Biochimica et Biophysica Acta, 484 (1977) 79—93

BBA 68192

STUDIES ON XYLAN DEGRADING ENZYMES

I. PURIFICATION AND CHARACTERIZATION OF ENDO-1,4-β-XYLANASE FROM *ASPERGILLUS NIGER* STR. 14

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(Received January 11th, 1977)

Summary

1. From an extract of a 48-h culture of *Aspergillus niger* str. 14 a crude preparation degrading xylan to xylose was obtained by isopropanol precipitation.

2. From 200 g of the crude xylanase preparation 21 mg of endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) were obtained by purification via 5 steps: ethanol precipitation, Sephadex G-50 gel filtration, chromatography and rechromatography on hydroxyapatite, and chromatography on CM-cellulose. The purification was 5000-fold with regard to the culture extract and 250-fold with regard to the crude enzyme preparation.

3. Endo-1,4-β-xylanase was homogeneous when examined by polyacrylamide gel electrophoresis and ultracentrifugation. The sedimentation coefficient of the enzyme was 1.46 S.

4. A pH optimum of 4.0 was found for the degradation of carboxymethylxylan by endo-1,4-β-xylanase. The temperature of 50°C was optimal for its activity. Endo-1,4-β-xylanase was stable at pH 3—8.

5. The molecular weight of higly purified endo-1,4-β-xylanase from *Asp. niger* str. 14 was determined by four methods: gel filtration on Sephadex G-200, electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate, sedimentation ultracentrifugation and via amino acid and carbohydrate analysis. These methods gave molecular weights of 24000; 33000; 24700; and 27000 respectively.

6. By isoelectric focusing the isoelectric point of endo-1,4-β-xylanase was found to be 4.2.

7. Amino acid analysis showed that the endo-1,4-β-xylanase molecule consisted of 212 amino acid residues, including a large number of aspartic (30) and glutamic acid (17) residues.

8. No free SH groups were detected in the experiments with *para*-chloromercuribenzene, which did not influence the enzyme activity.

9. By the anthrone method 20% carbohydrates were found in the endo-1,4-β-

xylanase molecule. After acid hydrolysis glucose and small quantities of galactose and glucosamine were detected in chromatograms.

Introduction

Endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) catalyzes hydrolysis of β-1,4-xylan at random, degrading inner bonds to oligosaccharides at first and then to xylobiose, xylose and xylotriose which are terminal products of hydrolysis of β-1,4-xylans.

Endo-1,4-β-xylanases are formed in large amounts by soil microorganisms that split plant residues and by phytopathogenic microorganisms.

Some investigators isolated fungal endo-1,4-β-xylanase which proved homogeneous during ultracentrifugation and electrophoresis, the level of purification being, however, inadequate [1—4]. Even highly purified enzymes contained admixtures of cellulase [5,6]. Physico-chemical and catalytic properties of endo-1,4-β-xylanase have been poorly studied and the molecular structure and active sites of the enzyme have not been examined at all.

The purpose of the present investigation was to obtain highly purified homogeneous endo-1,4-β-xylanase for further study of its physico-chemical and catalytic properties.

Materials and Methods

Preparation of the enzyme

From an extract of a 48-h culture of the mould fungus *Aspergillus niger* str. 14 a crude preparation of xylanase that degraded xylans to xylose was obtained by isopropanol precipitation [7].

Substrates

Arabinoglucuronoxylan, molecular weight 19400 was isolated from wheat straw. The content of monosaccharides in the hydrolyzate is as follows: xylose, 79.32%; arabinose, 12.07%; uronic acids, 6.61%. $[\alpha]\ D^{20} = -92°$.

Carboxymethylxylan, degree of substitution 0.5 was obtained from wheat straw xylan.

Glucuronoxylan, molecular weight 8600 and a xylose : uronic acids ratio of 10 : 1 was obtained from alder wood.

All xylan preparations were supplied by the Chair of Organic Chemistry of the Odessa Institute of Technology headed by Professor M.S. Dudkin.

Preparation of xylooligosaccharides

15 g 4-*O*-methylglucuronoxylan of alder were incubated with 0.06% solution containing endo-1,4,-β-xylanase (obtained through fractional precipitation of the crude preparation by three ethanol volumes) 0.03 M acetate buffer pH 4.2 for 3 h at 40° C. After incubation the hydrolyzate was boiled for 10 min on the water bath, cooled, centrifuged to remove non-soluble residue of glucuronoxylan and evaporated in a rotor vacuum evaporator. In order to separate acid oligosaccharides, water solution of the hydrolyzate was passed through a

Dowex column (I x 8) 100/200 mesh in the acetate form (1.7 × 50 cm). Acid oligosaccharides were adsorbed on an ion-exchanger. The solution of neutral xylooligosaccharides was concentrated in the rotor vacuum evaporator down to 10 ml and 3 ml were placed on the Bio Gel P-2 column. The column was 3.0 × 70 cm in size, fractions were 5 ml in volume, the elution rate was 25 ml/h; fractions 1—8 contained a mixture of xylooligosaccharides with DP (degree of polymerization) > 3, fractions 8—10 contained xylotriose, fractions 12—18 contained xylobiose.

The resultant fractions of oligosaccharides were separated from the admixture of other oligosaccharides on Whatman 3 MM paper (Whatman, England) in the system: butanol/pyridine/water (5 : 3 : 2) for xylooligosaccharides with DP $\leqslant$ 3 and in the system: ethylacetate/pyridine/acetic acid/water (5 : 5 : 1 : 3) for xylooligosaccharides with DP $\geqslant$ 3 by the descending method for 60 h. The control band was developed by aniline phthalate [7]. The undeveloped parts of the chromatogram were cut into 3 × 15 mm bands and eluted three times with 50—100 ml distilled water. The resultant solution was concentrated in the rotary vacuum evaporator and lyophilized. Xylooligosaccharides were treated by paper (Filtrak No. 3 of VEB Spezialpapierfabrik production) chromatography on obtain one spot. In order to identfy the resultant oligosaccharides, each xylooligosaccharide was hydrolyzed. Hydrolysis was done on a boiling water bath by 2% HCl for 4 h. The hydrolyzate was concentrated in the rotor vacuum evaporator, supplemented with 3—5 ml distilled water and concentrated again. The last operation was repeated three times to eliminate HCl. The dry residue was dissolved in 60% ethanol and exposed to paper chromatography. In all cases only one band with R_F of xylose was seen in the chromatogram.

To assay the degree of polymerization of xylooligosaccharides, 0.4 mg xylooligosaccharide in 5 ml of 2% HCl was boiled on the water bath for 4 h. HCl was removed as above. The resultant dry hydrolyzate was dissolved in 2 ml distilled water and reducing sugars were measured by the methods of Somogyi and Nelson [9,10]. As control 0.4 mg xylooligosaccharides in 2 ml H_2O were used. The results obtained are summarized in Table I.

The xylooligosaccharides with degrees of polymerization 1—6 were separated by paper chromatography and the value of log $1 - R_F/R_F$ was calculated. The correlation between the polymerization degree and log $1 - R_F/R_F$ was expressed graphically for each xylooligosaccharide.

TABLE I

ACID HYDROLYSIS OF XYLOOLIGOSACCHARIDES

Fraction	Reducing sugars in 1 ml before hydrolysis	Reducing sugars in 1 ml after hydrolysis	degree of polymerisation
1	0.21	0.66	3
2	0.03	0.13	4
3	0.126	0.66	5
4	0.09	0.54	6
5	0.06	0.55	9
6	0.57	1.38	2

Other materials

Sephadex G-50, Sephadex G-200 were purchased from the Pharmacia, Uppsala, Sweden, Bio-Gel P-2, Dowex 1 x 4 were obtained from Bio-Rad Laboratories, California, U.S.A.

Hydroxyapatite was prepared by the method of Levin [8], CM-Cellulose CM-32 Microgranul Standard produced by Whatman, U.K., was used.

Assay of enzyme activity and protein content

Protein content was determined by the method of Lowry, [9].

Assay of the enzyme capacity to degrade xylan and to form reducing sugars

In order to measure the saccharifying activity of the enzyme, 15 mg arabinoglucuronoxylan of wheat straw were incubated with 1 ml of the enzyme solution containing 0.1 mg protein, 0.5 ml 0.1 M acetate buffer pH 4.2 for 1 h at 40°C. After incubation the content of reducing sugars in 1 ml incubation mixture was estimated by the method of Somogyi and Nelson [10,11]. Activity was expressed as milligrams of xylose. The amount of the enzyme forming 1 mg xylose in the given incubation mixture for 1 h at 40°C was taken as the unit of activity.

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

This was carried out by the viscosimetric method in the Ostwald viscometer, measuring relative viscosity in the incubation mixture containing 3 ml 1% carboxymethylxylan solution and 1 ml enzyme solution in 0.1 M acetate buffer, pH 4.2, at 40°C.

Activity of endo-1,4-β-xylanase was determined with regard to the rate of increment of the inverse value of relative viscosity. The amount of enzyme allowing an increment of this value by one unit for 2 min was taken for the unit of activity, when the above reaction mixture was used at 40°C.

Assay of exo-1,4-β-xylosidase activity

In order to determine exo-1,4-β-xylosidase activity, 0.5 ml 0.01% xylobiose or methyl-β,D-xylopyranoside in 0.1 M acetate buffer, pH 4.2, was incubated with 0.5 ml enzyme solution for 1 h at 40°C. The content of reducing sugars in the reaction mixture was measured by the method of Nelson. Activity was expressed as milligrams of xylose. The amount of the enzyme forming 1 mg xylose in the above reaction mixture at a given temperature in 1 h was taken as the unit of activity.

Paper chromatography of products of the enzymic degradation of xylans

The mixture containing 15 mg xylan, 0.5 ml 0.1 M acetate buffer, pH 4.2, 0.02 M NaN_3 and 1 ml enzyme solution were incubated at 40°C for 5, 15, 30 min, 1, 6, 26 h. After incubation the mixture was boiled for 10 min on the water bath, 1-ml samples were taken and dried in the vacuum desiccator. The dry residue was dissolved in 0.05—0.1 ml ethanol (60%) and placed on FN-3 paper of VEB Spezialpapierfabrik production. To separate carbohydrates, chromatograms were put into the descending chromatography chamber with the following systems of solvents: butanol/pyridine/water (5 : 3 : 2) (I) and

ethyl acetate/pyridine/acetic acid/water (5 : 5 : 1 : 3) (II) for 60 h. After that chromatograms were air-dried and developed by aniline phthalate (1.66 g phthalic acid/0.92 ml freshly distilled aniline/100 ml ethanol), passing chromatographs through this solution in the cuvette. Then chromatograms were dried at room temperature and heated at 100°C for 5 min.

Purification of endo-1,4-β-xylanase

Step I. Separation of endo-1,4-β-xylanase and exo-1,4-β-xylosidase by cooled ethanol. 200 g of a crude xylanase preparation were dissolved in 1000 ml distilled water, centrifuged and the solution was kept overnight at 4°C. Then 1, 2 and 3 vols. of cooled (—15°C) ethanol were added one after another to the solution of xylanase. Each time the residue was separated by centrifugation, dissolved in 100—200 ml distilled water and lyophilized. As a result, three fractions containing different amount of enzymes: endo-1,4-β-xylanase and exo-1,4-β-xylosidase were obtained.

The fraction obtained upon addition of 3 vols. of ethanol contained the largest amount of endo-1,4-β-xylanase. This fraction was used for further purification.

The fraction obtained by precipitation with 1 vol. ethanol contained predominantly exo-1,4-β-xylosidase. The fraction obtained by precipitation with 2 vols. ethanol volumes contained endo-1,4-β-xylanase and exo-1,4-β-xylosidase in equal quantities.

Step II. Sephadex G-50 column chromatography. 6.5 g lyophilized fraction obtained by 3 vols. ethanol and containing 1.4 g protein were dissolved in 15 ml 0.005 M ammonium acetate solution and placed on a Sephadex G-50 column (7 × 35 cm) equilibrated with the same solution. 10-ml fractions were collected. The elution rate was 1 ml/min. Fractions 4—28 containing endo-1,4-β-xylanase were combined and concentrated in the rotary vacuum evaporator to 8.5 ml.

Steps III and IV. Hydroxyapatite column chromatography. 8.5 ml enzyme solution containing 171.1 mg protein were placed on the hydroxyapatite column (3 × 55 cm) equilibrated with 0.001 M phosphate buffer (NaH_2PO_4/Na_2HPO_4) pH 6.2. The enzyme was eluated with the same buffer and 10.5 ml fractions were collected, the elution rate being 0.5 ml/min. Fractions 9—15 were combined, dialyzed against distilled water, concentrated in the rotor vacuum evaporator to 2.5 ml and placed onto the hydroxyapatite column (2.5 × 44 cm) equilibrated with 0.001 M phosphate buffer pH 6.0 5 ml fractions were then collected.

The peak containing endo-1,4-β-xylanase (fractions 6—14) was combined, dialyzed at 4°C for 3 h in a Japanese collage film against distilled water and concentrated in the rotary vacuum evaporator to 3.1 ml.

Step V. Carboxymethylcellulose column chromatography. 3.0 ml enzyme solution were placed onto the CM-cellulose column (1.8 × 22 cm) equilibrated with 0.1 M acetate buffer, pH 4.2; 10.0 ml fractions were collected, the elution rate being 0.5 ml/min. The column was eluated with the initial buffer. Fractions 16—24 were combined and the resultant enzyme was examined with respect to its homogeneity, physicochemical and catalytic properties.

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

The activity of the enzyme was measured viscometrically at different pH values under standard conditions with sodium carboxymethyl xylan used as substrate. The activity of the enzyme at pH 2.2—6.8 was assayed, using phosphate-citrate buffer (McIlvain buffer).

Study of pH stability of endo-1,4-β-xylanase

The enzyme solution (0.003 mg/ml) was kept for 15 min at pH 2.2—7.7 (phosphate-citrate buffer). Then the activity of endo-1,4-β-xylanase at pH 4.2 was determined. The residual activity was expressed as percentage of the initial one.

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

Under standard conditions the activity of endo-1,4-β-xylanase was assayed at temperatures of 30, 40, 50, 60, 70, 80°C viscometrically, using carboxymethylxylan as substrate.

Study of the temperature stability of endo-1,4-β-xylanase

Under standard conditions the initial activity of endo-1,4-β-xylanase was assayed. The enzyme solution was (0.003 mg/ml) kept at different temperatures (30—80°C) for 15 min, and cooled down to 0°C. The activity of the enzyme was then assayed viscosimetrically, using carboxymethylxylan as substrate.

Polyacrylamide gel electrophoresis

Endo-1,4-β-xylanase was analyzed by disc electrophoresis in polyacrylamide gel, according to the method of Ornstein [12] and Davis [13]. Electrophoresis was carried out in the Reanal device (Hungary) at pH 4.3 and 8.9.

Study of endo-1,4-β-xylanase homogeneity in the ultracentrifuge

The rate of sedimentation of endo-1,4-β-xylanase was measured in the Spinco Model E ultracentrifuge at 39460 rev./min at 20°C. The concentration of the enzyme was 0.5% in distilled water in a cell with an interface of the capillary type.

Study of the degradation of CM-cellulose by endo-1,4-β-xylanase

1 ml of 1% CM-cellulose in 0.1 M acetate buffer, pH 4.2, and 1 ml of 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.

Determination of molecular weight by gel-filtration. [14]

On a Sephadex G-200 column (Pharmacia, Uppsala, Sweden) (1.7 × 65 cm), 5—10 mg protein were placed. The column was equilibrated with 0.1 M acetate buffer, pH 4.2. The fraction volume was 2.5 ml. The void volume of the column was measured with respect to the elution volume of Blue Dextran (dextran 2000). The molecular weight was derived from the formula $\log M_r = 6.698 - 0.987\ (V_e/V_0)$, where M_r is molecular weight, V_e is protein elution volume, and V_0 is elution volume of Blue Dextran. The column was calibrated on the basis

of the elution volume of proteins with a known molecular weight (chymotrypsinogen: 25000 (prepared in the Protein and Nucleic Acids Laboratory of the Bakh Institute of Biochemistry), lysozyme: 14300 (Gee Lowson Chemicals, Ltd., England), egg albumin: 43000 (British Drug Houses, Ltd., England), and human serum albumin: 68000 (Chemical Corporation, Calif., U.S.A.).

Determination of molecular weight by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate

The molecular weight was assayed by the method of Weber and Osborn [15]. The same proteins that were used in gel-filtration on a Sephadex G-200 column were used as markers. 20 μg protein were added to the gel.

Determination of molecular weight by sedimentation equilibration

The molecular weight was assayed in the Spinco Model E ultracentrifuge. The average speed was 13410 rev./min and the temperature was 24°C. The enzyme concentration was 5 mg/ml. The partial specific volume was taken as 0.75 cm^3/g [16].

Determination of the isoelectric point

The isoelectric point of endo-1,4-β-xylanase was determined according to the method of Vesterberg and Swensson [17] in a 110-ml column (LKB-Producter AB, Sweden) at 4°C. The density gradient was provided by sucrose in an automatic mixer (LKB-Producter AB, Sweden). The heavy solution contained 40% sucrose (g/vol). Carrier ampholytes with pH 3—5 were used to keep the pH value within this range. The concentration of ampholytes was 2.5% (vol./vol.). The cathode solution contained 1% NaOH and was placed in the upper part of the column whereas the anode solution contained 40% sucrose in 1% H_2SO_4 and was placed in the lower part of the column. The protein solution (10 mg) was placed in the heavy solution. The electric focusing was carried out for 48 h. The voltage was 300 V and the current was 40 mA. Fractions of 1.5 ml were collected from the lower part of the column upwards at a rate of one fraction per min. The resultant fractions were analysed for pH and endo-1,4-β-xylanase activity with respect to its capacity to degrade arabinoglucuronoxylan and to form reducing sugars assayed by the Somogyi method [10].

Amino acid analysis

5 mg protein were hydrolyzed for 22 h at 110°C in 6 M HCl in a molybdenum glass ampoule. The amino acid content was measured in a Hitachi KL A3 amino acid analyzer.

Free SH-group analysis

The content of free SH-groups was measured in the reaction with *p*-chloromercuribenzene (PCMB) [18].

Assay of PCMB effect on endo-1,4-β-xylanase activity

0.1 ml endo-1,4-β-xylanase solution containing 0.023 mg protein was incubated with 0.1 ml $5 \cdot 10^{-4}$ M PCMB at 20°C for 15 min and 18 h. Then the endo-1,4-β-xylanase activity was measured viscosimetrically [7]. The incubation mixture devoid of endo-1,4-β-xylanase was used as control.

Determination of the tryptophan content

The content of tryptophan was assessed according to the method of Messineo and Musarra [19].

Carbohydrate analysis

The carbohydrate content in the endo-1,4-β-xylanase preparation was measured by the anthrone method [20]. The sugars contained in the enzyme molecule were determined by means of endo-1,4-β-xylanase hydrolysis with 2% HCl and subsequent paper chromatography of hydrolyzates according to the previously described methods [7]. The carbohyrate content in endo-1,4-β-xylanase after disc electrophoresis in polyacrylamide gel was assayed by the Keyser method [21].

Results

Enzyme purification

During ethanol fractionation the major portion of endo-1,4-β-xylanase occurred in the fraction obtained after precipitation with 3 vols. of ethanol. The fraction obtained as a result of precipitation with one volume of ethanol contained largely exo-1,4-β-xylosidase.

The results of hydrolysis of arabinoglucuronoxylan of wheat straw done with fractions I and III (Fig. 1) show that under the influence of fraction I of exo-1,4-β-xylosidase a large amount of xylose and a small quantity of oligosaccharides are formed whereas under the influence of fraction III of endo-1,4-β-xylanase just the opposite occurs: a large amount of oligosaccharides and a small quantity of xylose are formed.

At this stage a 6-fold purification of endo-1,4-β-xylanase takes place (Table II).

The results of endo-1,4-β-xylanase purification on Sephadex G-50 column are given in Fig. 2 and in Table II.

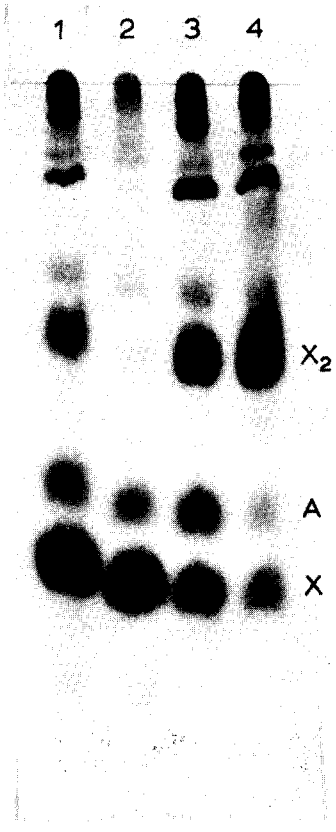
Fig. 2 shows that gel filtration through Sephadex G-50 column leads to the separation of endo-1,4-β-xylanase from smaller molecules of exo-1,4-β-xylosidase.

As follows from Table II, at the second stage of purification the specific activity of the enzyme increases more than 5-fold. When passed through hydroxyapatite the enzyme is not adsorbed and only contaminating proteins are sedimented on it. At this stage the specific activity doubles. During rechromatography of active fractions of the hydroxyapatite column the specific activity also doubles. Upon fractionation on the CM-cellulose column at room temperature and optimum pH the enzyme is probably eluated after endopolygalacturonase and cellulase occurring in the preparation. Thus, purification from these enzymes can be achieved. At this stage the specific activity doubles.

As a result of five stages of purification the specific activity of endo-1,4-β-xylanase increased 250-fold. The yield of the enzyme was about 13% (by activity) and 0.051% (by protein). (From 200 g preparation/42.4 g protein/21.7 mg endo-1,4-β-xylanase were obtained).

Criteria of purity

Disc electrophoresis in polyacrylamide gel. Examination of endo-1,4-β-



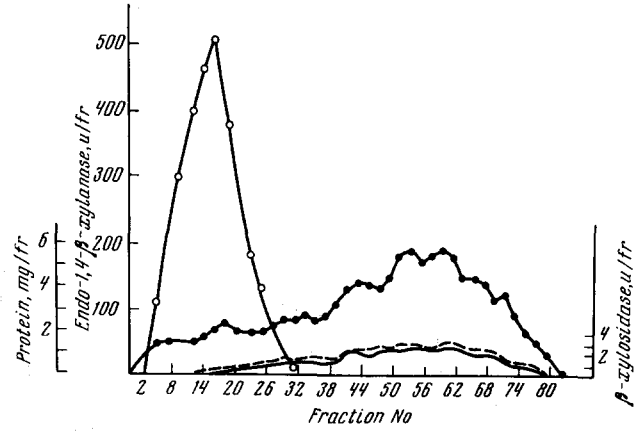


Fig. 1. Chromatogram of products of hydrolysis of wheat straw arabinoglucuronoxylan obtained via the action of 1, crude xylanase preparation; 2, fraction prepared by precipitation with one volume of ethanol; 3, fraction prepared by precipitation with two volumes of ethanol; 4, fraction prepared by precipitation with three volumes of ethanol. X: xylose; X_2: xylobiose; A: arabinose.

Fig. 2. Sephadex G-50 gel filtration. Column: 7 × 37 cm, fraction volume: 10 ml. Flow rate: 60 ml/h. The column was equilibrated and the protein was washed with 0.005 M ammonium acetate. ●———●, protein; ○———○, endo-1,4-β-xylanase; - - - - -, exo-1,4-β-xylosidase (activity on xylobiose); ———, exo-1,4-β-xylosidase (activity on methyl-β,D-xylopyranoside).

xylanase by disc electrophoresis in polyacrylamide gel at acidic and alkaline pH showed that endo-1,4-β-xylanase appeared as one band (Fig. 3).

Ultracentrifugation studies. Study of the sedimentation rate of the enzyme indicated that it precipitated as one peak with a sedimentation coefficient of 1.46 S (Fig. 4).

Degradation of CM-cellulose by endo-1,4-β-xylanase. Studies of the degradation of CM-cellulose by endo-1,4-β-xylanase demonstrated that no reducing sugars were formed as a result of 72-h incubation of 1% solution of the substrate with the enzyme; i.e. the enzyme was free from admixtures of cellulase which was detected in the initial preparation.

pH and temperature effect on the activity of endo-1,4-β-xylanase. Study of the pH effect on the activity of endo-1,4-β-xylanase has demonstrated that its optimum effect on carboxymethylxylan is at pH 4.0.

TABLE II

RESULTS OF ENDO-1,4-β-XYLANASE PURIFICATION

Stages of purification	Protein (mg)	Activity of endo-1,4-β-xylanase	Activity of exo-1,4-β-xylosidase	Specific activity of endo-1,4-β-xylanase	Factor purification	Endo-1,4-β-xylanase yield (%)
I. Crude preparation of xylanase	42 400	373 120	20 350	8.8	1.0	100
II. Fraction obtained after precipitation with three volumes of ethanol	1 400	77 000	256.5	55.0	6.25	20.7
III. Endo-1,4-β-xylanase after gel filtration through Sephadex G-50 column	171.2	51 682	Traces	302.0	34.3	13.8
IV. Endo-1,4-β-xylanase after chromatography on hydroxyapatite column	81.0	49 167	Traces	607.0	68.9	13.2
V. Endo-1,4-β-xylanase after rechromatography on hydroxyapatite column	41.6	48 172	0	1158.0	131.5	12.9
VI. Endo-1,4-β-xyla nase after chromatography on CM-cellulose column	21.7	47 880	0	2200.0	250	12.8

Study of pH stability of endo-1,4-β-xylanase has indicated that the enzyme is stable at pH 3—8.

Study of the temperature effect on the activity and stability of endo-1,4-β-xyalanase has indicated that the maximum activity of the enzyme occurs at 50°C. The enzyme is significantly inactivated upon 15 min of incubation at 40°C and becomes completely inactivated upon 15 min incubation at 80°C.

Physicochemical properties of endo-1,4-β-xylanase

By gel filtration the molecular weight of endo-1,4-β-xylanase was estimated to be 24000. Fig. 5 gives a graph of the elution volume of proteins as a function of their molecular weight.

The assay of the enzyme molecular weight by electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate showed that the protein mobility was as follows: lysozyme: 0.88; egg albumin: 0.41; chymotrypsinogen: 0.63; human serum albumin: 0.16; endo-1,4-β-xylanase: 0.50. The endo-1,4-β-xylanase mobility corresponded to a molecular weight of 33000.

By sedimentation ultracentrifugation the molecular weight of endo-1,4-β-xylanase was found to be 24700. By electric focusing the isoelectric point (p*I*) of endo-1,4-β-xylanase was shown to be 4.2. Fig. 6 presents the electric focusing data.

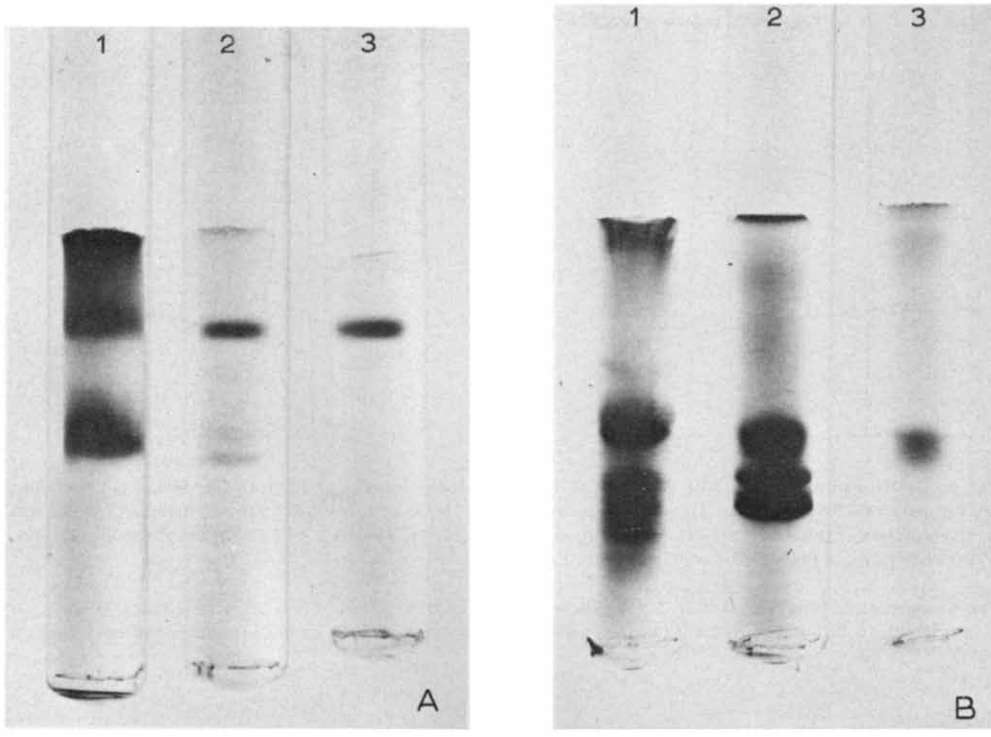


Fig. 3. Polyacrylamide gel electrophoresis of endo-1,4-β-xylanase. A: pH 4.3; B: pH 8.9; 1: crude preparation of xylanase; 2: fraction obtained by precipitation with 3 vols. ethanol; 3: highly purified endo-1,4-β-xylanase.

The content of amino acids in the endo-1,4-β-xylanase molecule is given in Table III.

Endo-1,4-β-xylanase was shown to contain 20% carbohydrates. After acid hydrolysis glucose and small quantities of galactose and glucosamine were detected in chromatograms. After gel electrophoresis and treatment by the method of Keyser a weakly stained band was found in the protein site. Table III shows that the endo-1,4-β-xylanase molecule contains 212 amino acid residues and a good number of dicarboxylic amino acids.

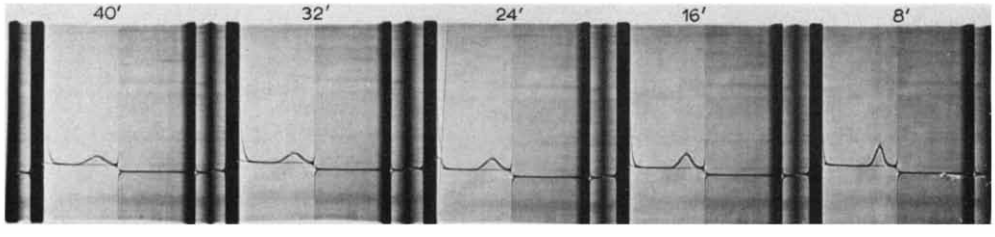


Fig. 4. Measurement of the sedimentation velocity of endo-1,4-β-xylanase. A model E analytical Spinco ultracentrifuge. The rate of rotation: 39460 rev./min. Interval between removals: 8 min. Temperature 20°C. Enzyme concentration: 0.5% in distilled water. The cell had an interface of the capillary type.

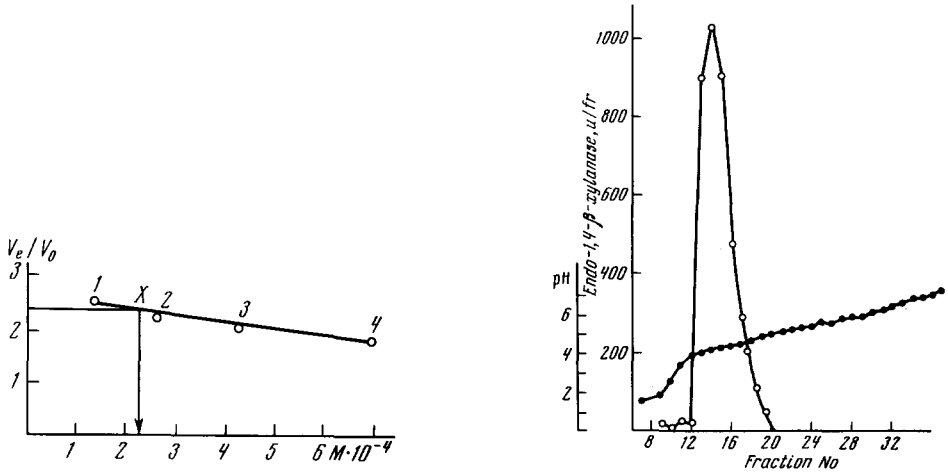


Fig. 5. Relationship between the elution volume and molecular weight of proteins during gel filtration on Sephadex G-200. Column: 1.75 X 65 cm. Acetate buffer 0.1 M, pH 4.2. 1, lysozyme, mol. wt. 14300 2, chymotrypsinogen, mol. wt. 25000 3, egg albumin, mol. wt. 43000 4, bovine serum albumin, mol. wt. 67000 X, endo-1,4-β-xylanase, mol. wt. 24000.

Fig. 6. Isoelectric focusing of endo-1,4-β-xylanase. Total volume of column: 110 ml; pH range: 3—5; separation time: 48 h; current: 40 mA; voltage: 300 V; temperature: 4°C; sucrose density gradient; fraction volume: 1.5 ml.

TABLE III

AMINO ACID COMPOSITION OF ENDO-1,4-β-XYLANASE FROM *ASPERGILLUS NIGER* STR. 14

Amino acid	Number of residues per molecule
Lysine	6
Histidine	2
Arginine	6
Aspartic acid	30
Threonine	32
Serine	28
Glutamic acid	17
Proline	5
Glycine	36
Alanine	13
Half-Cystine	traces
Valine	I
Methionine	2
Isoleucine	5
Leucine	5
Tyrosine	16
Phenylalanine	5
Tryptophan	3
Amide NH_3	not determined
Total number of amino acid residues:	212
Molecular weight:	22 000

Discussion

As a result of five stages of purification we obtained an enzyme endo-1,4-β-xylanase that was purifiifed 5000-fold as compared with the culture extract and

250-fold as compared with the crude preparation.

Endo-1,4-β-xylanase was homogeneous during polyacrylamidegel electrophoresis, ultracentrifugation, gel filtration and isoelectric focussing.

Homogeneous endo-1,4-β-xylanase from surface cultures of mould fungi was obtained previously [1,2]; however, the degree of its purification was rather low; the content of enzymes which normally accompany endo-1,4-β-xylanase was not measured in the resultant preparation. The study of selected purified preparations of endo-1,4-β-xylanase revealed admixtures of cellulase [5,6].

With respect to its properties the enzyme endo-1,4-β-xylanase we obtained was similar to endo-1,4-β-xylanase prepared by Swiss researchers from commercial cellulase [5]. Their enzyme was not adsorbed on hydroxyapatite column at pH 6. Our preparation is also similar to endo-1,4-β-xylanase from the fungus *Coniophora cerebella* that was precipitated with three ethanol volumes [22]. Unlike endo-1,4-β-xylanases examined by Swedish authors that had smaller molecules than xylosidases [23], endo-1,4-β-xylanase from *Asp. niger* str. 14 has a larger molecule than concomitant β-xylosidase occurring in the fraction that can be precipitated with 3 vols. of ethanol. Endo-1,4-β-xylanase was the first to be eluated from Sephadex G-50 column whereas exo-1,4-β-xylosidase was found in the second protein peak.

It was a good achievement to use chromatography on CM-cellulose column at the last stage of purification. Chromatography was done at room temperature and pH 4.2 which was optimal for the enzyme action. It seems very likely that under these conditions endo-1,4-β-xylanase was retained in the column and eluated later than glycosidases, endopolygonacturonases, pectinesterase and cellulase. This pH value was not optimal for cellulase-CM-cellulose interaction. An addition of this stage of purification allowed us to remove admixtures of these enzymes which other researchers failed to do [5,6].

The optimum value of pH for our enzyme is 4.0—4.2 which is very close to that for endo-1,4-β-xylanases from other mould fungi. For instance, optimum pH of 4.5 has been found by Swiss researchers [5], of 3.5 and 4.5 for *Trichoderma viride* have been indicated by Japanese authors [24], and 3.6, 5.1, 5.9 for three endo-1,4-β-xylanases from *Aspergillus niger* [1].

Endo-1,4-β-xylanases investigated by other scientists were stable at pH 3—11 (and most active at 50°C) [25]. The enzyme activity decreased during prolonged incubation at a temperature higher than 40°C. The enzymes were, as a rule, entirely inactivated upon incubation at 80°C [25].

The molecular weight of endo-1,4-β-xylanase as determined by gel filtration was 24000. This value is in agreement with the molecular weight assays by sedimentation ultra-centrifugation (24700). The molecular weight of endo-1,4-β-xylanase from *Stereum sanguinolentum* assayed by ultracentrifugation was found to be 21200. Thus, endo-1,4-β-xylanases from *St. sanguinolentum* and *Asp. niger* are of similar molecular weight. The molecular weight of other endo-1,4-β-xylanases studied was in the range of 16000—39000 [25]. The molecular weight of endo-1,4-β-xylanase was found to be 33000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

An analysis of the amino acid composition of endo-1,4-β-xylanase indicated that its polypeptide chain contained 212 amino acid residues. The enzyme contained traces of half-cystine and a good number of acid amino acids: aspartic

(30 residues) and glutamic (17 residues) acids.

Previously, the amino acid composition only of endo-1,4-β-xylanase from *St. sanguinolentum* [6] was studied. It was shown to contain 218 amino acid residues, 3 molecules of half-cystine, 19 aspartic acid residues and 15 glutamic acid residues.

Therefore, endo-1,4-β-xylanases from *Asp. niger* and *St. sanguinolentum* are similar in regard to their amino acid content. The molecular weight of endo-1,4-β-xylanase from *Asp. niger* str. 14 as calculated from the amino acid composition was 22000.

It was shown that *Asp. niger* str. 14 endo-1,4-β-xylanase contained 20% carbohydrates, and its molecular weight was estimated to be 27700 from the amino acid and carbohydrate analysis.

The presence and composition of carbohydrates in the molecule of endo-1,4-β-xylanase were not investigated in most endo-1,4-β-xylanases studied. Carbohydrates were found in some endo-1,4-β-xylanases; however, their composition was not examined [25]. In one endo-1,4-β-xylanase most carbohydrates (16.8%) occurred as glucose [26].

The isoelectric point of endo-1,4-β-xylanase from *Asp. niger* str. 14 is at pH 4.2. By electric focusing Algren, Eriksson and Vesterberg [15] determined isoelectric points of endo-1,4-β-xylanases contained in the crude preparation of cellulase 36 (Rohm and Haas Co., U.S.A.) to be 3.9 and 4.5. Swiss researchers [5] isolated from the crude preparation of cellulase produced by Miles Chemical Co., Caifton, N.Y., U.S.A., endo-1,4-β-xylanase with p*I* 4.5.

We detected no SH groups in endo-1,4-β-xylanase. We also demonstrated that PCMB did not influence endo-1,4-β-xylanase activity. However, these data are rather preliminary. Further study of the content of SH and S-S groups in the endo-1,4-β-xylanase molecule from *Asp. niger* str. 14 is planned. Thus, endo-1,4-β-xylanase we examined differs but slightly from endo-1,4-β-xylanases from other mould fungi with respects to its properties.

Our further goal is to investigate catalytic properties of endo-1,4-β-xylanase we have isolated.

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