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ENZYMATIC DEGRADATION OF LINSEED MUCILAGE

I. ISOLATION AND CHARACTERIZATION OF EXTRACELLULAR ENZYME PRODUCED BY *ASPERGILLUS NIGER*

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SUMMARY

A method is described for the isolation of the extracellular enzyme produced by *Aspergillus niger* which degrades linseed mucilage. The crude enzymatic preparation is obtained from the filtrate of the surface culture by salting out with ammonium sulphate and precipitating with ethanol after gel filtration through a Sephadex G-25 column. By means of chromatography on CM-cellulose column and by gel filtration through Sephadex G-75 and G-25 columns, a homogeneous preparation was obtained. The course of the enzymatic reaction with linseed mucilage is characterised by a rapid decrease of the viscosity of the solutions and by a slow increment of reducing groups. The properties of the isolated enzyme are characterised as endo-polyglycosidase.

INTRODUCTION

Structural studies on plant polysaccharides include many problems which are at present of great interest. Their solution requires the application of appropriate methods: physical and chemical, and also biochemical methods, namely using enzymes. Valuable results concerning the structure of glycogen and starch were obtained from studying the action of enzymes on them. The main advantages of the application of enzymes are a well defined course of degradation and simple constitutional studies on the degraded fragments. These problems were given little concern until recently when the need arose for homogeneous preparations and homogeneous polysaccharides as substrates. One of the few attempts is represented by the isolation of homogeneous enzymes produced by *Myrothecium verrucaria* fungi[1], attacking xylans. AHLGREN, ERIKSSON AND VESTERBERG[2] using gel filtration, isolated cellulase, mannanase, xylanase, β-glucosidase and some other enzymes from commercial preparations of Aspergillus enzymes.

Described in this paper is a separation and isolation method for the extracellular enzyme produced by *Aspergillus niger* which hydrolyses linseed mucilage.

MATERIAL AND METHODS

Linseed mucilage (acidic fraction), used as the substrate in the determination of the activity of the enzyme, was prepared from purified commercial linseed mucilage (variety Wiera) by precipitation with cetyltrimethylammonium bromide (Cetavlon, BDH, England)[3,4]. The isolated mucilage had a neutralisation equivalent of 810, viscosity of 13.20 centipoises/20° (*c* 0.5 in water), ash content, 0.60%, $[\alpha]_D^{20}$ + 32° (*c* 0.5 in 0.1 M NaOH) and uronic acid content, 30.4% (as anhydro D-galacturonic acid).

The neutralisation equivalent was determined by potentiometric titration; viscosity of the solutions, by means of Reoviskometer Höppler (cells 0.01 and 0.1, respectively; shear stress, 10 g/cm²).

Cultivation of Aspergillus niger

Aspergillus niger, strain No. 69 (obtained from Dr. F. STRMISKA, Faculty of Chemistry, Department of Food Technology, Bratislava) was grown in surface culture at 28° on synthetic Czapek-Dox medium, adjusted to pH 6.2, containing 1.0% of linseed mucilage instead of glucose as the only carbon source. Cultivation was interrupted after 10 days, the mycelium was separated by filtration through a layer of cotton wool and the filtrate was further worked up.

Separation and isolation of the enzyme

Crude proteins were salted out from 10 l of cold filtrate by gradually adding solid ammonium sulphate to a saturation degree of 0.9. The mixture was allowed to stand in the refrigerator for 24 h at 0°; the precipitate was then separated by centrifuging and dissolved in 250 ml water. The part insoluble in water was separated by centrifuging (0°). The supernatant was dialysed against distilled water for several days in the refrigerator (0°) and then poured into 4 vol. of ethanol cooled to − 15° and stirred intensively. The precipitate was centrifuged (− 5°) and dissolved in 50 ml water. After centrifugation the supernatants were collected and freeze-dried. Salts and some of the coloured substances were removed by gel filtration through a column of Sephadex G-25 (4 cm × 50 cm; Medium, Pharmacia, Uppsala, Sweden). The course of gel filtration was followed by measuring the absorbance of 15–20-ml fractions at 280 nm. The fractions containing proteins were combined and freeze-dried. The yield of the dry, crude product was 1.065 g; N content, 2.25%; activity expressed as decrease of viscosity in %, 53.03 (1% solution of linseed mucilage; pH, 4.0; temperature, 30°; duration of enzymatic reaction, 60 min). It still contained about 40% polysaccharide.

Purification of crude enzyme preparation

Separation of the crude enzyme and polysaccharide obtained from the crude preparation was effected by chromatography on a column of CM-cellulose (4 cm × 40 cm; Whatman CM-70) and by gel filtration through Sephadex G-75 and Sephadex G-25 medium columns, respectively (Fig. 1). 50 mg of the crude preparation dissolved in McIlvaine buffer solution (pH 4.5) were chromatographed on a CM-cellulose column at a flow rate of 0.5 ml per min. The course of fractionation was followed by measuring the absorbance of 10-ml fractions at 280 nm and part of these fractions

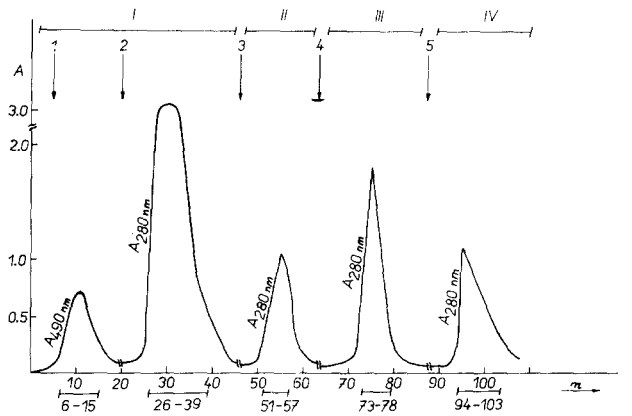


Fig. 1. Course of the separation of the crude enzyme preparation by chromatography on CM-cellulose and by gel filtration on Sephadex G-25 and G-75 columns. I, CM-cellulose column; II, Sephadex G-25 column; III, Sephadex G-75 column; IV, Sephadex G-25 column; 1, McIlvaine buffer solution, (pH 4.50); 2, McIlvaine buffer solution (pH 7.80); 3, distilled water; 4, phosphate buffer solution of ionic strength $I = 1.0$; 5, distilled water; A, absorbance at 280 and 490 nm, respectively; n, number of fractions.

was measured at 490 nm after reaction of 0.5-ml eluates with phenol and sulphuric acid[8]. If the second reaction was negative, eluation without interruption was followed with McIlvaine buffer solution (pH 7.8). Absorbance of each fraction was measured as previously. The fractions containing proteins were combined and freeze-dried and the salts from the buffer solutions were removed by gel filtration through Sephadex G-25 column. The full separation of the enzyme from traces of polysaccharide and coloured substances was effected by gel filtration on Sephadex G-75 column. Sephadex was previously suspended in a phosphate buffer solution of ionic strength $I = 1.0$. The same buffer solution was used for the eluation of the column and the fractions containing proteins were combined, freeze-dried and desalted on Sephadex G-25. The yield of freeze-dried enzyme was 350 mg (calculated to the crude product).

Determination of the activity of the enzyme

Determination of the activity was followed viscosimetrically[5], by measuring the increase in reducing power photometrically with 3,5-dinitrosalicylic acid[6] and by the analysis of the degraded mucilages, isolated from the substrates during the enzymatic reaction (180 min). In all cases the substrates were the 1.0% solutions of linseed mucilage (acidic fraction) in McIlvaine buffer solutions having a pH of 4.0. The temperature of the mixtures was 30°. The activity of the enzyme was expressed by the % decrease of viscosity of solutions and by the number of microequivalents of reducing groups. The unit of enzymatic activity is the quantity of enzyme releasing 1 μmole of reducing groups per min at 30°.

Separation of the degraded mucilage on DEAE-cellulose column

Stepwise elution: 30 ml of 1% solutions of degraded mucilage (30 min) were added to columns of DEAE-cellulose (3 cm × 30 cm; Whatman DE II) and eluted with water; 0.05, 0.1, 0.5, 1.0, 2.0 M sodium acetate solutions were adjusted to pH 6.0

by adding concentrated acetic acid and 0.5 M NaOH. The flow rate was 20 ml/h and tube volume was 10 ml. The total sugar content of eluates was determined by the phenol sulphuric acid method[8]. In all cases, the elution solutions were exchanged after negative reaction on sugars. The eluates with sugars were collected, then poured into 4 vol. of acidified ethanol, with 1.0% (v/v) hydrochloric acid. Each precipitate was washed with neutral ethanol, acetone, ether and vacuum dried above P_2O_5.

Paper chromatography

The samples of degraded polysaccharides were hydrolysed; HCl and H_2SO_4 hydrolysates were neutralised, filtered, vacuum evaporated above KOH and diluted with water. Paper partition chromatography was performed on Whatman No. 1 paper with the following systems of solvents: *n*-butanol–acetic acid–water (4:1:3, by vol.) ethylacetate–acetic acid–water (3:1:3, by vol.) and ethylacetate–pyridine–water (8:2:1, by vol.). Sugars were detected with aniline phtalate and with naphthoresorcinol, respectively.

Each mother liquor from the precipitation of polysaccharides was neutralised, vacuum evaporated, diluted with water and chromatographed.

Determination of substrate specificity

The substrate specificity of the enzyme was characterised by determination of the degradation of the linseed mucilage viscosimetrically and with 3,5-dinitrosalicylic acid.

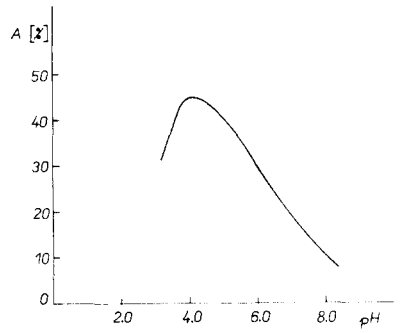
Determination of the purity of the enzyme

The purity of the enzyme was followed by horizontal paper electrophoresis (Whatman No. 3 paper; potential gradient, 5 V/cm; duration, 20 h) in buffer solutions of ionic strength $I = 0.1$, pH 4.5–9.6; furthermore, by the Tiselius electrophoresis procedure on the Kern L.K. 30 apparatus for microelectrophoresis in borate buffer solutions pH 6.5 and 9.3, respectively, and by ultracentrifugation on the analytical centrifuge MOM G-110 at 50 000 rev./min.

RESULTS AND DISCUSSION

Aspergillus niger growing in surface culture on a medium containing linseed mucilage as the carbon source produces an enzyme which hydrolyses linseed mucilage. By salting out with ammonium sulphate, by dialysis and precipitating with ethanol and desalting, a crude preparation was obtained. The crude preparation contains part of a polysaccharide which is simultaneously isolated with the protein product. The separation of these parts was effected by chromatography on CM-cellulose column and elution with buffer solutions above and below the isoelectric point of the enzyme. The first fraction is a remainder of the polysaccharide. The protein fraction (Fig. 1, the second curve) is enzymatically active, but it is not homogeneous. Gel filtration of the protein fraction on Sephadex G-75 column yields a specifically active preparation (Fig. 1, the fourth curve). Examination of this preparation by the Tiselius electrophoresis procedure and by sedimentation analysis indicated the presence of one component.

This enzyme preparation catalysing hydrolysis of linseed mucilage is charac-



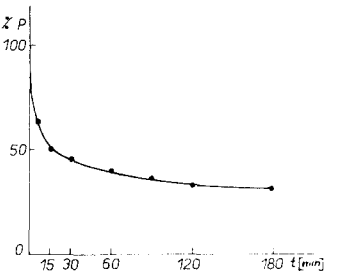


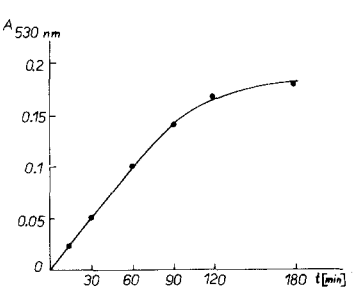
Fig. 2. Course of the activity of enzyme in dependence of pH. Length of the reaction, 30 min; temperature, 30°; reaction mixture contained 15 ml solution of the linseed mucilage and 5 ml (1 mg/ml) solution of the enzyme; A, decrease of the viscosity of the reaction mixture, expressed as 100 — P, where P = decrease in % (ref. 5).

Fig. 3. Course of the cleavage of the linseed mucilage by the isolated enzyme. Determination of the decrease of the viscosity: % P (ref. 5).

terised as follows. The optimum pH of the activity of the enzyme is 4.0 (Fig. 2). The viscosity declines rapidly during the first period of enzymatic reaction (Fig. 3), but the increment of the reducing groups is smaller under this condition (Fig. 4). The specific activity of the enzyme is 0.023 μequiv reducing groups released by 1 mg of the enzyme per min at 30°. The temperature dependence of the enzyme activity increases up to 40° and the optimum of activity ranges from 30 to 40°; exposure at 60° for 30 min inactivates the enzyme.

From the electrophoretic mobility of the enzyme, the isoelectric point was found to be in 7.0. The Michaelis constant determined from the course of the reaction velocity (v), expressed by the increment of reducing groups, dependent on the substrate concentration [S] (Fig. 5), gives value $3.14 \cdot 10^{-2}$ M (ref. 7). The enzyme has the only maximum absorbance in ultraviolet range at 280 nm.

By means of fractionating degraded mucilage isolated from the substrate after 30 min enzymatic reaction, 6 polysaccharides on a column of DEAE-cellulose were



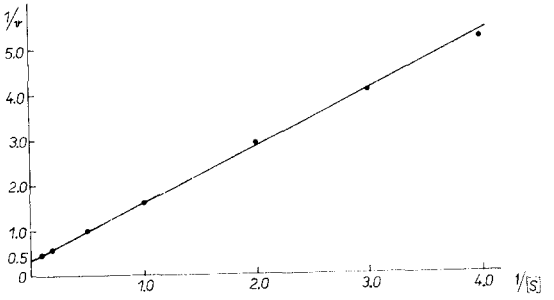


Fig. 4. Determination of the increment of the reducing groups during the enzymatic degradation of the linseed mucilage. Reaction mixture contained 1 ml of 1% solution of the linseed mucilage and 1 mg of the enzyme (in 1 ml).

Fig. 5. Determination of the Michaelis constant.

obtained. Each fraction was hydrolysed and the sugar components were identified by chromatography. The first 3 polysaccharides contain a higher proportion of pentoses (L-arabinose and D-xylose). These polysaccharides contain, as hexose, L-galactose in approximately similar quantities as L-arabinose. L-Rhamnose is present only in traces. The fourth to sixth fractions have more acidic character because D-galacturonic acid is the predominant component besides neutral sugars.

After precipitation of the polysaccharides obtained from the column of DEAE-cellulose, the supernatants do not contain any free sugar. However, by means of paper chromatography they yield a series of oligosaccharides so far unidentified.

The characteristic properties of the isolated, degraded polysaccharides in the course of the studied enzymatic reaction (180 min) are given in Table I. All these products contain the same sugar component though in different proportions.

TABLE I

CHARACTERISATION OF THE LINSEED POLYSACCHARIDES DEGRADED WITH THE ASPERGILLUS ENZYME

Duration of the enzymatic reaction	*Time (min)*					
	15	*30*	*45*	*60*	*120*	*180*
Neutralisation equivalent	360	540	540	620	640	560
Viscosity/20° (*c* 0.5 in water)	4.50	3.82	3.64	3.28	3.03	2.20
$[\alpha]_D^{20}$ (*c* 0.5 in 0.1 M NaOH)	+32°	+34°	+36°	+40°	+42°	+45°
Yield (%)	86.4	72.2	58.3	47.6	32.0	27.2
Sugar constituents						
L-Arabinose	+	++	++	++	++	++
L-Galactose	++	+++	+++	+++	++	++
D-Xylose	+++	++++	++++	+++	+++	++
L-Rhamnose	++	++	++	++	++	+
D-Galacturonic acid	++	++	++	++	++	+

During the entire process of enzymatic reaction, liberation of free sugars does not occur. It is obvious from the rapid decline of viscosity of the mucilage solution, as well as from the small rise of reducing groups, that cleavage of the mucilage molecule occurs mainly "inside". On the basis of these findings, it is suggested that the isolated enzyme has the activity of an endo-polyglycosidase.

Further studies concerning the effect of the isolated enzyme are in progress.

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