ACTA MICROBIOLOGICA BULGARICA Volume 33 / 1 (2017)

Influence of Enzymatically Hydrolysed Polysaccharide from *Plantago Major* L. on Beta-Xylosidase Activity in *Lactobacillus* Strains

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Abstract

The dynamics of hydrolysis of total acid-extractable polysaccharides from leaves of the medicinal plant *Plantago major* L. using hemicellulase and xylanase were studied. The degree of polysaccharide hydrolysis and the monomeric units were experimentally determined.

Hemicellulase and xylanase hydrolysis both indicated larger amounts of detected monosaccharides with increasing the reaction time. The enzymatic hydrolysis conducted with hemicellulase showed a higher degree of hydrolysis (93.7% after 28 h) compared to the hydrolysis carried out with the enzyme xylanase (27.8% after 28 h). Galactose was the prevalent monosaccharide identified by HPLC analysis. It could be proposed that in the total acid-extractable polysaccharides from *P. major* leaves a smaller number of the monomer residues are linked by β-1,4-glycosidic linkages. Probably β-1,3 or β-1,6-linkages prevail, which are the basis of arabinogalactan II, or α-1,4-linkages, building pectic type polysaccharides. Our results from the enzymatic reactions demonstrated the *in vitro* capacity of β-xylosidase from *L. plantarum* S26 and *L. brevis* S27 to hydrolyse xylobiose and polysaccharide hydrolysates from *P. major* leaves.

Key words: *Plantago major* L., total acid-extractable polysaccharides, enzyme hydrolysis, hemicellulase, xylanase, β-xylosidase, *Lactobacillus plantarum, Lactobacillus brevis.*

Резюме

В настоящата работа е изследвана динамика на хидролизния процес на тотално киселинно-екстрахируем полизахарид от листа на *Plantago major* L. посредством ензимите хемицелулаза и ксиланаза. Определена е степента на ензимна хидролиза на изолирания полизахарид, както и вида на съставящите го монозахаридни единици.

Проведената хидролиза с хемицелулаза и ксиланаза показа нарастване на количеството монозахаридни остатъци с увеличаване на реакционното време. При използването на хемицелулаза е отчетена по-висока степен на хидролиза (93.7% след 28 ч.) в сравнение с хидролизата, проведена с ксиланаза (27.8% след 28 ч.). Галактозата е основният монозахарид, идентифициран посредством HPLC анализ. От резултатите може да се предположи, че в тотално киселинно-екстрахируемия полизахарид от листа на *Plantago major* L. има по-малък брой мономерни остатъци, свързани с β-1,4-гликозидни връзки. Вероятно преобладават β-1,3 или β-1,6-връзки, които са в основата на арабиногалактан тип II или α-1,4-връзки, изграждащи пектиновия тип полизахарариди. Резултатите от *in vitro* ензимната хидролиза показват капацитет на β-ксилозидазата от *L. plantarum* S26 и *L. brevis* S27 да хидролизира ксилобиоза и получените полизахаридини хидролизати от листа на *Plantago major* L.

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Introduction

According to the latest definition of the International Scientific Association for Probiotics and Prebiotics, a dietary prebiotic is a selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health (Hill *et al*., 2014). The best known prebiotics are non-digestible oligosaccharides including galactooligosaccharides, fructooligosaccharides, glucooligosaccharides, isomaltooligosaccharides, xylooligosaccharides (Quiang, *et al*., 2009). Moreover, recent *in vitro* and *in vivo* experiments on the prebiotic potential of different oligosaccharides have demonstrated that functional oligosaccharides have antibacterial, antioxidant, antimutagenic properties and other pharmacological activities (Wu and Pan, 2012; Yao *et al*., 2013).

Plantago major L. (Greater plantain) is a medicinal plant, used for centuries in both traditional and contemporary medicine as a cure for a number of diseases related to the skin, respiratory and digestive organs, reproduction, and against infections. The leaves are a good source of polysaccharides, flavonoids, iridoid glycosides, phenolic acids and vitamins (Samuelsen, 2000; Jankovic *et al*, 2012; Gonçalves and Romano, 2016). The main polysaccharide fractions isolated from *Plantago major L*. leaves are xylogalacturonans, rhamnogalacturonans, galactans and arabinogalactans (Samuelsen *et al*., 1995; 1998). The polysaccharides have been found to possess immunostimulatory, antiviral, antioxidant and antitumor activities (Jin *et al*., 2013; Liu *et al.,* 2015).

Degradation and utilization of different oligosaccharides, especially xylooligosaccharides (XOS), by probiotic microorganisms began in the early work of Okazaki *et al*. (1990), who studied *in vitro* fermentations with *Bifidobacterium adolescentis*, *B. longum* and *B. infantis.* The degradation and utilization of oligosaccharides are strain-specific and are also affected by the degree of polymerization (DP) of the oligomers present in the oligosaccharide mixture. Later, Hopkins *et al.* (1998) carried out fermentations with commercial XOS (Suntory, Japan; 70% purity and DP 2-4) and found that the ability of bifidobacteria to grow on XOS depended on the considered strain. The influence of oat bran oligosaccharides on carbohydrate utilization and fermentation end-products was studied with *Lactobacillus rhamnosus*, *L. plantarum* and *L. lactis* (Kontula *et al*., 1998). The main products of *Lactobacillus* metabolism were lactic acid, acetic acid, formic acid and ethanol. The results indicated that XOS induce *Lactobacillus* to form end-products of typical mixed-acid fermentation, which was mainly due to the starvation of cells (Aachary and Prapulla, 2011).

Efficient and complete degradation of different prebiotic oligosaccharides requires the cooperation of different enzymes including α-glucosidase, β-glucosidase, β- glucanase, *β*-xylosidase, *α*-glucuronidase, *α*-L-arabinosidase, acetylxylan esterase and other enzymes.

The production of oligosaccharides from polysaccharides isolated from *P. major L.* leaves requires knowledge on the optimal polysaccharides extraction conditions and the enzymatic hydrolysis of polysaccharides into oligosaccharide fractions and their utilization by *Lactobacillus* strains with probiotic potential. The aim of the present work was to study the optimal conditions for enzymatic hydrolysis of the total acid-extractable polysaccharides (TAEPs) isolated from *P. major* L. leaves with hemicellulase and xylanase and to determine the degree of polysaccharide hydrolysis. Furthermore, enzyme activity in *Lactobacillus* strains related to the utilization of the obtained oligosaccharide fractions was studied.

Materials and Methods

Materials and reagents

P. major L. leaves were collected from the Thracian valley floristic region, Bulgaria, in the vegetative season of 2015. Species identification was carried out according to Tutin *et al*. (1976) and Delipavlov *et al*. (2011). Xylan (from oat-spelts), p-nitrophenyl-β-D-xylopyranoside (pNPX) and standards of monosaccharides were purchased from Sigma-Aldrich, US. Hemicellulase (EC 3.2.1.78) was obtained from Amano, Japan and xylanase (EC 3.2.1.8) was obtained from Pectopan, DSM. MRS (de Mann Rogosa Sharpe broth) was purchased from Merck, Germany. All other reagents used in this study were of analytical grade.

Extraction of polysaccharides

The extraction of polysaccharides from *P. major* L. leaves was carried out following the methodology of Kratchanova *et al*. (2008). Fresh sliced leaves of *P. major* were treated with 95% ethanol (1:2.5, *w/v*) for 1 hour at 70°C and then filtered. The filtrate was washed successively with 95% ethanol, chlorophorm-methanol solution (1:1, *v/v*) and acetone (Sengkhamparn *et al*., 2009). Dilute hydrochloric acid (pH 1) was used for extraction of poly-

saccharides. The extraction was conducted at 80°C for 1.5 h. Total acid-extractable polysaccharides (TAEPs) were precipitated with 95% ethanol (1:1, *v/v*) and washed with 70% ethanol to neutral pH.

Bacterial strains and culture conditions

In this study we used two *Lactobacillus* strains: *L. plantarum* S26 and *L. brevis* S27, which were kindly provided by the Department of Microbiology (Sofia University, Bulgaria). They were identified using 16S RNA techniques. The strains were cultured overnight (16-18 h) on MRS (de Mann Rogosa Sharpe broth) at 37ºC and in limitation of oxygen (BBL® Gas Pak anaerobic system Envelopes, Becton Dickinson, Franklin Lakes, NJ USA).

Fermentation

Lactobacilli were routinely grown in MRS broth. Overnight grown cells were washed twice in saline (0.85% NaCl solution) and 10% of the bacterial suspension (10^7 cfu/mL^{-1}) was used to inoculate mMRS broth medium (pH 6.8) 1% glucose and 1% xylobiose. The anaerobic fermentations were performed for lactobacilli in 50 mL polystyrene bottles at 37ºC for 48 h (Mandadzhieva *et al*., 2011).

Determination of enzyme activity

Hemicellulase and xylanase basic enzyme activity was determined spectrophotometrically according to the methodology of Bailey and Poutanen (1989). Xylan from oat-spelts was used as a substrate. The analyses were conducted at pH 5.45, temperature 50°C. One unit of hemicellulase or xylanase activity was defined as the amount of enzyme that produced 1 μmol of reducing sugars equivalent to xylose per min per mg of protein at 50°C and pH 5.45.

The β-xylosidase (EC 3.2.1.37) activity assays were carried out using p-nitrophenyl-β-D-xylopyranoside (pNPX) with substrate prepared in citrate-phosphate buffer solution. One β-xylosidase unit (U) was defined as the amount of enzyme which liberated 1 μmol of ONP (ortho-nitrophenyl) per min per mg of protein at 37°C and pH 6.0.

Proteins were assayed by the method of Bradford (1976) by using bovine serum albumin as a standard. The spectrophotometric analyses were carried out with Spectrophotometer Beckman Coulter DU 800.

Enzymatic hydrolysis of polysaccharide fractions isolated from P. major L. leaves

Enzymatic hydrolysis of 1% solution of TAEPs of *P. major* leaves was conducted with hemicellulase (2.5 U/ml) and xylanase (10 U/ml) at 45ºC, pH 5.45. The hydrolysis dynamic was performed for 1, 2, 3, 6, 15, 24 and 28 h (n = 3). After the reaction time had elapsed, the samples were heated for 10 min in a boiling water bath and then coagulated by two volumes of 95% ethanol. The samples were centrifuged and the supernatants were concentrated in a rotary evaporator.

Determination of the degree of hydrolysis

The amount of the released reducing sugars and the degree of polysaccharide hydrolysis were assessed by the 3,5-dinitrosalicylic acid (DNS) assay, described by Miller (1959). The absorbance was measured at a wavelength of 540 nm using glucose as a reference standard.

HPLC analysis

Carbohydrate composition in polysaccharide hydrolysates of *P. major* leaves was analysed using HPLC system Konik-Tech, with RI Detector Shodex R1-101 and Tracer Excel ODSB 120/5 μm (150 x 0.4 mm) column, mobile phase water, flow rate 0.5 mL/min, temperature 30°C. The registered peaks of the samples were evaluated using reference monosaccharide standards.

Preparation of resting cells

Bacterial cells of *L. plantarum* S26 *and L. brevis* S27, cultivated in mMRS-1% xylobiose in the logarithmic phase of growth, were harvested by centrifugation at 6000xg for 10 min (4°C) and then washed twice with physiological saline.

Hydrolysis of xylobiose and polysaccharide hydrolysates from P. major L. by resting cells

The hydrolysis of xylobiose and polysaccharide hydrolysates from *P. major* was performed with 5g-wet/L resting cells in physiological saline at 37°C for 60 min. After boiling for 10 min, the sample was analysed by HPLC for the detection of galactose, glucose, lactose, lactulose and oligosaccharides as described above.

Results and Discussion

Polysaccharides were extracted from plant cell walls of *P. major* leaves with dilute hydrochloric acid. Mineral acids, as a destructive type of extragents, led to breakage of the bonds in the polysaccharide molecule with other components of the cell wall and in parallel hydrolysed the glycosidic bonds in the macromolecule (Kratchanova *et al*., 2008). The yield of isolated total acid-extractable polysaccharides (TAEPs) was 2.47 ± 0.26% expressed as gram polysaccharide per 100 g of fresh leaves.

The results from the determination of hemicellulase and xylanase activities are presented in Table 1. It is known that as a complex enzyme and

Table 1. Enzyme activity of hemicellulase and xylanase

Enzyme	Substrate	pH	Temperature (°C)	Time (sec)	Basic enzyme activity (U/g product)	Specific enzyme activity (U/g protein)
Hemicellulase	Xylan	5.45	50	300	5 061	678
Xylanase	Xylan	5.45	50	300	19 966	962

depending on its production source, hemicellulase exhibits different activities (xylanase, arabinofuranosidase, galactomannase, cellulase). The studied hemicellulase definitely showed lower xylanase activity (5 061 U/g product) compared to the monocomponent enzyme xylanase (19 966 U/g product).

In *P. major* leaves, different polysaccharides were detected: xylans with β-1,4-linked xylofuranosidase residues; arabinogalactans with β-1,4, β-1,3 and β-1,6 glycosidic linkages and pectins with α-1,4-glycosidic linkages (Samulesen *et al*., 1995, 1998; Samuelsen, 2000; Paulsen and Hilde, 2005).

In order to achieve maximum degradation of the polysaccharide chain of TAEPs, the complex enzyme hemicellulase, which breaks down α- and β-glycosidic linkages, was used (Biely, 2003). The dynamics of hydrolysis process with hemicellulase (2.5 U/mL) and xylanase (10 U/mL) were performed to determine the optimal time of hydrolysis of TAEPs from *P. major*. The hydrolysis experiments were run for 28 h by taking samples at 0, 1, 2, 3, 6, 15, 24 and 28 h. It is known that xylanase breaks mainly the β-1,4-bonds between pentose residues in the polysaccharide chain (Motta *et al*., 2013). The obtained results showed a linear increase in the degree of hydrolysis with an increase in the time of the enzymatic reaction (Fig. 1).

Despite the high concentration (10 U/mL) of xylanase, the degree of hydrolysis was significantly lower (27.8% at 28 h) compared to the degree of hydrolysis using 2.5 U/mL hemicellulase (93.7% at 28 h). The significantly lower degree of hydrolysis obtained by the action of the enzyme xylanase and the high degree of hydrolysis using hemicellulase suggested that in *P. major* TAEPs a smaller number of the monomeric residues were linked with β-1,4-glysosidic linkages. Probably, the polysaccharide fractions in TAEPs are of type II arabinogalactan or pectic type. The above-mentioned polysaccharides can be hydrolysed by comercial hemicellulase with different activities (arabinofuranosidase, galactomannase, pectinase).

The results from the HPLC analysis of polysaccharide hydrolysates showed the presence of galactose as the main released monosaccharide. Contrary to the established linear gradation in the degree of hydrolysis with increasing the reaction time, no linear dependence was observed between the release of galactose units and the hydrolysis time (Table 2). The highest amount of released galactose units was registered between 3 and 15 hours

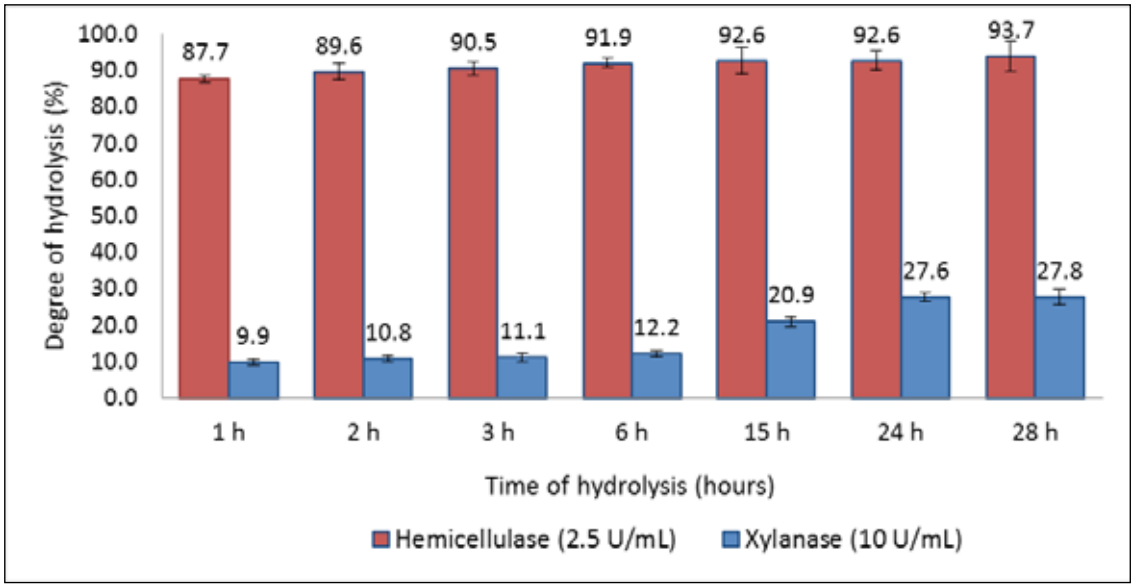

Fig. 1. Dynamic of degree of polysaccharide hydrolysis after treatment of 1% solution of TAEPs from *P. major* leaves with hemicellulase and xylanase.

Table 2. Galactose concentration in 1% solution of TAEPs from *P. major* leaves after enzymatic hydrolysis with hemicellulase (2.5 U/mL) and xylanase (10 U/mL).

Time (hours)	Galactose (mg/g TAEPs)	
	Hemicellulase (2.5 U/mL)	Xylanase (10 U/mL)
1 h	295.76	153.65
2 h	182.93	207.20
3 h	430.61	244.30
6 h	577.88	84.15
15 h	561.10	176.15
24 h	333.85	92.10
28 h	227.16	116.55

(430.61 - 577.88 mg galactose/g TAEPs) using the enzyme hemicellulase (Fig. 2).

The next studies were performed in order to prove the induction or inhibition of β-xylosidase activity in the presence of 1% xylobiose and polysaccharide hydrolysates of *P. major* leaves. We investigated the influence of the obtained polysaccharide hydrolysates on hydrolase activity of β-xylosidase from the studied strains (Table 3).

Xylose conversion by resting cells of *L. plantarum* S26 and *L. brevis* S27 was incubated at 37ºC for 24 h. The total activity was expressed as the concentration of xylose for the first 2 hours of the reaction. Xylose is a noncompetitive inhibitor of β-xylosidase which was confirmed by the results from 24 h of incubation.

Conclusion

From the enzymatic hydrolysis conducted in this study it can be concluded that the enzyme hemicellulase is a more suitable commercial enzyme for hydrolysis of TAEPs from *P. major* leaves

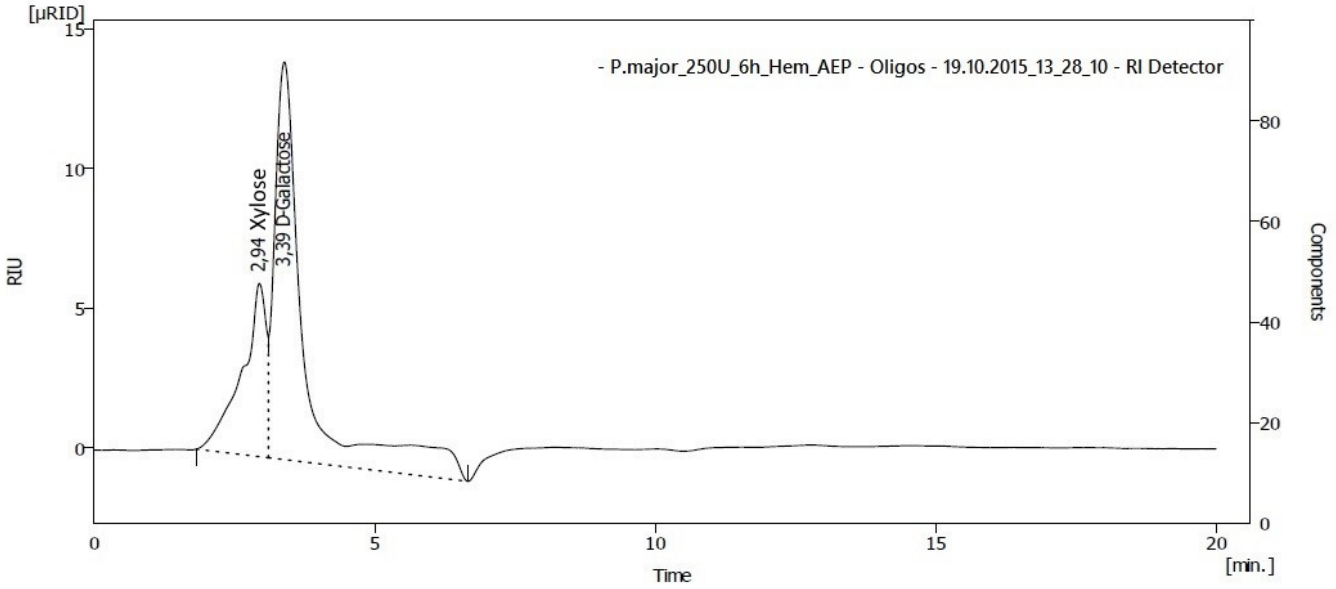

Fig. 2. HPLC profile of 1% solution of TAEPs from *P. major* leaves after enzymatic hydrolysis with hemicellulase (2.5 U/mL) for 6 h.

Table 3. Xylose concentration after hydrolysis of xylobiose and TAEPs from *P. major* leaves by resting cells of *L. plantarum S26* and *L. brevis S27* strains.

Strain	Time (min)	Xylose concentration (g/L)	
		Xylobiose	TAEPs from *P. major* leaves
Lactobacillus plantarum S26	0	0	0
	60	0.4	0.18
	120	0.9	0.35
Lactobacillus brevis S27	0	0	0
	60	0.7	0.25
	120	1.3	0.45

compared to xylanase. The prevalent monosaccharide registered by the HPLC analysis was galactose. The higher degree of hydrolysis using hemicellulase and the significant amounts of the released galactose units suggest that the TAEPs may be composed of β-1,3 or β-1,6-linked galactans or pectic polysaccharides with mainly galactan side chains. Our results from the enzymatic reactions demonstrated the *in vitro* capacity of β-xylosidase from *L. plantarum* S26 and *L. brevis* S27 to hydrolyse xylobiose and polysaccharide hydrolysates from *P. major* leaves.

Acknowledgments

The authors are grateful to the Medical University of Plovdiv for the provided financial support (University project SDP - 03/2015).

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