Studies on hemicellulose-hydrolyzing enzymes from Neurospora sp. : I. Some properties and partial purification

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Studies on hemicellulose-hydrolyzing enzymes from *Neurospora* sp.

I. Some properties and partial purification

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Hcmicellulosc-hydrolyzing enzymes (HHE) produced by microorganisms have been studied by many investigators.[1,2,3,4] Some of these enzymes were obtained as purified preparations and their modes of action toward substrates were clearly demonstrated.* However, the mechanism of hydrolysis of β-1,4-linkage between xylopyranose residues has not been intensively studied. The authors attempted to elucidate the mechanism of hydrolysis of hemicellulose by enzymes from *Neurospora* sp. in connection with the variety of the enzymes found in microorganisms. The present paper deals mainly with general properties and the partial purification of hemicellulose-hydrolyzing enzymes from ***Neurospora*** sp.

Experimental

Materials

A commercial crude enzyme preparation from ***Neurospora*** sp. and a crystalline n-amylase from B. *subtilis* were purchased from Nagase Sangyo Co. Ltd.

Methods

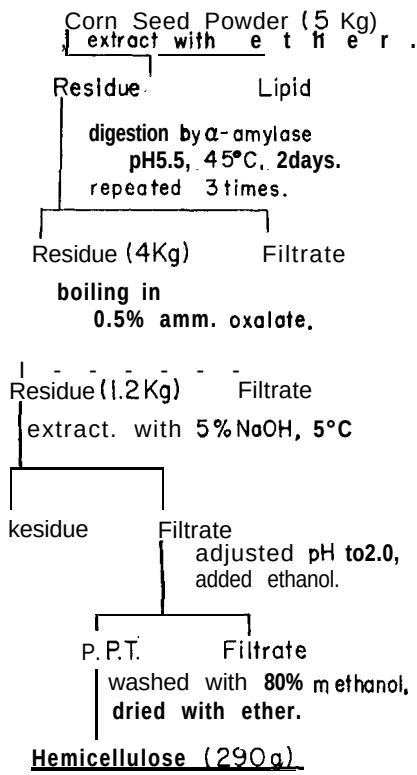
Preparation of corn seed hemicellulose (xylan)

Hemicellulose was prepared from corn seed powder by treating suc-

* These enzymes are usually classified to xylanase [EC 3. 2. 1. 8 β-1, 4-xylan xylanohydrolase]. However, the mode of action of enzymes from ***Neurospora*** sp. toward hemicellulose has not been studied. There remains doubt that these enzymes can simply be classified to xylanase. In the present paper, the authors used a tentative term "hemicellulose-hydrolyzing enzyme" for the enzymes from ***Neurospora*** sp., which hydrolyze some hemicelluloses.

cessively with α-amylase, ammonium oxalate and 5 % sodium hydroxide solution as shown in the Diagram. The hemicellulose obtained was water-soluble, being pale brown powder. This hemicellulose preparation consists mainly of xylose residues, but contains a trace amount of glucose residue.

Preparation of Corn Seed Hemicellulose



Soy bean hemicellulose C was prepared by extracting the rind of soy bean with 0.4 % sodium sulfite and 4 % sodium hydroxide solutions. Rice-straw holocellulose was prepared by removing lignin from rice-straw with sodium chlorite solution.") Rice-straw hemicellulose was extracted from the holocellulose with sodium hydroxide solution.

Purification of the enzymes

a). Precipitation with ammonium sulfate : Crude enzyme preparation

was dissolved in water, and the saturated ammonium sulfate solution was added to precipitate whole protein component. The precipitate was dissolved in 0.01 M acetate at pH 5.0. After removing the insoluble material by centrifugation at 7000 rpm for 20 minutes, the supernatant was dialyzed against the same buffer for three days using fish air bladder. The resulting solution was subjected to the purification by column chromatography.

b). Treatment with basic lead acetate: Partially purified HHE exhibited a broad absorption band near UV-region (300-400 mμ). In order to remove colored substances previous to column chromatography, the crude enzyme preparation was treated with basic lead acetate. To 100 ml of 2 % crude enzyme solution 14 ml of 40 % lead acetate solution was added. Precipitate was removed by centrifugation. Four milliliters aliquot of 40 % lead acetate soiution was again added to the supernatant. After centrifugation, the equal volume of 0.1 M dibasic sodium phosphate solution to the supernatant was added to precipitate the remaining lead ions as the phosphate salt.

c). Chromatography on column of DEAE-Sephadex A-25: Colored substances in the crude enzyme preparation can also be removed, in general, by addition of a basic substance, which is capable of forming the insoluble complex. Column chromatography with DEAE-Sephadex A-25 was adopted for the fractionation of the enzymes and for the removal of the colored substances. The crude enzyme dissolved in 0.01 M acetate buffer at pH 5.0 was applied on a column (3.5 x 40 cm) of DEAE-Sephadex A-25 and eluted stepwise at 9°C with the same buffer and 1.0 M sodium chloride solution. Flow rate was 20 ml per hour.

d). Chromatography on column of Amberlite IRC-50 XE 64 : Fraction FI obtained from DEAE-Sephadex A-25 column was applied on a column (2 × 30 cm) of Amberlite IRC-50 XE 64 equilibrated by McIlvain buffer at pH 5.0. Elution was done stepwise using 0.1 M McIlvain buffers at pH 5.0 and at pH 6.0.

e). Chromatography on column of hydroxylapatite: Fraction FI-1 obtained by chromatography on a column of Amberlite IRC-50 was dialyzed against 0.001 M phosphate buffer at pH 6.0, and then applied on a column (1.6 x 23 cm) of hydroxylapatite. Elution was done at 19°C with 0.01 M and 0.1 M phosphate buffers at pH 6.0. Flow rate was 10 ml per hour.

Assay of enzyme activity

Corn seed hemicellulose was suspended to 5 % in 0.1 M phosphate buffer at pH 6.0, and the suspension was allowed to stand in cold for overnight. Insoluble material was removed by filtration, and

the supernatant was used as a substrate solution. To 1 ml of substrate solution, 1 ml of the enzyme solution was added and the mixture was incubated at 40°C for one hour. Somogyi reagent (2 ml) was added to the reaction mixture, and the mixture was heated on a boiling water bath for 10 minutes, and cooled with tap water. After acidified by addition of 1.5 ml of 2 N sulfuric acid, iodine liberated was titrated with 0.005 N sodium thiosulfate solution. The reducing power was represented by the amount of xylose in μg units. Enzyme unit was conveniently defined as the activity that produces 1 μmole of xylosc per hour. The concentration of protein was represented by optical density at 280 mμ.

Results

The pH-dependence of the activity of crude enzyme solution

The pH-dependence of the activity of crude enzyme preparation was determined between pH 3.5 and pH 8.5 at 40°C. As shown in Fig. 1, pH-activity profile shows a peak at pH 6.0.

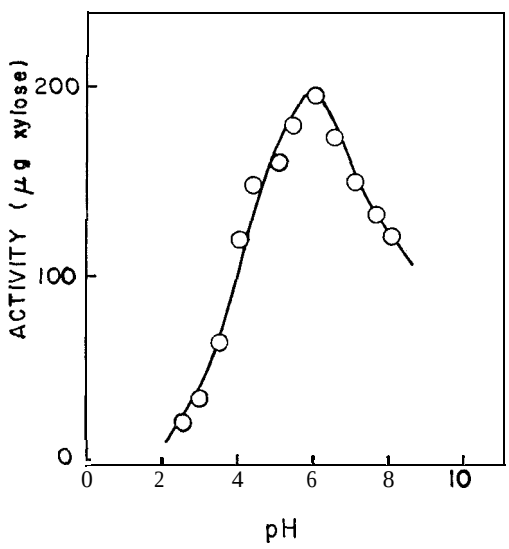


Fig. 1. The pH-dependence of the activity of the crude enzyme preparation. Reducing power was measured after incubating the reaction mixture at 40°C for 60 min.

The pH-stability of crude enzyme solution

To 1 ml of aqueous crude enzyme solution, 1 ml of each buffer solution was added, and the solution was allowed to stand at each individual temperature. The remaning activity was measured at 40°C, after the pH value of each solution had been adjusted to 6.0. The crude enzyme solution was stable between pH 4.0 and pH 6.0. Below pH 2.5, the enzymes were unstable and lost their activity almost completely by standing at 40°C for 2 hours, as shown in Fig. 2.

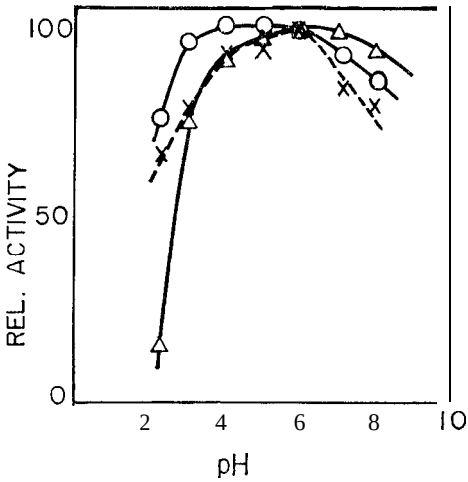


Fig. 2. The pH-stability profile of the crude enzyme preparation.
△: incubated at 40°C for 2 hr,
○: incubated at 9°C for 24 hr,
×: incubated at 14°C for 97 hr.
The activity was measured at 40°C after adjusting the pH value to 6.0.

Temperature-dependence of activity

Assay of the activity of crude enzyme preparation at pH 6.0 was carried out by incubating the reaction mixture at each desired temperature for 30 minutes. The crude enzyme preparation shows a broad temperature-activity profile with the maximum at 50°C, as shown in Fig. 3.

Heat-stability of crude enzyme preparation

The crude enzyme solution at pH 6.0 was prcincubated at various temperatures for one hour, and then the solution was rapidly cooled.

The activity was assayed at pH 6.0 and at 40°C. Fig. 4 shows that the crude enzymes are stable up to 40°C, while they lost nearly completely their activity by preincubation at 60°C.

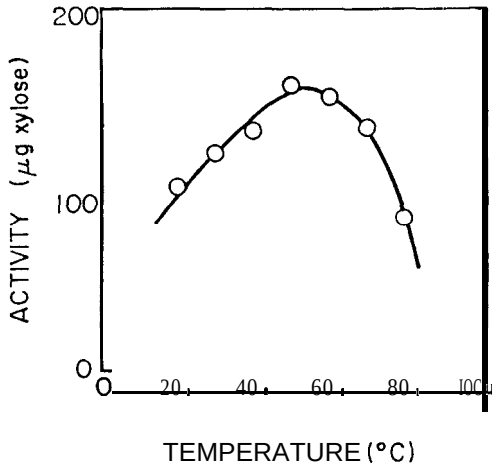


Fig. 3. Temperature-dependence of the activity. The mixture of enzyme and substrate was incubated for 60 min at each given temperature, and then reducing power was estimated.

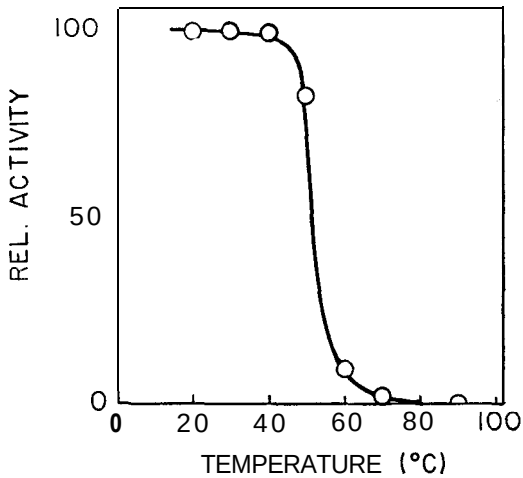


Fig. 4. Temperature-stability profile of the crude enzyme preparation. After preincubating the enzyme solution at each given temperature for 60 min, the activity was measured at 40°C and at pH 6.0.

Activity toward various substrates

Fig. 5 shows the actions of the crude enzyme preparation toward various substrates.

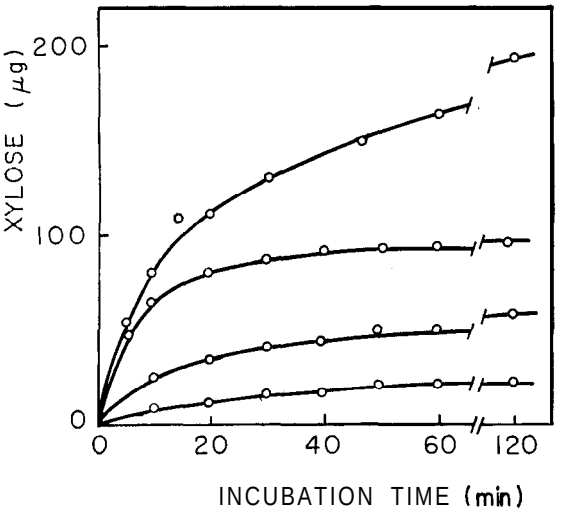


Fig. 5. Activities toward various substrates.
Top : rice-straw alkali-hemicellulose,
second : corn seed hemicellulose,
third : rice-straw holocellulose,
bottom: soy bean seed hemicellulose C.

Column chromatography on DEAE-Sephadex A-25

The crude enzyme preparation in 0.01 M acetate buffer at pH 6.0 was applied on a column of DEAE-Sephadex A-25. Chromatogram is shown in Fig. 6. The enzymes were separated into two active fractions, FI and FII. The colored substances were eluted in the last protein peak. Fifty five percent of the total activity in the solution applied was found in FI and 26 % in FII. Specific activities in (enzyme unit/OD_{280}) of FI and FII were 0.45 and 0.31, respectively, which are about ten times higher than that of the solution applied.

Colum chromotography on Amberlite IRC-50 XE 64

The fraction FI obtained from column of DEAE-Sephadex A-25 was applied on a column of Amberlite IRC-50 XE 64 equilibrated with 0.1 M McIlvain buffer at pH 5.0. The elution was carried out stepwise with 0.1 M McIlvain buffers at pH 5.0, 6.0 and 7.0. As shown in Fig 7, the

major part of enzyme activity (about 75 %) was found in FI-1 eluted by 0.1 M McIlvain buffer at pH 5.0.

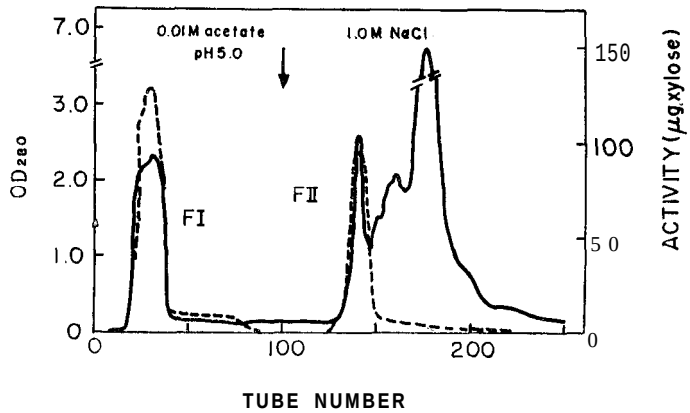


Fig. 6. Column chromntogram of the crude enzyme preparation on DEAE-Sephadex A-25. Solid line represents OD_{280} and broken line represents the activity measured by reducing power method. The eluent was changed to 1.0 M sodium chloride solution at point indicated by arrow.

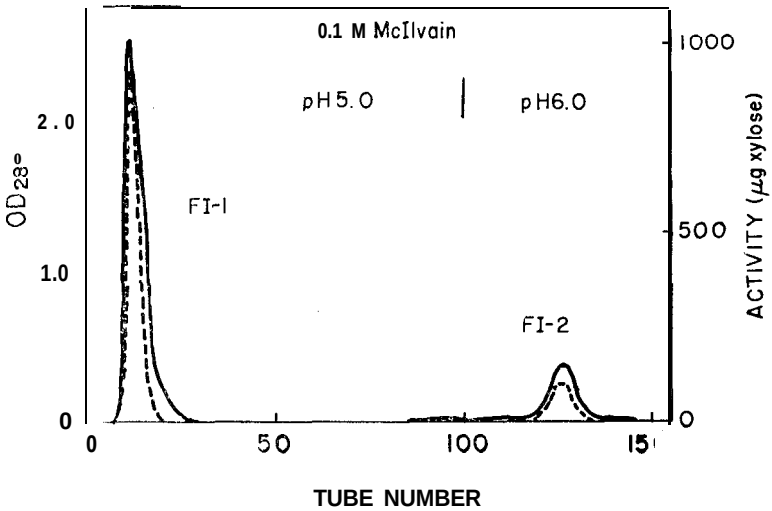


Fig. 7. Column chromatogram of FI on Amberlite IRC-50 XE 64. Solid and broken lines represent OD_{280} and activity, respectively. Activity was measured with 10 times diluted effluent,

Column chromatography on hydroxylapatite

The fraction FI-1 was applied on a column of hydroxylapatite and eluted successively with 0.001 M, 0.01 M and 0.1 M phosphate buffers at pH 6.0. As shown in Fig. 8, FI-1 was separated further into two fractions, Fl-l-l and FI-1-2. No remarkable increase in the specific activity was observed in this chromatography.

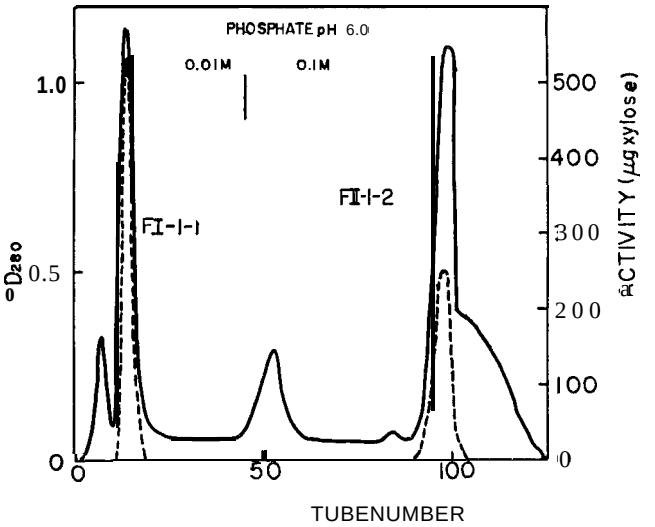


Fig. 8. Column chromatogram of FI-1 on hydroxylapatite. Solid and broken lines represent OD_{280} and activity, respectively.

LTV-absorption spectrum of FI-1-1 is shown in Fig. 9. The spectrum indicates that this preparation contains some impurities, which absorb lights near UV-region.

The specific activities are summarized in Table 1.

Discussion

An enzyme, which can hydrolyze β-1,4-linkage of corn seed hemicellulose, is usually classified to xylanase. The crude enzyme preparation from *Neurospora* sp. hydrolyzed most efficiently hemciellulose that consisted mainly of xylopyranose residue, and exhibited low activity toward soy bean hemicellulose C, which is believed to consist of galactose (40 %), pentose (20 %) and other (40 %).[6] This fact implies that

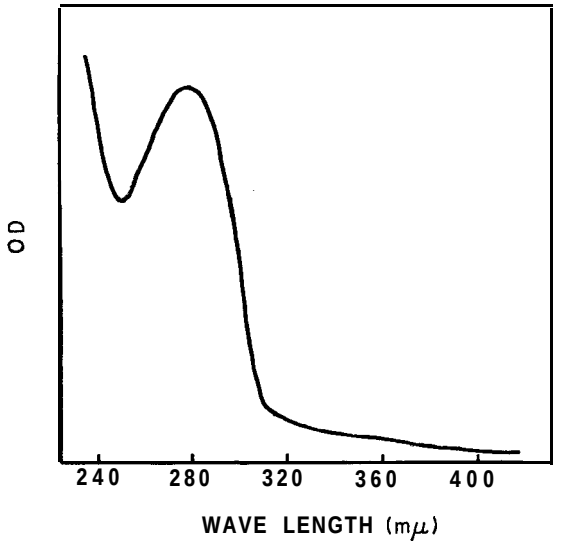


Fig. 9. Absorption spectrum of FI-l-l.

Table 1. Purifiation of HHE.

		Specific activity
Crude enzyme		0.046
DEAD-Sephadex A-25	FI	0.450
	FII	0.304
Amberlite IRC-50	FI-1	2.76
	FI-2	2.60
Hydroxylapatite	FI-l-1	3.00
	FI-1-2	1.16

enzymes from ***Neurospora*** sp. probably hydrolyze β-1, 4-glycoside linkage between xylopyranose residues in hemicellulose. Since detailed mode of action has not yet been studied, a tentative term "hemicellulose-hydrolyzing enzme" (HHE) was adopted to the enzymes from ***Neurospora*** sp.

General enzymatic properties of the crude enzyme preparation was not different from other enzymes except for a broad peak in the temperature-activity profile. This characteristic profile seems to be arisen from the multiplicity of HHE in the crude enzyme preparation from ***Neurospora*** sp. In column chromatography, it was evidenced that the

crude enzyme preparation contained at least four types of HHE, FII, FI-2, FI-1-l and FI-1-2. Such multiplicity of polyases in a microorganism has been well known. An enzyme, which can be distinguished chemically from the others, usually exhibits a different mode of action toward a common substrate for a group of enzyme. It is, therefore, necessary at the present step to characterize each of HHE from *Neurospora* sp. by means of its mode of action toward various hemicelluloses. Since the substrate hemicellulose is a complex polysaccharide, some aspect on applying hemicellulose as the substrate of HHE should first be considered in detail. In succeeding paper, the authors will discuss the preparation of a soluble substrate and its digestibility by HHE.

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