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PURIFICATION, PROPERTIES, AND MODE OF ACTION OF HEMICELLULASE II PRODUCED BY *Ceratocystis paradoxa**

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ABSTRACT

An extra-cellular endo-hemicellulase (HC-II) from a culture isolate of the fungal plant pathogen *Ceratocystis paradoxa* (CP_2) was purified 147-fold by ammonium sulphate precipitation, DEAE-Sephadex chromatography, iso-electric focusing at pH 3–10, and gel-permeation chromatography. The resulting enzyme preparation, which contained traces of invertase, gave a single protein-band on disc electrophoresis at pH 8.4, and was active towards sucrose, hemicellulose, and carboxymethylcellulose (CMC). HC-II randomly degraded hemicelluloses from several different sources, to xylose and to arabinose–xylose and xylose oligosaccharides of d.p. 3–6 and 2–5, respectively, and also produced a degraded hemicellulose which precipitated from the digest solution. The precipitated hemicellulose contained less arabinose and uronic acid than the original hemicellulose. When redissolved by alkali-treatment, it was susceptible to further degradation by hemicellulases HC-I and HC-II. CMC was degraded by HC-II, mainly to D-glucose and cellobiose, with trace amounts of unidentified higher oligosaccharides, while cellobiose remained unattacked. Xylotriose (Xyl_3) was the lowest homologue of the xylose oligosaccharides attacked by HC-II at a significant rate, yielding xylobiose [Xyl_2; β-D-Xyl*p*-(1→4)-D-Xyl] and xylose. $AraXyl_3$–$AraXyl_5$ were mainly hydrolysed to $AraXyl_2$, xylose, and Xyl_2 or Xyl_3. HC-II had a temperature optimum of 80°, and was stable for 1 h at temperatures up to 70°. The pH optimum was 5.1, and HC-II was stable between pH 5–10. The K_m was 0.267 mg of hemicellulose B/ml. The effects of mercury(II) ions and high concentrations of xylose on the activity of HC-II were also investigated.

INTRODUCTION

Hemicellulases, *viz.*, xylanases (E.C.3.2.1.8), are enzymes capable of hydrolysing (1→4)-β-D-xylopyranosyl linkages in hemicellulose. Recently, some highly purified hemicellulases (*e.g.*, refs. 1–7) have also been shown to be capable of hydrolysing the

*Studies on Hemicellulases: Part III. For Part II, see Ref. 1.

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(1→3)-α-L-arabinofuranosyl branch-points of arabinoxylans and arabinoglucuronoxylans.

In Part II of this series[1], we described the production of extra-cellular hemicellulases by a culture isolate of the fungus *Ceratocystis paradoxa* (*CP_2*), and the purification, properties, and mode of action of one of them (HC-I). We now report the purification of a second endo-enzyme, *viz.*, HC-II, from the same source, and its properties and mode of action on hemicelluloses and related oligosaccharides.

EXPERIMENTAL

Materials and methods. — The preceding paper in this series[1] describes the conditions for growth of the fungal pathogen *Ceratocystis paradoxa* (*CP_2* culture isolate) to produce a crude mixture of extracellular hemicellulases, and also the origin of the hemicelluloses and arabinose–xylose and xylose oligosaccharides. A commercial hemicellulose, "xylan", was obtained from Sigma Chemical Co., U.S.A., and yielded glucose, mannose, 4-*O*-methylglucuronic acid, and xylose on acid hydrolysis. The "xylan" therefore appears to contain a glucomannan in addition to the expected 4-*O*-methylglucuronoxylan. The different hemicellulose B fractions[8] obtained from spear grass, rumen-digested spear grass, and bovine faeces were isolated by Dr. R. J. Beveridge of this laboratory. Hemicellulose B from spear grass was used as substrate for assay of hemicellulase activity. Methods for assay of hemicellulase and invertase activities, protein assay, paper chromatography, disc electrophoresis at pH 8.4, ammonium sulphate precipitation, and DEAE-Sephadex chromatography are all described in the preceding paper[1].

Purification of hemicellulase II (HC-II). — The crude hemicellulases were precipitated with ammonium sulphate from the culture solution and resolved into two major fractions (HC-I and HC-II) by chromatography on DEAE-Sephadex (A-50), as described previously[1]. The purification of HC-I has already been described[1].

(*a*) *Iso-electric focusing at pH 3–10.* The fraction (II) eluted with sodium chloride from DEAE-Sephadex (A-50) (see Fig. 1, Part II) was dialysed against 0.5% aqueous glycine for 66 h at 5° prior to iso-electric focusing in a L.K.B. column of 440-ml capacity (L.K.B.-Producter, A.B., Bromma 1, Sweden). A pH gradient of 3–10 (Ampholine) was employed, and the density gradient was prepared with sucrose (50% w/v). After focusing for 67 h at 300 V and 5°, fractions (2 ml) were withdrawn from the bottom of the column, and assayed for reducing power and pH (Fig. 1). It was later discovered that invertases were present. The enzymic activities shown in Fig. 1 are therefore expressed in terms of reducing power, *i.e.*, total μmoles of reducing sugar (measured as xylose) per ml of enzyme, and include both HC-II and invertase (acting on the sucrose used to form the density gradient). The degradation of sucrose by invertase was relatively slight during isofocusing and storage at 5°, but when the fractions were assayed in the presence of hemicellulose B at 37°, the invertase action on sucrose became quite significant, making it difficult to distinguish HC-II

fractions from those of invertase. Fractions were therefore grouped, combined, dialysed against glycine solution for 72 h at 5°, and assayed for HC-II and invertase activities. The results are shown in Table I.

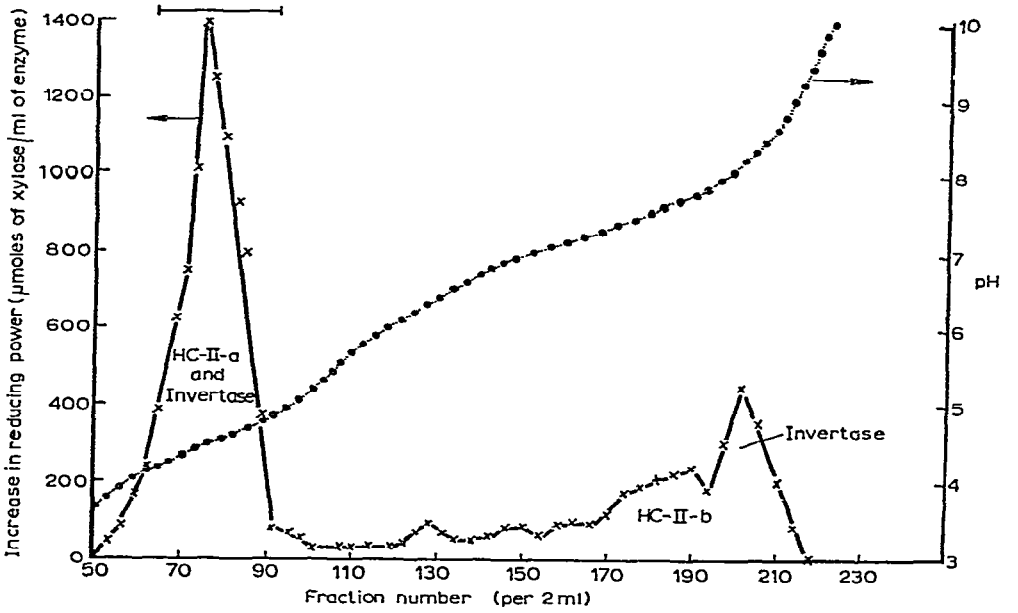


Fig. 1. Iso-electric focusing of hemicellulase II.

TABLE I

HEMICELLULASE AND INVERTASE ACTIVITIES IN ISO-ELECTRIC FOCUSED FRACTIONS

Fraction No.[a]	*pI*[b]	*Total hemicellulase activity*[d]	*Total invertase activity*[d]
50– 64	(3.70–4.20)[c]	40	33
65– 92	4.50	217	490
93–166	(5.0–7.25)[c]	0	97
167–194	(7.3–7.85)[c]	29	190
195–219	8.21	0	572

[a]2-ml Fractions. [b]Iso-electric point. [c]Indicates pH range. [d]Activity measured as μmoles of xylose/ml of enzyme/min.

(*b*) *Differential heat denaturation of invertase in the hemicellulase II fraction.* Enzyme solution (0.05 ml, fraction 65–92, Table I) and acetate buffer (0.1M, pH 5.5, 0.5 ml) were incubated at various temperatures for 1 h, and then at 37° with added sucrose or hemicellulose B solution (1%, 0.5 ml) for 5 h, followed by assay of reducing power (Table II).

TABLE II

DIFFERENTIAL THERMAL INACTIVATION OF INVERTASE IN THE HC-II FRACTIONS 65–92

Temperature (degrees)	*Hemicellulase II-a loss of activity*[a] (%)	*Loss of activity* (%)
5	0	0
37	8.6	5.6
45	10.1	11.3
51	N.d.[b]	15.0
55	N.d.	37.5
61	15.7	41.9
66	N.d.	50.6
71	N.d.	88.1
77	31.5	93.1
81	87.6	100.0
85	94.3	

[a]Relative to activity at 5°. [b]N.d., not determined.

(*c*) *Chromatography on Bio Gel P-200.* A column (2.6 × 96 cm) of Bio Gel P-200 was equilibrated with 0.01M phosphate buffer (pH 7.0). Fraction 65–92 (Table I) was dialysed against the same buffer (66 h/5°), concentrated by ultrafiltration to ~8 ml, applied to the bottom of the column, and eluted by upward flow with the same buffer at the rate of 9.8 ml/h. Fractions (5 ml) were monitored for protein by absorbance at

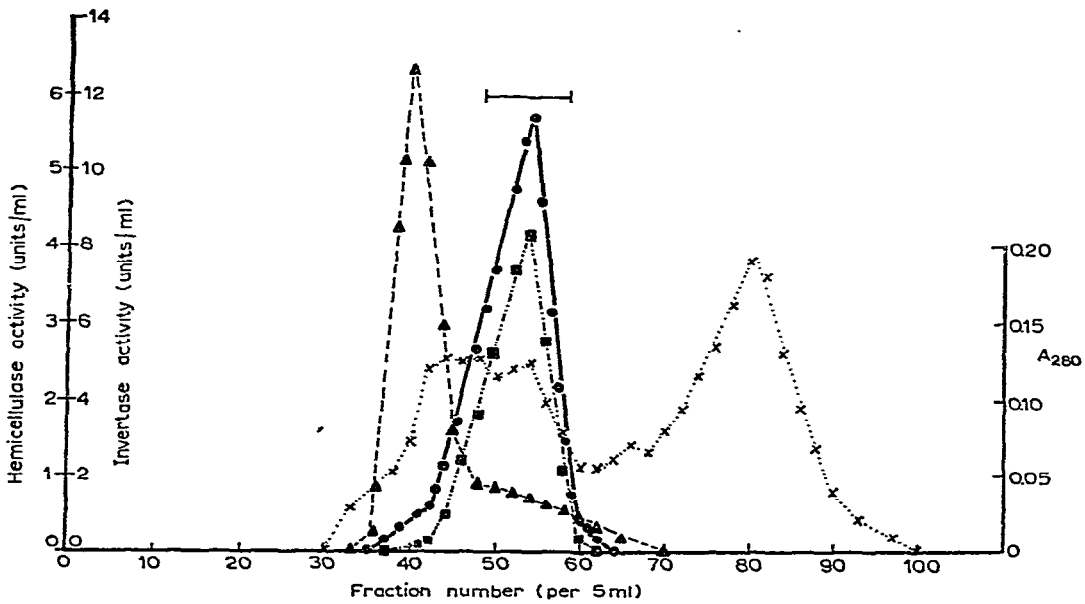


Fig. 2. Elution of hemicellulose II-a from Bio-gel P-200. ▲— — —▲, Invertase activity; ×·····×, absorbance at 280 nm; ●————●, hemicellulase activity; ■— · · —■, activity towards "xylan".

280 nm, and assayed for invertase and HC-II activities (Fig. 2). Fractions 35–44 were combined as acid invertase, while fractions 48–58 were combined as hemicellulase II (HC-II).

Isolation of an insoluble degraded-hemicellulose from the enzymic hydrolysate of spear-grass hemicellulose B. — Hemicellulose B (1 g) was solubilised in water (50 ml) by heating in a water bath (10 min). Acetate buffer (0.1M, pH 5.5, 50 ml) was added, and the solution centrifuged (1000 *g*/15 min) to remove traces of hemicellulose A. Enzyme solution (2 ml) and a few drops of toluene were then added, and the mixture was incubated at 35° until ~30% apparent conversion into xylose had occurred. The hydrolysate was then centrifuged (1000 *g*/30 min), and the precipitate was thoroughly washed with cold distilled water, slurried in water, dialysed against water for 48 h at 5°, and freeze-dried. The resulting white solid (yield, 13.2%) was called the "insoluble degraded-hemicellulose" (IH).

Substrate studies on the purified HC-II preparation. — Unless otherwise specified, substrate studies were carried out by incubating the various substrate solutions (1%, 0.5 ml for polysaccharides; and 2mM, 0.5 ml for arabinose–xylose and xylose oligosaccharides), acetate buffer (0.1M, pH 5.5, 0.5 ml), and enzyme solution (0.01 ml) at 35° for the times indicated.

The insoluble degraded-hemicellulose (0.1 g) was solubilised by trituration in M sodium hydroxide (1 ml), and the solution was diluted to 9 ml with water and neutralised with M acetic acid (1 ml). Attempts to terminate the enzymic digestions by heating the tubes in a boiling-water bath gave misleading results because significant HC-II action occurred during the "warm-up" period. The reactions were therefore terminated by addition of mercury(II) acetate (0.1 ml, 0.1M). This procedure was shown to terminate HC-II activity in less than 1 min at 35°.

The hydrolysates were deionized with mixed Amberlite IRC-50(H^+) and IR-45(HO^-) resins, filtered, and examined by paper chromatography. Authentic samples of oligosaccharides were used as reference standards to identify the products of hydrolysis. The results are shown in Tables III–V.

Composition analysis. — Total acid hydrolysis of IH and "xylan" (Sigma) was carried out as described by Dekker *et al.*[9], and the neutral "anhydro-glycose" composition of IH was determined by g.l.c. of the derived alditol acetates, using *myo*-inositol as internal standard. Uronic acid contents of hemicellulose B (spear grass) and IH were determined by the method of Galambos[10], using D-glucuronic acid as standard. The results are shown in Table VI.

Viscometry. — Viscosities were determined by using an Ubbelohde viscometer at 25°. Solutions of spear-grass hemicellulose B and IH (1%, w/v) in M potassium hydroxide were filtered through a G-3 porosity sinter and placed in the viscometer under nitrogen. Serial dilutions were made with the same solvent. The results are shown in Fig. 3.

Properties of the enzyme preparation. — (*a*) *Effect of temperature on activity.* The temperature optimum (Fig. 4) was obtained by incubation of enzyme solution

TABLE III

HYDROLYSIS PRODUCTS[a] FROM THE DEGRADATION OF SPEAR-GRASS HEMICELLULOSE B BY HC-II

Hydrolysis products	*Time of hydrolysis (h)*								
	0.25	*0.50*	*1.0*	*2.0*	*4.0*	*6.0*	*24.0*	*96.0*	*112.5*
Xylose	—	—	—	—	tr	1	5	8	10
$AraXyl_2$	—	—	—	—	1	2	5	6	4
Glucose	—	—	tr	1	3	5	4	7	6
Xyl_2	tr[b]	1	3	4	6	8	9	10	8
$AraXyl_3$	—	tr	1	2	3	4	3	2	1
Xyl_3	—	tr	2	3	4	5	4	2	2
$AraXyl_4$	—	tr	1	2	3	2	1	1	tr
Xyl_4	tr	1	4	6	6	6	tr	tr	tr
$AraXyl_5$	1	2	4	6	6	6	tr	tr	tr
Xyl_5	2	2	4	6	6	6	2	1	tr
Higher than Xyl_5 (at origin)	10	10	10	10	10	10	5	3	—

[a]The figures in the columns represent the relative amount of each oligosaccharide estimated by visual observation following paper chromatography; [b]tr, trace amounts.

TABLE IV

DEGRADATION PRODUCTS FROM THE ACTION OF HC-II ON DIFFERENT HEMICELLULOSES

Hemicellulose source	*Time of hydrolysis (h)*	*Hydrolysis products*						
		Xyl	*$AraXyl_2$*	*Xyl_2*	*$AraXyl_3$*	*Xyl_3*	*$AraXyl_4$*	*>$AraXyl_4$*
Wheat-endosperm arabinoxylan	4	tr	5	10	4	7	5	10
	24	5	4	8	3	3	4	4
	49.5	6	4	8	1	4	3	—
	166	6	4	8	2	1	—	—
Setaria sphacelata hemicellulose B	4	tr	4	10	5	8	6	8
	24	4	3	10	3	4	3	3
	49.5	5	3	20	2	5	2	—
	166[a]	6	3	10	—	1	—	—
Sugar-cane bagasse hemicellulose B	4	—	1	8	5	7	2	6
	24	1	2	5	tr	3	4	2
	124[a]	5	1	10	—	2	—	—
Commercial "xylan"	1	—	—	3	—	2	5[b]	10
	3	tr	—	8	—	4	tr[b]	10
	5	1	—	6	—	3	tr[b]	8
	24	5	—	8	—	2	tr[b]	—
Spear-grass hemicellulose B	24	tr	1	4	tr	4	—	3
(*a*) from grass digested in bovine rumen (72 h)	96[a]	8	4	10	2	3	1	tr
(*b*) from bovine faeces	24	2	3	6	2	4	—	1
	96[a]	8	4	10	2	3	1	tr

[a]Appearance of glucose in hydrolysates. [b]Indicates Xyl_4.

TABLE V

DEGRADATION PRODUCTS FROM ENZYMIC HYDROLYSIS OF ARABINOSE–XYLOSE AND XYLOSE OLIGOSACCHARIDES BY HC II

Oligosaccharide[a]	*Increase in reducing power* (%)[b]	*Hydrolysis products*						
		Xyl	*AraXyl*$_2$	*Xyl*$_2$	*AraXyl*$_3$	*Xyl*$_3$	*AraXyl*$_4$	*Xyl*$_4$
AraYyl$_2$	0	—	—	—	—	—	—	—
AraXyl$_3$	10	1	1	—	—	—	—	—
AraXyl$_4$	83	5	2	4	5	—	—	—
AtaXyl$_5$	66	2	2	7	6	3	2	—
Xyl$_2$	0	—	—	—	—	—	—	—
Xyl$_3$	18	1		1				—
Xyl$_4$	50	1		7		2		—
Xyl$_5$	57	3		8		1		1

[a]AraXyl$_m$, branched arabinose–xylose oligosaccharides of d.p. *m*; Xyl$_n$, linear xylose oligosaccharides of d.p. *n*. [b]Hydrolysis at 35° for 4 h.

TABLE VI

COMPOSITIONAL ANALYSIS OF SPEAR-GRASS HEMICELLULOSE B, AND THE "INSOLUBLE DEGRADED-HEMICELLULOSE"

Glycose constituent	*Relative composition* (% *glycan*)			
	Hemicellulose B[a]		*Insoluble degraded-hemicellulose*	
Arabinose (Ara)	14.0	Xyl/Ara = 5.3	9.4	Xyl/Ara = 9.3
Xylose (Xyl)	73.4		87.2	
Galactose	2.7		0	
Glucose	5.0		0.5	
Uronic acid (UA)	4.9[b]	Xyl/UA = 15	3.6	Xyl/UA = 24

[a]See ref. 8. [b]Found: uronic acid by the method of Galambos[10], 4.78%.

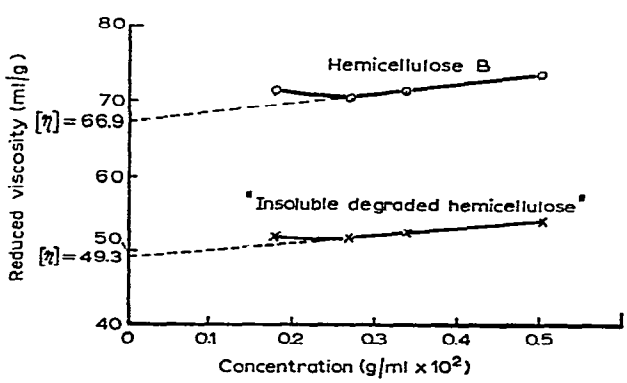


Fig. 3. Viscosity of spear-grass hemicellulose and enzyme-degraded insoluble hemicellulose. [η] = Intrinsic viscosity.

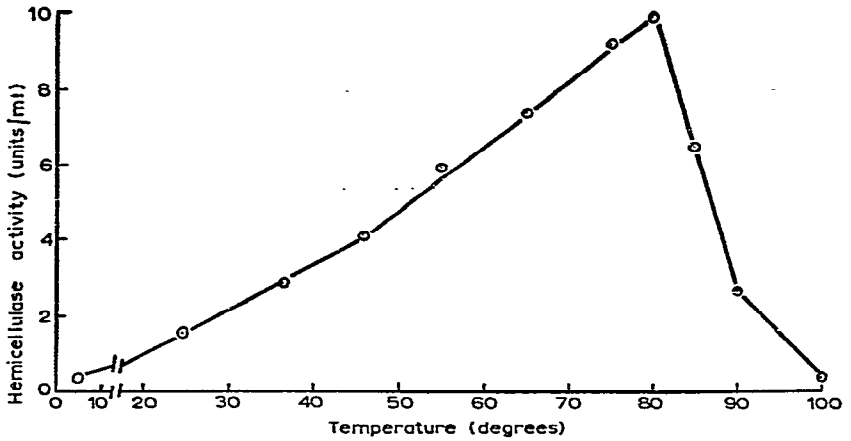


Fig. 4. Temperature optimum.

(0.01 ml), acetate buffer (0.1M, pH 5.5, 0.5 ml), and hemicellulose B solution (1%, 0.5 ml) for 30 min at various temperatures, followed by assay of reducing power.

(*b*) *Temperature stability.* The temperature stability (Fig. 5) was determined by incubating the enzyme solution (0.01 ml) and acetate buffer (0.1M, pH 5.5, 0.5 ml) for 1 h at various temperatures, then at 45° with added hemicellulose B (1%, 0.5 ml) for 30 min, followed by assay of reducing power. The stability of the enzyme for more prolonged periods was also examined at lower temperatures. The enzyme, when incubated in buffer for 4.5 h at 35 and 45°, lost ~56 and ~80%, respectively, of its initial activity. The thermal stability of HC-II in the presence of EDTA (2mM) in Tris-HCl buffer (0.05M, pH 8.4) is also shown in Fig. 5.

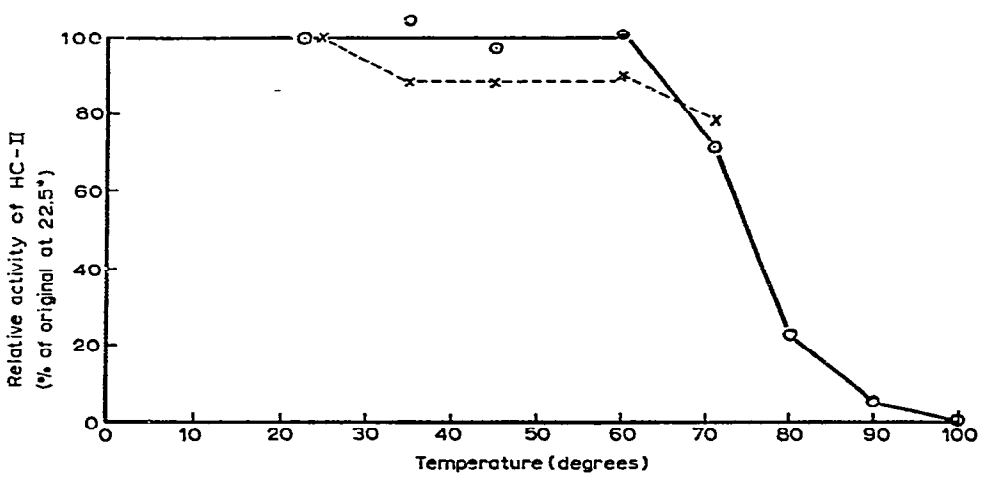


Fig. 5. Thermal stability (1 h). ⊙———⊙, No EDTA (pH 5.5); × – – – ×, EDTA (2mM, pH 8.4).

(*c*) *pH Optimum.* The pH optimum (Fig. 6) was determined by incubation of enzyme solution (0.01 ml) and hemicellulose B solution (1%, 0.5 ml) in Universal buffer solution (0.5 ml) of various pH values for 30 min at 45°, followed by assay of reducing power.

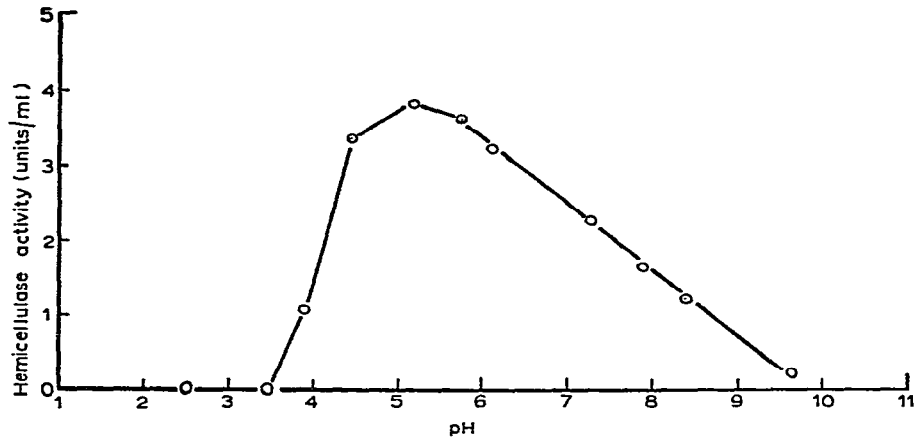


Fig. 6. pH Optimum.

(*d*) *pH Stability.* The pH stability (Fig. 7) was determined by incubation of enzyme solution (0.01 ml) in Universal buffer solution (0.5 ml) of various pH values for 1 h at 24°. The pH value of each solution was next adjusted to pH 5.0 by addition of NaOH (0.2M) or H_3PO_4 (0.2M), and the final volume adjusted to 2 ml with water. An aliquot (0.5 ml) of each solution was then removed, added to hemicellulose B solution (1%, 0.5 ml), incubated at 45° for 30 min, and assayed for reducing power.

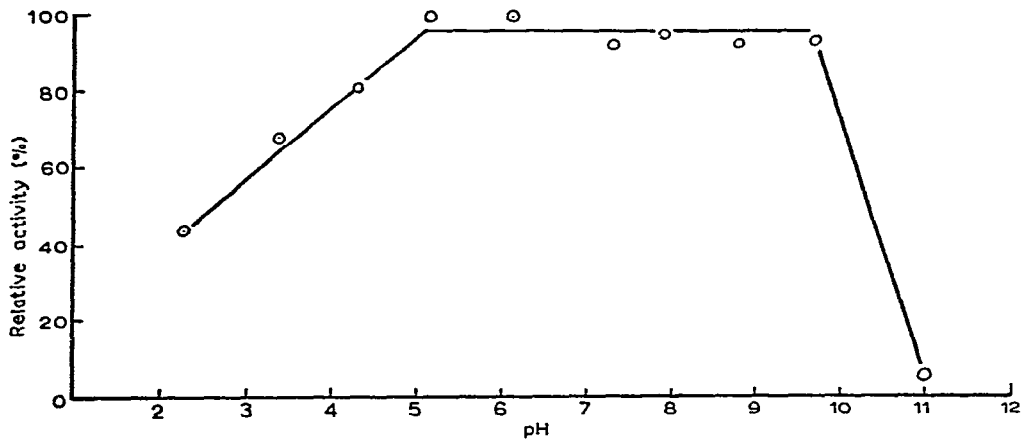


Fig. 7. pH Stability.

(*e*) *Determination of* K_m. Solutions (1.01 ml) containing acetate buffer (0.1M, pH 5.5, 0.5 ml) and enzyme solution (0.01 ml) were incubated with various concentrations of hemicellulose B (spear grass, 0.6–40 mg/ml) at 35° for 20 min, followed by assay of reducing power. The line of best fit for the Hane's plot was obtained by the least-squares method, and is given as $S/v = 1.685(S) + 0.450$ (correlation coefficient was 0.98) [Fig. 8(b)].

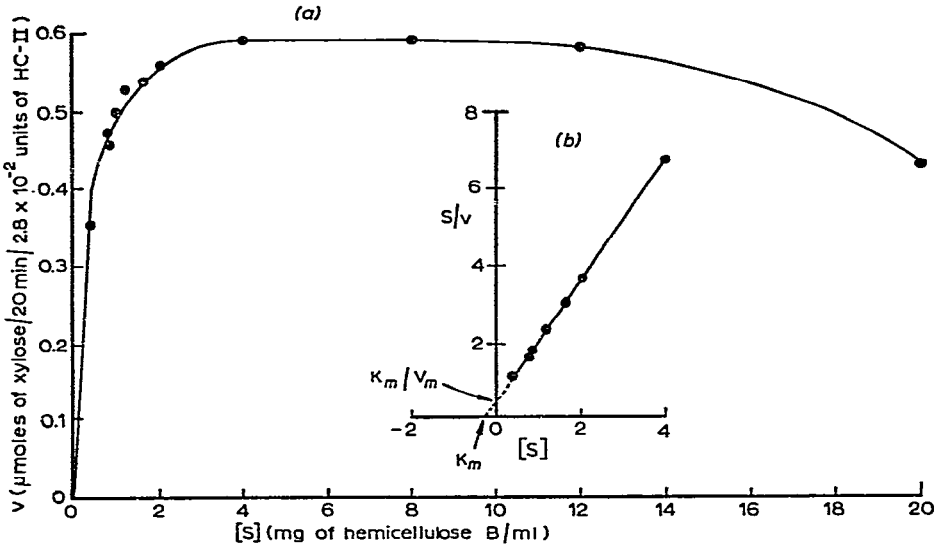


Fig. 8. (*a*) Michaelis–Menten plot for HC-II; (*b*) Hane's plot for HC-II; $K_m = 0.267$ mg of hemicellulose B/ml; $V_m = 32.3$ μmoles of xylose produced/mg of protein/min.

(*f*) *The effect of xylose on the enzymic hydrolysis of hemicellulose B.* Acetate solutions (0.1M, pH 5.5, 0.5 ml) containing various concentrations of xylose (0–10mM) were incubated with enzyme solution (0.01 ml) and hemicellulose B solution (1%, 0.5 ml) for 24 h at 35° and assayed for reducing power (Table VII).

TABLE VII

THE EFFECT OF XYLOSE ON THE ACTIVITY OF HEMICELLULASE II

Xylose (mM)	0	5	10
Relative activity[a]	100	81.4	63.7

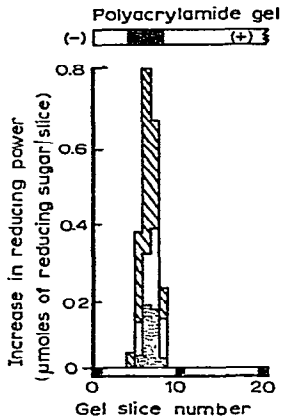
[a]Incubated at 35° for 24 h.

RESULTS AND DISCUSSION

In Part II of this series[1], we described the separation of hemicellulases HC-I and HC-II by chromatography on DEAE-Sephadex. Iso-electric focusing of the latter fraction at pH 3–10 has now revealed two peaks of hemicellulase activity (HC-II-a

and HC-II-b), each containing invertase activity (Table I, Fig. 1). The presence of invertase in these fractions interfered with the assay procedure for hemicellulase activity because of the presence of sucrose used to prepare the density gradient in the iso-electric focusing column. The fractions, shown in Table I, were therefore grouped and combined, and exhaustively dialysed against buffer prior to assay for hemicellulase and invertase activities. The bulk of the hemicellulase activity was found in fractions 50–90, but was accompanied by considerable invertase activity. The invertase could be deactivated by heating, but this resulted also in considerable losses of hemicellulase activity (Table II), invertase being completely inactivated at 81° at the expense of 88% loss in hemicellulase activity.

Gel-permeation chromatography of the major hemicellulase fraction from iso-electric focusing (Fig. 2) resulted in the elution of a major peak of invertase activity, followed by further invertase-active component(s) which overlapped the peak containing hemicellulase activity. The latter, named HC-II (fractions 48–58), showed a broad, single protein-band on gel electrophoresis at pH 8.4 (Fig. 9). Eluates of HC-II from the polyacrylamide gel, after electrophoresis, were found to be active towards hemicellulose B, sucrose, and CMC (Fig. 9). Other polysaccharides listed in Table IX were not attacked. Activity towards sucrose was presumably due to the presence of a small amount of invertase (see Fig. 2). The hydrolysis of CMC was probably catalysed by HC-II and not by a separate cellulase component. This is considered possible because the only structural difference between xylan and cellulose is in the nature of the substituent at C-5 of the pyranoid ring. The converse has been shown[11] to occur when a highly purified cellulase component of *Trichoderma viride* was found to hydrolyse xylan at the same active site as CMC.



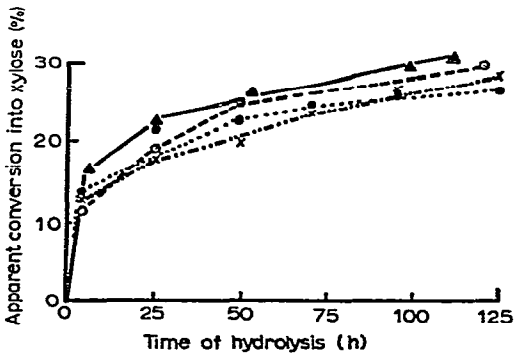


Fig. 9. Distribution of enzyme activities after electrophoresis on polyacrylamide gel. ▨, Activity towards hemicellulose B; □, activity towards sucrose; ▩, activity towards CMC.

Fig. 10. Time course of hydrolysis of several hemicelluloses. ▲———▲, Spear-grass hemicellulose B; ⊙— — —⊙, bagasse hemicellulose B; ●·····●, wheat arabinoxylan; × —·· — ×, *S. sphacelata* hemicellulose B.

TABLE VIII

PURIFICATION OF HEMICELLULASE II

Purification procedure	*Specific activity (units/mg of protein)*	*Recovery yield (%)*	*Purification (-fold)*
Cell-free culture fluid	0.202	100	1
$(NH_4)_2SO_4$ precipitate (50–90% saturation)	2.73	95	14
DEAE-Sephadex (A-50) Fr. 78–95, HC-II	18.20	16	90
Iso-electric focusing, pH 3–10 Fr. 65–92, HC-II-a	N.d.[a]	11	N.d.
Bio Gel P-200 Fr. 48–58, HC-II-a (HC-II)	29.7	7.6	147

[a]N.d., not determined.

TABLE IX

HEMICELLULASE II ACTIVITY TOWARDS OTHER POLYSACCHARIDE SUBSTRATES

Polysaccharide	*Hemicellulase activity[a] (units/ml)*
Control Hemicellulose B (Spear grass)	1.54
Carboxymethylcellulose (d.s., 0.7–0.8)	0.12
"Xylan" (Sigma)	1.01

[a]Measured as xylose. No activity was detected against soluble starch (potato), apple pectin, sugar-beet arabinan, dextran (B-512 *Leuconostoc*), larch galactan and arabinogalactan, and yeast mannan.

The purification procedure of HC-II at this stage represented a 147-fold purification, with a recovery yield of 7.6% (Table VIII). No further attempts were made to remove the small amount of invertase activity. HC-II showed optimal activity at 80° and pH 5.1 (Figs. 4 and 6), and was stable over a broad pH-range of 5–10 (Fig. 7). Incubations were normally carried out at 35° as the enzyme was found to lose activity with prolonged incubation (*e.g.*, 4 h) at higher temperatures. The enzyme was, however, stable up to 70° when kept at this temperature for 1 h (pH 5.5), and maintained this stability even in the presence of EDTA (2mM, pH 8.4) (Fig. 5), thus suggesting that polyvalent metal ions may not be involved in this rather high thermal stability. Because of the high degree of thermal stability of HC-II, it was not satisfactory in rate studies to attempt to terminate enzyme action by the usual method of heating to 100°. Instead, the reactions were terminated by addition of mercury(II) acetate (0.01M), which removed all enzyme activity in less than 1 min at 35°. HC-II inhibition occurred at high concentrations of substrate [Fig. 8(a)], and data from the plot of S/v against S (Hane's Plot) gave a K_m value of 0.267 mg of hemicellulose B/ml for spear-grass hemicellulose [Fig. 8(b)].

The time course of hydrolysis for several hemicelluloses from different plant sources, and from spear grass digested in the bovine rumen is shown in Figs. 10 and 11. In all cases, an apparent conversion into xylose of 25–30% occurred after ~100 h of hydrolysis. Fig. 11 also shows that the spear-grass hemicellulose B components were hydrolysed to a greater extent by HC-II than by HC-I. The experiments on hemicellulose from bovine faeces [Fig. 11(b)] are relevant to earlier studies of factors that limit the digestion of hemicelluloses in the bovine rumen[8]. They show that the hemicellulose in the faeces is attacked by HC-I and HC-II to an extent equal to the hemicellulose of the fodder that produced the faeces. The two types of hemicellulose are therefore very similar, and we confirm the earlier conclusion that the hemicellulose of the faeces has survived digestion because of physical protection by lignin in the grass[16].

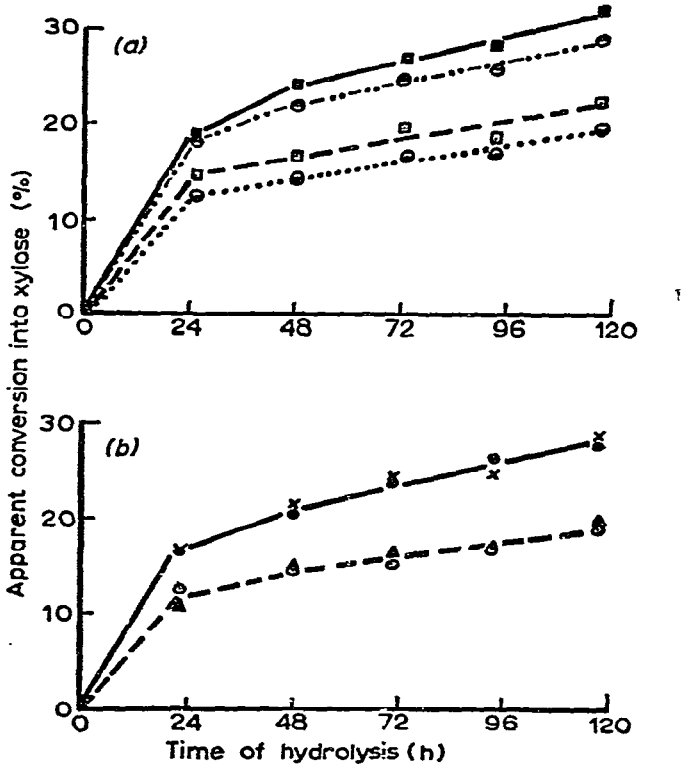
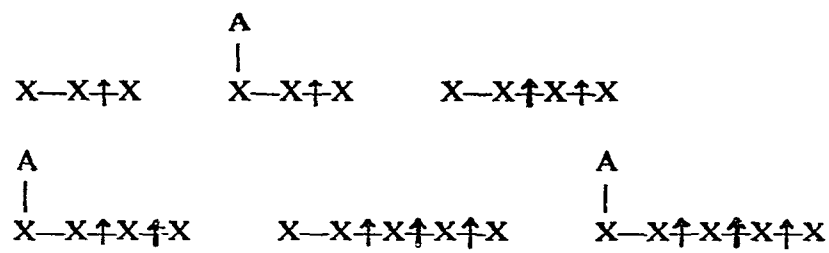


Fig. 11. (a) Time course of hydrolysis of hemicellulose B from spear grass and from rumen-digested spear grass by HC-I and HC-II. HC-II: ■———■, rumen-digested spear-grass hemicellulose B: ●–··–●, spear-grass hemicellulose B. HC-I: □– – –□, rumen-digested spear-grass hemicellulose B; ◒·····◒, spear-grass hemicellulose B. (b) Time course of hydrolysis of hemicellulose B from spear grass and from corresponding bovine faeces by HC-I and HC-II. HC-II: ×———×, spear-grass hemicellulose B; ●———●, bovine faeces hemicellulose B. HC-I: O– – –O, spear-grass hemicellulose B; ▲– – –▲, bovine faeces hemicellulose B.

Three of the hemicelluloses, *viz.*, *Heteropogon contortus*[8] (spear grass), *Setaria sphacelata*[12], and bagasse[13] (sugar cane) are predominantly arabino-4-*O*-methylglucuronoxylans. Arabinoxylan isolated from wheat endosperm is highly branched (xylose/arabinose = 1.77), and its structure has been described by Perlin[14]. The enzymic-degradation products of these polysaccharides are shown in Tables III and IV, and clearly indicate that HC-II is an endo-enzyme. In this work, we have neglected products containing uronic acid groups, as these would have been removed in the deionisation treatment. Initial attack of the enzyme preparation on hemicellulose B and arabinoxylan liberated Xyl_2 and a series of mixed arabinose–xylose and xylose oligosaccharides of d.p. greater than 3. Glucose also appeared in the hydrolysates of the hemicelluloses, indicating the degradation of a minor glucan component, which may be either a degraded cellulose or another type of glucan[15]. A glucose disaccharide, which co-chromatographed with cellobiose, was also liberated. D-Xylose and an arabinose–xylose trisaccharide (xylose:arabinose = 2:1; $AraXyl_2$) appeared after 4 h. A more detailed investigation of the structure of the latter product will be described in a later paper. Arabinose was not detected, but after 24 h, a precipitate, which we have called an "insoluble degraded-hemicellulose", appeared in the hydrolysate. Prolonged incubation progressively decreased the mean d.p. of the liberated oligosaccharides, and after 5–6 days, the main products of hydrolysis were D-xylose, a trisaccharide ($AraXyl_2$), D-glucose, and Xyl_2. The absence of any oligosaccharide product containing more than one arabinose residue (*e.g.*, Ara_2Xyl_3) is significant, and we conclude that, in the portions of the hemicellulose molecules attacked by the enzyme, there were no instances where L-arabinofuranose substituents occurred on contiguous xylose residues of the hemicellulose backbone.

Similar studies using arabinose–xylose and xylose oligosaccharides of d.p. 3–6 and 2–5, respectively, are shown in Table V. Xyl_3 was the lowest homologue attacked, liberating D-xylose and Xyl_2, while $AraXyl_3$ yielded $AraXyl_2$ and D-xylose. The most likely mode of action of HC-II on these oligosaccharides is shown below (see also Table V for the products arising from hydrolysis).



where X = β-(1→4)-linked D-Xyl*p*, A = α-(1→3)-linked L-Ara*f*, ↑ = linkages readily attacked by HC-II, and ↑ = linkages attacked at a slower rate.

Alternative modes of attack on the xylose oligosaccharides would, of course, give the same products (*e.g.*, X↑X—X), but the analogy with the reactivities of the arabinose–xylose oligosaccharides suggests the indicated preferential attack near the

reducing end. The arabinose branch-point in $AraXyl_2$ and $AraXyl_3$ has been shown by methylation studies to be attached to the terminal, non-reducing xylosyl unit, and a more detailed account of their structures will be described elsewhere, as also will evidence to suggest that $AraXyl_4$ and $AraXyl_5$ similarly carry the arabinose substituent on the non-reducing xylose end-residue.

The relative rates of hydrolysis of xylose oligosaccharides by HC-II (Table V) suggest that the binding site of the enzyme is effectively filled by a chain of four xylose residues. Apparently, the same "fit" can be achieved by $AraXyl_4$, as the latter is hydrolysed even more rapidly than Xyl_4. It seems, therefore, that the presence of arabinose substituents may not impede enzyme binding to the hemicellulose in the vicinity of the substituent although, of course, the xyloside linkage to the "right" of the substituent (normal formulae conventions) is resistant to hydrolysis and hence not at the active site (*i.e.*, $AraXyl_2$ is stable). These conclusions are compatible with the rather unusual suggestion that the more highly substituted regions of the hemicellulose may be preferentially attacked by HC-II (see below).

The degraded hemicellulose (IH) which precipitated from solution during the course of enzymic hydrolysis of spear-grass hemicellulose B had an intrinsic viscosity (Fig. 3), and hence probably a molecular weight, which was significantly lower than that of the original hemicellulose B (49.3, *cf.* 66.9 $ml.g^{-1}$). The compositional analysis of IH (Table VI) indicated that it also contained smaller proportions of arabinose, glucose, galactose, and uronic acid than the original material. Since no free arabinose was liberated during hydrolysis, the reduction in arabinose content in the polymer must have resulted from formation of arabinose–xylose oligosaccharides. When IH was redissolved in alkali and the solution neutralised, it was further attacked by HC-II, liberating the same oligosaccharides as those arising from attack on the original hemicellulose B (Table X). During the second hydrolysis, a hemicellulose precipitate again formed; this precipitate could be redissolved and further hydrolysed by HC-II, yielding similar products, including the precipitate. HC-I, which is capable[1] of hydrolysing both the (1→3)-α-L-arabinofuranosyl and (1→4)-β-D-xylopyranosyl linkages in hemicellulose B, also degraded IH, yielding arabinose, xylose, xylose oligosaccharides of d.p. 2–4, and the characteristic precipitate (Table X). However, oligosaccharides containing both arabinose and xylose could not be detected in the hydrolysate, which suggests that the arabinose residues were hydrolysed prior to the attack on the xylan moiety. The separation of the IH precipitate during the enzymic hydrolyses is evidently a time-dependent retrogradation phenomenon which appears to be favoured by reduction in the proportion of non-xylose constituents. It is not surprising to find that the less-substituted xylan chains retrograde more rapidly. However, in order to explain our total observations, we are forced to the surprising conclusion that the enzymes tend preferentially to attack the hemicellulose molecules (or portions of molecules) which are most substituted with arabinose and uronic acid residues, leaving a less-substituted and lower molecular weight xylan which retrogrades and thus becomes resistant to further attack.

TABLE X

DEGRADATION PRODUCTS ARISING FROM THE HYDROLYSIS OF "INSOLUBLE DEGRADED-HEMICELLULOSE" BY HC-I AND HC-II

Degradation products	*Relative amounts*	
HC-I	*27 h (5.4%)*[a]	*78 h (14.0%)*[a]
Xylose	0	1
Arabinose	2	8
Xyl_2	4	10
Xyl_3	6	8
Xyl_4	4	1
HC-II	*27 h (10.5%)*[a]	
Xylose	4	
Arabinose	0	
$AraXyl_2$	2	
Xyl_2	8	
$AraXyl_3$	2	
Xyl_3	3	

[a]Time of hydrolysis; extent of apparent hydrolysis in brackets.

The effect of high concentrations of xylose on the activity of HC-II is shown in Table VII. Enzyme activity is suppressed at higher concentrations of xylose, thus suggesting the possibility of product inhibition in the action of the enzyme on hemicelluloses.

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