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Influence of composition on the biological activity of pectic polysaccharides from leek

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ABSTRACT

Water-soluble and acid-soluble pectic polysaccharides with significant immunostimulating activity were isolated from alcohol-insoluble solids (AIS) of leek. Pectic polysaccharides characterized with high anhydrouronic acid content and common pectic sugars – L-arabinose, D-galactose, D-glucose, and L-rhamnose. It was accomplished that D-galactose content was the highest, which allowed the assumption that pectin sugar side chains were from galactan type. Enzyme modification of acid-extracted pectin was realized. Different pectin-degrading enzymes were used. The first modified pectic fraction, obtained after endopolygalacturonase action and alcohol precipitation contained rhamnogalacturonan I and had high D-galactose content. The second fraction obtained from the first, after β-galactosidase treatment characterized with low D-galactose content. Probably this was result from β-galactosidase hydrolysis of pectin side chains. It was proved by the action of β-galactosidase that galactan side chains were mainly composed of β-(1→4)-linked D-galactose. It was accomplished that rhamnogalacturonan I had higher immunostimulating activity than the initial pectic polysaccharide, which confirmed the statement that pectin hairy regions were responsible for the biological activity. The second fraction had lower D-galactose content and lower activity than the first. Probably galactan side chains were responsible for the immunostimulating activity.

Key words: leek, pectic polysaccharides, pectic enzymes, immunostimulating activity

Introduction

Pectins are the most structurally complex polysaccharides in plant cell walls and determining their chemical structure and precise biological roles still provides a significant challenge. However, in the last decade the information available on pectin structure has widened considerably, and our understanding of the structure-function relationships of pectins.

Their structure generally encompasses homogalacturonan (HG), rhamnogalacturonan type-one (RG-I) and rhamnogalacturonan type-two (RG-II) (Figure 1). These three pectin constituents are covalently linked to one another to form the pectin macromolecule. HG consists of an unbranched molecule composed of 1,4-linked α-D-galacturonic acid (GalA) and may have a chain length of ~100 consecutive GalA residues (Thibault et al., 1993). The GalA residues can be methyl-esterified at C-6, and carry acetyl groups at O-2 and O-3. RG I is a side-chain-containing polysaccharide, whose backbone is believed to be consist of [→4-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→] disaccharide repeating units (Albersheim et al., 1996). L-Rhamnosyl residues can be substituted at O-4 with neutral sugars (McNeil et al., 1980; Lau et al., 1987). The side chains are mainly composed of D-galactosyl and/or L-arabinosyl residues. They can be single-unit, but also neutral polymers such as arabinan, galactan, arabinogalactan I (AG-I) and arabinogalactan II (AG-II). AG-I is composed of a β-D-(1→4)-linked galactose unit, but α-L-Ara*f* residue can be attached to O-3 of the D-galactosyl residue (Carpita &

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Gibeaut, 1993; Ridley et al., 2001).

Rhamnogalacturonan II is conservative structure, which consists of a backbone of about nine α-(l→4)-linked D-galacturonosyl residues carrying four side chains, containing a number of rare sugars (apiose, aceric acid, KDO and DHA). The general basic structure of RG-II is published by Vidal et al. (2000).

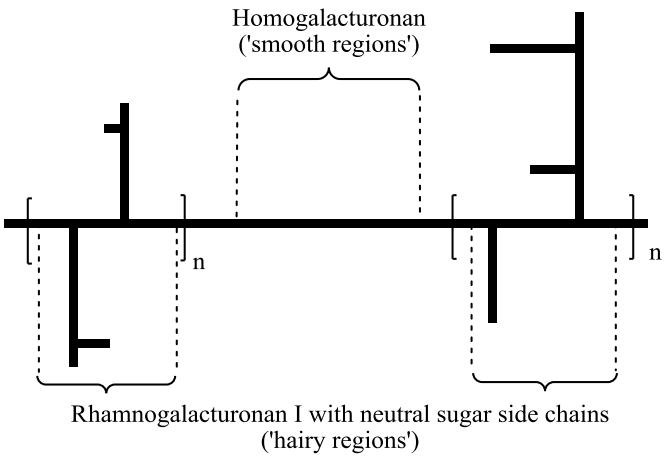

Figure 1. *Schematic structure of pectic polysaccharides with homogalacturonan ("smooth regions") and rhamno-galacturonan backbone with neutral sugar side chains ("hairy regions"), adapted from Schols et al., 2009 (with modification).*

Pectins are used as natural food ingredients for their gelling and thickening properties (Kertesz, 1951). Food applications of pectins have been revised exhaustively by Voragen et al. (1995).

In the last three decades there is an increasing interest in pectins as prebiotics in general (Dongowski & Lorenz, 1998; Amado, 2003) and more specifically, for their different types of biological activity. Over the past 25 years, several pharmacological activities, including immunostimulating activity (Yamada & Kiyohara, 2007), anti-ulcer activity (gastroprotective action), total cholesterol-lowering effect (Cerda, 1990; Groudeva et al., 1997), anti-metastasis activity, hypoglycemic activity (Hockaday, 1987) were discovered. The ability to eliminate toxic substances, heavy metals and radioactive elements has been reported and widely studied (Stantschev et al., 1979; Tahiri et al., 2000; Yaneva et al., 2002). Most of these activities are reviewed well by Yamada (1996), Yamada & Kiyohara (2007), and Paulsen & Barsett (2005).

From all reports above, it may be concluded that the general structure and bioactivity of pectin are known in much detail, but the structure-biological activity relationship is still under debate. Also not all pectins have anti-complementary activity (Samuelsen et al., 1996). It is generally admitted that the biological activities of polysaccharides depend on their molecular structure, including monosaccharide composition, glycosidic bond of the main chain, and degree of substitution, degree of branching, sugar component and conformation of the main chain. Therefore, increasing attention was attracted to molecular modification and structure-activity relationship of pectic polysaccharides.

The purpose of the present study is to elucidate the relationship between polysaccharide structure and expressed biological activity.

Materials and Methods

Materials

Samples were of known moisture content and all calculations were on a dry-weight basis.

All enzymes used in these experiments were commercial preparations and were purchased from Megazyme International Ireland Ldt. (Bray, Co. Wicklow, Ireland). They were used without further purification. The monosaccharide standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Trifluoroacetic acid (TFA) and sulfuric acid were from Fluka (Switzerland). HPLC grade acetonitrile (Sigma) was used for preparation of mobile phase. Pullulan standards (Shodex Standard P-82) were from Showa Denko K.K. All other chemicals and reagents were commercial products of the highest purity available and were purchased locally.

The leek (*Allium ampeloprasum* var. *porrum* (L.)) was bought from a local market (Plovdiv, Bulgaria) and stored at -18°C until use.

Isolation of alcohol-insoluble solid (AIS)

AIS from leek was prepared as described previously (Kratchanova et al., 2008).

Sequential extraction of leek AIS

The water- and acid-extractable polysaccharides were isolated from AIS as reported in our previous paper (Kratchanova et al., 2010).

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Chemical analysis of pectic polysaccharides from leek

The degree of esterification (DE) of pectic polysaccharides was determined by the titrimetric method, according to the Food Chemical Codex, slightly modified for using Hinton's indicator, which gives a magenta end point at pH 7.5. Total neutral sugar content was determined with orcinol-sulfuric acid method, according to Shannon (1972).

Sugar composition analysis and protein content

The polysaccharide sample (20 mg) was hydrolyzed with 2M CF_3CO_2H (10 mL) in a closed screw-cap test tube at 121°C for 3 h (Quemener & Thibault, 1990). The hydrolyzates were evaporated to dryness in a rotary evaporator under reduced pressure and temperature (40°C). The dried sample was resuspended in distilled water (1 mL) for HPLC analysis. Neutral sugars and uronic acids were analyzed by HPLC equipped with a refractive index detector. The products were identified by their typical retention times (Kratchanova et al., 2010).

Protein content was determined by Lowry method with bovine serum albumin as a standard (Lowry et al., 1951).

High performance size exclusion chromatography (HPSEC)

HPSEC was performed on a HPLC (Waters) equipped with two Ultrahydrogel™ columns (each 300×7.8 mm) in series (500 and 120) and elution at 25°C with 0.1M $NaNO_3$ at 0.8 mL/min. The eluate was monitored using a Waters R-401 refractive index detector. The system was calibrated with pullulan standards, having molecular weight values in the range 5.9÷788 kDa.

Enzymatic degradation of pectic polysaccharides from leek

A saponification step was performed for removing methyl ester groups before incubation with enzymes. Acid-extracted leek pectin was saponified with 0.1M NaOH for 24 h at 4°C. Then the samples were neutralized with 3M HCl and precipitated with two volumes 96% ethanol for 1 h at 4°C. The mixture was filtered through a G2 sintered glass. The precipitate was squeezed free of alcohol, purified by washing in more alcohol. The insoluble residue was washed with 96% (v/v) ethanol and freeze-dried and milled prior to further analysis. The DE value of saponified AEPL from leek was also determined by the titrimetric method (see before).

Polygalacturonase treatment

Acid-extractable pectic polysaccharide isolated from leek was dissolved (5 mg/ml) in 50 mM NaOAc buffer, at pH 5.0 and incubated with 0.32 units of polygalacturonase from *A. aculeatus*. The incubations were performed at 40°C for 24 h under continuously stirring, and the digests were subsequently heated for 5 min at 80°C to inactivate the enzyme (optimum pH and temperature conditions specified by the manufacturer). The enzyme and degraded polysaccharide were precipitated by the addition of 96% ethanol (2 vol.) (see below). The variations in molecular weight (Mw) and the release of oligomeric products were analyzed by HPSEC.

Treatment with galactan-degrading enzymes

The alcohol precipitate residue after PG-ase action was used as substrate for further treatment with galactan-degrading enzymes. The incubations were performed at 40°C for 24 h with the following enzymes individually: 2.5 units of endo-β-(1→4)-galactanase (*Asp. niger*), 0.8 units of β-galactosidase (*Asp. niger*), α-galactosidase (from guar seed) and the digests were subsequently heated for 5 min at 80°C to inactivate the enzyme. The undegraded polysaccharide was precipitated with two volumes of 96% ethanol (see below).

Alcohol precipitation procedure

The pectic polysaccharide in the reaction mixture was isolated and purified by precipitation and washed with two volumes of cold 96% (v/v) ethanol. The mixture was stored at 4°C at least for an 1 hour to allow complete formation of insoluble complexes. Then it was centrifuged for 20 min (6000 rpm) to separate the precipitate formed from the supernatant. The sediment was washed twice with the same concentration of ethanol. The precipitate was resuspended in water, freeze-dried, and weighed. The dried pectin was stored at room temperature, until use. The pectin so obtained was referred to as alcohol-precipitated residue (APR).

Determination of the complement hemolysis through the classical pathway (TCH_{50})

The effect of polysaccharides on complement activity were tested using a micro-assay method according to Klerx et al. (1983) and Kiyohara et al. (1989).

Results and Discussion

Sequential extraction of pectic polysaccharides from AIS of leek

Extraction of pectins from plant material is difficult because of their complicated chemical nature and their association with other cell wall materials. Very often the

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plant material is preheated with ethanol to deactivate the endogenous enzymes and to remove some soluble components, including free sugars, amino acids and some phenols, and pigments. With the hypothesis that a sequential extraction scheme using various chemical extracting agents may solubilise, pectins of different structural features, water and 0.5% hydrochloric acid were used to obtain water- and acid-soluble pectin from AIS of leek (Voragen et al., 1995; Kratchanova et al., 2008). In the text these two pectins are referred to as WEPL and AEPL. The yields and results of the composition analysis of isolated pectic polysaccharides from leek are summarized in Table 1.

Table 1. *Chemical characteristics of the initial pectic samples, extracted from leek AIS (% w/w).*

Polysaccharides	Yield	Uronide content	DE	NS	Protein
WEPL	2.8	79	74	12	9
AEPL	4.8	49	65	45	6

The yield of acid-extracted pectin is higher than water-extracted pectin. The degree of esterification (DE) of water and acid-extracted pectic polysaccharides were 74% and 65%, respectively. The water-extracted pectin characterized with high content of D-galacturonic acid (79%) and a relatively low content of neutral sugars (12%). On the other hand D-galacturonic acid content of the acid-extracted pectic polysaccharide decreased to 49%, and the total neutral sugar content increased to 45%. Pectic polysaccharide extracted at lower pH-values has higher neutral sugar content than pectin extracted at higher pH-values. Apparently the use of hydrochloric acid for extraction led to a breakage of the bonds of pectin molecule with other high molecule components of the cell wall. Also we could consider the influence of neutral sugar content on the expressed biological activity.

The protein content in acid-soluble pectin was 6.0%, but corresponding protein concentration in water-soluble pectin increased to 9.0%.

Sugar composition of extracted polysaccharides from AIS of leek

The monosaccharide compositions of water- and acid-extractable fractions are shown in Table 2. In addition to the predominance of uronic acids (WEPL=79%; AEPL=49.8%), the fractions contained also other monosaccharide usually found in pectins, such as L-rhamnose, D-galactose, L-arabinose and D-glucose. These monosaccharides constitute the rhamnogalacturonan (RG) regions of pectin. Our results suggested that galactans and arabinogalactans were side chains in RG regions of these water and acid-soluble pectins from leek. The sugar composition suggested that leek pectin consisted of different types of neutral sugar side chains. In addition to the carbohydrates typical of pectins, the WEPL and AEPL fractions also contained small amounts of D-ribose, D-xylose and D-mannose. The sugar composition (Table 2) of the extracted pectins were rather similar to those reported in our previous studies (Kratchanova et al., 2000).

Table 2. *Sugar composition (mol %) of WEPL and AEPL*[*].

Sugar	Polysaccharides	
	WEPL	AEPL
Rha	2.77	14.13
Rib	1.30	1.34
Ara	1.61	3.82
Xyl	0.27	0.37
Man	Trace	Trace
Gal	13.30	25.96
Glc	1.72	4.57
GalA	71.10	37.48
GlcA	7.3	12.33

[*] These data were taken from Kratchanova et al., 2010

Enzymatic hydrolysis of pectic polysaccharides from leek

The enzymatic hydrolysis of AEPL from leek was made according to Figure 2. The DE of saponified pectic polysaccharide was 6.0%.

Endo-polygalacturonases (EC 3.2.1.15, PG-ases) randomly cleave α-(1→4)-D-galacturonosidic linkage in homogalacturonan by hydrolysis. PG-ases generally prefer the nonesterified substrate, polygalacturonic acid, and show decreasing activities with increasing degree of methyl-esterification (Voragen et al., 2001). Digestion with endo-α-(1→4)-polygalacturonase after deesterification gives ramified fraction, which is enzyme-resistant and several oligogalacturonides.

The separation of hydrolysis products was carried out by alcohol precipitation (AP). It is generally assumed that oligosaccharides above a certain molecular weight will be precipitated by the addition of 96% ethanol, and those below that Mw will remain in solution. The biological activities are greatly influenced by molecular mass and polydispersity, therefore, it is necessary to determine the molecular mass of the alcohol precipitated residue after PG-ase treatment.

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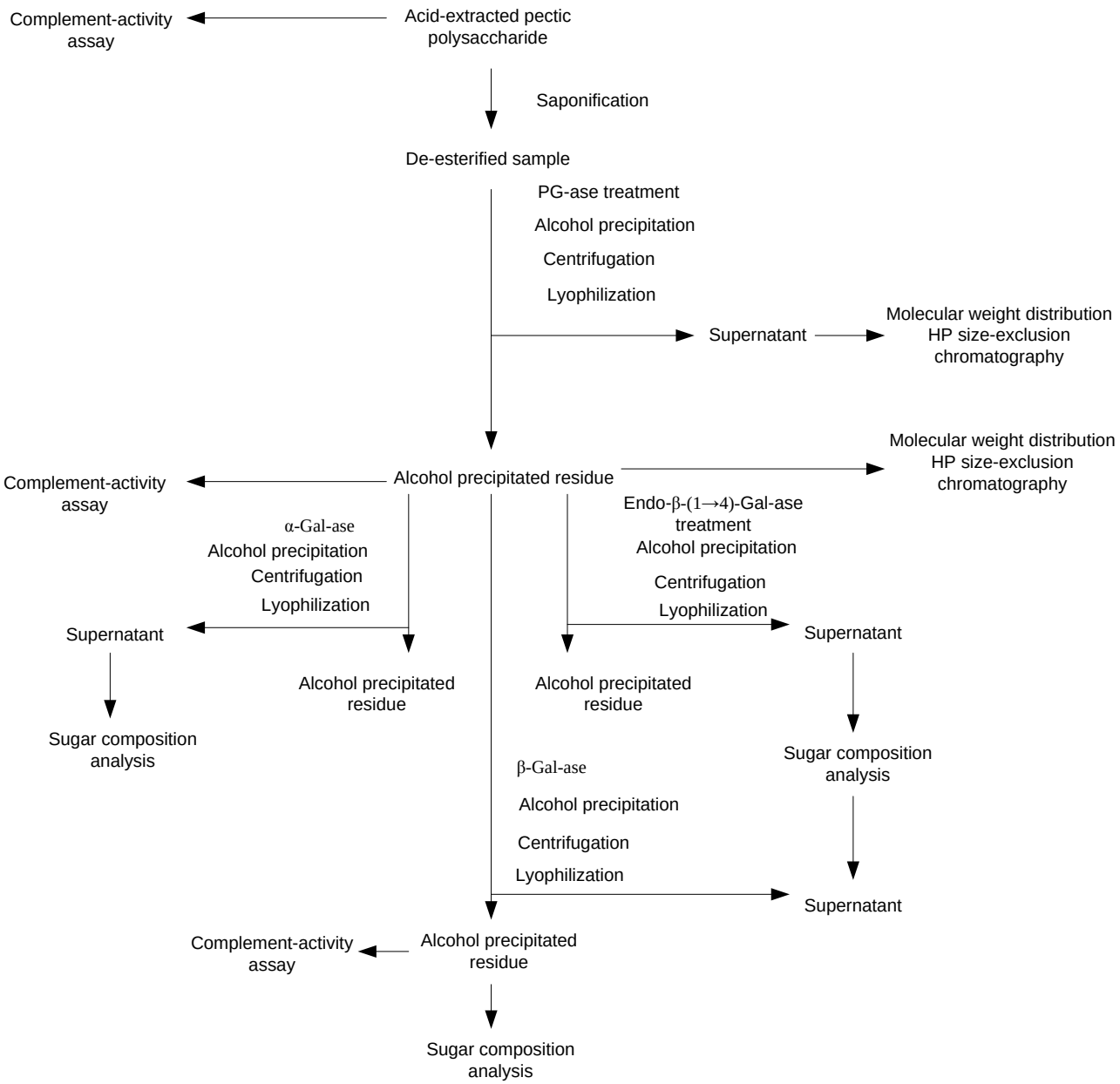

Figure 2. *Flow diagram of pectin modification procedures with pectin-modifying enzymes.*

Supernatant solutions were also analyzed by high performance size-exclusion chromatography, in order to determine the size of the components that were precipitated. Mw distribution of the polysaccharides obtained from AEPL after PG-ase action is shown in Table 3. Polysaccharide fractions obtained after hydrolysis with PG-ase differed in molecular weight and homogeneity. Enzyme modification with PG-ase showed a significant effect on molecular weight distribution and molecular weight averages of acid-extracted pectic polysaccharide. In result of the hydrolysis of homogalacturonan with PG-ase we obtained four low molecular products with Mw from 10^2 to about 10^4 Da in the ethanol solution. However, significant amounts (48%) of molecules with Mw $5.1\times10^4\div4.1\times10^5$ Da remained in the

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alcohol precipitated residue. In an additional experiment PG-ase also was found in the supernatant after alcohol precipitation. The Mw of PG-ase is 42 000 Da submitted by the producer and this value is not included in Table 3.

Table 3. *Molecular heterogeneity of APR and supernatant obtained from acid-extracted pectin after saponification and degradation with PG-ase.*

Alcohol precipitated pectin/residue		Supernatant	
Mw, Da	**%**	**Mw, Da**	**%**
4.1×10^5	13.0*	1.0×10^4	10.1*
5.1×10^4	35.0	5.0×10^3	10.0
	48.0	2.0×10^2	16.2
		1.0×10^2	15.2
			51.5

* % of totals

Sugar composition of the saponified AEPL and alcohol precipitated residue after degradation with PG-ase is shown in Table 4. L-Arabinose and D-galactose presented in reasonable amounts. The APR was relatively enriched in L-Rha, L-Ara, and D-Gal, which suggested higher proportions of hairy regions.

Table 4. *Sugar composition (% w/w) of the saponified acid-extracted pectin and alcohol precipitated residue after degradation with PG-ase.*

Sugar	AEPL	
	Initial	De-esterified and digested with PG-ase
Rha	1.6	6.4
Ara	27.2	10.5
Gal	35.1	13.0
GalA	14.0	10.3
GlcA	5.6	3.9
Neutral oligomers	5.0	6.0
Uronide oligomers	11.6	50.0

To obtain more information about the structure of D-galactose-containing side chains, the alcohol precipitated residue after PG-ase digestion was incubated separately with endo-β-(1→4)-galactanase (EC 3.2.1.89, endo-β-(1→4)-Gal-ase) from *A. aculeatus*, α-galactosidase (EC 3.2.1.22, α-Gal-ase), β-galactosidase (EC 3.2.1.23, β-Gal-ase). These enzymes cleave 1→4 linkage between α- and β-linked galactosyl residues respectively within galactan chains, and release mono-galactose and galactose oligomers. β-Galactosidase shows some activity on α-L-arabinosides, β-fucosides, and β-galactans, because of the wide tolerance for the structure of the aglycone (Mahoney R., 2003; de Vries & Visser J., 2003).

The highest degree of degradation of the substrate (RG-I) was reached by β-galactosidase – over 40% of the substrate was converted into monosaccharides (Table 5). D-Galactose (24.5%) and L-arabinose (12.6%) were mainly accumulated sugars in the supernatant. Based on the specificity of β-Gal-ase and endo-β-(1→4)-Gal-ase activity, we concluded that the individual monosaccharide units, constituting the side chains of galactan pectic polysaccharide fraction were connected by β-(1→4)-glycosidic bonds. The data from Table 5 showed that α-Gal-ase is hardly able to degrade the substrate side chains. These results pointed out that α-glycoside binding of D-Gal did not exist in the research pectin polysaccharide from leek. Surprisingly, only okra pectin showed that terminal α-linked galactosyl groups were presented as neutral side chain substituents (Sengkhamparn et al., 2009). Attention was given to the fact that products obtained after endo-β-D-(1→4)-Gal-ase action contained significant amount of D-Gal. The absence of L-rhamnose after hydrolysis with enzyme was a confirmation that the enzyme is not able to degrade rhamnogalacturonan I core. This is possible due to the specificity of the enzyme to degrade β-D-(1→4)-linked galactose units in neutral galactan polymers. This is a proof that the composition of AEPL from leek present structure, corresponding to the β-(1→4)-galactan as a side chain.

Table 5. *Sugar composition (% w/w) of the supernatant obtained after degradation with galactan-degrading enzymes and alcohol precipitation.*

Sugar	Supernatant AEPL		
	endo-β-(1→4)-Gal-ase	α-Gal-ase	β-Gal-ase
Rha	nf	0.8	3.2
Ara	6.0	3.6	12.6
Gal	9.4	nf	24.5
Xyl	nf	1.5	1.5
Total	15.4	5.9	41.8

nf, not found

Anti-complementary activity

It was interestingly to investigate the influence of obtained pectic fractions after enzymatic digestion on the

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biological activity and especially the complement activation through the classical pathway (Table 6).

Table 6. *Anti-complementary activity of the acid-extracted polysaccharide and the enzyme digestion products (AEPL-PG-ase and β-Gal-ase) through the classical pathway.*

Polysaccharide	Treatment	Concentration (μg/mL) 1250	625	312
		Inhibition of TCH_{50} (%)		
AEPL	Original (No treatment)	14.3	13.8	9.6
	De-esterified Enzyme digested[a]	44.3	27.2	13.3
	De-esterified Enzyme digested[b]	23.1	4.6	9.4

[a] digested with PG-ase;
[b] after PG-ase treatment APR was digested with *β*-Gal-ase.

The anti-complementary activity of the samples was dose-depending. The obtained after hydrolysis with PG-ase fraction (rich in RG-I) was with higher activity than the initial polysaccharide. The same tendency remained at the three different doses. This result showed that ramified regions ("hairy regions") were responsible for the expressed immunnostimulating activity and it also confirmed Kiyohara's observations that "hairy regions" have stronger complement activating ability (Kiyohara et al., 1988, 1989; Yamada & Kiyohara, 2007). After β-Gal-ase action galactan side chains were degraded, which led to decrease in activity of the newly obtained pectic fractions. Compared to the starting pectin, the anti-complementary activity of the residue after β-Gal-ase treatment was decreased approximately 2 times at 1250 μg/mL. Therefore galactan chains were responsible for complement activation through the classical pathway. Another structure–activity study showed that pectin with unbranched regions had low activity, while highly branched regions had higher activity (Samuelsen et al., 1996; Paulsen & Barsett, 2005). De-esterification and de-acetylation did not alter the anti-complementary activity (Samuelsen et al., 1996).

Conclusion

Using specific enzymes (endo-polygalacturonase, endo-β-(1→4)-galactanase, α-galactosidase and β-galactosidase) led to obtaining of hydrolysis products, representing individual fractions from pectic macromolecule of leek. In result of this it was appeared the influence of individual structural fragments from the macromolecule on its immunostimulating activity. Obtained hydrolysis products rich in RG-I participated at most in the activation process of the immune system.

Under the action of β-galactosidase on the fraction rich in RG-I was obtained new fraction with reduced D-Gal content. This fraction was composed of β-(1→4) glycosidic linked galactosyl residues and it had lower immunostimulating activity, which proved the role of galactan side chains on the complement activation through the classical pathway.

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