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Characteristics of pectic polysaccharides from leek obtained through consecutive extraction with various reaction agents

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Dedicated to Academician Ivan Juchnovski on the occasion of his 70th birthday

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Five polysaccharide fractions of commensurable by yield, but different in composition were obtained through consecutive extraction with water, solutions of ammonium oxalate, sodium carbonate, hydrochloric acid and sodium hydroxide from the alcohol-insoluble residue (AIR) of leek. In the polyuronide part of these fractions besides galacturonic acid was found also glucuronic acid. In the neutral sugar fraction, the prevailing sugar was galactose, followed by rhamnose. The water-extractable pectic polysaccharide was highly homogenous (93.3% of it had molecular mass of 1.3×10^6 kDa) and protein content of 8% (the highest compared to the other extracted polysaccharides). Extraction with diluted hydrochloric acid yielded polysaccharide with the highest neutral sugar content of 71.1% and a low uronic acids content. The water- and chelate-extractable fractions had a lower L-rhamnose content (2.7% and 2.9%, respectively) and the other polysaccharide fractions from leek were characterized by a high L-rhamnose content (from 14 to 28%). The pectic polysaccharides obtained from leek have shown good immunostimulating properties. The highest immunostimulating activity has been shown by the water- and chelate-extractable polysaccharides, which are also characterized by a high polyuronic acid content and polysaccharides with molecular mass over 10^6 Da.

Key words: leek, pectic polysaccharides, consecutive extractions, chemical compositions, immunological activity.

INTRODUCTION

Pectic polysaccharides along with cellulose, hemicelluloses, glycoproteins, proteins and lignin build up the plant cell wall [2–6]. The pectic polysaccharides are complex biopolymers made up of α-(1→4) bound residues of D-galacturonic acid, the so-called "smooth region" or homogalacturonan and branched regions of rhamnogalacturonans. Rhamnogalacturonans contain side chains of neutral sugars such as arabinans, galactans, arabinogalactans, *etc*., which account for the haired layout ("hairy region") of the pectic macromolecule [1].

The branched pectic molecules are highly entwined in the cell wall, which hampers their extraction from the plant material. Various approaches for isolation of pectic substances have been applied. Voragen *et al*. [1] have introduced a method for obtaining pectic polysaccharides through consecutive extraction with water, chelate substances, acids and alkali. The scheme for consecutive fractional extraction also gives information on the type of bonding of the isolated pectic polysaccharides with the other components in the cell wall [1, 7, 8].

The leek is a vegetable known from hoary antiquity. It has been used for food, which is beneficial for the human health. The influence of some of the components of leek, such as polysaccharides, flavonoids, saponins, sulphur-containing compounds, minerals and vitamins, on the human health is well known, but there is a lack of information for the composition and biological importance of pectic polysaccharides [9].

It has been found that pectic polysaccharides isolated from different plant sources have diverse biological properties. They and their derivatives have antitumour, anticomplement, antiinflamatory, immunological and antivirus activity [10].

The aim of the present investigation is the isolation of pectic polysaccharides from AIR of leek through consecutive fractional extraction, their

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characterization and determination of their immunostimulating activity.

EXPERIMENTAL

Obtaining of alcohol-insoluble substance (AIS) from leek: Extraction and characterization of the pectic polysaccharides were conducted with leek (*Allium porrum*) from the region of Plovdiv, Bulgaria. 1 kg fresh leek was cut into small pieces of 8–10 mm and mixed with 96% ethanol pre-heated to 65ºC. The ratio between the raw materials and alcohol was 1:2.5. The obtained mass was kept for 1 h at 65ºC and for 24 h at room temperature. Then it was filtered through cloth and washed twice with 200 ml 96% ethanol. The obtained alcohol-insoluble substance was dried at 60ºC.

Fractionation of pectic polysaccharides from leek by means of consecutive extraction with various extractants (Figure 1):

1. Water extraction – carried out twice. As a first step, 70 g AIS from leek were extracted with 1400 ml water at 80ºC at continuous stirring for 45 min. The obtained mixture was filtered through cloth. A second extraction was applied for 20 min with 700 ml water, at 80ºC and then the mixture was filtered again. The two combined filtrates of pH~6 were precipitated with ethanol to isolate the water soluble pectic polysaccharide from leek;

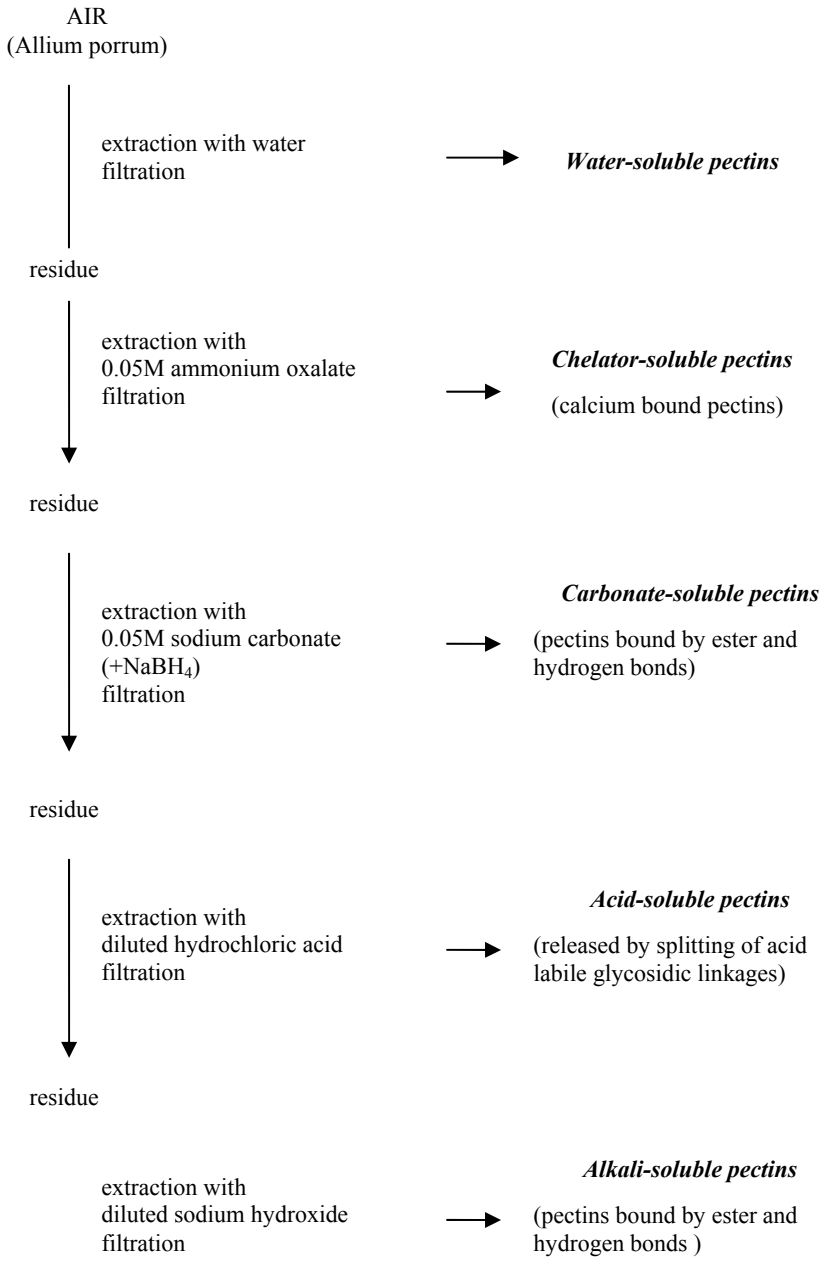

Fig. 1. Scheme for extraction of pectins proposed by Voragen *et al*. [1].

2. Chelate extraction – for this purpose 930 ml 0.05 M aqueous solution of ammonium oxalate was added to the residue obtained after the water extraction. The extraction took place for 4 h at 20ºC followed by continuous stirring at pH~6. The obtained mixture was filtered through cloth. The residue was subjected to a second extraction with 760 ml 0.05 M aqueous solution of ammonium oxalate for 30 min at the same temperature. The combined filtrates were precipitated with ethanol to obtain the chelate-extractable pectic polysaccharide;

3. Carbonate extraction – it was conducted twice with 0.05 M aqueous solution of sodium carbonate. 930 ml were added to the residue from the chelate extraction at 1ºC followed by continuous stirring for 17h at pH~10. The first extraction with carbonates was conducted in the presence of 280 mg sodium borehydride ($NaBH_4$). The obtained mixture was filtered through cloth. A second extraction was conducted for 3 h with 885 ml 0.05 M solution of sodium carbonate at 20ºC, but without sodium borehydride. The obtained mixture was filtered through cloth. The residue from the second extraction with sodium carbonate was washed with H_2O at a ratio of 1:10 (470 ml water, respectively) for 20 min at room temperature at stirring. The combined filtrates were precipitated with ethanol to obtain the carbonate-soluble pectic polysaccharide;

4. Acid extraction was performed twice. The first extraction was conducted with 930 ml 0.1 M solution of hydrochloric acid added to the residue from the previous extraction for 1 h followed by continuous stirring, at 80ºC and pH~1.5. The obtained mixture was filtered through cloth. A second extraction was conducted with 450 ml water for 30 min at 80ºC. The combined filtrates were precipitated with ethanol to obtain the acid-soluble pectic polysaccharide;

5. Extraction with alkali – conducted with 0.05 M solution of sodium hydroxide (590 ml) added to the residue from the previous extraction at 0ºC followed by continuous stirring for 17 h at pH~11.8. The obtained mixture was filtered through cloth and the filtrate was precipitated with ethanol to obtain alkaline-soluble pectic polysaccharide.

Isolation of the polysaccharides: Each of the obtained filtrates was precipitated with 96% ethanol at a ratio of 1:1 for 1h. The coagulated polysaccharides were isolated after filtration through cloth. The obtained pectic polysaccharides were washed consecutively with 100 ml 70% hydrochloric ethanol, with 70% ethanol to a neutral reaction and with 100 ml 96% ethanol. The washed pectic polysaccharides from leek were dried at 60ºC.

Determination of polyuronic content, degree of esterification and protein content: The assay of polyuronic content (PUC) and degree of esterification (DE) of the polysaccharides was determined by the method of Owens *et al*. [11], the protein content of leek – by Kjlthal and the polysaccharides – by Lowry [12].

Determination of monosaccharide composition: Neutral sugars were measured as alditol acetates after hydrolysis of samples. The crude extracted pectic polysaccharides (20 mg) were pretreated with 2 M trifluoroacetic acid (TFA) for 3 h at 120°C before conversion to alditol acetates according to the method described in [13].

The monosaccharide composition of pectic polysaccharides was assessed on a gas-mass chromatography system 6890 GC System Plus/5793 Mass Selective Detector (Hewlett Packard) with a column SP-2380 Supelco, 200°C for 3 min, then 5°C/min to 250°C; injector temperature 250°C, detector temperature 280°C; helium as a carrier gas at a flow rate of 1 ml/min. Peak identification was based on retention times, using myo-inositole as an internal standard.

Determination of molar mass: The molar masses of the crude polysaccharides were assayed through HPSEC on a Waters (Millipore) system. The assay was made on Ultrahydrogel™ 120 and Ultrahydrogel™ 500 column (7.8×300 mm, Waters) with bidistilled water as eluent at an eluation rate of 0.8 ml/min. The column was calibrated using the Shodex standard P-82 (Showa DENKO, Japan).

Determination of biological activity: The immunological activity of pectic polysaccharides was assayed applying the following biological tests: protective activity of the polysaccharides in an experimental bacterial infection, killing capacity of the peritoneal macrophages obtained from mice treated with polysaccharides [14] and activation of the complement system in blood serum by a classical pathway [15] and by an alternative pathway [16].

RESULTS AND DISCUSSION

The analysis of AIR obtained from leek, crop 2002, from the region of Plovdiv, Bulgaria showed that the major polysaccharides in the leek were pectic substances – 23.3%, which had a high degree of esterification (73–74%) and cellulose – 21.7%. The protein content of the leek was 24%. The results from the consecutive fractional extraction of AIR from leek carried out following the scheme used by

Voragen [1] showed that five pectic polysaccharide fractions of commensurate yields (2.4 – 3%) were obtained (Table 1). The obtained pectins differed in their polyuronic content, degree of esterification, neutral sugar content and protein content. The highest PUC was observed in the water- and chelate-extractable fractions. The acid- and alkaline-extractable pectic polysaccharides contained about 30% uronic acids. In the polysaccharide fractions, a presence of glucuronic acid was observed. Its content was about 10% of the total polyuronic acid content. Only the first two fractions had a high degree of esterification: 76.59 and 61.53%. The first fraction had also very high protein content (8%). In the other polysaccharides the protein content was between 1.3 and 2.2%.

Table 1. Yield and characteristics of pectic polysaccharides obtained by a consecutive extraction with different reaction agents from AIS of leek.

Polysaccharides*	yield, per100 g AIR	Polyuronic content, %	Degree of esterification, %	Neutral sugar, %	Protein by Lowry, %
A	3.39	73.6	76.6	18.4	8.0
B	3.11	64.1	61.5	34.3	1.6
C	3.60	49.3	32.1	48.5	2.2
D	3.60	27.5	26.1	71.1	1.5
E	2.94	28.0	12.6	70.8	1.3

* A – water-extractable pectic polysaccharide, B – chelate-extractable pectic polysaccharide, C – carbonate-extractable pectic polysaccharide, D – acid-extractable pectic polysaccharide, E – alkaline-extractable pectic polysaccharide.

Interesting data on the neutral sugars in the obtained pectic polysaccharides were found as it is seen in Table 2. It is assumed that the extraction with water and chelate reagents was not destructive and, therefore, the amount of the neutral sugars in the first two polysaccharide fractions was lower (less than 35%). It must be noted, however, that the polysaccharide obtained with a chelate extractant (ammonium oxalate) had almost twofold more neutral sugars than the water-extractable pectic polysaccharide. Twice as high was the content of galactose (increased from 13 to 24%), arabinose (1.6 and 3.7%) and glucose (1.9 and 3.4%). It is well known that polysaccharides extracted by aqueous solutions of chelate extractants are mostly pectic chains of homogalacturonan linked to each other through calcium bridges, which form the so called "egg-box" systems in the cell walls of plants [1, 17]. Obviously, in our case the degradation of these "egg-box" systems through the linkage of Ca^{2+} by the chelating extractant and the degradation of homogalacturonan, were accompanied by the degradation of part of the rhamnogalacturonan, containing galactose and arabinose. This led to a relative increase in the amount of the neutral sugars in the second fraction and probably to a higher molecular heterogeneity of this fraction (Figure 2). During the extraction of pectin from tomatoes with a chelating extractant, Round et al. have also found out that the extracted pectic polysaccharide contains ~15% of the branched part of the pectic molecule [18].

Table 2. Monosaccharide composition of pectic polysaccharides from leek, obtained through a consecutive extraction with different extractants (as % of the total amount of carbohydrates).

Monosaccharides*	A	B	C	D	E
rhamnose	2.4	3.0	14.4	17.0	28.3
fucose	-	-	1.1	-	-
ribose	0.8	-	1.2	-	-
arabinose	1.6	3.7	9.5	5.1	5.9
xylose	0.1	0.2	0.3	2.1	2.2
manose	trace	trace	trace	trace	trace
galactose	13.2	24.7	20.0	45.0	30.7
glucose	1.9	3.4	3.3	3.2	4.7
Σ neutral sugar	20.0	35.0	49.8	72.4	71.8
Σ uronic acid	80.0	65.0	50.2	27.6	28.2
GalA	68.2	50.2	40.3	19.3	18.4
GluA	11.8	14.8	9.9	8.3	9.8
GalA/Rha	28.4	16.7	2.8	1.1	0.7

* A – water-extractable pectic polysaccharide, B – chelate-extractable pectic polysaccharide, C – carbonate-extractable pectic polysaccharide, D – acid-extractable pectic polysaccharide, E – alkaline-extractable pectic polysaccharide.

The pectic polysaccharides in the residue after the second extraction were more strongly linked to the cell wall and because of that, they could be extracted only using hydrolyzing extracting agents. By extraction with sodium carbonate, a pectic fraction containing higher amount of neutral sugars was obtained, as the content of L-arabinose and particularly that of L-rhamnose sharply increased. Presence of fucose and ribose was also observed (Table 2). The molecular mass of this polysaccharide was lower compared to the chelate-extractable polysaccharide (Figure 2), but it was more homogenous (80% of it had 1.55×10^6 Da) in comparison with the next two pectic polysaccharides. According to Selvendran and O'Neill [17], during extraction with aqueous solution of sodium carbonate, pectins, bound to the other components of the cell wall through ester and hydrogen bonds, are released.

During As a result of extraction with dilute solution of hydrochloric acid (0.1 M), a pectic polysaccharide with a low polyuronic content (27.5%) and a very high amount of neutral sugars (71.1%) was obtained. This fraction was characterized by the highest galactose content and by the higher amount of L-rhamnose and xylose compared to the previous polysaccharide fractions, while the amount of arabinose was lower (Table 2). Apparently, the use

of hydrochloric acid for the purposes of extraction led to a breakage of the bonds of the pectin molecule with other components of the cell wall. In parallel, a hydrolysis of the glycosidic bonds in the macromolecule itself took place, which was registered by the decrease in the molecular mass (Figure 2). This observation was in correspondence with the different stability of glycosidic bonds observed at heating the pectic polysaccharides with a solution of dilute mineral acid, established by BeMiller [19] and Neukom et al. [20]. In pectins, particularly, the arabinofuranosyl linkages are most labile, followed by the glycosidic linkages between the other neutral sugar residues as well as between the neutral sugars and the galacturonic acid residues (pseudo-aldobiuronic acid linkages), glycosidic linkages between the galacturonic acid and the neutral sugars residues (aldobiuronic acid linkages). The most stable are those between the galacturonic acid residues.

As a result of the extraction with diluted solution of sodium hydroxide (at pH~11.8), a polysaccharide with a high content of neutral sugars, low content of uronide and the lowest amount of protein was obtained (Table 1). There was a considerable increase in the amount of L-rhamnose in comparison with the other pectic fractions (Table 2). This polysaccharide contained similar amounts of uronic acids (28%), L-rhamnose (27.9%) and galactose (30.3%). At these conditions, degradation also took place in terms by hemicellulose breakdown [6]. This was confirmed by the data on the relative increase of the amount of glucose and xylose as compared to the other pectic polysaccharides. The molecular mass (Figure 2) of the alkaline-extractable polysaccharide from leek was between 4.0×10^6 and 2.9×10^4 Da.

The data presented in Table 2 show that when water and chelating extractants were used, pectic fractions with a smaller content of L-rhamnose were obtained, as the ratio GalA/Rha varied from 16.7 to 28.4. According to Redgwell and Selvendran [9], under these conditions the pectic polysaccharides from the middle lamella of the plant cell wall dissolved. With the other extraction agents, pectic polysaccharides from the primary cell wall were isolated. Their content of L-rhamnose increased, as the ratio GalA/Rha became between 2.8 and 0.7. These facts gave us ground to assume that the pectin macromolecule of leek had areas of different composition, which also differed in terms of linking to the cell wall of leek.

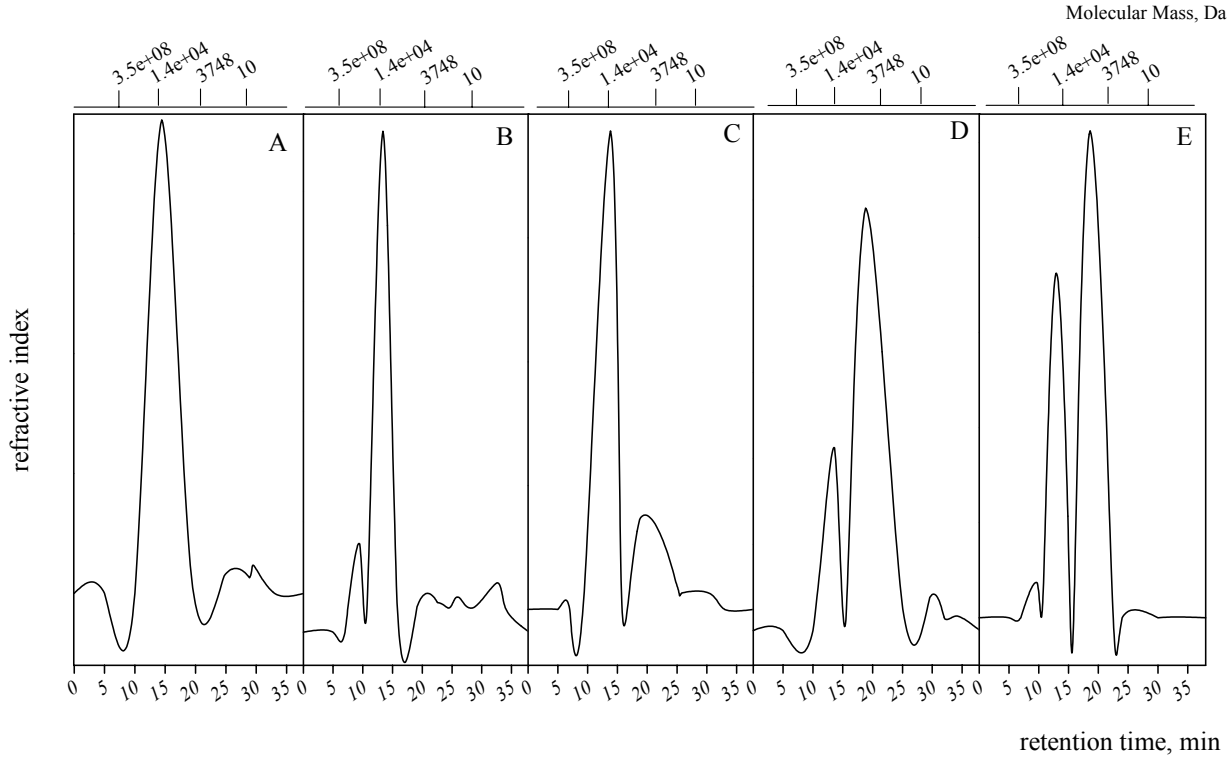

Fig. 2: HPSEC elution patterns of fractionated pectic polysaccharides from leek by means of consecutive extraction with various extractants: A - water extraction; B - chelate extraction; C - carbonate extraction; D - acid extraction; E - alkali extraction.

Table 3. Biological activity of pectic polysaccharides from leek obtained through consecutive extraction with various extractants.

Polysaccharide*	Survivability, days	Complement activation, % inhibition of the hemolysis					Killing index
		Alternative pathway			Classical pathway		
		1000 μg/ml	500 μg/ml	250 μg/ml	1000 μg/ml	500 μg/ml	
A	17.2±3.2	95.9±3.8	35.4±4.1	19.6±2.3	34.7±2.4	0	12.5±1.8
B	12.2±2.5	62.1±8.2	27.4±4.2	9.3±1.5	18.8±4.2	0	10.5±1.6
C	14.8±3.4	79.9±11.4	28.9±4.4	0	6.7±0.8	0	13.8±1.2
D	11.8±2.1	25.3±3.5	6.5±0.8	0	11.2±3.9	0	14.4±3.2
Control	6.6±2.1						5.4±0.9

A – water-extractable pectic polysaccharide, B – chelate-extractable pectic polysaccharide, C – carbonate-extractable pectic polysaccharide, D – acid-extractable pectic polysaccharide.

The obtained five pectic polysaccharides were biologically active. The data presented in Table 3 show that they are good immunostimulators. Treating a test mice with the pectic polysaccharides caused a significant increase in the number of the peritoneal macrophages, which are effector cells of the natural resistance, unlike the control, non-treated mice. The data correlate with the observed protection in the experimental salmonella infection manifested in increased survivability of the mice treated with polysaccharides. At a concentration of 1000 μg/ml, the polysaccharides resulted in a considerable activation of the serum complement, which was more distinct in the alternative rather than in the classical pathway. Trials with a reducing concentration of the polysaccharides distinguished the water- and chelate-extractable polysaccharides, characterized by the highest PUC and molecular mass, as most strongly activating the complement.

CONCLUSION

The approach of consecutive extraction of AIR from leek led to obtaining pectic polysaccharides of different composition. Water- and chelate-extractable polysaccharides present in the middle lamella had the highest polyuronic acid content and molecular mass. Extraction with solution of sodium carbonate led to obtaining pectic polysaccharides with increased total amount of monosaccharides and higher homogeneity than the chelate-extractable polysaccharide. Application of hydrochloric acid as extracting reagent showed a sharp increase in the amount of neutral sugars. In each fraction the prevailing monosaccharide was galactose and its content increased with every extraction step. It can be assumed that this is due to the presence of polysaccharide built by galactose residues in the cell wall of leek bound to the pectin macromolecule. The glucuronic acid could be found in every isolated fraction.

This investigation demonstrated also the heterogeneity of the pectic molecule. The pectic polysaccharides isolated from leek had sections differing in terms of composition. In some of them, the galacturonic acid prevailed, as the ratio between GalA/Rha in water- and chelate-extractable polysaccharides varied from 28.4 to 16.7 and in the other isolated polysaccharides it was between 2.8 and 0.7.

The pectic polysaccharides isolated through consecutive extraction from the cell wall of leek possess biological activity and are good stimulators of the immune system. Pectic polysaccharides with a higher uronic content and molecular mass show also higher immunostimulating activity.

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ХАРАКТЕРИЗИРАНЕ НА ПЕКТИНОВИ ПОЛИЗАХАРИДИ ОТ ПРАЗ ПОЛУЧЕНИ ЧРЕЗ ПОСЛЕДОВАТЕЛНО ЕКСТРАХИРАНЕ С РАЗЛИЧНИ РЕАГЕНТИ

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Посветена на акад. Иван Юхновски по повод на 70-та му годишнина

Постъпила на 7 февруари 2008 г.; Преработена на 18 март 2008 г.

(Резюме)

Получени са пет съизмерими по добив, но различни по състав пектинови полизахаридни фракции чрез последователна екстракция с вода, разтвори на амониев оксалат, натриев карбонат, солна киселина и натриева основа от алкохолно неразтворима част (АНЧ) на праз. В полиуронидния състав на тези фракции освен галактуронова киселина се открива и наличие на глюкуронова киселина. В състава на неутралните захари (НЗ) доминира галактозата последвана от рамнозата. Водно-екстрахируемият пектинов полизахарид е високо хомогенен (93.3% с молекулна маса 1.3×10^6 Da) и с белтъчно съдържание 8% (най-високо в сравнение с останалите полизахариди). При екстракцията с разредена солна киселина се получава полизахарид с най-високо съдържание на НЗ (71.1%) и ниско количество на уронови киселини. Водно- и хелатно-екстрахируемите фракции имат по-ниско съдържание на L-рамноза (съответно 2.7% и 2.9%), а останалите полизахаридни фракции от праз се отличават с високо съдържание на L-рамноза(от 14 до 28%). Пектиновите полизахариди на праза са добри имуностимулатори. Най-висока имуностимулираща активност показват водно- и хелатно-екстрахируемите полизахариди, които се характеризират с високо съдържание на полиуроними и молекулна маса над 10^6 Da.