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

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ABSTRACT

A culture isolate (CP_2) of the fungal plant pathogen *Ceratocystis paradoxa* produces at least five extra-cellular hemicellulases when grown on a medium containing a commercial hemicellulose as inducer. One of the five enzymes, hemicellulase I (HC-I), was purified by ammonium sulphate precipitation, ion-exchange chromatography (DEAE-Sephadex and then Cellex-CM), and iso-electric focusing at pH 3–10 and 8–10. HC-I behaves as a single protein on electrophoresis at pH 6.0 and 8.4. The enzyme degrades hemicellulose B (an arabino-4-*O*-methylglucurono-xylan) and arabinoxylan to arabinose, xylose, xylobiose (Xyl_2; β-D-Xyl*p*-(1→4)-D-Xyl), and a mixture of arabinose–xylose and xylose oligosaccharides ($AraXyl_n$ and Xyl_n, where $n = 3$, 4, or 5). The enzyme is deduced to be an endo-enzyme. Xylotetraose (Xyl_4) was the lowest homologue of the xylose oligosaccharides attacked, yielding xylobiose and xylotriose (Xyl_3) only. A mechanism is postulated for this reaction. $AraXyl_2$–$AraXyl_5$ were slowly hydrolysed to arabinose and the respective xylose saccharide (Xyl_2–Xyl_5), and thence to Xyl_2 and Xyl_3. Hydrolysis of the arabinofuranosyl linkage probably does not occur at the same active site as for the xylose oligosaccharides. Hemicellulose B fractions from different sources appeared to be degraded by HC-I. The enzyme showed optimum activity at pH 5.5 and 40°, and K_m was 4.24 mg of hemicellulose/ml.

INTRODUCTION

Hemicellulases, *viz.* xylanases (E.C.3.2.1.8), are capable of hydrolysing (1→4)-β-D-xylopyranosyl linkages in hemicellulose. Preparations of highly purified hemicellulase have also been shown to be capable of hydrolysing the (1→3)-α-L-arabinofuranosyl branch points of arabinoxylans[1–3]. Whether the two types of linkage are hydrolysed at the same active site has not been shown, but it seems likely that both types of hydrolysis are catalysed by the same enzyme. A similar effect has been

*Studies on Hemicellulases: Part II. For Part I, see Ref. 30.

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demonstrated for a highly purified cellulase component isolated from *Trichoderma viride*[4]. Both xylan and carboxymethylcellulose were shown, by kinetic studies, to be hydrolysed at the same active site of the one enzyme. This is not entirely unexpected, since the two types of polysaccharide differ only in the nature of the substituent at C-5 of the pyranoid ring.

Recent reviews on the degradation of hemicellulose by enzymes of microbial origin have been reported by Sorensen[5] and Timell[6]. Since then, purified hemicellulases of fungal origin have been prepared and used[1–3] in studies of the degradation of hemicelluloses (see below).

Hemicellulases from rumen bacterial (*Bacteroides amylogenes*[7] and *Butyrivibrio fibrisolvens*[7,8]) and protozoal (*Epidinium ecaudatum*[9] and *Polyplastron multivesiculatum*[10]) cell-free extracts are of the following types: xylobiase (β-D-xylosidase), xylodextrinase, arabinofuranosidase, and xylanase. The xylobiases hydrolyse xylobiose (Xyl_2; β-D-Xyl*p*-(1→4)-D-Xyl) and D-xylose oligosaccharides of d.p. 3–6 to D-xylose. Xylodextrinases[9] hydrolyse D-xylose oligosaccharides of d.p. >2 to D-xylose and Xyl_2, but do not attack Xyl_2 or xylan, whereas the xylanases degrade xylan, arabinoxylan, and D-xylose oligosaccharides mainly to Xyl_2 and D-xylose by an endo-type mechanism. Xyl_2 was not attacked, and the xylanase attacked linear xylan more readily than the branched hemicelluloses, *viz.*, arabinoxylan (wheat flour) and a branched B fraction from *Trifolium pratense*. The presence of arabinose, galactose, and uronic acids as side branch-points on the xylan backbone appears to inhibit the action of the xylanases. This has also been demonstrated[11] when a commercial hemicellulase preparation was used to degrade the highly ramified xylan of sapote gum (*Sapota achras*). With the rumen microbial system, the xylan chain was not effectively hydrolysed by xylanase until the arabinofuranosidase had removed the arabinose residues from the arabinoxylans. The different types of rumen hemicellulases therefore appear to work in a synergistic manner in degrading arabinoxylan to D-xylose and L-arabinose[7–10].

Hemicellulases have also been isolated and purified from different bacteria (*e.g.*, *Streptomyces*[5,12], *Cellvibrio*[5], *Clostridium*[5], *Micromonospora*[5], and *Bacillus*[13,14] species), and their action pattern has been shown to be predominantly of the endo-enzyme type, degrading xylan and arabinoxylan to D-xylose, L-arabinose, D-xylose oligosaccharides of d.p. 2–6, and oligosaccharides containing both D-xylose and L-arabinose. Xyl_2 was not attacked, but the endo-enzymes were capable of hydrolysing Xyl_3–Xyl_6 to Xyl_2 and D-xylose. Cell material and the culture fluid of a *Cellvibrio* species, isolated from soil, also possess[5] an exo-enzyme capable of hydrolysing xylan and Xyl_2 to D-xylose. It is possible, however, that the observed results may have been due to the presence of both an endo-xylanase and a β-D-xylosidase.

The most common sources of hemicellulases have been the fungi, and enzyme preparations therefrom have been more extensively studied than from any other genera. Some of the fungal hemicellulase systems studied include *Aspergillus batatae*[14], *A. foetidus*[15], *A. niger*[1–3], *A. oryzae*[14], and *A. wentii*[16], *Coniophora*

cerebella[17], *Chaetomium globosum*[5,16], and *C. trilaterale*[18], *Diplodia viticola*[19], a *Neurospora* sp.[20], *Penicillium janthinellum*[5,21], *Schizophyllum commune*[22], and *Trichoderma viride*[4,23]. Some of these fungi, *e.g.*, *A. niger*[1,3] and *P. janthinellum*[21], produce up to three different extra-cellular hemicellulases, each capable of degrading hemicellulose by a different mode of attack. The above-mentioned fungi all produce hemicellulases of the endo-type, and only two have been conclusively shown to produce exo-type hemicellulases, *viz.*, *A. batatae*[14] (xylanase B) and *A. foetidus*[15] (Fraction 1-A). The latter enzymes were isolated from the culture fluid and mycelia, respectively, and were shown to degrade xylan and xylose oligosaccharides of d.p. 2–6, completely to xylose.

Commercial preparations, *e.g.*, pectinase[6] and cellulase[23–25], have also been used as sources of hemicellulases and, when purified[23,24] or used as supplied[6,25], degrade hemicellulose by an endo-type mechanism. Hemicellulase preparations have also been employed to furnish structural information about arabinoxylans[3,26–29], 4-*O*-methylglucuronoxylans[6,17], and complex hemicelluloses such as sapote gum[11].

We now report on the purification of one of several extra-cellular hemicellulases (hemicellulase I, HC-I) produced by the fungal pathogen *Ceratocystis paradoxa* (isolate[30] CP_2), and its mode of action on hemicellulose and related oligosaccharides.

EXPERIMENTAL

Materials. — The fungal plant pathogen, *Ceratocystis paradoxa* (CP_2 culture isolate), used for the production of hemicellulases has been described in Part I of this series[30]. Arabinoxylan (*ex.* wheat endosperm) was kindly provided by Professor B. A. Stone. Hemicellulose B from spear grass (*Heteropogon contortus*)[31], $AraXyl_3$–$AraXyl_5$[32], and Xyl_4 and Xyl_5 were isolated by Dr. R. J. Beveridge. Hemicellulose B from *Setaria sphacelata*[33] was isolated by Dr. N. W. H. Cheetham. Xylobiose (Xyl_2) and xylotriose (Xyl_3) were isolated by paper chromatography from enzymic hydrolysates of commercial "xylan"[30] (Calbiochem, Calif., U.S.A.). $AraXyl_2$ was isolated from the hemicellulase II hydrolysate of hemicellulose B (Part III of this series).

Cellex-CM (exchange capacity, 0.68 mequiv./g), a cation-exchange cellulose, was obtained from Bio-Rad Laboratories, (California, U.S.A.). The iso-electric focusing equipment (440-ml capacity) used along with the gradient mixing device and the Ampholine Carrier Ampholytes (40% solutions, w/v) of pH range 3–10 and 8–10 were obtained from L.K.B.-Produkter, A.B., Bromma (Sweden). Amicon ultrafiltration units (stirred cell, model 52; and the propellent-pressurized cell, model 10PA) and Diaflo membranes (UM20E and UM10) were obtained from Amicon Corporation, Lexington, Mass. (U.S.A.).

General methods. — (*a*) *Assay for hemicellulase activity.* Hemicellulase activity was assayed in 50mM acetate buffer (0.5 ml, pH 5.5) containing 0.5% of hemicellulose B from bagasse. After incubation for 30 min at 37°, samples were withdrawn into alkaline Nelson reagent C to stop the reaction, and assayed[34] for reducing sugars,

using anhydrous D-xylose as reference standard. The unit of hemicellulase activity is defined as that amount of enzyme which produces one μmole of D-xylose per min under the above conditions, and specific activity as the units of activity per mg of total protein.

(*b*) *Protein assays.* Protein eluted during chromatography on DEAE-Sephadex (A-50) and Cellex-CM was monitored by absorbance at 280 nm. Protein was also determined on enzyme fractions, after concentration by ultrafiltration, by the Lowry method[35] as modified by Hartree[36], using bovine serum albumin (Sigma, Fraction V) as reference standard.

(*c*) *Chromatography.* Degradation products from the enzymic hydrolysis of hemicellulose and homologues of arabinose–xylose and xylose oligosaccharides (d.p. 2–6) were separated and identified on Whatman No. 1 paper, using ethyl acetate–pyridine–water (10:4:3) and detection with silver nitrate–sodium hydroxide[37] and *p*-anisidine hydrochloride[38]. Samples from hemicellulase digests were deionized prior to chromatography by using Amberlite IRC-50(H^+) and IR-45(HO^-) mixed resins.

(*d*) *Disc electrophoresis of enzymes.* The method used for discontinuous polyacrylamide-gel electrophoresis of enzyme at pH 6.0 (citrate buffer), and pH 8.4 (borate–HCl buffer) is described elsewhere[39].

(*e*) *Determination of the hemicellulase activities distributed along a polyacrylamide gel after electrophoresis.* The polyacrylamide gel was transectionally sliced into 1-mm sections by using a slicer made from razor blades (30), separated by spacers. Each gel slice was then placed in a tube containing borate–HCl buffer (0.5 ml, 12.5mM, pH 8.4) and left at 5° for 24 h. Acetate buffer (0.5 ml, 0.1M, pH 5.5) and hemicellulose B solution (0.5 ml, 1%) were next added, and the mixture was incubated at 37° for 30 min, followed by assay of reducing power.

(*f*) *Growth of fungal pathogen.* Six 1-litre cultures of *Ceratocystis paradoxa* (CP_2) were grown on commercial "xylan" as inducer at 32° under conditions for optimal enzyme production, as described in Part I of this series[30].

Purification of hemicellulases. — (*a*) *Precipitation by ammonium sulphate.* The fungal cultures (6 litres) were harvested by centrifugation at 20,000 *g* for 30 min at 5°, when hemicellulase activity was at a maximum, usually after ~48 h of growth (see Table I). The cell-free culture fluid was buffered (0.02M, pH 7.0) by the addition of solid sodium dihydrogen phosphate dihydrate (1.21 g/l) and anhydrous disodium hydrogen phosphate (1.73 g/l) prior to the addition of solid ammonium sulphate. This step was undertaken since considerable loss of hemicellulase activity was observed when ammonium sulphate was added to the unbuffered culture fluid.

TABLE I

PRODUCTION OF HEMICELLULASE DURING GROWTH OF *Ceratocystis paradoxa* (CP_2) AT 32°

Time of growth (h)	24	48	67	90
Hemicellulase activity (unit/ml)	0.614	0.712	0.710	0.662

The buffered culture fluid was then brought to 50% saturation by slow addition of solid ammonium sulphate, and the solution was left for 6 h at 5°. A low-density precipitate resulted, and after separation this showed no hemicellulase activity either before or after dialysis against 0.01M acetate buffer (pH 5.5). The 50% saturated ammonium sulphate solution was next brought to 90% saturation, and kept at 5° for 17 h prior to centrifugation. A low-density precipitate was collected from the surface, and a more dense one by centrifugation. Both precipitates had hemicellulase activity, (28% and 72% of the total hemicellulase activity, respectively) and were combined, resuspended in sodium acetate buffer (0.01M, pH 5.5), dialysed against the same buffer, and concentrated by ultrafiltration to ~20 ml prior to chromatography on DEAE-Sephadex.

(*b*) *Chromatography on DEAE-Sephadex (A-50).* A column (2.6 × 40.0 cm) of DEAE-Sephadex (A-50) was equilibrated with 0.01M sodium acetate buffer (pH 5.5). A sample (19 ml) of the concentrated solution of enzyme was applied to the bottom of the column and eluted by upward flow with the same buffer, at the rate of ~20 ml/h. After elution of ~4 void volumes of buffer (600 ml), a 0–0.5M linear gradient of sodium chloride in the same buffer was applied. Fractions were assayed for protein and hemicellulase activity, and the results are shown in Fig. 1.

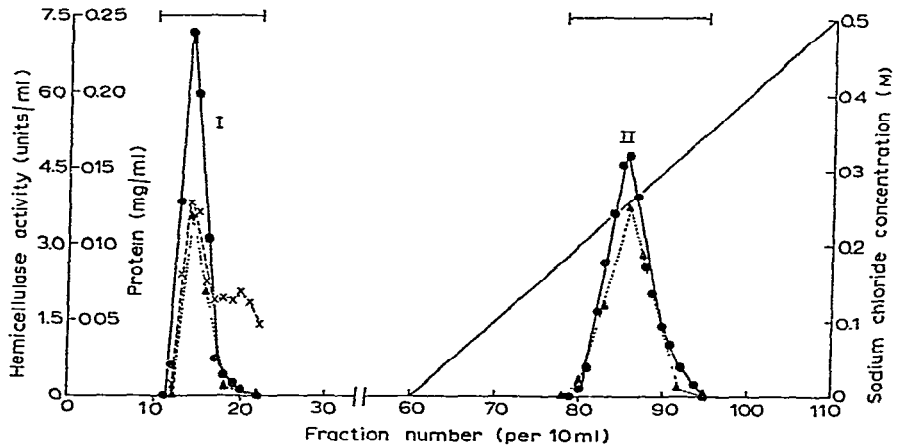

Fig. 1. Elution pattern of extra-cellular hemicellulases from DEAE Sephadex A-50. (●—●), Hemicellulase activity; (▲.... ▲), activity towards "xylan"; (×----×), protein.

(*c*) *Chromatography on Cellex-CM.* A Cellex-CM column (1.3 × 31.0 cm) was equilibrated with 0.02M sodium phosphate buffer (pH 7.0). Fractions 10–22 (hemicellulase I) from chromatography on DEAE-Sephadex were combined, dialysed against the same phosphate buffer, concentrated by ultrafiltration to 6 ml, applied to the top of the column, and eluted with the same buffer. After elution of ~3 void volumes (60 ml), a 0–0.5M linear gradient of sodium chloride in the same buffer was

applied. Fractions were assayed for hemicellulase activity and protein, and the results are shown in Fig. 2.

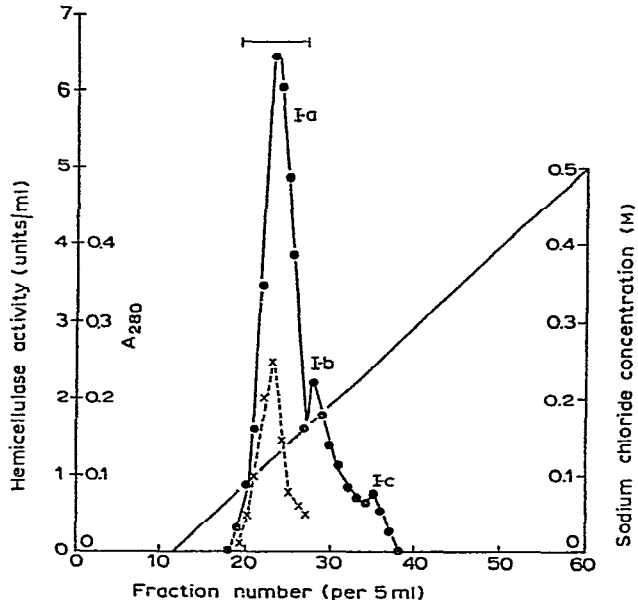

Fig. 2. Elution pattern of hemicellulase fraction I from Cellex-CM. (●—●), Hemicellulase activity; (×-----×), absorbance at 280 nm.

(d) Fractionation by iso-electric focusing. The hemicellulase fractions 19–27 (Ia, Fig. 2) were combined, dialysed against several changes of 0.5% aqueous glycine for 60 h, and concentrated by ultrafiltration. This fraction was then assayed for invertase activity in 50mM acetate buffer (0.5 ml, pH 5.5) containing 0.5% and 25% of sucrose. No invertase activity was detected and it was concluded that sucrose was a suitable material to form the density gradient for the isoelectric focusing.

Isoelectric focusing was performed in the standard L.K.B. 8102 apparatus at 5°, using a sucrose gradient which varied from 50% of sucrose at the bottom of the column surrounding the dense electrode solution, to 25% at the top. The procedure used for the preparation of electrode solutions and density gradients, and for the general operation of the isofocusing apparatus was as outlined in the L.K.B. 8100 Ampholine instruction manual (L.K.B. Produkter A.B., Bromma, Sweden). The total volume of the solution in the electrofocusing apparatus containing the sucrose gradient was ~400 ml, and the concentration of the Ampholine ampholyte carriers was 1%.

Isoelectric focusing of hemicellulase Ia (Fig. 2) was carried out by using ampholyte carriers of pH 3–10. The voltage applied was initially 150 volts for 3 h at 5° (3.5 watts), followed by 300 volts until the final power was constant, (64 h, 0.42 watt). The column was emptied by pumping water (2 ml/min) into the top of the column,

and fractions were collected from the bottom. These were assayed for hemicellulase activity, and their pH values measured. The results are shown in Fig. 3.

Fractions 158–185 (Fig. 3) were combined, dialysed against aqueous glycine (0.5%), and electrofocused using ampholyte carriers of pH 8–10. The initial voltage was 200 volts (0.92 watt) for 2 h at 5°, and was increased to 400 volts until the final power remained constant, (137 h, 0.24 watt). The results are shown in Fig. 4. Fractions 63–82 (hemicellulase I) were combined, dialysed against acetate buffer (0.01M, pH 5.5, 3 days), and stored at −20° or 5°.

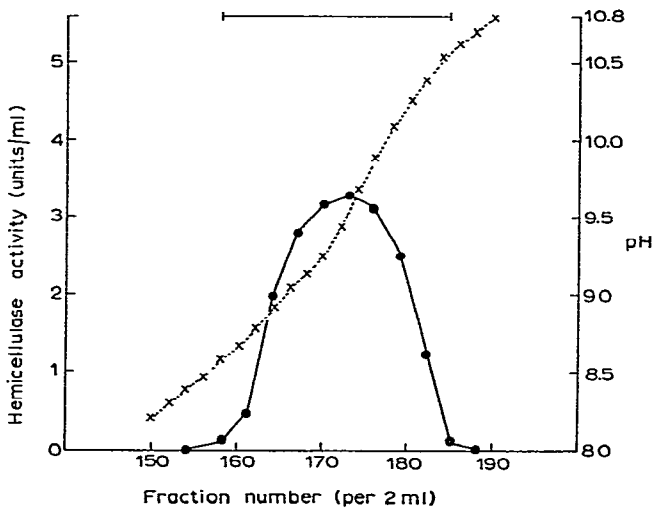

Fig. 3. Iso-electric focusing of hemicellulase I-a for 67 h at 300 volts, pH 3–10. (●—●), Hemicellulase activity; (× • • • • ×), pH profile.

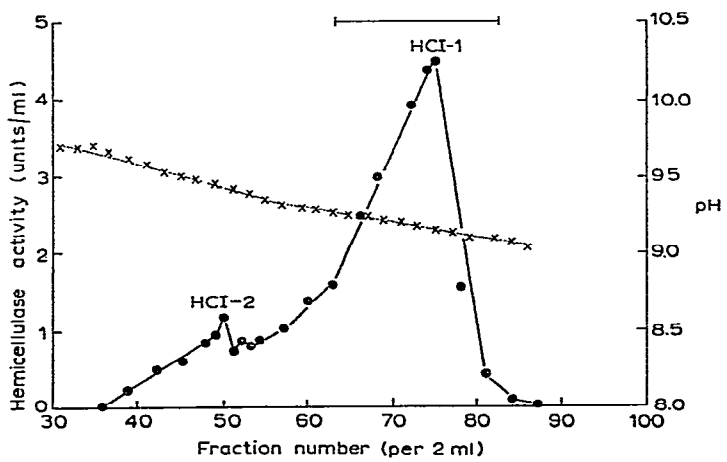

Fig. 4. Iso-electric focusing of hemicellulases for 139 h at 400 volts, pH 8–10. (●—●), Hemicellulase activity; (× • • • • ×), pH profile.

General properties of the purified enzyme preparation. — (*a*) *Effect of temperature on activity.* The enzyme solution in acetate buffer (pH 5.5, 0.01M) was relatively stable when stored at 5°. Freezing and thawing of such a solution caused 12% loss in activity per cycle.

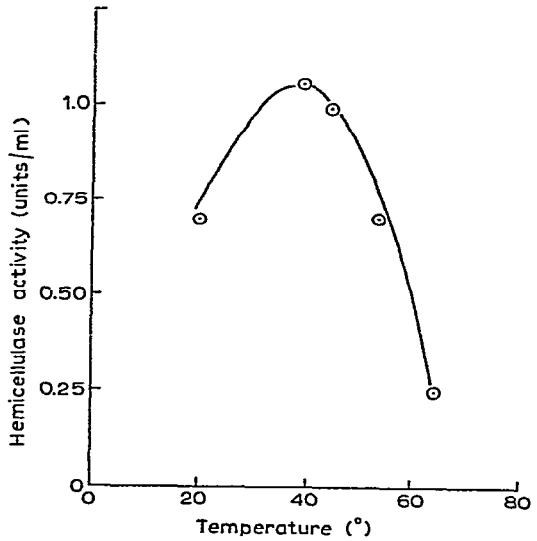

Fig. 5. Temperature optimum.

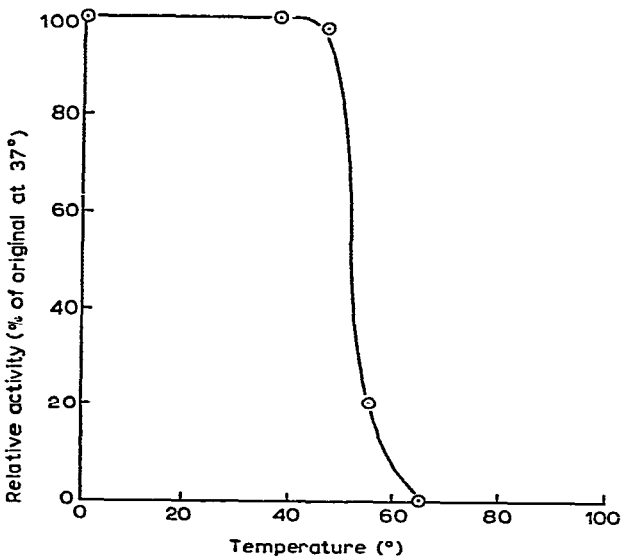

Fig. 6. Temperature stability.

The temperature optimum (Fig. 5) was obtained by incubating the enzyme solution (0.02 ml), acetate buffer (pH 5.5, 0.10M, 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 enzyme solution (0.02 ml) was incubated with acetate buffer for 1 h at various temperatures, then at 37° with added hemicellulose B solution (1%, 0.5 ml) for 1 h, followed by assay of reducing power. The results are shown in Fig. 6.

Incubation of the enzyme solution (0.02 ml) at 30° and 37° in acetate buffer (0.01 and 0.1M, pH 5.5) for >4 h, followed by assay for hemicellulase activity, showed (Table II) that 84 and 89%, respectively, of its activity was lost.

TABLE II

THE RELATIVE STABILITY OF HEMICELLULASE I AT 30 AND 37°

Temperature (degrees)	*Time (h)*	*Loss of activity*[a]	
		A	*B*
30	0	0	0
	3	76	69
	6	84	88
37	0	N.d.[b]	0
	1	N.d.	16
	4	N.d.	89

[a]A, 0.01M acetate buffer; B, 0.1M acetate buffer. [b]N.d., not determined.

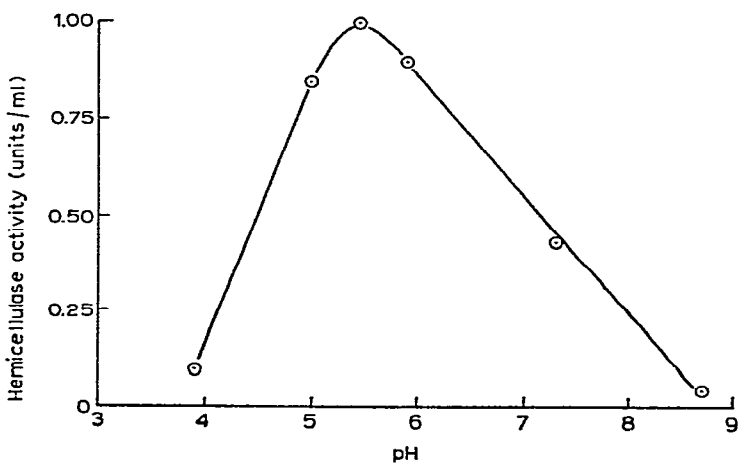

Fig. 7. pH optimum.

(*c*) *pH Optimum.* Enzyme solution (0.02 ml) and hemicellulose B solution (1%, 0.5 ml) were incubated in Universal buffer (citrate–phosphate–borate–barbiturate) solution (0.5 ml) of various pH values for 30 min at 37°, followed by assay of reducing power. The results are shown in Fig. 7.

(*d*) *Determination of* K_m *value.* Solutions containing acetate buffer (1.0 ml, 0.05M, pH 5.5), enzyme solution (0.02 ml), and various concentrations of hemicellulose B (0.6 to 40 mg/ml) were incubated at 37° for 20 min, followed by assay of reducing power. The production of reducing sugars was linear (Fig. 8*a*) during the 20-min incubation period. The line of best fit for the Lineweaver–Burk reciprocal plot was obtained by the least-squares method, and is given as $1/v = 2.186\,(1/S) + 0.459$ (correlation coefficient = 0.98), and the results for this plot are shown in Fig. 9*b*.

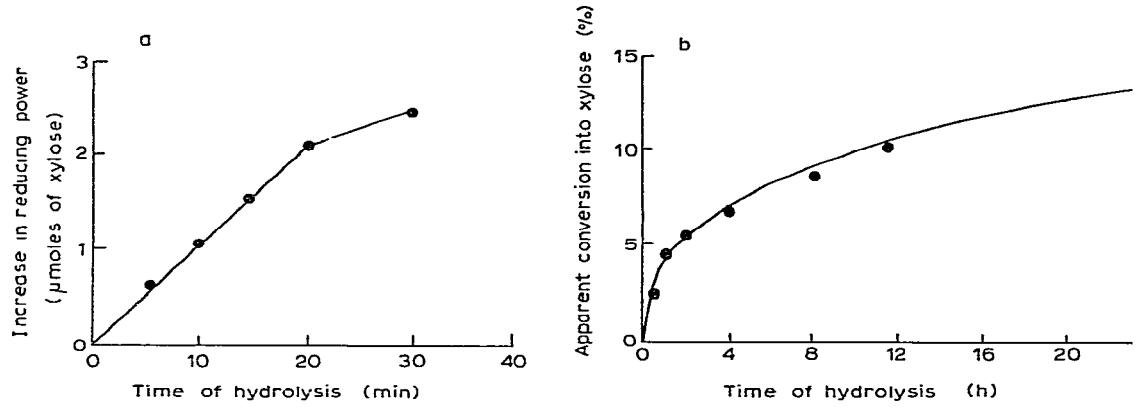

Fig. 8. Time course of hydrolysis of spear-grass hemicellulose B.

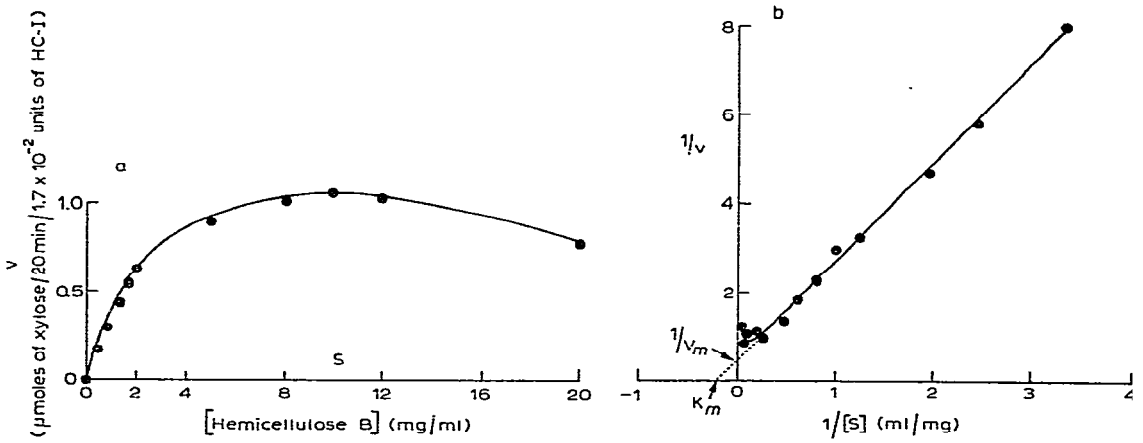

Fig. 9a. Michaelis–Menten plot for HC-I.

Fig. 9b. Lineweaver–Burk reciprocal plot for HC-I. $K_m = 4.24$ mg of hemicellulose B/ml (Spear grass); $V_m = 72.9$ μmoles of xylose/min/mg of protein.

Substrate studies on the purified hemicellulase 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 the xylose oligosaccharides), acetate buffer (0.5 ml, 0.1M, pH 5.5), and enzyme solution (0.02 ml) at 37° for the times indicated, followed by assay of reducing power. Figs. 8 and 10 show the time course of hydrolysis of several hemicelluloses. Fresh enzyme (0.02 ml) was added after 4, 8, and 11.5 h (Fig. 8), or 6, 26, 49, and 76 h (Fig. 10), since it was shown (Table II) that prolonged incubation of the enzyme caused considerable loss of activity. Tables III and IV show the degradation products arising from the enzymic hydrolysis of the xylose oligosaccharides and of several hemicelluloses from different sources, respectively. The products of hydrolysis were identified by paper chromato-

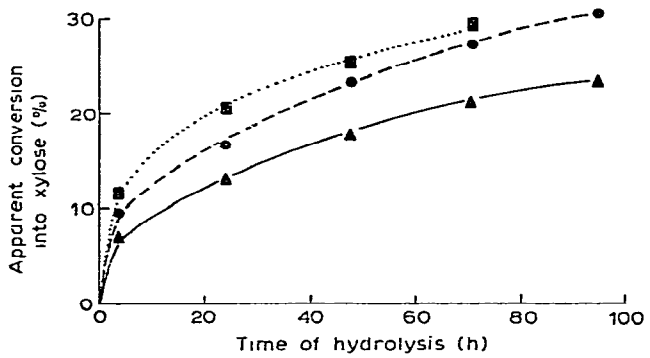

Fig. 10. Time course of hydrolysis of several hemicelluloses. (■.... ■), Hemicellulose B (bagasse); (●----●), arabinoxylan (wheat endosperm); (▲—▲), hemicellulose B (*S. sphacelata*).

TABLE III

PRODUCTS ARISING FROM THE DEGRADATION OF ARABINOSE–XYLOSE AND XYLOSE OLIGOSACCHARIDES BY HEMICELLULASE I

Oligosaccharide[a]	*Increase in reducing power* (%)[c]	*Degradation products*[b]						
		Xyl	*Ara*	Xyl_2	$AraXyl_3$	Xyl_3	Xyl_4	Xyl_5
$AraXyl_2$	N.d.	0	1	1	0	0	0	0
$AraXyl_3$	10.2	0	1	0	0	1	0	0
$AraXyl_4$	36.6	0	2	1	0	1	3	0
$AraXyl_5$	31.1	0	2	3	2	1	0	tr
Xyl_2	0	0	0	0	0	0	0	0
Xyl_3	0	0	0	0	0	0	0	0
Xyl_4	22.6	0	0	2	0	1	0	0
Xyl_5	72.6	0	0	2	0	3	0	0

[a]$AraXyl_n$, branched arabinose–xylose oligosaccharide; Xyl_n, linear xylose oligosaccharide. [b]The figures represent relative amounts of oligosaccharide present, as judged visually on paper chromatograms. [c]Hydrolysis at 37° after 4 h. N.d., not determined.

TABLE IV

DEGRADATION PRODUCTS ARISING FROM THE HYDROLYSIS OF HEMICELLULOSES FROM DIFFERENT SOURCES BY HEMICELLULASE I

Hemicellulose source	*Time of hydrolysis (h)*[a]	*Hydrolysis products*[b]				
		Xyl	*Ara*	*Xyl_2*	*$AraXyl_3$*	*Xyl_3*
Wheat-endosperm arabinoxylan	4	—	2	3	—	3
	24	trace	5	5	—	3
	49.5	2	8	10	—	6
Sugarcane-bagasse hemicellulose B	24.5	trace	3	4	2	4
Heteropogon contortus hemicellulose B	4	—	trace	2	—	4
	24	1	4	5	—	3
Setaria sphacelata hemicellulose B	4	—	2	3	—	6
	24	—	5	6	—	6
	49.5	1	7	10	—	8

[a]Fresh enzyme was added to the digest every 24 h. [b]Complex mixtures of higher oligosaccharides (>Xyl_3) were also observed in all digests.

graphy, and the relative amounts of each product were judged visually by the intensity of staining after spraying with alkaline silver nitrate[37].

The effect of Xyl_3 on the enzymic degradation of hemicellulose B. — Xyl_3 solution (0.105 ml containing 1.4 mg by weight), enzyme solution (0.02 ml), and acetate buffer (0.25 ml, 0.1M, pH 5.5) were left to equilibrate at 5° for 1 h to form the enzyme–substrate complex. Hemicellulose B solution (0.14 ml, containing 1.4 mg by weight) was then added, and the mixture was incubated for 24 h at 37°. Similarly, controls containing (*a*) enzyme and hemicellulose B, and (*b*) enzyme and Xyl_3 were also set up and conducted under identical conditions. Hydrolysis was terminated by heating to 100°/10 min, the hydrolysates were deionized as described above, and the products examined by paper chromatography. The results are shown in Table V.

TABLE V

THE EFFECT OF Xyl_3 ON THE ENZYMIC DEGRADATION OF HEMICELLULOSE B BY HEMICELLULASE I

Glycose product	*Relative amounts*	
	Without added Xyl_3	*Xyl_3 added*
Xylose	trace	trace
Arabinose	4	trace
Xyl_2	5	6
Xyl_3	3	10
Xyl_4Ara	1	4

The effect of L-*arabinose and* D-*xylose on the enzymic degradation of hemicellulose B.* — Sodium acetate solutions (0.1M, pH 5.5, 0.5 ml) containing various concentrations of L-arabinose and D-xylose (0–20mM) were incubated with enzyme solution (0.02 ml) and hemicellulose B solution (1%, 0.5 ml) for 24 h at 35°, and assayed for reducing power (Table VI).

TABLE VI

THE EFFECT OF D-XYLOSE AND L-ARABINOSE ON THE ACTIVITY OF HEMICELLULASE I

	Glycose concentration (mM)	*Relative activity* (%)
Xylose		
	0	100
	5	90.6
	10	73.1
Arabinose		
	0	100
	10	67.2
	20	52.6

RESULTS AND DISCUSSION

In Part I of this series[30], we determined, in small-scale experiments, the conditions necessary for optimal growth and production of hemicellulases by the culture isolate, CP_2. When this organism was cultured on a large scale, using Calbiochem "xylan" as inducer, hemicellulase activity was maximal after 48 h of growth (Table I). In six different culture-vessels after 48 h, the hemicellulase activity of 1-litre cultures varied between 0.627 and 0.761 unit/ml (mean 0.712).

Initial attempts to fractionate hemicellulases in the unbuffered culture fluid by precipitation with ammonium sulphate resulted in low recoveries of the enzymes. However, when the culture fluid was buffered (*e.g.*, 0.02M phosphate, pH 7.0), 95% of the original enzyme activity was recovered in the 50–90% ammonium sulphate fraction.

Chromatography of the ammonium sulphate precipitate on DEAE-Sephadex gave two well-separated hemicellulase peaks (fractions I and II, Fig. 1). Fraction I was eluted with the void volume, while fraction II was eluted by using a linear gradient (0–0.5M) of sodium chloride. The purification of the latter fraction will be the subject of a further paper. The purity of hemicellulase fraction I was investigated by polyacrylamide gel-electrophoresis at pH 8.4. Four protein bands were detected on the gel (Fig. 11) by staining with Coomassie Brilliant Blue, three of which possessed hemicellulase activity (Fig. 11).

Fig. 2 shows the elution profile for fraction I when chromatographed on a Cellex-CM column with a linear gradient of sodium chloride. Three poorly resolved

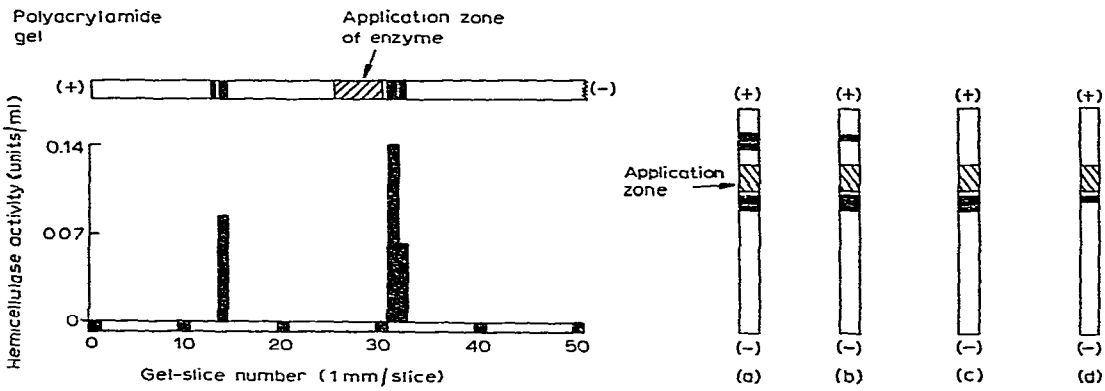

Fig. 11. The distribution of hemicellulase activities after electrophoresis of Fraction I in polyacrylamide gel.

Fig. 12. Diagramatic representations of electrophoretic patterns during the purification stages of hemicellulase I. (*a*) Fraction I from DEAE-Sephadex chromatography (See Figs. 1 and 11). (*b*) Fraction I-a from Cellex-CM chromatography (See Fig. 2). (*c*) Fraction I-a from iso-electric focusing at pH 3–10 (See Fig. 3). (*d*) Fraction I-1 (HC-I) from iso-electric focusing at pH 8–10 (See Fig. 4).

peaks, one major (fraction Ia) and two minor (Ib and Ic, respectively), resulted. Gel electrophoresis of fraction Ia at pH 6.0 and 8.4 showed the presence of three protein bands (Fig. 12).

Isoelectric focusing of fraction Ia for 67 h at 300 volts in a pH gradient of 3–10 resulted in a single, broad peak of hemicellulase activity (Fig. 3). Gel electrophoresis at pH 6.0 and 8.4 showed two protein bands in this peak, the third protein band of fraction Ia having disappeared (Fig. 12). When this hemicellulase fraction was electro-focused in a pH gradient of 8–10 for 139 h at 400 volts, two incompletely

TABLE VII

PURIFICATION OF HEMICELLULASE I FROM CELL-FREE CULTURE MEDIUM

Procedure	*Specific activity (units/mg of protein)*	*Recovery yield (%)*	*Purification*
Cell-free culture medium	0.202	100	1
Ammonium sulphate (50–90% saturation)	2.73	95	14
DEAE-Sephadex (A-50) Fraction (10–22), I	4.79	37	24
Cellex-CM I-a Fraction (19–27)	12.90	7.5	64
Iso-electric focusing pH 3–10, I-a, Fraction (158–185)	13.55	6.3	67
pH 8–10, I-1, Fraction (63–82)	34.60	2.7	171

resolved peaks resulted, HC I-1 (pI 9.17) and HC I-2 (pI 9.44) (Fig. 4). Fractions 63–82 were combined, and named hemicellulase I (HC-I). Gel electrophoresis at pH 6.0 and 8.4 showed a single protein band, but the area surrounding this band was also slightly stained (Fig. 12). This preparation was considered to be pure, and was used for physico-chemical and substrate studies. The above procedures represent a 171-fold purification, with a recovery yield of 2.7% of total hemicellulase activity (Table VII).

HC-I showed optimal activity at 40° and pH 5.5 (Figs. 5 and 7). The enzyme was stable when kept at 46° for 30 min (pH 5.5), but lost all of its activity after 30 min at 65° (Fig. 6). The enzyme lost >80% of its original activity when incubated at 30° and 37° for periods greater than 4 h (Table II). Therefore, in subsequent substrate studies, incubations were limited to 4 h where possible; for more prolonged incubations, fresh enzyme was added regularly (*e.g.*, Figs. 8 and 10). The activity of HC-I against other substrates is shown in Table VIII. The slight activity on pectin and arabinan resulted in the release of arabinose which was detected by paper chromatography. The rate of hydrolysis of hemicellulose B decreased in the higher range of substrate concentration (Fig. 9a), presumably because of substrate inhibition. The data from the plot of 1/v against 1/S (Fig. 9b) gives a value for K_m of 4.24 mg of hemicellulose B/ml for spear-grass hemicellulose.

TABLE VIII

HEMICELLULASE I ACTIVITY TOWARDS OTHER POLYSACCHARIDE SUBSTRATES

Polysaccharide substrate	*Source*	*Hemicellulase activity (units/ml)*
Hemicellulose B (*ex.* Spear grass)		0.61
Soluble starch	B.D.H. Ltd., England	0
CM cellulose, sodium salt (d.s. 0.7–0.8)	B.D.H.	0
Pectin (*ex.* apple)	B.D.H.	0.02
Arabinan (araban)	Koch-Light Labs., Ltd., England	0.02
Galactan (*ex.* Larch)	Koch–Light	0
Mannan (*ex.* Yeast)	Koch–Light	0
Arabinogalactan	Calbiochem, La Jolla, Calif., U.S.A.	0

The time course of hydrolysis for several hemicelluloses from different plant sources (Figs. 8 and 10) shows that, under similar conditions of hydrolysis, arabinoxylan and hemicellulose B (bagasse) were more extensively hydrolysed (~30% apparent conversion into xylose) than hemicellulose B from spear grass (*Heteropogon contortus*) or *Setaria sphacelata*. The three samples of hemicellulose B have each been shown to be predominantly arabino-4-*O*-methylglucuronoxylans (Refs. 41, 40, and 33, respectively), and they have quite similar, relative glycose compositions. The dif-

ferences in their degree of conversion may therefore indicate some relatively subtle differences in molecular architecture. Arabinoxylan has a highly branched structure (xylose–arabinose ratio, 1.77), but for every 25 xylose–arabinose residues there occurs an "open segment" of 5 unbranched xylose residues[42]. This polysaccharide has been shown by Perlin[42] to contain both α-(1→3)- and α-(1→2)-linked L-arabinose residues attached to the same D-xylose residue, as well as single α-(1→3)-linked L-arabinose residues on the xylan backbone. The enzymic degradation products of these polysaccharides are shown in Table IV, and demonstrate that HC-I is an endo-enzyme. Initial attack of the enzyme on arabinoxylan and the hemicellulose B samples liberated mixed oligosaccharides. Arabinose appeared after 2 h, while xylose and an "insoluble, degraded hemicellulose" appeared on prolonged incubation. The latter may have been the result of the removal of arabinose residues, producing a "xylan" of low arabinose content which precipitated from solution. This product will be the subject of further investigation.

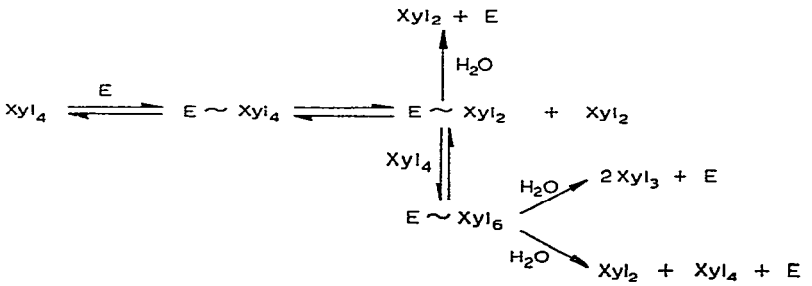

Fig. 13. Mechanism proposed for hydrolysis of Xyl_4 by hemicellulase I. E, enzyme; E~Xyl_4, enzyme–substrate complex.

Studies of the degradation of pure arabinose–xylose and xylose oligosaccharides of d.p. 3–6 and 2–5, respectively, are shown in Table III. Xyl_4 was the lowest xylose oligosaccharide attacked, and both it and Xyl_5 yielded Xyl_2 and Xyl_3. The mechanism proposed for the hydrolysis of Xyl_4 is represented in Fig. 13. It is envisaged that Xyl_4 and the enzyme first combined to form the enzyme–substrate complex ($E \sim Xyl_4$), which establishes a reversible equilibrium with $E \sim Xyl_2$ and Xyl_2. In the reverse of this reaction, it is then conceivable that Xyl_4 may replace Xyl_2 with resultant formation of the Xyl_6 complex, which would degrade rapidly to Xyl_2, Xyl_3, and Xyl_4. This type of action pattern has also been observed when a bacterial dextranase attacked isomaltotetraose[43]. HC-I degraded the arabinose–xylose oligosaccharides to arabinose and the parent xylose saccharides which (if greater than Xyl_3) were subsequently further hydrolysed. $AraXyl_5$ also yielded some $AraXyl_3$ which was subject to further degradation to arabinose and Xyl_3. This was rather surprising, since the rate and product studies with xylose saccharides suggest that the binding site of HC-I "fits" a chain of five D-xylose residues, and the active site is evidently situated within the binding subsites. The relatively rapid attack on $AraXyl_5$

to produce $AraXyl_3$ and Xyl_2 suggests, therefore, that the presence of the arabinose substituent may not interfere with the "fit" of the (1→4)-β-D-xylose chain into the binding site. We have evidence, to be detailed in a subsequent paper, that the arabinose substituent in $AraXyl_5$ is probably present on the xylose residue at the non-reducing end, but the above argument is valid wherever the arabinose is placed on the xylose chain.

The effect of pre-incubation of enzyme and Xyl_3 on the enzymic hydrolysis of hemicellulose B is shown in Table V. The results indicate that arabinosidase activity of HC-I is suppressed, whereas hydrolysis of hemicellulose B continued, and this suggests that the two types of hydrolysis are probably catalysed at different catalytic sites. However, more-detailed kinetic studies would have to be performed to confirm this suggestion. The effect of added arabinose and xylose on the enzymic hydrolysis of hemicellulose B is shown in Table VI. The results show that enzyme activity is suppressed at high concentrations of both L-arabinose and D-xylose. It is probable, therefore, that product inhibition is an important factor in limiting the action of HC-I on hemicelluloses. Paper-chromatographic examination of the oligosaccharide products of hemicellulose degradation in the presence of added D-xylose or L-arabinose showed no significant differences with control digests. It is possible that the addition of L-arabinose might specifically have suppressed the arabinosidase activity, but this effect could not be detected in chromatography of the products because of the added L-arabinose.

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