Bulgarian Chemical Communications, Volume 46, Special Issue A (pp. 71 – 78) 2014

Purification and biological activity of pectic polysaccharides from leek

M. Nikolova[1], M. G. Kratchanova[1]*, E. V. Pavlova[1], I. Ianakieva[1], V. Kussovski[2]

[1]*Laboratory of Biologically Active Substances, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 139 Ruski blv., 4000 Plovdiv, Bulgaria*
[2]*Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl. 26, 1113 Sofia, Bulgaria*

Received May 20, 2014; Revised July 04, 2014

Dedicated to Acad. Dimiter Ivanov on the occasion of his 120th birth anniversary

The pectic polysaccharides isolated from leek through consecutive water and acid extractions with 0.5% HCl were purified by ion-exchange chromatography on DEAE-Sepharose fast flow. As a result four fractions – W_1, A_1, A_2 and A_3 were obtained, which were partitioned with gel-chromatography on Sephadex G-200 and other fractions were obtained - W_{11}, A_{11}, A_{21} and A_{31}. All fractions differ on their monosaccharide compositions. In the A_1 fraction obtained through chromatography purification of the acid-extractable polysaccharides prevail neutral sugars (> 58%) whereas the other fractions have higher uronic acid content. It was found that fractions having higher uronic acid content also contain glucuronic acid. The dominant neutral monosaccharides in the pectic polysaccharides from leek are galactose and rhamnose. The molecular mass and the polydispersity of the polysaccharide fractions were determined by HPLC. The purified polysaccharide fractions from leek showed immunomodulating activity, which was determined by the alternative and classical pathway. The activity of the purified polysaccharides increase compared to the activity of the initial polysaccharides.

Key words: pectic polysaccharides, purification, biological activity, leek

INTRODUCTION

The effect of pectic polysaccharides on the physiological processes in the human body, such as lipid exchange [1-4], carbohydrate exchange [2], bile acid metabolism [5,6], etc. has been the subject of a host of experimental and clinical observations. It has been found by international [1,3,7] and local [8,9] studies that the intake of pectin significantly reduces the serum cholesterol levels in the body, which is especially important in cardiovascular diseases. Other biological activities of the pectic polysaccharides, such as immunomodulating, anti-complementary and anti-inflammatory, have also been reported in the recent years [10-13].

Two pectic polysaccharides - W_0 and A_0, were obtained through sequenced water and acid extraction with 0.5% HCl in the course of our previous studies [14]. They were characterised and identified as polysaccharides of a pectic type with a pronounced immunomodulating activity. Definitely, the individual structural fragments from the macromolecule of leek influence on its immunomodulating activity after enzymatic hydrolysis. Obtained hydrolysis products rich in RG-I participated in the activation process of the immune system [15]. The purification process played an important role in determining the relationship between the structure of the polysaccharides and their biological properties. The purification of the active polysaccharides was carried out through various chromatographic methods depending on the composition of the crude polysaccharides.

The aim of this work was chromatographic purification by means of ion-exchange and gel chromatography of the biologically active pectic polysaccharides from leek and the comparison of their immunomodulating activities.

EXPERIMENTAL

The trials were carried out with leek (*Allium porrum* L.) purchased on the vegetable market. The alcohol insoluble substances (AIS) of leek were obtained by the method described in a previous article of us [16].

Isolation and purification of polysaccharides from leek

Two pectic polysaccharides were isolated by means of a sequenced water and acid extraction with 0.5% HCl from the AIS of leek. The purifycation procedure is presented in Fig. 1.

* To whom all correspondence should be sent:
E-mail: lbas@plov.omega.bg

The two isolated polysaccharides W_0 and A_0 were subjected to fractionating by means of ion-exchange chromatography on a DEAE-Sepharose fast flow using a semipreparative chromatographic system Pharmacia, Sweden, equipped with a XK 2.6/40 cm column (Pharmacia, Sweden). The eluent was a buffer solution of sodium acetate with pH=4.8 and a linear gradient (0.05-1.0 M). The elution rate was 1 ml/min. Fractions of 10 ml were collected and analyzed for content of neutral sugars and galacturonic acid.

120 mg of polysaccharide B_0 were diluted in 15 ml sodium acetate buffer (pH=4.8), and the solution was centrifuged at 15 000 rpm. 10 ml of the obtained supernatant containing 66.5 mg of B_0 was applied to a column with DEAE-Sepharose fast flow. Chromatography on the polysaccharide led to the emergence of a peak - W_1, which was collected, dialyzed for 48 h against distilled water and lyophilized on a Cryodos 50 system (Telstar, Spain).

120 mg of polysaccharide A_0 were dissolved in 15 ml sodium acetate buffer (pH=4.8) and the solution was centrifuged at 15 000 rpm. 10 ml of the supernatant containing 71.7 mg of polysaccharide A_0 were subjected to chromatographic purification on a DEAE-Sepharose fast flow. Chromatography on A_0 led to the distinction of three peaks: A_1, A_2, A_3, respectively. The isolated peaks were collected, dialyzed for 48 h against distilled water and lyophilized on a Cryodoc 50 system (Telstar, Spain).

The chromatographically purified fractions W_1, A_1, A_2 and A_3 were subjected to gel chromatographic separation on Sephadex G-200, on column XK 1.6/100 cm (Pharmacia, Sweden). The eluent was phosphate buffer (pH=6), and the elution rate was 0.2 ml/min. Fractions of 10 ml were collected in the process of elution and were analyzed for neutral sugars and galacturonic acid.

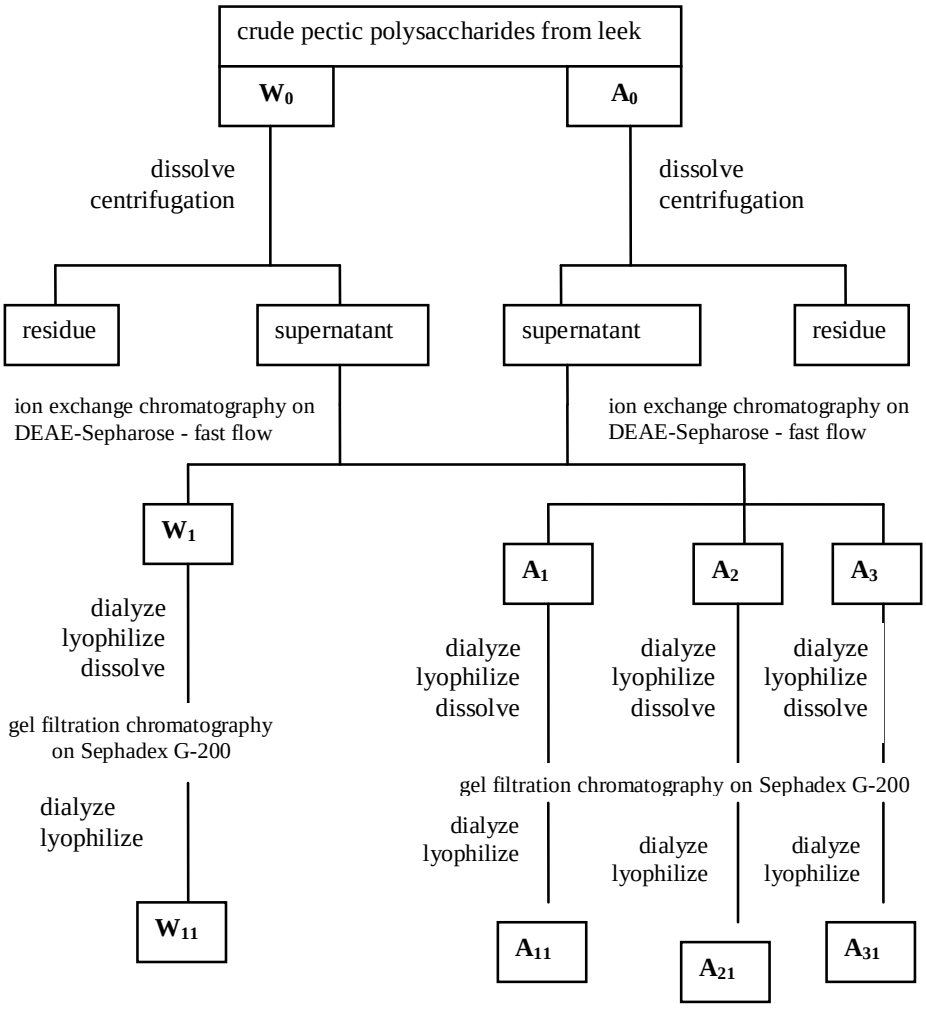

Fig. 1. Purification procedure of anti-complementary pectic polysaccharides from leek.

60 mg of each purified fraction (W_1, A_1, A_2 and A_3) were dissolved in 8 ml distilled water. 7 ml of the solution obtained (containing a sample of 52.2 mg) was applied to a column with Sephadex G-200. Fractions W_{11}, A_{11}, A_{21} and A_{31} were isolated during the gel chromatography separation. Each fraction was collected, dialyzed for 48h and lyophilized.

Major analytical methods

Assessment of the total neutral sugars, uronic acids, and protein

The total neutral sugars and uronic acids were assessed by the orcinol [17] and meta-hydroxybiphenyl methods [18], respectively, using arabinose and galacturonic acid as a standard. The separation of galacturonic acid and glucuronic acid was implemented on a Waters HPLC system with RI detector – R401. HPLC analysis was conducted on an Aminex HPX - 87H column with 0.04M H_2SO_4 and the elution rate was 0.3 ml/min. Galacturonic acid and glucuronic acid were identified by the retention time.

The protein content was analyzed by the Lowry method [19] against a standard of bovine serum albumin.

Determination of neutral sugars

Neutral sugars were measured as alditol acetates after hydrolysis. The crude extracted pectic polysaccharides (20 mg) were pretreated with 2M trifluoroacetic acid (TFA) for 3 h at 120°C before conversion to alditol acetates according to the method developed by Blakeney *at al.* [20].

The monosaccharide composition of the pectic polysaccharides was determined by HP gas chromatograph 6890 Series Plus coupled with a 5973 mass-selective detector (Helwett–Packard, Palo Alto, CA), on a column SP-2380 (Supelco). The oven temperature was programmed to 200°C for 3 min, then to 250°C with a rate of 5°C/min. Helium was carrier gas at 1 ml/min. Peak identifycation was based on retention times, using myo-inositole as internal standard.

Determination of molecular mass

The molar masses of the polysaccharides were assayed through HPSEC on a Waters (Millipore) system. The assay was made on Ultrahydrogel^{TM} 120 and Ultrahydrogel^{TM} 500 column (7.8 x 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 anti-complementary activity

Complement activity was measured using a microassay method according to Klerx *et al.* [21,22]. The test is based on the colorimetric measurement of hemoglobin released from target erythrocytes after incubation with normal human serum. In the classical pathway assay (CP), veronal buffered saline (25 mM, pH 7.4) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ served as an diluent. Sheep red blood cells (SRBC) sensitized with rabbit anti-SRBC antibodies at a concentration of $2x10^8$cells/ml were used as targets. In the alternative pathway (AP) assay, uncoated (without anti-RRBC antibodies) rabbit erythrocytes (RRBC) at a concentration of $1x10^8$cells/ml as targets were used. Activation of the calcium-dependent classical pathway is prevented by the addition of calcium chelator ethyleneglycol-bis(2-aminoethyl) tetra-acetic acid (EGTA) to the assay veronal buffer containing 5 mM $MgCl_2$ and 8 mM EGTA.

The effect of polysaccharides on complement activity was determined after pre-incubation for 45 min at 37°C of equal volumes of normal human serum (dilution giving about 50% lysis of the target cells) and two-fold dilutions of polysaccharides (usually starting with 2 mg/ml). After incubation (1.5h at 37°C) of target erythrocytes and treated sera, hemolysis in cell supernatants was measured at 541 nm. The anti-complementary activity of the samples was expressed as the percentage inhibition of the total complement hemolysis (TCH_{50}) of the control serum given by the formula:

Inhibition of TCH_{50} (%) = [(TCH_{50} of control serum - TCH_{50} treated with pectin serum)/ TCH_{50} of control] x 100.

RESULTS AND DISCUSSION

The isolated pectic polysaccharides, obtained by a sequenced extraction with water were name W_0 and 0.5% hydrochloric acid were name A_0 from AIS of leek, were purified by the procedure shown in Fig. 1.

The polysaccharides were applied to a column with ion-exchange resin DEAE–Sepharose – fast flow and eluted with sodium acetate buffer at a linear gradient (0.05-1.0 M). One fraction, W_1, was obtained from the water extractable polysaccharide, while three fractions, A_1, A_2, A_3 (Fig. 2), were obtained from the acid extractable polysaccharide.

The chemical composition of these fractions is presented in Table 1.

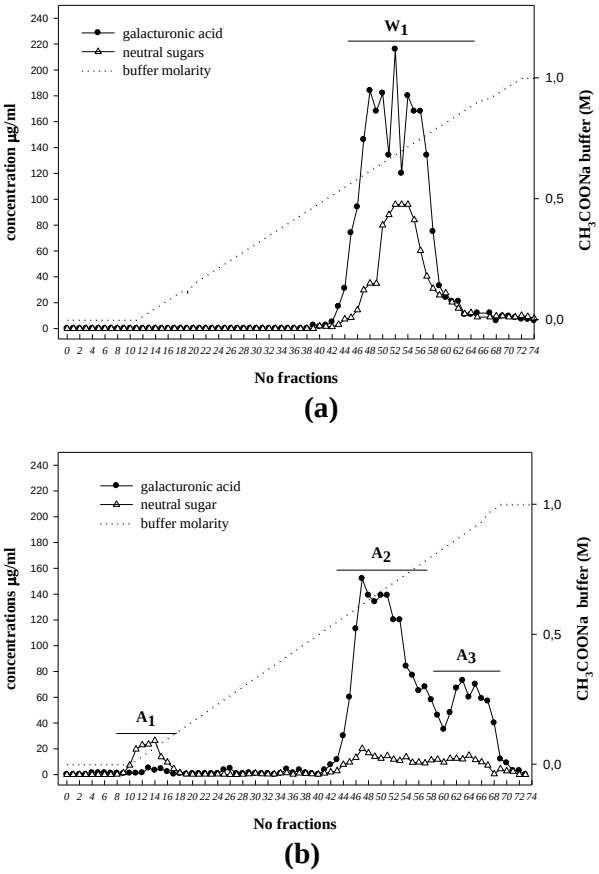

Fig. 2. Elution profile of water extractable (a) and acid extractable (b) pectic polysaccharides on DEAE-Sepharose fast flow.

The water extractable purified fraction – W_1 had a high content of uronic acids 66.4% (59.7% galacturonic acid and 6.7% glucuronic acid). Galactose (21.2%) was the prevailing neutral monosaccharide in the composition of W_1.

Fraction A_1 from the acid extractable polysaccharide contained mostly neutral monosaccarides (59.9%) and 38.8% uronic acids, as it can be seen from Table 1. No glucuronic acid was found in this fraction. This polysaccharide had a high molecular mass and the major neutral monosaccharide was galactose (47%). Most probably, it was a polysaccharide of galactan type.

The other two fractions A_2 and A_3 were acidic polysaccharides because they had a higher content of uronic acids: 50.5% and 61.2%, respectively. In both polysaccharides (A_2 and A_3), glucuronic acid was found in addition to galacturonic acid, as it occurred in higher amounts in A_3. Galactose and rhamnose accounted for the largest share of the monosaccharide.

Dialyzed and lyophilized polysaccharide fractions purified by ion-exchange chromatography (W_1, A_1, A_2 and A_3) were subjected to gel chromatography on Sephadex G-200. Each fraction was eluted with a phosphate buffer at pH=6, where fractions W_{11}, A_{11}, A_{21} and A_{31} were obtained (Fig. 3).

Fraction W_{11} was characterized by a higher content of galacturonic acid than W_1 and a lower quantity of glucuronic acid (Table 2). The nonuronide ingredients of W_{11} were dominated by the galactose monosaccharide.

In the purified fraction A_{11} the amount of galacturonic acid increased from 38.8% in A_1, to 50.7%, respectively. Total neutral monosaccharide in the purified fraction A_{11} was 48.0% mostly that of galactose (42.8%). In the other two SEC purified acidic polysasccharides - A_{21} and A_{31}, a lower content of glucuronic acid was obtained as compared with A_2 and A_3. As for the nonuronide monosaccharide, the rhamnose content in A_{31} increased to 16.3% in comparison with A_3 (10.7%).

The polysaccharide fraction A_{21} was dominated by the monosaccharide galactose.

Table 1. Chemical compositions of purified anti-complementary extractable polysaccharides on DEAE-Sepharose fast flow.

	W_1	**A_1**	**A_2**	**A_3**
Uronic acid, %	66.4	38.8	50.5	61.2
Total neutral sugar, %	33.1	59.9	48.0	37.8
Protein,%	0.5	1.3	1.5	1.0
Monosaccharides composition,%				
Rhamnose	5.8	8.2	16.0	10.7
Arabinose	5.2	4.2	8.0	2.5
Xylose	0.8	-	0.4	0.5
Mannose	trace	trace	trace	trace
Galactose	21.2	47.0	19.2	23.6
Glucose	trace	trace	4.5	trace
Galacturonic acid	59.7	38.8	41.2	43.5
Glucuronic acid	6.7	-	9.3	17.7

Table 2. Chemical compositions of purified anti-complementary extractable polysaccharides on Sephadex G-200.

	W_{11}	A_{11}	A_{21}	A_{31}
Uronic acid, %	66.2	50.7	60.6	67.1
Total neutral sugar, %	33.4	48.0	38.2	31.9
Protein,%	0.4	1.3	1.2	1.0
Monosaccharides composition,%				
Rhamnose	0.6	2.2	5.0	16.3
Arabinose	0.9	1.0	6.2	trace
Xylose	0.8	-	0.4	0.5
Mannose	trace	trace	trace	trace
Galactose	27.9	42.8	24.4	15.1
Glucose	3.2	2.4	2.2	trace
Galacturonic acid	61.6	50.7	53.7	58.2
Glucuronic acid	4.6	-	6.9	8.9

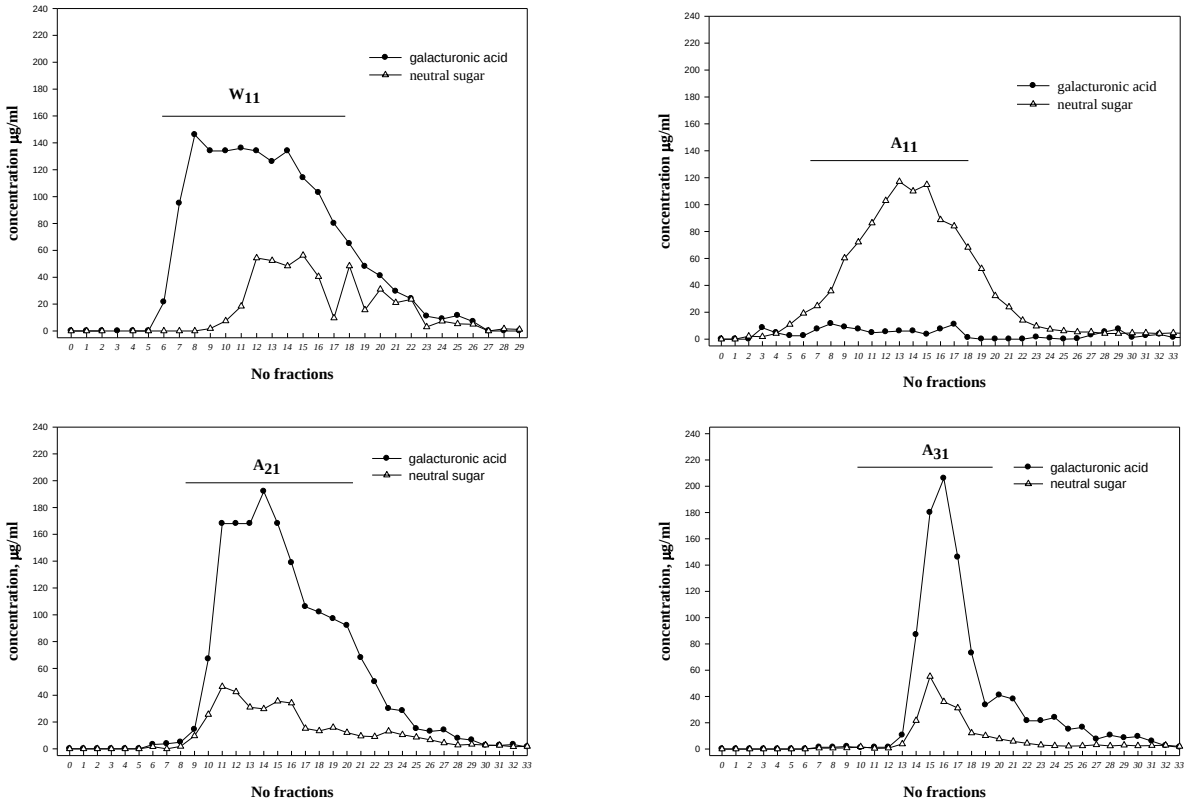

Fig. 3. Elution profile of purified pectic polysaccharides on Sephadex G-200.

The follow-up analyses of the purified samples were related to determining their molecular mass values and their molecular homogeneity (Fig. 4). Fractions W_1 and W_{11} obtained as a result of the purification and separation of the water-extractable pectic polysaccharide had the highest molecular mass, as well as the highest homogeneity.

Fractions A_1 and A_{11} made up mostly of neutral monosaccharide showed a high molecular mass and were considerably more homogeneous than the other polysaccharide fractions of the acid extractable pectic polysaccharide. Fractions A_2 and A_{21} had a higher molecular mass than A_3 and A_{31}.

All polysaccharides tested had some activity in complement-fixation test (Fig. 5). The fractions W_0 and W_1 showed the same low activity. W_{11} was signifycantly more active than W_0 and W_1. Neither the anticomplementary activity values of A_0, A_1, and A_2 nor those of A_3 showed significant differences. A_{11}, A_{21} and A_{31} were the most active fractions.

The obtained results indicate that the activation of the complement system by these polysaccharides occurs *via* both the classical and alternative pathways.

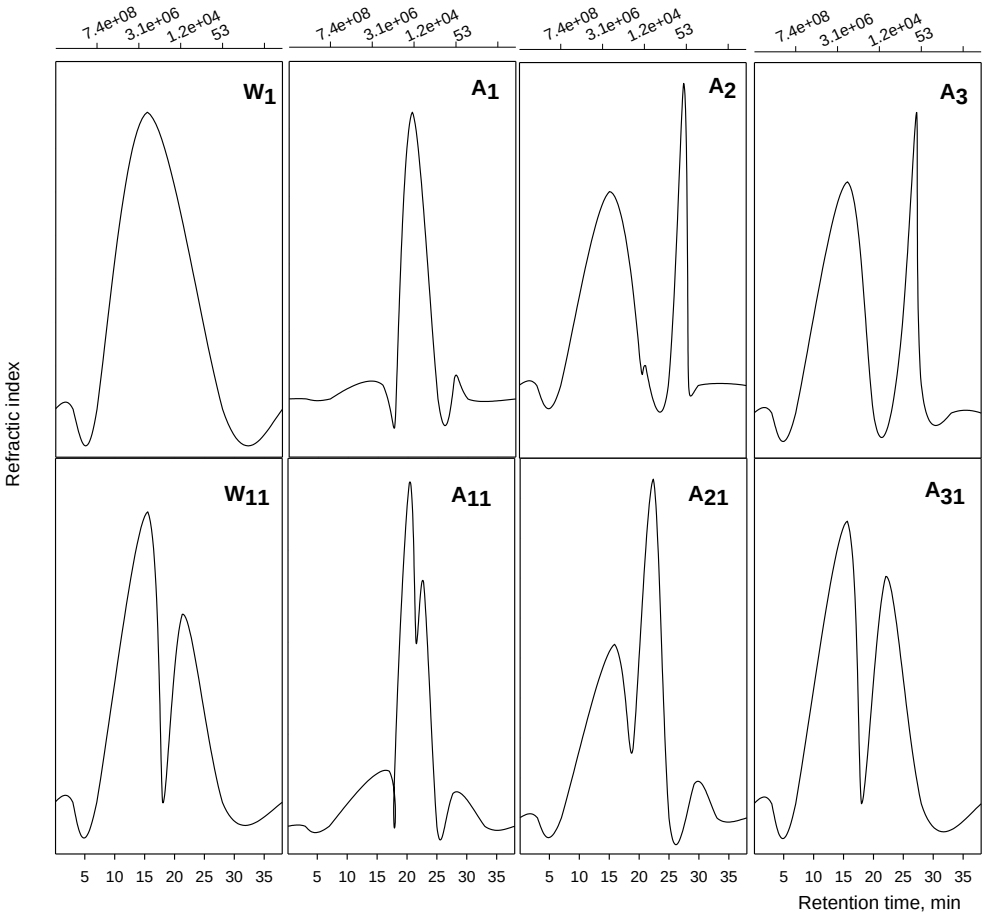

Fig. 4. HPSEC elution patterns to molecular weight of purified on DEAE-Sepharose fast flow and Sephadex G-200 pectic polysaccharides from leek.

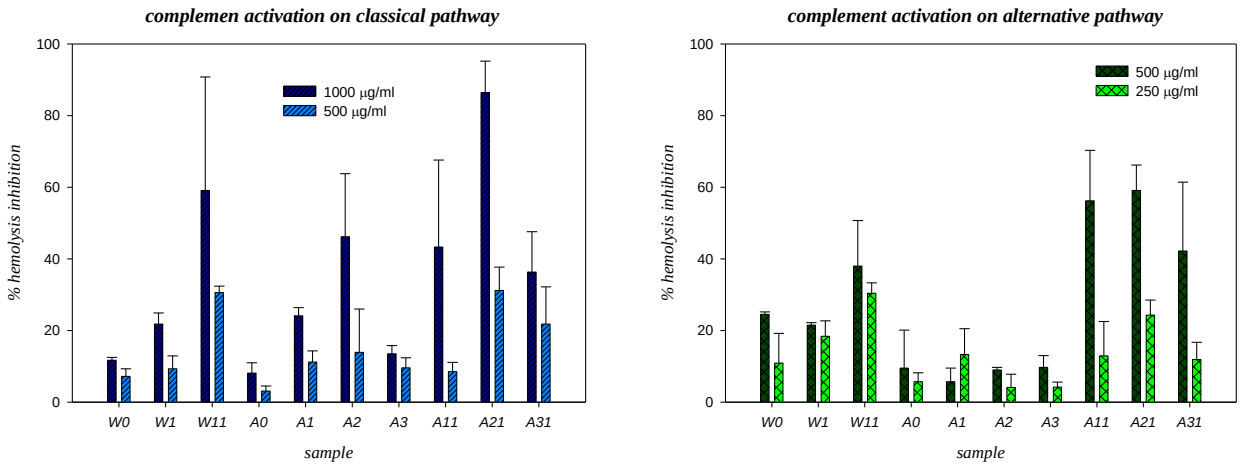

Fig. 5. Immunomodulating activity of purified pectic polysaccharides from leek.

CONCLUSION

It was found, based on the analysis of the polysaccharide fractions obtained by ion-exchange and gel chromatography, that fractions A_2 and A_3 had a higher uronic acid content. The composition of those uronic acids included galacturoniuc acid and glucuronic acid. In the process of ion-exchange chromatography, a fraction was isolated that had a high content of neutral monosaccharides, such as fraction A_1 (59.9% neutral sugars). This fraction contained 47.0% galactose. Most probably, there was a presence of galactan type of polysaccharide in the leek.

The analyzed polysaccharide fractions that were by ion-exchange and gel chromatography purified were characterized by a pronounced biological activity which increased in comparison with the source polysaccharides.

Acknowledgements: *This work was funded by project No BG161PO003-1.1.05-0024-C0001 "Development of nutraceuticals with antioxidant and immune-stimulating action" of Operational Program "Competitiveness" of the EU.*

REFERENCES

1. M. Fernandes, D. M. Sun, M. A. Tosca, D. J. McNamara, *A.m. J. Clin. Nutr.*, **59**, 869 (1994).
2. R. D. Hopewell, R. Yeater, I. Ullrich, *Prog. Food. Nutr. Sci.*, **17**, 159 (1993).
3. M. Thorogood, R. Carter, L. Benfield, *Brit. Med. J.*, **259**, 351 (1987).
4. A. S. Truswell, A. C. Beynen, in: Dietary Fibre, A component of Food Nutrional Function in Health and Disease, Schweizer T.F., Edwards C. A. (eds.), Springer Verlag, 1999.
5. C. Huang, N. Dural, *J. Food Process. Eng.*, **18**, 234 (1995).
6. T. Ide, M. Horil, K. Kawasluma, T. Yamamoto, *Br. J. Nutr.*, **62**, 539 (1989).
7. T. D. Hockaday, *Scand. J. Gastr. Suppl*., 124 (1987).
8. J. Grudeva, M. Kratchanova, I. Panchev, C. Kratchanov, *Z. Lebensm. Unters. F. A.*, **204**, 374 (1997).
9. J. Grudeva-Popova, M. Kratchanova, A. Djurdjev, C. Kratchanov, *Folia Medica*, **XXXIX**, 1 (1997).
10. R. Labadie, in: Bioactive natural products, S. M. Colegate, R. S. Molyneux (eds.), CRC Press Inc. London, 1993, p. 297.
11. A. Sendi, N. Mulinacci, F. Vincieri , H. Wagner, *Phytochemistry*, **34**, 1357 (1993).
12. R. Srivastara, D. K. Kulshrestha, *Phytochem.*, **28**, 2877 (1989).
13. H. Yamada, H. Kiohara, T. Matsumoto, in: Advances in Pectin and Pectinase Reserch, F. Voragen, H. Schols, R. Visser (eds.), The Netherlands: Kluwer Academic Publishers, 2003, p. 481.
14. M. Kratchanova, M. Nikolova, E. Pavlova, I. Yanakieva, V. Kussovski, *J. Sci. Food Agr.*, **90**, 2046 (2010).
15. M. Ognyanov, M. Nikolova, I. Yanakieva, V. Kussovski, M. Kratchanova, *J. BioSci. Biotech. SE/ONLINE*, **2**, 13 (2013).
16. M. Kratchanova, M. Gocheva, E. Pavlova, I. Yanakiev, D. Nedelcheva, V. Kussovski, A. Slavov, *Bulg. Chem. Commun.*, **40**, 561 (2008).
17. D. W. F. Shannon, *Analyst*, **97**, 209 (1972).
18. M. Blumenkrantz, G. Asboe-Hansen, *Anal. Biochem.*, **54**, 484 (1973).
19. O. H. Lowry, N. J. Rosenbrough, A. L. Farr, R. L. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
20. A. Blakeney, P. Harris, R. Henry, B. Stony, *Carbohyd. Res.*, **113**, 291 (1983).
21. J. P. A. M. Klerx, C. J. Beukelman, V. H. Dijk, J. M. N. Willers, *J. Immunol. Methods*, **63**, 215 (1983).
22. J. P. A. M. Klerx, C. J. Beukelman, H. V. Dijk, F. J. Van Overveld, W. J. Van der Maaden, J. M. N. Willers, *Infect. Immun.*, **49**, 841 (1985).

ПРЕЧИСТВАНЕ И БИОЛОГИЧНА АКТИВНОСТ НА ПЕКТИНОВИ ПОЛИЗАХАРИДИ ОТ ПРАЗ

М. Николова[1], М. Крачанова[1]*, Е. Павлова[1], И. Янакиева[1], В. Късовски[2]

[1]*Лаборатория Биологично Активни Вещества, Институт по Органична химия с Център по Фитохимия, Българска Академия на Науките, бул. Руски 139, 4000 Пловдив, България*
[2]*Институт по микробиология, Българска Академия на Науките, ул. Акад. Г. Бончев, бл. 26, 1113 София, България*

Постъпила на 20 май 2014 г.; Коригирана на 04 юли 2014 г.

(Резюме)

Осъществено е хроматографско пречистване на пектинови полизахариди от праз, изолирани при последователно екстрахиране с вода и 0,5% солна киселина. Посредством йонообменна хроматография върху DEAE-Sepharose fast flow от водно и киселинно екстрахируемите полизахариди се получават четири фракции– W_1, A_1, A_2 и A_3, всяка от които е гел храмотографски пречистена на Sephadex G-200, при което са обособени фракциите W_{11}, A_{11}, A_{21} и A_{31}. Тези фракции се различават по монозахаридния си състав. Във фракцията K_1, получена при йонообменното пречистване на киселинния екстракт, преобладават неутралните захари (> 58%), докато останалите фракции са с високо уронидно съдържание. Установено е, че високо уронидните фракции съдържат и глюкуронова киселина. Доминиращи монозахариди в пектиновите полизахариди от праз са галактоза и рамноза. Чрез HPLC е определена молекулна маса на полизахаридните фракции и тяхната дисперсност. Хроматографски пречистените полизахаридни фракции от праз показват имуномодулираща активност, определена чрез активиране на комплемента по класическия и алтернативния път. В сравнение с изходните полизахариди, активността на пречистените се увеличава.