99. G. F. Fild and W. Caldwell, U.S. Patent 4,054,740, cl. 548-303 (1977).
100. M. Ternaki and S. Yoshinari, Japanese Patent 76,138,692 (1976); Chem. Abstr., 87, 53927 (1977).
101. J. Vasilevskis, J. A. Gualtieri, S. D. Hutchings, R. C. West, J. W. Scot, D. R. Parrish, F. T. Bizzarro, and G. F. Fild, J. Am. Chem. Soc., 100, 7423 (1978).
102. K. Folkers, R. Mozingo, and D. E. Wolf, U.S. Patent 2,442,681, cl. 260-309.7 (1948).
103. K. Folkers, D. E. Wolf, and N. J. Rahway, U.S. Patent 2,441,141, cl. 260-309.7 (1948).

A STUDY OF THE PRODUCTS OF THE HYDROLYSIS OF THE XYLAN OF *Melilotus albus* BY ENDO-1,4-β-XYLANASE

M. S. Dudkin, N. A. Rodionova, I. S. Kazanskaya, I. V. Gorbacheva, E. I. Kozarez, and N. A. Denisyuk

UDC 547.458.8:577.154.33/34

The enzymatic hydrolysis of a 4-O-methylglucuronoxylan of white sweetclover (*Melilotus albus*) by a highly purified homogeneous endo-1,4-β-xylanase from *Aspergillus niger* 14 has shown that the enzyme hydrolyzes 97% of the polysaccharide in 72 h. Acidic and neutral oligosaccharides were found in the hydrolysate after the action of the enzyme. An investigation of the hexauronic acid isolated has shown that the glucuronic acid is attached to the nonreducing end of the xylooligosaccharide, which demonstrates the specific action of the enzyme on the polysaccharide.

The action of an endo-1,4-β-xylanase with a molecular weight of 24,000-27,000 produced by the fungus *Aspergillus niger* 14 in surface cultivation on wheat bran has been studied [1]. The endo-1,4-β-xylanase was purified by precipitation from an extract of the culture with isopropanol, fractionation with ethanol, gel filtration through a column of Sephadex G-50, chromatography and rechromatography on a column of hydroxylapatite, and chromatography and rechromatography on CM-cellulose [2, 3]. The degree of purification was 5000.

The homogeneity of the enzyme used was established by electrophoresis in polyacrylamide gel and isoelectric focusing, by the use of an ultracentrifuge, and by gel filtration through a column of Sephadex G-200 [2].

The endo-1,4-β-xylanase did not cleave xylobiose and methyl D-xyloside and did not hydrolyze the (1→4) bonds between the β-D-glucopyranose residues in the molecules of cotton fiber, filter paper, and sodium carboxymethylcellulose and the 1→3 bonds in laminarin and did not hydrolyze the 1→4 and 1→6 bonds in starch molecules, andit did not contain as impurities pectolytic and proteolytic enzymes, β-1,4-mannanases and α-1,6-galactosidases.

In experiments in which it was incubated with the arabinoglucuronoxylan isolated from wheat straw and with [^{14}C]xylose the endo-1,4-β-xylanase from the fungus *Aspergillus niger* 14 exhibited no transglycosylase activity, and it hydrolyzed xylotriose and xylotetraose to xylobiose and xylose, hydrolyzed xylopentaose mainly to xylotetraose, and hydrolyzed xylohexaose to xylopentaose and xylotetraose. The rate of cleavage of the oligosaccharides rose with an increase in the degree of polymerization.

Under conditions of the continuous removal of the hydrolysis products, using dialysis bags of collagen film placed in a vessel containing 0.01 M acetate buffer at pH 4.2 with continuous stirring of the solution and the replacement of the buffer every 3 h, after 72 h almost complete (97.7%) cleavage by the endo-1,4-β-xylanase of the 4-O-methylglucurono-β-D-xylan isolated from the stems of the herb white sweetclover (*Melilotus albus*) took place.

The unhydrolyzed insoluble residue contained a polysaccharide with a molecular weight of 7400. Its degree of polymerization was 56, $[\alpha]_D^{20}$ −37°. It was constructed of β-D-xylose residues linked to one another by (1→4)-bonds together witha small amount of D-glucuronic and 4-O-methyl-D-glucuronic acids.

M. V. Lomonosov Odessa Technological Institute of the Food Industry. A. N. Bakh Institute of Biochemistry, Academy of Sciences of the USSR, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 4, pp. 477-483, July-August, 1980. Original article submitted January 10, 1980

 0009-3130/80/1604-0338$07.50

After enzymatic hydrolysis for 72 h, 10 g of the 4-O-methylglucuronoxylan yielded 5.8 g of neutral and 3.7 g of acidic xylooligosaccharides. Among the neutral hydrolysis products xylooligosaccharides with the following R_{Xyl} values were detected by paper chromatography: 1, 0.044; 2, 0.083; 3, 0.146; 4, 0.48; and 5, 1.0 (xylose). From a consideration of the dependence of $\log R_{Xyl}/(1 - R_{Xyl})$ on m (degree of polymerization), having compared the compounds isolated with authentic markers calculated from the results of thin-layer chromatography, we identified xylobiose and xylotriose, small amounts of xylotetraose and xylopentaose, and traces of xylose in the neutral oligosaccharides (Fig. 1a).

The presence of a number of compounds among the oligosaccharides was established by paper chromatography. Below we give the composition of the acidic oligosaccharides in a hydrolyzate of the 4-O-methylglucuronoxylan of white sweetclover after the action of the endo-1,4-β-xylanase (A, aldouronic acid)

R_{Xyl} of the oligosaccharides investigated	R_{Xyl} of acidic oligosaccharides according to Timell [5]
0.19	0.17 (A_7)
0.30	0.28 (A_6)
0.32	0.43 (A_5)
0.57	0.59 (A_4)
0.72	0.77 (A_3)

A graph of the dependence of the mobility of the aldouronic acids on the degree of their polymerization (Fig. 1b) and a comparison of the values for the compounds found with R_{Xyl} of markers shows that among the acidic products of the hydrolysis of the 4-O-methylglucuronoxylan of white sweetclover there are a number of substances including aldobiuronic, aldotriuronic, etc., up to aldoheptauronic acids. Among them the oligomers with the highest molecular weight, particularly the hexauronic acid, predominate. After chromatography, it was eluted with water. The extract was evaporated in vacuum, and the residue was identified.

The high amount of aldopenta-, aldohexa-, and aldoheptauronic acids in the enzymatic hydrolysate of the glucuronoxylan of white sweetclover is in harmony with the characteristics of the oligomers found after hydrolysis in the presence of the endo-1,4-β-xylanase of xylans isolated from other types of raw material [4] and is explained by the fact that residues of a glucuronic acid and its methyl derivative sterically hinder the action of the enzyme on certain bonds of the polyxyloside chain of the polymer.

Timell [5] has studied the products of the hydrolysis of the 4-O-methylglucuronoxylan isolated from the wood of the European white birch, which contained branches in a proportion of one for every 10 β-xylose residue, in the form of glucuronic and 4-O-methylglucuronic acid residues. In addition to neutral oligosaccharides, acidic oligomers with degrees of polymerization of from four to eight were present.

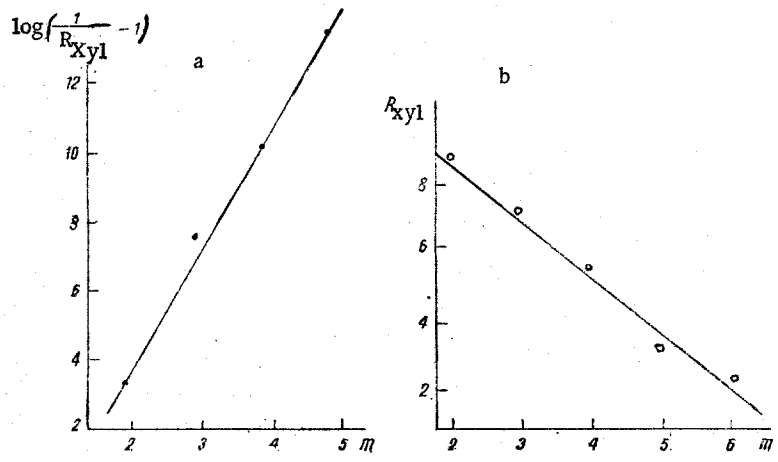
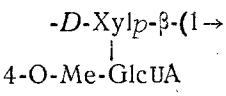


Fig. 1. Dependence of $\log[(1/R_{Xyl}) - 1]$ on the number of xylose residues m in a homologous series of xylooligosaccharides (a) and the dependence of R for xylose on the number of xylose residues m in a homologous series of aldouronic acids (b).

After the action of endo-1,4-β-xylanase present in the cellulase preparations from Merck AG and Onozuka on the 4-O-methylglucuronoxylan isolated from the wood of *Fagus sylvatica* L., it was established [6] that these enzymes differ according to their origin in the nature of their action and give different hydrolysis products. Thus, after the treatment of the 4-O-methylglucuronoxylan from beech wood with the endo-1,4-β-xylanase present in the Merck AG cellulase preparation, D-xylose and xylooligosaccharides with a degree of polymerization of 2 and 4-O-methylglucuronic and aldotetrauronic acids were found in the solution. After the action on the same xylan of the xylanase of the Onozuka cellulase preparation, the hydrolysate contained xylose, xylooligosaccharides with degrees of polymerization of from 2 to 10, 4-O-methylglucuronic acid, and a series of acidic oligosaccharides with degrees of polymerization of 3-8.

It is known that under the action of β-D-xylanase isolated from *Coniophora arrebella* the 4-O-methylglucuronoxylan of the poplar *Populus tremuloides* Michx., which has one branch per 10 xylose residues on an average, is hydrolyzed with the formation of a series of acidic oligomers having a degree of polymerization of 4 and D-xylose and neutral oligomers with degrees of polymerization of 2-5. Their structure has not been studied. A comparison of our results with those of Dekker and Richards [4] permits the statement that the structure of the acidic oligosaccharides formed in the enzymatic hydrolysis of 4-O-methylglucuronoxylans is characterized by the following general formula:



where n is the number of residues.

In the molecules, the branching is present at the nonreducing end, and the bond located to the left of the β-xylose residue with branching is most resistant to hydrolysis in the presence of the enzyme. This fact also confirmed that the aldohexauronic acid that we isolated is also located at the nonreducing end of the molecule.

EXPERIMENTAL

As the substrate for studying enzymatic hydrolysis we used the 4-O-methylglucuronoxylan from the stems of white sweetclover (*Melilotus albus*). The macromolecule of this polysaccharide consists of β-D-xylose residues linked by β-(1→4)-bonds. For every six anhydroxylose residues of the main chain, on an average, there is one branch in the form of D-glucuronic and 4-O-methylglucuronic acid residues. The degree of polymerization of the xylan is 120, its molecular weight 15,900, $[\alpha]_D^{20}$ 95.2° (c 0.2%, 4% NaOH) [7].

Enzymatic Hydrolysis. To hydrolyze the xylan we used a highly purified homogeneous endo-1,4-β-xylanase isolated from an *Aspergillus niger* preparation [2]. The incubation mixture consisted of 2 g of xylan, 6 ml of enzyme solution containing 1.5 mg of endo-1,4-xylanase, and 50 ml of 0.01 M acetate buffer with pH 4.2. The specific activity of the xylanase was 2200 units/mg [2, 3]. The mixture was placed in a dialysis bag made from Japanese collagen film. Twelve such bags were introduced into a vessel containing five liters of 0.01 M acetate buffer with pH 4.2. Hydrolysis was carried out for 72 h at 40°C with continuous elimination of the cleavage products by dialysis [5] with stirring. The buffer was changed every 3 h. The dialysates were evaporated in vacuum and the amount of sugars in them was determined by the anthrone method [8]. After hydrolysis for 24 h, 31.25% of oligosaccharides had been formed; after 48 h, 18.12%; and after 72 h, 6.87%.

The dialysates obtained after hydrolysis for 24, 48, and 72 h contained, according to chromatographic results, the same oligosaccharides, and therefore in the subsequent investigations they were combined. To free them from hydrogen ions, concentrated solutions of the oligosaccharides were passed through a column (1.5 × 40 cm) containing KU-2 resin, and to free them from hydroxyl ions they were passed through a column containing AV-60 G resin.

The separation of the xylooligosaccharides into acidic and neutral components was performed on a column (1.2 × 10.0 cm) of Dowex 1 × 8 resin in the acetate form. For separation, the previously desalted dialysate was introduced into it and it was left for 12 h. The oligosaccharides were eluted with distilled water. The completeness of elution was monitored by the anthrone reaction. After the elimination of the neutral xylooligosaccharides, the acidic xylooligosaccharides were extracted with a 25% solution of acetic acid. The separation of 10 g of the total oligosaccharides yielded 5.6 g of neutral and 3.7 g of acidic products.

Natural Xylooligosaccharides. The eluate containing the neutral xylooligosaccharides was evaporated in vacuum to the state of a viscous syrup. Part of it was dissolved in water and was investigated by descending paper chromatography in the mobile solvent system pyridine—butan-1-ol—benzene—water (3:5:1:3). The presence of xylooligosaccharides with the following R_{Xyl} values was established (Fig. 1a): 1, 0.044 (xylopentaose); 2, 0.088 (xylotetraose); 3, 0.146 (xylotriose); 4, 0.48 (xylobiose); 5, 1.00 (xylose).

Acidic Xylooligosaccharides. The fraction of acidic oligosaccharides was evaporated to dryness, KOH being added to form an alkaline medium in order to prevent lactonization. The resulting solution was chromatographed on paper using the ethyl acetate—acetic acid—water (3:1:3) solvent system. From the results of chromatography using the graphical dependence of the mobility on the degree of polymerization of the oligosaccharides (Fig. 1b) we established the presence of acidic xylooligosaccharides — aldouronic acids — with R_{Xyl} 0.19 (heptauronic acid), 0.30 (hexauronic acid), and 0.32 (penturonic acid), with a predominance of the hexauronic acid in the mixture. Then this product was characterized in more detail. For this purpose, it was eluted with distilled water and was evaporated in vacuum.

Structure of the Hexauronic Acid. The substance isolated was studied in accordance with a known scheme: methylation of the oligosaccharide, its reduction, methanolysis, and identification of the methyl glycosides obtained. Methylation was performed with dimethyl sulfate in the presence of caustic soda [9]. A solution of 0.084 g of the oligosaccharide in 20 ml of 40% sodium hydroxide was treated with 15 ml of dimethyl sulfate, and the mixture was stirred for 3 h and was then left for 24 h. After two methylations, the mixture was neutralized, with cooling, and was extracted with chloroform. The extract was dried over anhydrous sodium sulfate. The chloroform was distilled off in vacuum, and the residue was dissolved in dry tetrahydrofuran and was treated with 5 g of powdered NaOH and 4 ml of dimethyl sulfate. The mixture was stirred at 30-40°C for 6 h and was kept in the cold for another 12 h. Then it was neutralized and the methylated oligosaccharide was extracted with chloroform and was isolated further as described above. The completeness of methylation was checked by thin-layer chromatography and IR spectroscopy.

The syrup obtained was mixed with dry ether and was reduced with $LiAlH_4$. The reducing agent was added in portions of 50-60 mg every 15 min for 1 h. The mixture was then stirred for another 4 h, and it was cooled to 0°C and the excess of $LiAlH_4$ was decomposed by the addition of distilled water (until the solution had become decolorized). The resulting solution was neutralized with sulfuric acid. The reduced methylated oligosaccharide was extracted with chloroform and was remethylated as described above.

The substance obtained after the chloroform had been distilled off was subjected to methanolysis with 4% HCl in methanol for 5 h. The methanolysate was neutralized with AV-17 anion-exchange resin and was evaporated. The product obtained was investigated by gas—liquid chromatography on an LKhM-8 MD instrument with a flame-ionization detector at 150°C under isothermal conditions. Column 200 × 0.5 cm, solid phase Chromaton N-AWDMCS; liquid phase 5% XE-60; carrier gas helium; rate of flow 35 ml/min. From the results of chromatography in comparison with model substances the presence of the 2,3-di-O-methylxyloside, the 3,4-di-O-methylxyloside, and the 2,3,4,6-tetra-O-methylglucopyranoside was established. The molar ratio of the combined di-O-methylxylosides and the tetra-O-methylglucopyranoside was 5:1. This shows that the oligosaccharide isolated was a hexauronic acid. The presence in the methanolisate of the 3,4-di-O-methylxyloside shows the addition of the uronic acid to the nonreducing end of the oligosaccharide.

Characterization of the Solid Residue after the Enzymatic Hydrolysis of the Xylan. The solid residue after enzymatic hydrolysis was washed, dried, and purified by reprecipitation via the copper complex. After two treatments, the amount of readily hydrolyzable polysaccharides in the product had stabilized. It contained a small amount of ash and of nitrogenous substances. From the results of viscometry the molecular weight of the polysaccharide was 7400 (DP 56), $[\alpha]_D^{20}$ 37° (c 0.2; 4% NaOH). According to chromatographic analysis of a hydrolysate, the polysaccharide contained xylose and a small amount of glucuronic and 4-O-methylglucuronic acids.

The IR spectra contained absorption bands at 899 cm^{-1} (corresponding to a β-linkage between the monose residues in the polysaccharide [10]), 1716 cm^{-1} ($-C(=O)-O-$ groups) and

2876 cm^{-1} ($-OCH_3$ groups), confirming thepresence of glucuronic and 4-O-methylglucuronic acids in the xylan. Consequently, the solid residue is a low-molecular-weight xylan with a small content of uronic acids.

SUMMARY

1. A highly purified homogeneous endo-1,4-β-xylanase isolated from *Aspergillus niger* 14 hydrolyzes 97% of the 4-O-methylglucuronoxylan of the stems of white sweetclover in 72 h.

2. The 4-O-methylglucuronic and glucuronic acid branches in the chain of the sweetclover xylan sterically hinder its hydrolysis in the presence of an enzyme, which leads to the formation of increased amounts of hexauronic acids in the hydrolysate.

3. The results of a study of the structure of the hexauronic acid produced by the enzymatic decomposition of the xylan show its attachment to the nonreducing end of the xylooligosaccharide.

4. The solid residue after the enzymatic hydrolysis of the glucuronoxylan of white sweetclover — a low-molecular-weight polysaccharide constructed of the β-D-xylose residues — contains side chains consisting of glucuronic and 4-O-methylglucuronic acids.

LITERATURE CITED

1. N. A. Rodionova, R. V. Feniksova, and I. V. Itomlenskite, Khim. Drev., 8, 127 (1971).
2. I. V. Gorbacheva and N. A. Rodionova, Biochem. Biophys. Acta, 484, 79 (1977).
3. N. A. Rodionova, I. V. Gorbacheva, and V. A. Buivid, Biokhimiya, 42, No. 4, 659 (1977).
4. R. F. H. Dekker and G. N. Richards, Adv. Carbohydr. Chem., 32, 346 (1976).
5. T. E. Timell, Svensk. Papperstidn., 65, 435 (1962).
6. M. Sinner, Mitt. Bundesforschunganstalt Forst, Holz-Wirtsch., 93, 257 (1973).
7. M. S. Dudkin and N. G. Shkantova, Abstracts of Lectures at an All-Union Conference on Processing Products of the Timber and Agricultural Industries [in Russian], Riga (1973), p. 24.
8. G. H. Roe, J. Biol. Chem., 212, 335 (1955).
9. E. L. Hirst and E. Percival, in: Methods of Carbohydrate Chemistry (R. L. Whistler, ed.), Vol. 5, Academic Press, New York (1965), pp. 289-290.
10. C. J. Giang and R. H. Marchessault, J. Polymer Sci., 39, 269 (1959).