialyzed (dialysis membranes Sigma–Aldrich, D9652-100FT, Mr. cut-off 12,000) for 48 h against distilled water. Non-dialyzable portion was freeze dried, and crude polysaccharide fraction (RP-1) was obtained.

2.4. Fractionation and purification of polysaccharides from RP-1

RP-1 (5000 mg) was fractionated by anion-exchange chromatography on DEAE Sepharose FF (HCO_3^- form) column (5 cm × 50 cm) by stepwise manner. After the neutral polysaccharide fraction was obtained as unabsorbed fraction (RP-1-I) with elution of water, absorbed acidic polysaccharide fractions were eluted gradually with increasing ionic strength of NH_4HCO_3 solutions (50, 100, 200, 300, 400, 500, 600, 800 and 1000 mM), and gave twelve polysaccharide fractions (RP-1-IIa, IIb and IIc from 50 mM eluate; IId from 100 mM eluate; IIe from 200 mM eluate; IIf from 300 mM eluate; IIg from 400 mM eluate; IIh from 500 mM eluate; IIi and IIj from 600 mM eluate; IIk from 800 mM eluate and IIl from 1000 mM eluate, respectively). The obtained fractions were each pooled and concentrated by evaporation *in vacuo* (temperature 40 °C). Then the polysaccharide fractions were dialyzed (dialysis membranes from Sanko Junyaku Co. Ltd., Japan, Mr. cut-off 12,000) against distilled water for 72 h, and the non-dialyzable fractions were lyophilized.

Major pectic polysaccharide fractions (RP-1-IId, RP-1-IIe, RP-1-IIf, RP-1-IIg and RP-1-IIh) obtained by anion-exchange chromatography, were further purified by gel filtration on Sepharose CL-6B column (2.6 cm × 95 cm) with 0.2 M NaCl as eluant. The fractions obtained were pooled, concentrated by evaporation *in vacuo* (temperature 40 °C), dialyzed (dialysis membranes from Sanko Junyaku Co. Ltd., Japan, Mr. cut-off 12,000) for 72 h against distilled water and lyophilized. As a result 12 subfractions were obtained. RP-1-IId gave four fractions – RP-1-IId-1, RP-1-IId-2, RP-1-IId-3 and RP-1-IId-4. RP-1-IIe gave two fractions – RP-1-IIe-1 and RP-1-IIe-2. RP-1-IIf gave two fractions – RP-1-IIf-1 and RP-1-IIf-2. RP-1-IIg gave two fractions – RP-1-IIg-1 and RP-1-IIg-2. RP-1-IIh gave two fractions – RP-1-IIh-1 and RP-1-IIh-2.

2.5. Chlorite treatment of crude polysaccharide fraction

Chlorite treatment was performed by the procedure as described previously [28]. To RP-1 (100 mg) solution in 4% acetic acid (100 ml) was added $NaClO_2$ (500 mg), and incubated at 70 °C for 40 min. After neutralization with 3 M NaOH, the solution was dialyzed (dialysis membranes from Sanko Junyaku Co. Ltd., Japan, Mr. cut-off 12,000) against distilled water for 72 h and the non-dialyzable portion was concentrated and lyophilized. As a result $NaClO_2$-treated RP-1 was obtained (yield 86.5%).

2.6. Periodate oxidation of crude polysaccharide fraction

Periodate oxidation was performed by the procedure as described previously [28]. RP-1 (50 mg) was treated with 100 mM $NaIO_4$ (10 ml) in acetate buffer (pH 4.6, 50 mM) at 4 °C for 96 h. To stop the reaction, ethylene glycol (1 ml) was added and the mixture was stirred for 1 hr at room temperature. After dialysis (dialysis membranes from Sanko Junyaku Co. Ltd., Japan, Mr. cut-off 12,000) for 72 h, the non-dialyzable portion was concentrated to half volume and $NaBH_4$ was added. Then the mixture was stirred for 8 h at room temperature. After careful neutralization with concentrated acetic acid, the solution was dialyzed against distilled water and non-dialyzable portion was lyophilized. As a result $NaIO_4$-treated RP-1 was obtained (yield 82.6%).