*Bulgarian Chemical Communications, Volume 46, Special Issue A (pp. 79 – 87) 2014*

# Chemical composition and anti-complementary activity of enzyme-modified citrus pectins

M. H. Ognyanov[1], Y. N. Georgiev[1], I. Z. Yanakieva[1], V. K. Kussovski[2], M. G. Kratchanova[1]*

[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]*Department of Infectious Microbiology, Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bonchev str., bl. 26, 1113 Sofia, Bulgaria*

Received July 03, 2014; Revised July 26, 2014

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

A consecutive enzyme modification of citrus pectin series with *endo*-polygalacturonase (EPG) and combination of *β*-galactosidase and *endo*-*β*-(1→4)-galactanase was conducted. Alcohol precipitation with two volumes of 96% ethanol was used for fractionation of the enzyme hydrolyzate, resulting to recover of alcohol-insoluble and alcohol-soluble (AS) parts. Alcohol-precipitated (AP) rhamnose-enriched fragments were obtained after homogalacturonan (HG) degradation with EPG. Furthermore, it was found that citrus pectins contained xylogalacturonan fragments liberated after EPG treatment. After modification with galactan-degrading enzymes galactose content was reduced between 50-89%, which confirmed predominance of *β*-(1→4)-linked galactose in ramified hairy regions of citrus pectins. Anti-complementary activity of investigated pectins was higher through the classical pathway in comparison with alternative ones. Enzyme modification influenced to different extent on the anti-complementary activity. Alcohol precipitation approach let to obtaining of complex mixtures with pectic fragments, hindering the interpretation of biological activity results.

**Key words**: citrus pectin, enzyme modification, pectinases, monosaccharide composition, immunomodulating polysaccharides

## INTRODUCTION

Citrus peels are very rich in pectic constituents and are one of the best sources for commercial pectin production. Pectin is used mainly as a gelling agent in the food industry for manufacturing of jams, jellies and marmalades [1]. As well as pectin has potential uses in many other fields such as pharmaceutical and healthy supplement. For example, modified citrus pectin (MCP) obtained by acid, alkali or enzymatic treatment possesses considerable therapeutic effects on tumor metastasis and apoptosis in experimental animal models and in clinical trials with humans [2]. Regarding the study of anti-complementary activity of citrus pectins there is no enough investigation. During the past few years many efforts for MCP preparation were referred to application of enzymatic and physicochemical techniques. Alcohol precipitation is a method used for isolation and fractionation of polysaccharides from different extracts and at the same way for purification from low molecular weight substances. This approach is used also for isolation of GalA oligomers with different degree of polymerization after EPG treatment [3,4]. Additionally, alcohol precipitation was used also for separation of mono- and oligosaccharides from stepwise acid hydrolyzed pectic polysaccharide from Siberian fir [5]. In our work alcohol precipitation was used for separation of enzyme-modified high molecular weight fragments from low molecular weight mono- and oligomers. In our previous publication we described the chemical composition, molecular weight and complement-fixing activity of initial water- and acid-extracted orange and lemon peel pectins. All investigated pectins were modified by EPG and AP residues were assessed for their anti-complementary potential [6]. In other earlier studies, Kratchanova *et al*. [7-9] have established a favorable effect of microwave pre-treatment of fresh citrus peels on the yield and quality of pectin. Data on the influence of microwave heating on biological activity was not published.

The aim of the present study was to further elucidate the composition and anti-complementary activity of enzymatic modified by EPG and galactan-degrading enzymes orange and lemon peel pectins, obtained through extraction from alcohol-insoluble solids (AIS) or microwave pretreatment.

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

## EXPERIMENTAL

### *Preparation of AIS*

AIS from orange and lemon peels were prepared as described previously [6].

### *Microwave pretreatment of fresh orange peels*

Fresh orange peels (200 g) were placed in a glass vessel and heated in a microwave oven with duration of exposure 10 min, power 0.63 kW, and operating frequency 2450 MHz. Microwave treatment conditions were selected according to Kratchanova *et al*. [9].

### *Sequential extraction*

Sequential extraction of AIS from orange and lemon peels as well as from microwave pretreated fresh orange peels with hot water and 0.5% hydrochloric acid was carried out as previously reported [6]. Water- and acid-extracted orange (WEOP and AEOP), lemon (WELP and AELP) pectins as well as microwave pretreated (AEOP MW) were obtained.

### *Enzymatic hydrolysis*

All enzymes used in these experiments were commercially prepared and were purchased from Megazyme International Ireland Ltd. (Bray, Co. Wicklow, Ireland). They were used without further purification. EPG modification was carried out as previously described elsewhere with slight modification [6]. Briefly: saponified orange or lemon pectins (5 mg/ml) were dissolved in 50 mM sodium acetate buffer (pH 5.0) and incubated with 0.042 IU/5 mg pectin at 40ºC for 24 h. Further AP residues after EPG treatment were modified by galactan-degrading enzymes. Modification was performed by combination of *endo*-$\beta$-(1→4)-galactanase (2.5 IU/5 mg substrate) and $\beta$-galactosidase (0.8 IU/5 mg substrate) at 40ºC for 24 h according to Ognyanov *et al*. [10]. After this modification AP residues were obtained. All enzyme units for applied modifying enzymes were selected after some pretests at different enzyme dosages with saponified AEOP. According to the final kinetic investigation (data not included) the most significant changes in the saponified AEOP after EPG modification were until 7 h. For the subsequent modification with $\beta$-(1→4)-D-Gal-releasing enzymes the most significant changes were observed until 2 h.

### *Monosaccharide composition*

Determination of neutral monosaccharides and glucuronic acid content of AP enzyme-modified pectins were carried out after $CF_3CO_2H$ acid hydrolysis by HPLC without derivatization as previously described else-where [6]. Sugar composition analysis of alcohol-soluble parts was done without acid hydrolysis. Anhydrouronic acid content (AUAC) was assayed by *m*-hydroxy-biphenyl method [11]. Galacturonic acid was calculated on the base of difference between total anhydrouronic acid content and glucuronic acid.

### *Size-exclusion chromatography (SEC)*

SEC was performed on Pharmacia Biotech system. Two milliliters of each sample (3 mg/ml) were centrifuged and loaded on HK 16/100 column filled with Sephacryl S-300 HR and eluted with 0.05 M NaOAc buffer (pH=5.0) at 1.0 ml/min flow rate. Fractions (5.0 ml) were collected and analyzed for uronic acids by a microplate variant of carbazole-sulfuric acid method, using D-GalA as a standard [12]. Additionally molecular weight of separated fractions was determined, using dextran standards (35-200 kDa).

### *Anti-complementary activity*

Anti-complementary activity of alcohol-insoluble residues was measured through the classical and alternative pathway, using a micro-assay method, according to Klerx *et al*. [13]. The anti-complementary activity of the samples was expressed as a percentage inhibition of the total complement hemolysis ($TCH_{50}$) of target rabbit and sensitized mutton erythrocytes from normal human serum.

## RESULTS AND DISCUSSION

In a previous publication we reported sequential extraction of water- and acid-soluble pectins from AIS of orange and lemon peels. Data on the content and further chemical characterization of the initial pectins were published elsewhere [6]. Furthermore in the case of microwave treated peels a higher yield of pectin (5.18% on the fresh peel base) was obtained in comparison with both AIS (4.70%) and control directly dried peels (3.47%). This observation was in agreement with the results published by Kratchanova *et al*. [8,9], and Fishman *et al*. [14]. Keeping in mind higher yield and anti-complementary activity of AEOPMW than water extracted

one, the first was chosen for further investigations. AEOPMW was characterized with high AUAC (80.11%), high degree of methylesterification (58.69%) and low acetyl ester content (0.96%). Sugar composition analysis revealed that the main monosaccharide in AEOPMW was Ara (15.58%), followed by Gal (9.94%), Rha (5.47%) and Xyl (0.22%). It was detected 3.15% GlcA that represented a minor portion of the acidic part. AEOPMW consisted of two populations with different molecular weight $8.4\times10^5$ Da (91.3%) and $6.0\times10^3$ Da (8.7%).

All initial pectins were subjected to saponification and enzyme modification with EPG and further with galactan-degrading enzymes as mentioned above. The whole enzyme modification scheme is presented on Fig. 1.

In Table 1 are summarized the yield and monosaccharide composition of $AP_1$ EPG modified pectins. The yield of modified products varied between 28-41%. Modified products contained Rha, Gal, Ara, GalA and GlcA, as their Rha content was higher than those in initial pectins (except for AELP). Also, the amount of GalA decreased between 18% (WEOP) and 60% (AEOPMW) on the base of GalA content in native pectins (68-81%) [6]. These data illustrated that degradation was performed more fully in AEOPMW, contrary to WEOP that was fairly resistant to enzyme action, though saponification. During the degradation of

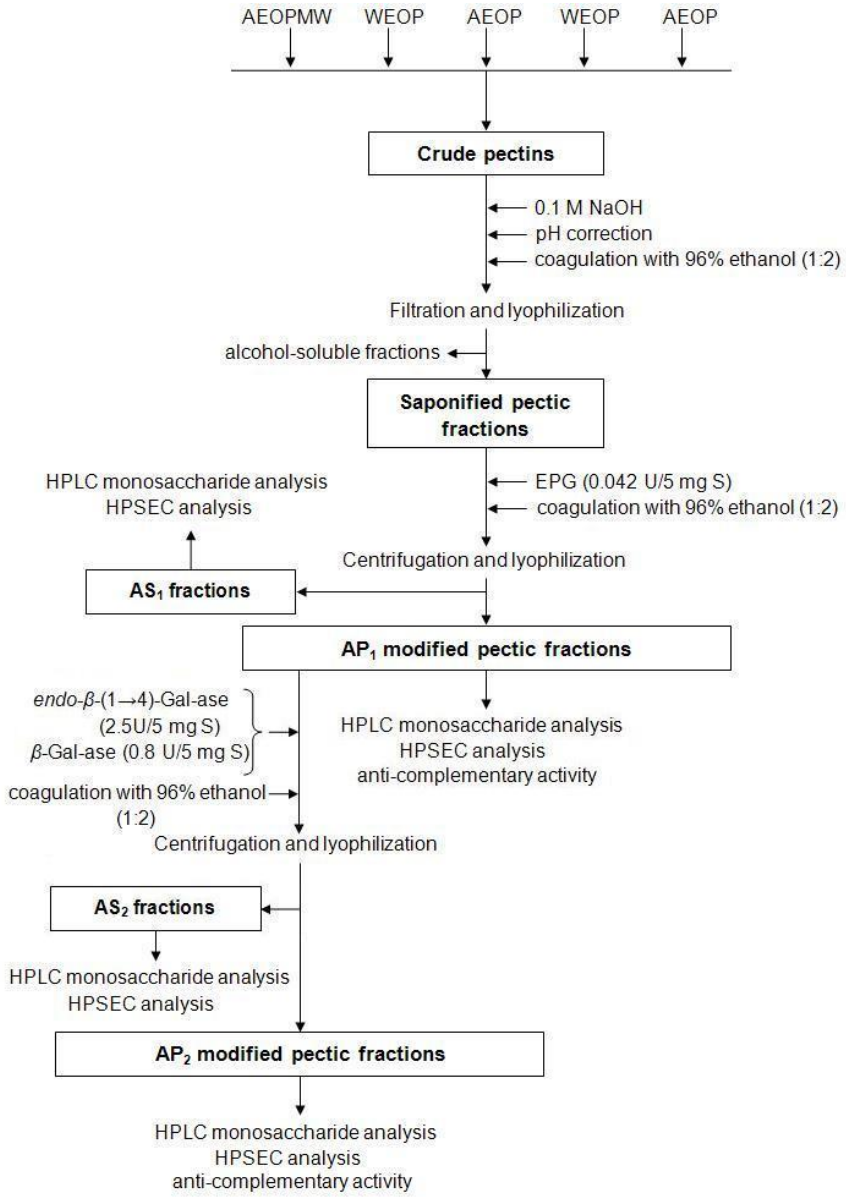


**Fig. 1.** Scheme for enzyme modification and analysis of different citrus pectins. Extraction procedure was described well in [6]. *Abbreviation*: EPG (*endo*-polygalacturonase), *endo*-$\beta$-(1→4)-Gal-ase (*endo*-$\beta$-(1→4)-galactanase) and $\beta$-Gal-ase ($\beta$-galactosidase); Alcohol-precipitated (AP); Alcohol-soluble (AS).

citrus pectins EPG activity could be hindered by neutral sugar side chains and resistant to degradation alcohol-insoluble residue could be isolated. Rhamnogalacturonan I (RG) fraction that was not free of the HG components may be accumulated in $AP_1$ residue. Indication for this was GalA presence in $AP_1$ fraction after enzyme treatment (Table 1).

**Table 1.** Yield and sugar composition (% w/w) of the alcohol-precipitated ($AP_1$) residue after degradation of citus pectins with EPG.

| | AEOPMW | WEOP | AEOP | WELP | AELP |
|---|---|---|---|---|---|
| Yield, % | 39 | 28 | 39 | 41 | 29 |
| Monosaccharides* | | | | | |
| Rha | 9.1 | 11.2 | 11.0 | 10.4 | 12.5 |
| Ara | 2.1 | 1.4 | 1.5 | 4.0 | 2.2 |
| Gal | 20.1 | 9.5 | 15.6 | 7.4 | 11.5 |
| GalA | 31 | 56.4 | 44.4 | 53.1 | 49.2 |
| GlcA | 2.6 | 2.7 | 3.5 | 0.7 | 3.4 |

*Values are the average of two replicates.

These results were in good agreement with mode of EPG action. The enzyme catalyzes hydrolytic depolymerization of linear HG regions in pectic polysaccharides to oligomers and monomers, but ramified hairy regions remain non-degraded [15]. Therefore a high proportion of pectic RG region is obtained. Surprisingly, after modification Gal and Ara contents were decreased (except AEOPMW). Interestingly, Xyl was not detected in all $AP_1$ residues, contrary to its presence in $AS_1$ residue (see Table 2) and this showed that the monosaccharide was in some way released. Quite possible could be the presence of short EPG resistant Xyl-substituted galacturonan segments interspersed with long EPG-degradable HG segments. Thus, it appears that after digestion intact xylosilated oligomers could be accumulated in $AS_1$ supernatant. Rather, it would be confirmed the statement for Xyl-GalA containing units in initial pectins than for co-extracted hemicelluloses. The presence of mixed AS oligomers could be seen in Table 2, where oligomer's raw data represent the sum of acidic and neutral oligomers. Schols *et al*. [16] and Mort *et al*. [17] have obtained and well-explained the phenomenon with Xyl-substituted HG subunit. Sugar composition was in agreement with another our previous publication, where we reported that in AP part after EPG treatment of leek pectin Rha amount was increased contrary to Gal and Ara contents that were decreased [10]. Suh *et al*. [18] have modified with EPG pectin isolated from mandarine and they obtained a fraction with increased Rha, Gal and Ara contents in comparison with the initial pectin. Inngjerdingen *et al*. [19] have observed a considerable Xyl decrease in one of the chromatographically purified fractions of EPG treated pectic polysaccharide from *Biophytum petersianum* Klotzsch. It could also be observed that GlcA content after this enzyme modification was increased for some of the products [19]. It was expected because it is well-known that GlcA presents in the RG side chains [20]. Inngjerdingen *et al*. [21] have also found increase of GlcA in a chromatographically purified fraction of pectic polysaccharide from *Glinus oppositifolius* after EPG modification.

Neutral monosaccharides and uronic acids in $AS_1$ parts after EPG treatment were determined without further acid hydrolysis to investigate enzyme-liberated free monomers. The results are presented in Table 2. Small amounts of free neutral sugars were quantified in all $AS_1$ products. Data for GalA content illustrated that EPG modification was performed. The presence of Ara and Gal in all samples could be explained with external enzyme activities mentioned in the certificates of used enzymes. Glucuronic acid was not detected in most of the $AS_1$ parts (except WEOP) as was expected.

**Table 2.** Sugar composition (% w/w) of the alcohol-soluble ($AS_1$) part after degradation of citrus pectins with EPG.

| Monosaccharides | AEOPMW | WEOP | AEOP | WELP | AELP |
|---|---|---|---|---|---|
| Rha | 1.7 | 3.4 | 2.5 | 1.3 | 1.7 |
| Xyl | 1.2 | 0.9 | 1.2 | 1.3 | 1.2 |
| Ara | 0.7 | 0.4 | 1.1 | 0.8 | 0.7 |
| Gal | 0.3 | 0.3 | 1.6 | 0.5 | 0.3 |
| GalA | 46.5 | 10.2 | 26.9 | 27.6 | 22.1 |
| GlcA | - | 0.4 | - | - | - |
| Oligomers* | 70.4 | 67.2 | 69.3 | 69.7 | 68.7 |

* Data for oligomers are directly extracted from chromatograms as % area. Values are the average of two replicates.

For expectation, as many enzymes are involved in the successive stepwise degradation of pectin as more substantial is the reduction in molecular weight and increase of molecular heterogeneity. Because of this fact lower AP products were recovered. The yield of $AP_2$ products varied in a narrow range between 49-54% calculated on EPG $AP_1$ residue base (Table 3). Generally, citrus pectins contain predominatly arabinogalactan type I, which is a linear chain of $\beta$-(1→4)-linked Gal residues with 20-40% Ara monomers connected mostly to C3 of Gal units [22]. To study the influence of this type of galactan chains on anti-complementary activity of citrus pectins treatment with $\beta$-(1→4)-

galactan-degrading enzymes was performed. Beta-Galactosidase (EC 3.2.1.23) catalyzes hydrolytic decomposition of *β*-(1→4)-galactosidic bonds in lactose and different *β*-D-galactosides towards non-reducing end. The enzyme acts in a synergistic manner with *endo-β*-(1→4)-D-galactanase (EC 3.2.1.89). The latter catalyzes hydrolytic degradation of different galactans to galactooligosaccharides by *endo* mechanism. These enzymes play a key role in decomposition of *β*-(1→4)-D-galactan side chains in pectic arabinogalactan type I from RG I [23,24]. The results in Table 3 show that Gal amount was considerably lower after synergistic action of *endo-β*-(1→4)-galactanase and *β*-galactosidase. From Table 1 and 3 could be calculated that Gal content was reduced between 50% (WELP) and 89% (WEOP). The detected Gal amount after this treatment could be related to non-degraded *β*-(1→4) glycosidic bonds and the presence of highly branched arabinogalactan type II with chains of galactose residues joint by *β*-(1→3,6) linkages. Zhang *et al*. [25] have found that after *endo-β*-(1→4)-galactanase digestion of arabinogalactan from *Angelica acutiloba* Gal amount was reduced with 30%. Characteristically, pectic ramified regions and more specifically galactan side chains could be substituted with GlcA and/or 4-O-methyl-GlcA, mainly as a non-reducing terminal residue [26]. Therefore digestion with galactan-degrading enzymes leads to liberation of AS galactooligomers substituted with GlcA, which is the reason for its lower quantity in $AP_2$ products (Table 3). Grønhaug *et al*. [27] have found that after modification with *endo-β*-(1→4)-galactanase of EPG treated pectic polysaccharide from *Biophytum petersianum* Klotzsch Rha, Gal and Ara contents were increased, but no GlcA was detected. Raw data expressed as %Area and extracted from chromatograms, showing the sum of acidic and neutral AS oligomers are also presented in Table 4. Additionally a small amount of Rha was detected in these fractions because most of the released RG backbone fragments could be AS. Contradictory to previous results for EPG $AP_1$ residues (Table 1) Xyl was detected in trace amounts in both products after galactose-releasing enzymes (Table 3).

The results, as shown in Table 4, confirmed that the same quality sugar composition was detected as before modification (Table 2). Galactose was the most abundant free neutral monosaccharide because of specificity of conducted enzyme modification. This simply showed that most of Gal was bound by *β*-(1→4) linkages.

It should be noted that the absence of monomeric GlcA in $AS_2$ parts (except AEOPMW) could be explained with direct sugar analysis without further acid hydrolysis of solubilized GlcA-substituted (galacto)oligomeric residue. This leads to underestimation and masking the GlcA amount in the sample. Detected amounts of GalA in all samples could be related to the mentioned minor external EPG activity in *endo-β*-(1→4)-galactanase certificate. Actually the same could be said for detected low (trace) amounts of Ara and Xyl.

**Table 3.** Yield and sugar composition (% w/w) of the alcohol-precipitated ($AP_2$) residue after degradation of citrus pectins with galactan-degrading enzymes *endo-β*-(1→4)-galactanase and *β*-galactosidase.

| | AEOPMW | WEOP | AEOP | WELP | AELP |
|---|---|---|---|---|---|
| Yield, % | 49 | 54 | 5152 | 54 | |
| Monosaccharides* | | | | | |
| Rha | 0.7 | 1.0 | 1.0 | 1.2 | 0.5 |
| Xyl | 0.4 | 0.1 | - | trace | 0.2 |
| Ara | 10.6 | 13.2 | 10.5 | 11.9 | 11.8 |
| Gal | 5.3 | 1.0 | 4.3 | 3.7 | 4.0 |
| GalA | 28.6 | 54.1 | 24.8 | 36.3 | 21.1 |
| GlcA | 0.5 | 0.6 | 0.3 | 1.0 | - |

*Values are the average of two replicates.

Generally, comparing molecular heterogeneity of all citrus pectins after EPG action $AP_1$ residues contained higher proportion of molecular fragments with Mw $10^5$ Da and smaller $10^4$ Da. Oppositely, $AS_1$ parts contained mainly $10^4$ Da population. In result of enzyme hydrolysis with galactose-releasing enzymes $AP_2$ residues contained predominantly fragments with Mw $10^4$ Da and smaller proportion with $10^5$ Da.

**Table 4.** Sugar composition (% w/w) of the alcohol-soluble ($AS_2$) part after degradation of citrus pectins with galactan-degrading enzymes *endo-β*-(1→4)-galactanase and *β*-galactosidase.

| Monosaccharides | AEOPMW | WEOP | AEOP | WELP | AELP |
|---|---|---|---|---|---|
| Rha | 1.3 | 0.5 | 0.1 | 0.8 | 1.6 |
| Xyl | trace | 0.1 | - | 0.2 | trace |
| Ara | 0.4 | 0.7 | 0.9 | 1.0 | 1.1 |
| Gal | 11.4 | 4.9 | 15.5 | 1.4 | 7.5 |
| GalA | 2.0 | 2.3 | 2.1 | 2.0 | 1.0 |
| GlcA | 0.4 | - | - | - | - |
| Oligomers* | 45.2 | 80.1 | 57.6 | 85 | 69.7 |

*Data for oligomers are directly extracted from chromatograms as % area. Values are the average of two replicates.

To access the molecular weight distribution of the initial AEOP and its alcohol-insoluble enzyme-modified products they were analyzed by SEC. AEOP was selected for fractionation because of its higher anti-complementary activity. Fig. 2 shows the elution pattern obtained by fractionation of

AEOP sample before and after enzyme treatment. Also, the found molecular weight value for different peaks was annotated. It was shown by the lower molecular mass of the $AP_1$ residue that initial high molecular weight substrate was degraded with EPG. In general, initial AEOP consisted of one distinct population in the high molecular weight region and was eluted as a broad peak faster than oligomers.

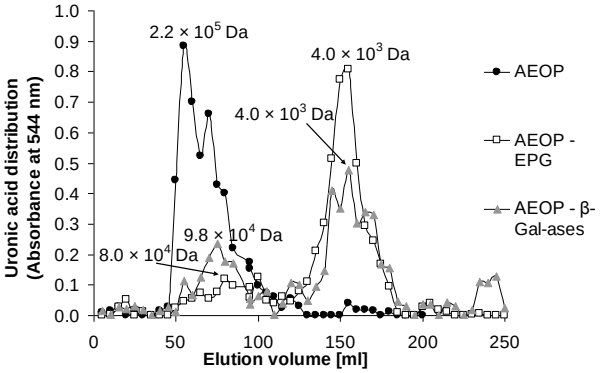


**Fig. 2.** Size-exclusion chromatography of AEOP and its alcohol-insoluble residues after a consecutive modification with EPG and galactan-degrading enzymes (*β*-Gal-ases).

Interestingly, two subfractions were separated from $AP_1$ residue. The higher molecular weight fraction was eluted as a rather broad peak between 50 and 110 ml consisted of lower amount of uronic acids, contrary to the second population that gave a high absorbance, but was eluted later with higher elution volume. Presumably, the first peak represented enzyme-resistant polymeric material and the second either galacturonic acid oligomers with higher molecular weight or mixture of lower molecular weight ramified hairy region fragments that co-eluted with larger oligogalacturonides. The molecular weight distribution and pattern of $AP_1$ residue after EPG were comparable with this after galactan-degrading enzymes, but as can be seen a small fraction was eluted at elution volume of 250 ml. It could be alcohol co-precipitated acidic oligomers with lower degree of polymerization. Schols *et al*. [28] have shown that by SEC on Sephacryl S-300 was possible the separation of apple pectin EPG hydrolizate to rich in neutral sugar regions and acidic oligogalacturonides. Authors have determined by HPSEC that molecular weight of obtained hydrolysis products were in the range $10^3$ - $10^5$ Da. This was in a good agreement with our results. Additionally our results and assumptions were comparative with these of Ros *et al*. [29]. It was also seen that after treatment with galactan-degrading enzymes the peak at elution volume under 80 ml was retained and absorption maximum of detected uronic acids was slightly increased. This was probably a result from change of uronic acid to neutral sugar ratio in this population as a consequence from Gal release.

The complement system is a basic antimicrobial defense mechanism in humans and normally present in blood in an inactive form. It can be activated through three different enzyme cascades - classical, alternative and lectin pathways by microbial cell wall carbohydrate structures (lipopolysaccharides, mannans, poly-N-acetylglucosamine) and by some plant polysaccharides (pectin, inulin, glucans). It is well-known that the anti-complementary activity of the most biologically active pectins is due to the containing arabinogalactan II side chains in RG I and that pectin could stimulate both classical and alternative pathway [30,31]. For investigation of the influence of linear acidic HG and RG I side chains on the activity was conducted modification by EPG and a successive modification with combination of galactan-degrading enzymes. EPG was used for obtaining of active ramified pectic RGs.

In Table 5 are presented the results from the complement activation test for different citrus pectins and their enzyme-modified alcohol-insoluble products through the classical and alternative pathways. All polysaccharides expressed higher anti-complementary activity through the classical pathway. Acid-extracted pectins were more active than water-extracted ones through this pathway. For example, acid-extracted citrus pectins inhibited mutton erythrocyte hemolysis over 50% at 2500 μg/ml. Furthermore, AEOP inhibited rabbit erythrocyte hemolysis over 80% at 5000 μg/ml through alternative pathway. Additionally, polysaccharides were investigated at 2500 μg/ml, but they did not show any activity through this pathway. Microwave pretreatment of orange peels did not negatively influence on the activity of AEOPMW. Studies dealing with anti-complementary activity of unmodified pectic polysaccharides from different plant sources have been published. For example, Kiyohara *et al*. [32] have obtained by ion-exchange chromatography four acidic fractions AR-2IIa-2IId from *A. acutiloba* with above 90% AUAC and anti-complementary activity over 50% at 1000 μg/ml. Paik *et al*. [33] have isolated the biologically active acidic fraction HCAP-0 with hot water from pepper (*Capsicum annuum*), which expressed 85% anti-complementary activity through the classical pathway at 1000 μg/ml. In comparison with our results AEOP possessed about 80% activity through both pathways, but in significantly higher concen-

**Table 5.** Anti-complementary activity of citrus pectins and their alcohol-insoluble ($AP_1$ and $AP_2$) enzyme-modified products. Mean ± SD.

| | **Inhibition of total complement hemolysis of target animal erythrocytes, %** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Classical pathway** | | **Alternative pathway** | **Classical pathway** | | **Alternative pathway** | **Classical pathway** | |
| | **2500 µg/ml** | **1250 µg/ml** | **5000 µg/ml** | **2500 µg/ml** | **1250 µg/ml** | **5000 µg/ml** | **2500 µg/ml** | **1250 µg/ml** |
| **Pectins** | **Initial** | | **EPG modified pectins** | | | | **β-Gal-ases modified pectins** | |
| AEOPMW | 74.8±13.8 | 14.2±5.8 | 20.7±6.4 | 34.8±6.3 | 4.2±0.8 | 35.1±15.9 | 60.7±6.2 | 10.6±1.6 |
| WEOP | 41.4±10.7 | 8.7±0.9 | 22.6±7.6 | 54.6±6.6 | 34.4±10.1 | 27.7±8.4 | 55.2±21.4 | 25.2±9.8 |
| AEOP | 85.8±7.4 | 12.6±1.7 | 81.5±15.1 | 37.3±4.7 | 7.7±4.2 | 22.4±2.2 | 46.4±15.3 | 19.1±11.7 |
| WELP | 31.5±5.6 | 5.3±0.6 | 56.9±16.2 | 25.5±5.8 | 14.2±4.1 | 63.6±14.6 | 64.3±11.4 | 35.3±13.2 |
| AELP | 57.1±2.5 | 11.4±2.9 | 19.1±4.4 | 28.7±4.1 | 14.2±5.8 | 29.2±1.8 | 44.8±7.3 | 25.8±4.6 |

trations. Furthermore, Diallo *et al*. [34] have investigated two very potent crude polysaccharide extracts from the leaves of *Trichilia emetica* that showed $ICH_{50}$ at 35 µg/ml and 45 µg/ml, respecttively. After ion-exchange chromatography of both extracts they isolated the most potent fraction "Te 100 fraction acidic 4" with $ICH_{50} < 15$ µg/ml rich in GalA, Gal and Rha. Investigated citrus pectins were with considerably lower activity than the mentioned above because of different plant source and polysaccharide preparation.

Clear positive influence after modification with EPG was observed in WEOP through the classical pathway. Except AEOP modification was not negative through the alternative pathway. Interestingly, AEOP was the most potent sample through this pathway before EPG digestion. In agreement with our results Kiyohara *et al*. [32] have observed the same phenomenon after EPG digestion of AR-2IIa and AR-2IIb because their anti-complementary activity decreased from 59.5% to 29.0% and 62.6% to 42.8% at 1000 µg/ml. But after separation of oligogalacturonides from enzyme resistant fraction on Sephadex G-50 the latter showed higher activity in comparison with the original fraction. Inngjerdingen *et al*. [21] have isolated a pectic polysaccharide GOA2 from *G. oppositifolius*, which inhibited hemolysis of sheep sensitized erythrocytes to about 40% under 1000 µg/ml. After modification by EPG they obtained chromatographically fractions GOA2-I, II, III, and oligogalacturonides as the first one inhibited hemolysis to 90% and the next two expressed very low activity. As is shown in Table 5 anti-complementary activity is either decreased or not substantially changed. Accumulation of alcohol-insoluble oligogalacturonides could not be excluded because of the mode of action of EPG. The latter may have a negative masking effect on the activity expression of arabinogalactans. The best way for elucidation of this problem could be fractionation of the obtained products to oligogalacturonides and rich of neutral sugar fraction by ion-exchange chromatography and further investigation of their activity.

Also it was interestingly to study the influence of *β*-(1→4)-linked Gal on the anti-complementary activity through the classical pathway. Generally, it could be concluded that this modification did not influence negatively on the activity of citrus pectins (Table 5). For example, WELP (initial) increased its activity from 31.5±5.6% to 64.3±11.4%. The higher observed activity for most of the samples could be interpreted with concentration of biologically active oligomers with glycosidic bonds resistant to the catalytic activity of applied enzymes. They should be the active *β*-(1→3)-galactans branched with *β*-(1→6)-galactans and/or biologically active *β*-(1→4)-galactooligosaccharides accumulated in AP residue. Ognyanov *et al*. [10] have determined for acid-extracted leek pectin that modification with EPG led to 3 fold increase in anti-complementary activity of AP residue through the classical pathway, otherwise digestion with *endo-β*-(1→4)-galactanase led to twofold decrease in activity. Grønhaug *et al*. [27] have reported for pectic polysaccharides from *B. petersianum* that additionally to (1→3,6)-galactans *β*-(1→4)-galactans could also contribute to the immunomodulating activity against immunocompetent Peyer's patch cells. They have shown this fact by digestion with *endo-β*-(1→4)-galactanase and the aforementioned specific Gal-releasing enzymes and further chromatographic separation. This was in contradiction with our results.

## CONCLUSION

In the present study an attempt for characterization of AP enzyme-released pectic oligosaccharides was made. $AP_1$ rhamnose-enriched fragments were obtained by EPG treatment. It was indicated that investigated citrus pectins contained xylogalacturonan fragments. The predominant presence of $\beta$-(1→4)-linked Gal in investigated pectins using $\beta$-(1→4)-Gal-releasing enzymes was confirmed. Enzyme modification influenced to different extent anti-complementary activity of investigated pectins. Alcohol precipitation is a suitable and relatively prompt strategy for fractionation of enzyme-digested pectins. Introduced approach led to obtaining of a complex mixture of mono- and oligosaccharides that hindered appropriate estimation of influence of enzyme modification on anti-complementary activity. On the base of our investigations it could be concluded that for obtaining more clear results alcohol precipitation procedure should be optimized by applying a stepwise procedure with different alcohol concentrations. For elucidation of the relationship between carbohydrate composition and anti-complementary activity enzymolysis and glycosidic linkage analysis of chromatographically purified pectic arabinogalactan chains are necessary to be used. Moreover for better understanding of pectic monosaccharide composition and structure investigation of more complex AS oligomers after appropriate acid hydrolysis by modern analytical tools (HILIC-ELSD/ESI-$MS^n$, CE-MS/CE-LIF, HPAEC-PAD) are needed.

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

## REFERENCES

1. Z. I. Kertesz, The Pectic Substances, Interscience Publishers, New York, 1951.
2. P. S. Bhimanagouda, J. S. Brodbelt, E. G. Miller, N. D. Turner, in: Potential Health Benefits of Citrus, *ACS Sym. Ser*., **936**, 1 (2006).
3. R. Cameron, G. Luzio, E. Baldwin, J. Narciso, A. Plotto, *Proc. Fla. State Hort. Soc.*, **118**, 406 (2005).
4. R. Cameron, G. Luzio, B. Savary, A. Nuñez, K. Goodner, *Proc. Fla. State Hort. Soc.*, **122**, 295 (2009).
5. E. Makarova, O. Patova, E. Shakhmatov, S. Kuznetsov, Yu. Ovodov, *Carbohydr. Polym*., **92**, 1817 (2013).
6. Y. Georgiev, M. Ognyanov, I. Yanakieva, V. Kussovski, M. Kratchanova, *J. BioSci. Biotech*., **1**, 223 (2012).
7. M. Kratchanova, I. Panchev, E. Pavlova, L. Schtereva, *Carbohydr. Polym*., **25**, 141 (1994).
8. M. Kratchanova, E. Pavlova, I. Panchev, Chr. Kratchanov, *Prog. Biotech*., **14**, 941 (1996).
9. M. Kratchanova, E. Pavlova, I. Panchev, *Carbohydr. Polym*., **56**, 181 (2004).
10. M. Ognyanov, M. Nikolova, I. Yanakieva, V. Kussovski, M. Kratchanova, *J. BioSci. Biotech.*, **2**, 13 (2013).
11. N. Blumenkrantz, G. Asboe-Hansen, *Anal. Biochem*., **54**, 484 (1973).
12. M. Cesaretti, E. Luppi, F. Maccari, N. Volpi, *Carbohydr. Polym*., **54**, 59 (2003).
13. J. Klerx, C. Beukelman, V. Dijk, J. Willers, *J. Immunol. Methods*, **63**, 215 (1983).
14. M. Fishman, H. Chau, P. Hoagland, A. Hotchkiss, *Food Hydrocolloids*, **20**, 1170 (2006).
15. J. A. E. Benen, J. Visser J. 2003. in: Handbook of Food Enzymology, J. R. Whitaker, A. G. J. Voragen, D. W. S. Wong (eds.), Marcel Dekker, Inc., 2003, ch. 69.
16. H. A. Schols, E. J. Bakx, D. Schipper, A. G. J. Voragen, *Carbohydr. Res*., **279**, 265 (1995).
17. A. Mort, Y. Zheng, F. Qui, M. Nimtz, G. Bell-Eunice, *Carbohydr. Res*., **343**, 1212 (2008).
18. H.-J. Suh, H.-S. Yang, K.-S. Ra, D.-O. Noh, K.-H. Kwon, J.-H. Hwang, K.-W. Yu, *Food Chem*., **138**, 1079 (2013).
19. M. Inngjerdingen, K. T. Inngjerdingen, T. R. Patel, S. Allen, X. Chen, B. Rolstad, G. A. Morris, S. E. Harding, T. E. Michaelsen, D. Diallo, B. S. Paulsen, *Glycobiology*, **18**, 1074 (2008).
20. J. An, M. A. O'Neill, P. Albersheim, A. G. Darvill, *Carbohydr. Res*., **252**, 235 (1994).
21. K. T. Inngjerdingen, T. R. Patel, X. Chen, L. Kenne, S. Allen, G. A. Morris, S. E. Harding, T. Matsumoto, D. Diallo, H. Yamada, T. E. Michaelsen, M. Inngjerdingen, B. S. Pausen, *Glycobiology*, **17**, 1299 (2007).
22. G. Beldman, H. A. Schols, S. M. Pitson, M. J. F. Searle-Van Leeuwen, A. G. J. Voragen, in: Advances in Macromolecular Carbohydrate Research, R. J. Sturgeon (ed.), vol. 1, 1997, Jai Press, Inc., London, 1997, p. 1.
23. R. R. Mohoney, in: Handbook of Food Enzymology, J. R. Whitaker, A. G. J. Voragen, D. W. S. Wong (eds.), Marcel Dekker, Inc., 2003, ch. 65.
24. R. de Vries, J. Visser, in: Handbook of Food Enzymology, J. R. Whitaker, A. G. J. Voragen, D. W. S. Wong (eds.), Marcel Dekker, Inc., 2003, ch. 70.
25. Y. Zhang, H. Kiyohara, M. H. Sakurai, H. Yamada, *Carbohydr. Polym*., **31**, 149 (1996).
26. M. Haque, T. Kotake, Y. Tsumuraya, *Biosci. Biotech. & Biochem*., **69**, 2170 (2005).

27. T. E. Grønhaug, H. Kiyohara, A. Sveaass, D. Diallo, H. Yamada, B. S. Paulsen, *Phytochem*., **72**, 2139 (2011).
28. H. A. Schols, E. Vierhuis, E. J. Bakx, A. G. J. Voragen, *Carbohydr. Res*., **275**, 343 (1995).
29. J. M. Ros, H. A. Schols, A. G. J. Voragen, *Carbohydr. Polym*., **37**, 159 (1998).
30. H. Yamada, H. Kiyohara, in: Immunomodulatory agents from plant, H. Wagner (ed.), 1999, Birkhäuser, Basel, p. 161.
31. H. Yamada, H. Kiyohara, in: Comprehensive Glycoscience – From Chemistry to Systems Biology, J. P. Kamerling (ed.), 2007, Elsevier, Oxford, p. 663.
32. H. Kiyohara, J. C. Cyong, H. Yamada, *Carbohydr. Res*., **182**, 259 (1988).
33. S.-Y. Paik, K. S. Ra, I. S. Chang, Y. C. Park, H. S. Park, H. S. Baik, J. W. Yun, J. W. Choi, *J. Biochem. Mol. Biol*., **36**, 230 (2003).
34. D. Diallo, B. S. Pausen, T. H. A. Liljebäck, T. E. Michaelsen, *J. Ethnopharmacol*., **84**, 279 (2003).

# ХИМИЧЕН СЪСТАВ И АНТИКОМПЛЕМЕНТАРНА АКТИВНОСТ НА ЕНЗИМНО-МОДИФИЦИРАНИ ЦИТРУСОВИ ПЕКТИНИ

М. Х. Огнянов[1], Й. Н. Георгиев[1], И. Ж. Янакиева[1], В. К. Късовски[2], М. Г. Крачанова[1]*

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

Постъпила на 03 юли 2014 г.; Коригирана на 26 юли 2014 г.

(Резюме)

В настоящото изследване беше проведена последователна ензимна модификация на серия от цитрусови пектини с *ендо*-полигалактуроназа и комбинация от *β*-галактозидаза и *ендо*-*β*-(1→4)-галактаназа. За фракциониране на ензимните хидролизати беше използвано утаяване с двукратен обем 96% етанол, което доведе до получаването на алкохолно-разтворима и алкохолно-неразтворима част. След действието на *ендо*-полигалактуроназа бяха получени алкохолно утаими фракции с повишено съдържание на рамноза и понижено на галактуронова киселина (между 18-60%). Освен това беше установено, че цитрусовите пектини съдържат ксилогалактуронанови фрагменти, които се освобождават след третирането с *ендо*-полигалактуроназа. След последващата ензимна модификация галактозното съдържание на изследваните пектини беше намалено между 50 и 89%, което потвърждава преимуществото на *β*-(1→4)-свързаната галактоза в разклонените вериги на цитрусовите пектини. Антикомплементарната активност на цитрусовите пектини беше по-висока по класическия път в сравнение с алтернативния път на активиране на комплемента. Използваният подход за фракциониране с алкохол след ензимната модификация доведе до получаването на комплексни смеси от пектинови фрагменти, проявяващи различно влияние върху антикомплементарната активност на модифицирания продукт.