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STUDIES ON XYLAN-DEGRADING ENZYMES

II. ACTION PATTERN OF ENDO-1,4-β-XYLANASE FROM *ASPERGILLUS NIGER* STR. 14 ON XYLAN AND XYLOOLIGOSACCHARIDES

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Summary

1. The degree with which endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) from *Aspergillus niger* str. 14 degrades xylans, arabinoxylans, arabinoglucuronoxylans and 4-*O*-methylglucuronoxylans was studied. Xylans were degraded by 14—30%. After elimination of products of xylan degradation during hydrolysis, 4-*O*-methylglucuronoxylans were degraded by 97%.

2. Among the neutral products of glucuronoxylan degradation xylobiose > xylotriose > xylose were the major products.

3. Among the products of arabinoglucuronoxylan degradation xylobiose > xylotriose > xylose and xylooligosaccharides with the polymerization degree of over 3 were in predominance.

4. Hydrolyzation of arabinoxylans from rye seeds yielded little xylobiose and xylose and the major products were oligosaccharides with a polymerization degree of over 3.

5. Endo-1,4-β-xylanase from *Asp. niger* str. 14 did not degrade xylobiose but hydrolyzed xylotriose to form xylobiose and xylose; xylotetraose to xylobiose and minor quantities of xylose; xylopentaose to xylotetraose and minor amounts of xylobiose and xylose; xylohexaose to xylopentaose > xylotetraose and minor amounts of xylobiose and xylose.

6. Experiments with [C^{14}]xylose did not reveal transglycosilase activity of endo-1,4-β-xylanase. However, the fact that xylotetraose and xylopentaose were the major products of xylopentaose and xylohexaose degradation suggested transglycosilase activity of endo-1,4-β-xylanase.

7. The K_m value was determined, for the action of endo-1,4-β-xylanase on carboxymethylxylan, to be $3 \cdot 10^{-3}$ g/ml and for xylotetraose, $1.04 \cdot 10^{-3}$ M.

8. The relative volocities of glucuronoxylane, xylonanoose and xylotetraose were 100, 10 and 7.4, respectively.

9. Endo-1,4-β-xylanase from *Asp. niger* str. 14 did not degrade starch, cellu-

lose, pectin, pectic acid, laminarin, methyl-β,D-xylopyranoside, methyl-β,D-arabinopyranoside or galactomannan.

Introduction

A preparation of xylanase that contained a large number of xylan-degrading enzymes was obtained from the strain *Asp. niger* 14 was detected [1]. From the enzyme preparation a highly purified endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) with a molecular weight of 24000 was isolated (See Part I). The enzyme was homogeneous during ultracentrifugation, polyacrylamide gel disc electrophoresis, gel filtration and isoelectric focusing. The purpose of the present investigation was to study the action pattern of this endo-1,4-β-xylanase from *Asp. niger* str. 14 on xylans and xylooligosaccharides.

Materials and Methods

Substrates

Glucuronoxylan from sweet clover. Molecular weight: 15900. Xylose : uronic acids ratio = 6 : 1. Ash content: 0.38%. $[\alpha]\ D^{20} = -95.21°$.

Arabinoglucuronoxylan from timothy grass. Molecular weight 18480. Xylose : arabinose : glucuronic acid ratio = 20 : 3 : I. $[\alpha]\ D^{20} = -69°$.

Arabinoglucuronoxylan from wheat straw. Molecular weight: 19400. The content of monosaccharides: xylose, 79.32%; arabinose, 12.07%; uronic acids, 6.61%. $[\alpha]\ D^{20} = -92°$.

Glucuronoxylan from alfalfa stems. Molecular weight: 12600. Xylose : uronic acids ratio = 18 : 1.

Crystalline xylan. Molecular weight: 1840. Xylose alone was found during hydrolysis.

Glucuronoxylan from alder wood. Molecular weight: 8600. Xylose : uronic acids ratio = 10 : 1.

Sodium carboxymethylxylan. Substitution degree: 0.5; obtained from wheat straw xylan.

Pectic acid. Isolated from pectic substances of beet pulp. Acid hydrolyzate was found to contain 70% reducing substances, 60% uronic acids and small amounts of galactose, xylose and rhamnose.

Laminarin. Isolated from the algae *Laminaria saccharina* and *L. digitata.* Acid hydrolyzate was found to contain 96% reducing substances, 95.5% glucose; $[\alpha]\ D^{20} = -14°$ (in water).

Galactomannan. Isolated from alfalfa seeds; had a galactose : mannose ratio of 1 : 1; $[\alpha]\ D^{20} = +89°$ (in water). All these preparations were kindly supplied by the Chair of Organic Chemistry of the Odessa Institute of Technology headed by Professor M.S. Dudkin.

4-O-methylglucuronoxylan from birch wood. Polymerization degree: 140; containing xylose and 4-*O*-methylglucuronic acid, ratio 9 : 1. This was supplied by Dr. R.G. Katkevich from the Institute of Wood chemistry of the Latvian Academy of Sciences.

4-O-methylglucuronoxylan from sunflower seed husk. Polymerization degree

of 136, $[\alpha]\ D^{20} = -65.9°$. This was kindly supplied by Dr. T.A. Pavlova from the Leningrad Research Institute of Hydrolysis.

Arabinoxylan from rye seeds. Prepared by the method of Preece [2].

Arabinoxylan from rye seeds. This was kindly supplied by Dr. V.F. Golenkov from the Research Institute of Grain.

Sodium carboxymethylcellulose. Substitution degree: 0.65; polymerization degree: 374. This was obtained and supplied by the Laboratory of Water-Soluble Esters of Cellulose, of the Institute of Oil and Gas Industry.

Methyl-β,D-xylopyranoside and methyl-β,D-arabopyranoside were purchased from Chemapol Co., Czechoslovakia.

Pectin. This was purchased from the Schuchard Co. (München). Content of galacturonic acid: 78—86%; that of methoxyl groups: 9.5—9.7%.

Xylooligosacchardides. Polymerization degree: 2—6. There were obtained via enzymic hydrolysis of endo-1,4-β-xylanase and consecutively separated by Dowex I x 8 chromatography, Bio-Gel P-2 chromatography and accumulative paper 3 chromatography and were characterized as previously described. (See Part I).

Study of the effect of endo-1,4-β-xylanase action on xylans of various structure and different origin

The level of hydrolysis of xylans of various structure and different origin as well as hydrolyzates formed as a result of degradation of these xylans by endo-1,4-β-xylanase during hydrolysis were studied by paper chromatography. 15 mg substrate (1%), 0.5 ml 0.1 M acetate buffer pH 4.2 and 1 ml enzyme (0.01 mg protein) were incubated at 40°C for 24, 48 and 120 h, then 0.5 ml was removed to measure reducing sugars. 0.5 ml of 24-h hydrlyzate was boiled for 10 min on a water bath, dried in a vacuum desiccator, dissolved in 0.05 ml 60% ethanol and put on paper for further chromatography.

Comparative study of the viscosity decrease of sodium carboxymethylxylan solution and formation of reducing sugars upon the action of endo-1,4-β-xylanase

3 ml 2% sodium carboxymethylxylan solution, 1 ml 0.1 M acetate buffer pH 4.2, 0.5 ml enzyme solution (0.001 mg protein) were incubated at 40°C for 10, 20, 30 min and 1, 2, 3, 5, 10, 20 h. At the above intervals the viscosity of the incubation mixture and the content of reducing sugars were measured by the method of Somogyi [3].

Study of the degradation of various polysaccharides and glycosides by endo-1,4-β-xylanase

1 ml 1—0.5% substrate solution in 0.1 M acetate buffer, pH 4.2, and 1 ml enzyme solution containing 0.02 mg protein were incubated at 40°C for 1, 22 and 72 h. After that the content of reducing sugars was assayed in the reaction mixture.

Study of the effect of endo-1,4-β-xylanase on xylooligosaccharides

0.02 mg enzyme in 1 ml 0.1 M acetate buffer, pH 4.2, containing 1% xylo-oligosaccharides with a polymerization degree of 2—6 was incubated at 40°C

for 16 h; the incubation mixture was then boiled on the water bath for 10 min and dried in the vacuum desiccator. The dry residue was dissolved in 0.05 ml of 60% ethanol and placed onto chromatographic paper FN-3 manufactured by VEB Spezialpapierfabrik (D.D.R.). As markers xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose were put on paper.

In order to separate oligosaccharides, the chromatogram was placed into a descending chromatography chamber with butanol/pyridine/water (5 : 3 : 2) solvents for 60 h. After the solvents had passed through, the chromatogram was dried in air and then taken in the cuvette through phthalate aniline, air-dried and heated at 105° C for 5 min.

Assay of transglycosilase activity of endo-1,4-β-xylanase

0.45 mg endo-1,4-β-xylanase was incubated in 1 ml 0.1 M acetate buffer, pH 4.2, with 0.5% glucuronoxylan (birch), 2.5 mg xylose and 0.8 mg [C^{14}]xylose (0.017 mC, Amersham, U.K.) at 40° C for 24 h. In parallel an identical mixture containing no enzyme was incubated. After incubation the reaction mixtures were evaporated, dissolved in 0.05 ml 60% ethanol and placed onto chromatographic paper. The resultant oligosaccharides were separated by descending paper chromatography for 60 h in the system: *n*-butanol/pyridine/water (5 : 3 : 2) solvents. After drying chromatograms were analyzed radiometrically. X-ray films PM-1 were kept with chromatograms for 11 days. Chromatograms were developed with phthalate aniline to determine xylose and xylooligosaccharides. [C^{14}]Xylose was quantitated with the aid of an automatic counter, Gamma-F-067 (Hungary).

Determination of K_m upon the action of endo-1,4-β-xylanase on carboxymethylxylan

1 ml carboxymethylxylan of varying concentrations was incubated with 0.9 ml of 0.1 M acetate buffer, pH 4.2, and 0.1 ml enzyme solution containing 0.0001 mg protein at 40° C for 10 min. Then content of reducing sugars was measured in 1 ml sample by the method of Somogyi. The reaction rate was expressed as μg of reducing sugars in 1 ml released in 10 min. K_m was derived from the Michaelis-Menten equation and determined by the method of double inverse values of Lineweaver and Burk [4,5].

Results

As shown in Fig. 1 the viscosity of a solution of sodium carboxymethylxylan was very rapidly reduced on incubation with the endo-1,4-β-xylanase, whilst reducing sugars were only slowly released.

The results of studying the degradation degree of arabinoxylans, arabinoglucuronoxylans, glucuronoxylans and xylans by endo-1,4-β-xylanase are given in Table I.

The tabulated data indicate that endo-1,4-β-xylanase degraded to the greatest glucuronoxylan from alfalfa stems (30%), arabinoglucuronoxylan from timothy stems and crystalline xylan from wheat straw made of xylose residues only (22%); endo-1,4-β-xylanase degraded arabinoxylan from rye seeds in the slowest and least degree (for 48 h 5.8% and for 120 h 14%).

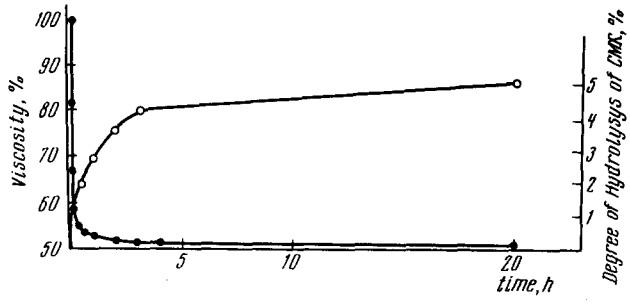

Fig. 1. Decrease of viscosity and formation of reducing sugars of sodium carboxymethylxylan (CMX)-solution upon the action of endo-1,4-β-xylanase. A reaction mixture of total volume 4.5 ml was taken for viscosity measurement and some was used for measurement of reducing sugar released as described in Materials and Methods (Part I); ●———● viscosity, %; ○———○ degree of hydrolysis of sodium carboxymethylxylan (CMX).

After elimination of products of xylan degradation during hydrolysis the residue of 4-*O*-methylglucuronoxylan which remained nonhydrolyzed made 2.3% and, therefore, the degradation level of this xylan amounted to 97%.

Fig. 2 shows chromatograms of hydrolyzates of different xylans. It can be seen that glucuronoxylans are degraded by endo-1,4-β-xylanase from *Asp. niger* str. 14 to form large quantities of xylobiose > xylotriose > xylose and small quantities of xylooligosaccharides with a higher degree of polymerization; the products of hydrolysis of arabinoglucuronoxylans are largely xylobiose > xylotriose > xylose and traces of arabinose; however, hydrolyzates contain large amounts of xylooligosaccharides with a degree of polymerization over three. Hydrolysis of arabinoxylans from rye seeds yields small amounts of xylobiose, xylotriose, xylose, oligosaccharides with a degree of polymerization over three being the major products.

The results of studying hydrolyzates of oligosaccharides with a degree of polymerization 2—6 are presented in Fig. 3.

It has been shown that endo-1,4-β-xylanase does not degrade xylobiose. Hydrolysis of xylotriose yields xylobiose and xylose. Xylotetraose is degraded to form xylobiose and lower quantities of xylose, xylopentaose is degraded to form xylotetraose and small quantities of xylobiose and xylose, xylohexaose is degraded to form xylopentaose, xylotetraose and small quantities of xylobiose and xylose.

The study of the products formed in the hydrolysis of xylan with endo-1,4-

TABLE I

PERCENTAGE HYDROLYSIS

Preparations of xylans	Incubation time (h)		
	24	48	120
Arabinoxylan from rye seeds	5.2	5.8	14.3
Arabinoglucuronoxylan from wheat straw	14.8	19.0	19.0
Arabinoglucuronoxylan from timothy stems	22.1	22.7	25.4
Glucuronoxylan from alfalfa stems	28.5	29.0	30.0
4-O-methylglucuronoxylan from birch wood	15.8	17.6	17.6
Crystalline xylan	17.4	21.0	22.0

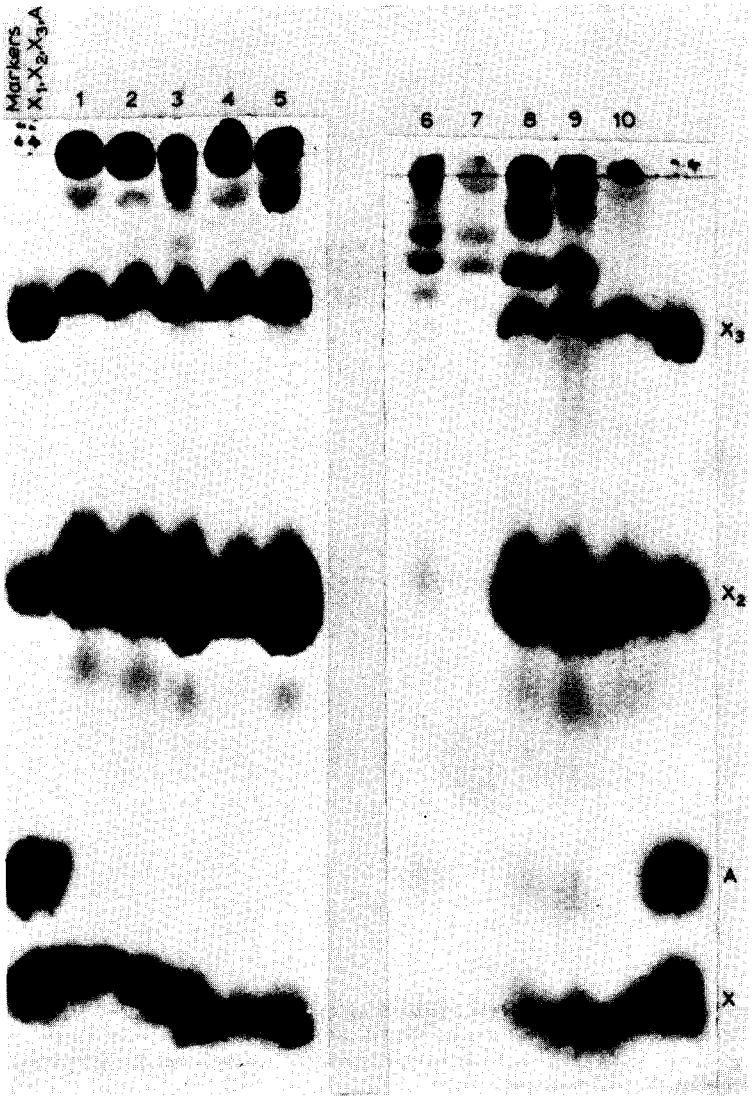

Fig. 2. Chromatogram of products of hydrolysis of xylans of different structure by endo-1,4-β-xylanase. 15 mg substrate, 0.5 ml 0.1 M acetate buffer pH 4.2 and 1 ml enzyme solution containing 0.01 mg protein withdrawn to place onto chromatographic paper. 1. 4-*O*-methylglucuronoxylan from sweetclover stems 2. 4-*O*-methylglucuronoxylan from birch wood 3. 4-*O*-methylglucuronoxylan from alder wood 4. 4-*O*-methylglucuronoxylan from sunflower seed husk 5. Glucuronoxylan from alfalfa stems 6. Arabinoxylan from rye seeds 7. Arabinoxylan from rye seeds 8. Arabinoglucuronoxylan from timothy grass 9. Arabinoglucuronoxylan from wheat straw 10. Crystalline xylan made of xylose only X - xylose, X_2 - xylobiose, X_3 - xylotriose, A - arabinose

β-xylanase in the presence of [^{14}C]xylose did not reveal transglycosilase activity of endo-1,4-β-xylanase. However, xylotetraose and xylopentaose were the major products in chromatograms of hydrolyzates of xylopentaose and xylohexaose, respectively. These results are indicative of transglycosilase activity of endo-1,4-β-xylanase upon its action on xylopentaose and xylohexaose.

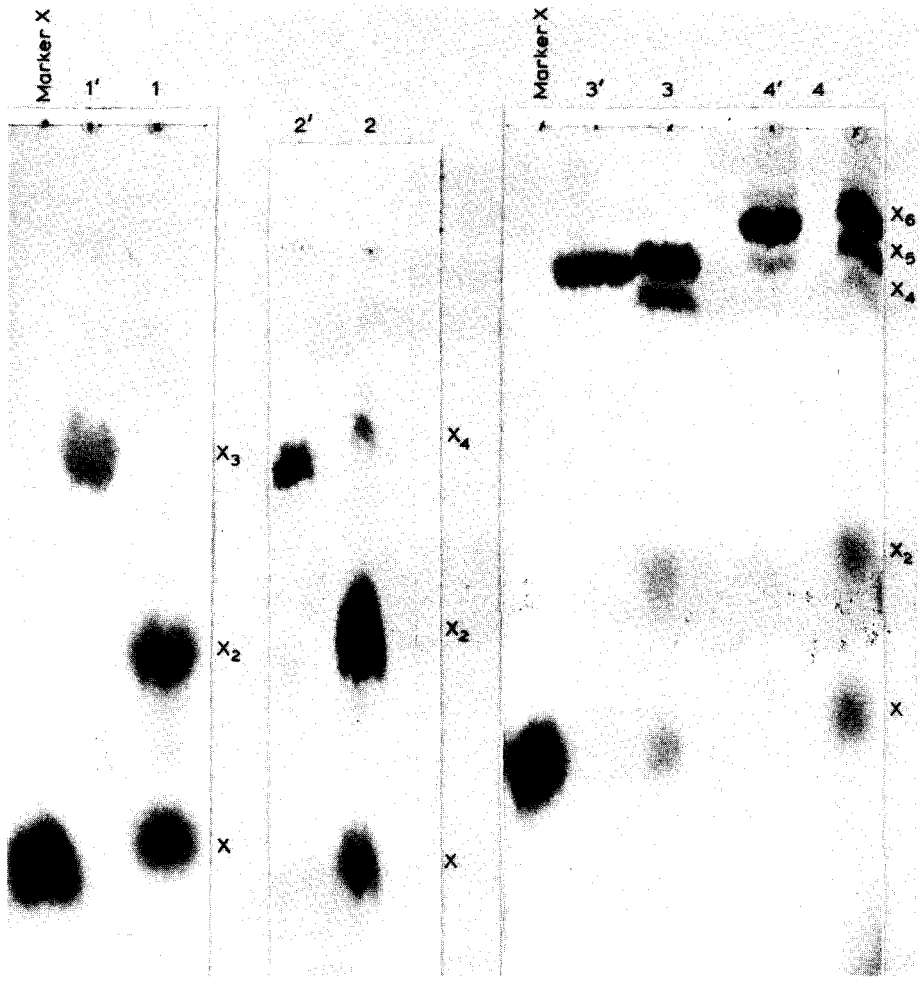

Fig. 3. Chromatogram of products of hydrolysis of xylooligosaccharides by endo-1,4-β-xylanase. 1 ml 1% xylooligosaccharide in 0.1 M acetate buffer pH 4.2 was incubated with 0.02 mg endo-1,4-β-xylanase at 40°C for 16 h. 1, X_3; 2, X_4; 3, X_5; 4, X_6; 1' 2' 3' 4', control. X, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose; X_6, xylohexaose.

K_m of endo-1,4-β-xylanase in its interaction with carboxymethylxylan was $3 \cdot 10^{-3}$ g/ml.

K_m of endo-1,4-β-xylanase for its effect on xylotetraose was $1.04 \cdot 10^{-3}$ M.

Relative velocities of degradation of 1% glucuronoxylan, xylonanoose and xylotetraose with 0.002% endo-1,4-β-xylanase at 40°C for 16 h were 100, 10 and 7.4, respectively. The degradation rate of glucuronoxylan expressed as mg xylose was taken as 100.

The specificity of endo-1,4-β-xylanase was examined upon its action on various polysaccharides and glycosides. Endo-1,4-β-xylanase does not degrade α, 1 → 4 or β, 1 → 4; the β, 1 → 3 bond remains between glucose residues in starch, carboxymethylcellulose and laminarin; the β, 1 → 4 bond remains between mannose residues in galactomannan and the α, 1 → 4 bond remains

between residues of galacturonic and methylgalacturonic acids in pectin and pectic acid as well as methylxyloside and methylarabinoside.

Discussion

The endo-1,4-β-xylanase from *Asp. niger* str. 14 which we have studied reduced rapidly the viscosity of carboxymethylxylan solution and degraded it insignificantly into reducing agents. This property of endo-1,4-β-xylanase of *Asp. niger* str. 14 indicates that it is a typical endo-enzyme.

Endo-1,4-β-xylanase did not degrade starch, pectic substances, carboxymethylcellulose, laminarin, galactomannan, methyl-β,D-xyloside or methyl-β,D-arabinoside, i.e. it did not contain the admixtures of enzymes that occurred in the xylanase crude preparation.

Xylans of various structure and different origin were degraded by 14—30% with endo-1,4-β-xylanase from *Asp. niger* str. 14 and by 97% after elimination of products formed as a result of hydrolysis of xylans.

It seems very likely that the degradation of xylans by endo-1,4-β-xylanase depends on the number and position of side branches in the molecule of the polysaccharide. The side branches are spatial obstacles that prevent the formation of the enzyme-substrate complex during the catalytic act.

On the other hand, in the course of xylan degradation end products of hydrolysis are accumulated which also impede the action of the enzyme. Inhibition of endo-1,4-β-xylanase by hydrolyzates is suggested by the fact that during their dialysis in the course of hydrolysis a stronger degradation of xylans takes place.

The literature data concerning investigations into the degradation of xylans of various structure with highly purified endo-1,4-β-xylanases are scarce. Xylans from woods of deciduous trees were degraded by some endo-1,4-β-xylanases of fungal and bacterial origin by 25—50% [6]. No comparison of the products of degradation of xylans of different structure from other sources was made.

The final neutral products of hydrolysis of xylans by endo-1,4-β-xylanase from *Asp. niger* str. 14 were xylobiose, xylotriose and xylose. During hydrolysis of arabinoglucuronoxylans traces of arabinose were found.

The previously described endo-1,4-β-xylanases from *Asp. niger* and *Penicillium janthinellum* differed in the hydrolyzates they produced from arabinoxylan from rice straw. Some of them degraded arabinoxylan to xylose, xylobiose and xylooligosaccharides whereas others did not form xylose and yielded xylobiose as the least hydrolyzate. Some highly purified endo-1,4-β-xylanases from *Asp. niger* degrade the bondage between residues of xylose and arabinose [7,8,9].

K_m of endo-1,4-β-xylanase from *Asp. niger* str. 14 for its action on carboxymethylxylan was $3 \cdot 10^{-3}$ g/ml. K_m for endo-1,4-β-xylanase action on sodium carboxymethylxylan was measured only in one endo-1,4-β-xylanase from commercial cellulase and estimated to be $1 \cdot 10^{-3}$ g/ml [10].

Endo-1,4-β-xylanase from *Asp. niger* str. 14 did not degrade xylobiose; it degraded xylotriose and xylotetraose to form xylobiose and xylose. During hydrolysis of xylopentaose and xylohexaose transglycosilase activity of endo-

1,4-β-xylanase seemed to develop since xylotetraose and xylopentaose were accumulated among the end products.

The degradation rate of xylooligosaccharides by endo-1,4-β-xylanase from *Asp. niger* str. 14 grew with an increase in length of the carbohydrate chain. K_m for endo-1,4-β-xylanase action on xylotetraose was $1.04 \cdot 10^{-3}$ M.

The degradation of xylooligosaccharides by highly purified endo-1,4-β-xylanase has been inadequately studied. Only one group of authors investigated the effect of three endo-1,4-β-xylanases from *Asp. niger* (14—80-fold purified) on xylotriose, xylotetraose and xylopentaose. These authors showed that the rate of oligosaccharide degradation elevated with an increase of the polymerization degree. K_m for endo-1,4-β-xylanase action on xylotetraose was $1 \cdot 10^{-3}$—$1.4 \cdot 10^{-3}$ M. Two out of the three endo-1,4-β-xylanases tested showed transglycolase activity when affecting xylotetraose and xylopentaose [11].

Thus, we have investigated in a more detailed and thorough way a highly purified endo-1,4-β-xylanase from *Asp. niger* str. 14 that was homogenous when studied by different techniques. Until now such comprehensive studies have not been carried out with any endo-1,4-β-xylanase.

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