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Enzymatic Hydrolysis of Water Extractable Polysaccharides from Leaves of *Plantago major* L.

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Background: *Plantago major* L. leaves have been used for centuries by the traditional medicine in the treatment of infectious disorders of the respiratory, urinary and digestive tracts. Researchers have reported that hot water extracts of *Plantago major* possess a broad-spectrum of anticancer, antioxidant and antiviral activities, as well as activities which modulate cell-mediated immunity. Their beneficial properties may be due to the significant content of polysaccharides. The polysaccharides that have been isolated from the leaves of *Plantago major* L. have different structures – pectic substances, galactans, arabinogalactans, glucomannans.

Aim: The aim of this paper was to study the correlation between the structure of the water extractable polysaccharides isolated from *Plantago major* L. leaves and their enzymatic hydrolysis with different carbohydrate hydrolases.

Materials and methods: The hydrolysis reactions were performed with the enzymes hemicellulase and mannanase. Spectrophotometric total reducing sugars assay was used to examine the hydrolysis yield. The monosaccharide and oligosaccharide compositions were determined using HPLC analysis.

Results: The highest hydrolysis yield of the water extractable polysaccharides from *Plantago major* leaves was obtained by treatment with hemicellulase. The hydrolysis yield increased with the augmentation of the ratio of enzyme to polysaccharide. Galactose was the prevalent monosaccharide identified in the composition of the isolated polysaccharides. Oligosaccharides with different degree of polymerization were also detected.

Conclusion: The enzymatic hydrolysis of water extractable polysaccharides from *Plantago major* leaves allows us to obtain different types of oligosaccharides with beneficial effects on both human health and industry.

BACKGROUND

Plantago major L. (Greater plantain) is an herbaceous perennial member of the *Plantaginaceae* family.¹ It is a well-known medicinal plant, used worldwide for treating skin problems and diseases related to the respiratory, urinary and digestive tracts.² A wide range of activities have been reported for *Plantago major* leaves extracts – anticancer, anti-inflammatory, antibacterial, antiviral, analgesic, antioxidant, anti-ulcerogenic, hepatoprotective and immune-modulating activities.²⁻⁹ Some of their beneficial properties may be due to the considerable content of polysaccharides. The main polysaccharides isolated from *P. major* leaves are rhamnogalacturonans, galactans, arabinogalactans and xylogalacturonans.¹⁰⁻¹³

Gorin (1966) isolated a pectic acid polysaccha-

ride, a galactoarabinan and a galactan (commonly referred to as ‘plantaglucid’) from *P. major* leaves and reported these to have spasmolytic and anti-ulcerogenic effects on animals.² By water extraction from *P. major* leaves, Samuelsen isolated a highly esterified pectic polysaccharide (PMII)^{10,11} and an acidic arabinogalactan (PMIa)¹². PMII contains a smooth polygalacturonan and two ramified regions, composed of arabinose and galactose, linked in positions 3, 4 and 6 to the backbone. PMIa is composed of arabinose (31%), galactose (32%), rhamnose (6%) and galacturonic acid (7%).¹⁰⁻¹²

AIM

The aim of this paper was to study the correlation between the structure of the water extractable

polysaccharides (WEP) isolated from *Plantago major* L., their enzymatic hydrolysis with different carbohydrate hydrolases and subsequently the determination of the obtained monosaccharide and oligosaccharide composition.

MATERIALS AND METHODS

PLANT MATERIAL

The leaves of *Plantago major* L. (*Plantago major* ssp. *major* L.) were harvested from the region of Plovdiv in May 2015 and stored at -18°C until used.

SUBSTRATES AND ENZYMES

Xylan from oat-spelts, Sigma, US. Galactomannan (Locust Bean Gum), Sigma, US. Mannanase (EC 3.2.1.78), Amano, Japan. Hemicellulase (EC 3.2.1.8), Amano, Japan. The monosaccharide and oligosaccharide standards were obtained from Sigma, US.

PREPARATION OF ALCOHOL-INSOLUBLE RESIDUE (AIR) FROM *PLANTAGO MAJOR* L. LEAVES

The alcohol-insoluble residue was prepared according to the methodology described by Kratchanova.^{14,15} The obtained AIR was purified from the chlorophyll according to the procedure reported by Sengkhampan.¹⁶

EXTRACTION PROCEDURE

The alcohol-insoluble residue from *P. major* leaves was extracted with distilled water (1:25) in boiling water bath at continuous stirring for 2 hours. The obtained mixture was filtered through cloth. The filtrate was coagulated with 95% ethanol (1:1) to isolate the water soluble polysaccharides.^{14,15} The loss on drying of AIR was determined using Moisture Analyzer Kern MLB50-3.

MEASUREMENT OF ENZYME ACTIVITY

The enzyme activity of hemicellulase and mannanase was determined by the method of Bailey & Pou-tanen.¹⁷ The specific enzyme activity was measured additionally using the protein assay of Bradford.¹⁸

ENZYMATIC HYDROLYSIS OF THE WATER EXTRACTABLE POLYSACCHARIDE FROM *P. MAJOR* LEAVES

Dry fraction of polysaccharide samples (1g) was wetted by careful dropwise addition of 10 ml of sodium acetate buffer (0.05 M, pH 5.45). The resulting gel was suspended in additional buffer (90 ml). The enzymatic hydrolysis of the polysaccharide suspension was conducted with hemicellulase (50 U and 250 U) and mannanase (0.2 U and 4 U) at 45°C for 40 h. After the reaction time passed the

samples were heated for 10 min at 100°C and then coagulated by the addition of 95% ethanol (1:2). The samples were centrifuged; the supernatants were separated and subsequently concentrated. The hydrolysis yield of WEP was assessed by 3,5-dinitrosalicylic acid (DNS) assay for determination of the released reducing sugars.¹⁹ Additionally, enzymatic hydrolysis was carried out with 100 and 500 U hemicellulase.

HPLC ANALYSIS

Determination of the monosaccharide composition was conducted with HPLC system Konik-Tech, with RI Detector Shodex R1-101 and Tracer Excel ODSB 120/5 µm (150×0.4 mm) column, mobile phase water, flow rate 0.5 ml/min, temperature 30°C. The oligosaccharides were analysed by HPLC system using a Polyamine II, 12 nm, 5 µm (250×4.6 mm) column, with RI Detector Shodex R1-101, mobile phase acetonitrile:water (60:40), flow rate 1 ml/min, temperature 30°C. The samples were determined by the retention time of galactose, xylose, arabinose, sucrose, maltose, maltotriose and raffinose standards.

RESULTS

PREPARATION OF WATER EXTRACTABLE POLYSACCHARIDES AND ALCOHOL-INSOLUBLE RESIDUE FROM *P. MAJOR* L. LEAVES

The AIR yield from 1 kg of *P. major* fresh leaves was 216 g kg⁻¹. The loss on drying of AIR was 10.02%. The yield of WEP from 100 g of *P. major* fresh leaves was 1.84 g ± 0.2 g.

ENZYME ACTIVITY

The enzymes used in this study exhibited different activity under different substrates. In standardizing the conditions for enzymatic hydrolysis of the WEP was determined the enzymatic activity of the two used enzymes under the substrates xylan and galactomannan (Locust Bean Gum). In the complex enzyme hemicellulase (Amano) were detected two enzyme activities: β-xylanase with activity 5000 U/g, using xylan as a substrate and β-mannanase with activity 990 U/g, using galactomannan as a substrate (**Table 1**). In the second studied enzyme mannanase (Amano), β-mannanase as the main enzyme activity was detected. Additionally, very low activity of β-xylosidase and α-galactosidase was detected.

DETERMINATION OF THE HYDROLYSIS YIELD

The carbohydrate amount and hydrolysis yield of WEP from *P. major* are shown in **Fig. 1**. The hydrolysis conditions were selected after preliminary

experiments. The highest carbohydrate amount and hydrolysis yield were obtained after treatment with 250 U hemicellulase (61.3%). The monosaccharide

main monosaccharide component and also a minor amount of xylose (**Fig. 2B**).

In order to optimize the conditions of oligosac-

Table 1. Measurement of enzyme activity of mannanase and hemicellulase

Enzyme	Substrate	Enzyme activity (U/g product)	Specific enzyme activity (U/g protein)
Hemicellulase	Xylan	5 061	678.2
Mannanase	Galactomannan	991	9.8

amount was the lowest after 0.4 U mannanase hydrolysis.

DETERMINATION OF THE COMPOSITION OF THE HYDROLYSIS PRODUCTS

HPLC analysis of 1% solution of WEP of *P. major* leaves after incubation for 40 h with mannanase showed the presence of the monosaccharide galactose. Another peak with a retention time of 13 min was observed, which probably corresponded to semi-hydrolysed oligosaccharide with a DP = 2-10 (**Fig. 2A**).

HPLC analysis of the hydrolysis products of 1% solution of WEP from *P. major* leaves with hemicellulase showed the presence of galactose as

charide production hydrolysis of 1% solution of WEP from *P. major* leaves was carried out with different concentrations of hemicellulase (50, 100, 250 and 500 U). The main obtained hydrolysis product was galactose, whose concentration increased with augmentation of the enzyme concentration. The hydrolysis conducted with 50 U hemicellulase showed the presence of the monosaccharide arabinose as well as a trisaccharide (DP = 3). At a higher enzyme concentration (100, 250 and 500 U) were reported disaccharides (DP = 2) and trisaccharides (DP = 3) (**Fig. 3**).

Beside the influence of the enzyme concentration, the impact of the time of hydrolysis reaction on the degree of enzymatic hydrolysis was also

Figure 1. Degree of polysaccharide hydrolysis after treatment of 1% solution of water extractable polysaccharide from *P. major* leaves with mannanase (**A**) and hemicellulase (**B**) for 40 h.

Figure 2. HPLC profiles of 1% solution of water extractable polysaccharide from *P. major* leaves after enzymatic hydrolysis for 40 h with 2 U mannanase (A) and 250 U hemicellulase (B).

investigated. Galactose was the main hydrolysis product obtained from the WEP after enzymatic hydrolysis with 100 U hemicellulase for 1h, 3h, 4h, 6h, 9h and 24 h. Small amounts of xylose, a disaccharide and trisaccharides with linear and branched structure were established (Fig. 4). The amount of detected carbohydrates increased with the reaction time passed.

DISCUSSION

The determination of considerable amounts of galactose after the enzymatic hydrolysis of WEP from *P. major* leaves proved the presence of galactans. Galactose was most likely released from the side chain of the polysaccharide molecule as a result of the galactosidase activity of hemicellulase. The monosaccharide xylose was assumed to be obtained

by the cleavage of β -1,4-linkages under the xylanase activity of the enzyme.

The β -xylosidase activity detected in hemicellulase did not act efficiently since the xylobiose had not been degraded to xylose. Due to the low amounts of detected arabinose probably in the studied hemicellulase α -arabinofuranosidase activity was not able to hydrolyse the WEP effectively.

As a result of the significantly lower hydrolysis yield with the enzyme mannanase and the high degree of hydrolysis using the enzyme hemicellulase we can conclude that in the WEP from *P. major* leaves a smaller number of the monomer residues are linked by β -1,4-linkages. Probably prevail β -1,3 or β -1,6-linkages, which are the basis of arabinogalactan II or α -1,4-linkages, building the pectic polysaccharides.

Figure 3. HPLC profiles of 1% solution of water extractable polysaccharide from *P. major* leaves after enzymatic hydrolysis with 50, 100, 250 and 500 U hemicellulase for 4 h.

Figure 4. HPLC profiles of 1% solution of water extractable polysaccharide from *P. major* leaves after enzymatic hydrolysis with 100 U hemicellulase.

We supposed that the detected disaccharides and the linear trisaccharides may be β -1,4-linked galactose residues resulting from the hydrolysis of arabinogalactan and some of the obtained branched trisaccharides may consist of residues of galactose, mannose and arabinose or xylose.

The Greater plantain is a rich source of pectic polysaccharides and hemicelluloses. According to Paulsen²⁰, the predominating oligosaccharides in *P. major* are arabinogalactan type II and a pectic type polymer with mainly a galacturonic acid backbone. The pectic polysaccharides are structurally built by different types of monosaccharides, they may be linear or branched, neutral or acidic. A wide range of biological activities have been reported for oligosaccharides, many of them having a great industrial value.^{21,22} Polysaccharide-degrading and modifying enzymes are an advantageous method to produce these molecules of interest. The hydrolysis can be conducted with a single enzyme or with a combination of enzymes and the total degradation of polysaccharides yield numerous different oligosaccharides and monosaccharides present in various quantities according to the source considered.

CONCLUSION

By enzymatic hydrolysis of polysaccharides we can obtain a wide range of effects beneficial for both human health and industry. The leaves of *P. major* are a rich source of polysaccharides. Polysaccharide-degrading and modifying enzymes can be used

in a controlled way to modulate polysaccharide properties or oligosaccharides of interest.

The enzyme hemicellulase was found to be a suitable commercial enzyme for hydrolysis of WEP from *P. major* leaves, with the hydrolysis yield increasing with augmentation of the ratio of enzyme to polysaccharide. Galactose was the prevalent monosaccharide detected in the composition of the isolated polysaccharides. After the enzymatic hydrolysis with hemicellulase and mannanase a different type of oligosaccharides were established. Further studies will tackle optimization of the use of polysaccharide-degrading and modifying enzymes and the determination of the obtained monosaccharide and oligosaccharide composition.

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Ферментативный гидролиз водорастворимых полисахаридов листьев *Plantago major* L

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Введение: Листья *Plantago major* L. имеют более чем вековую историю применения в традиционной медицине для лечения инфекционных состояний дыхательных путей и желудочно-кишечного тракта и мочевыводящих путей. Исследователи упоминают о том, что экстракты *Plantago major* в горячей воде обладают широким спектром антираковых, антиоксидантных и противовирусных свойств, а также свойств, которые приводят к изменению кле-

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точно-модулированного иммунитета. Вероятно, упомянутые полезные свойства могут быть связаны со значительным количеством содержащихся в них полисахаридов. Полисахариды, изолированные из листьев *Plantago major L.* отличаются различными структурами – пектиновые вещества, галактаны, арабиногалактаны, глюкоманнаны.

Цель: Целью данной работы являлось исследование взаимосвязи между структурой водорастворимых полисахаридов, изолированных из листьев *Plantago major L.* и их ферментативного гидролиза в различных углеводных гидролизах.

Материалы и методы: Гидролизные реакции были осуществлены с ферментами гемицеллюлаза и маннаназа. Спектрофотометрия общего количества редуцирующих сахаров была проведена с целью исследовать продукты гидролиза. Состав моносахаридов и олигосахаридов был определён при помощи высокоэффективной жидкостной хроматографии (ВЭЖХ).

Результаты: Наибольшее количество гидролизного продукта водорастворимых полисахаридов из листьев *Plantago major* было получено при обработке гемицеллюлазой. Гидролизный продукт увеличился путём наращивания соотношения фермента и полисахарида. Галактоза стала преобладающим моносахаридом, идентифицированным в составе изолированных полисахаридов. Олигосахариды с различной степенью полимеризации тоже были установлены.

Заключение: Ферментативный гидролиз водорастворимых полисахаридов из листьев *Plantago major* позволил нам получить различные виды олигосахаридов с полезным эффектом как для человеческого здоровья, так и для индустрии.