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Note

Observations on the degradation of hemicellulose B by hemicellulases produced from *Cephalosporium sacchari*, and inhibitory effect of sulphydryl reagents

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The activity of glycanhydrolases may be increased, decreased, or not affected by the presence of inorganic, organic, or organometallic reagents[1,2]. Iwasaki, Ikeda, Hayashi, and Funatsu[2] observed an increase in activity of cellulase I in the presence of cobalt ions, but a decrease in activity in the case of cellulase II. A cellulase preparation by Ikeda, Yamamoto, and Funatsu[1] lost 54.5% of its activity in the presence of *p*-chloromercuribenzoic acid (PCMB), whereas cellulases I and II retained all of their activities[2] in the presence of the same concentration of PCMB[2]. The loss of activity in the presence of sulphydryl reagents could be due to blocking of the sulphydryl group(s) involved in the active site, or to denaturation caused by the added reagent(s). Mercury(II) ions have been observed to cause a decrease in activities of cellulases I and II, but the same cellulases showed no loss of activity in the presence of PCMB, a specific sulphydryl reagent[2].

The effect of mercury(II) ions on the cellulases could be attributed to nonspecific binding of mercury(II) ions to the protein group(s), rather than reaction with sulphydryl group(s) at the active site. Loss of activity due to reaction of sulphydryl group(s) with sulphydryl reagents could be restored by addition of excess cysteine, which results into a reversible reaction, thereby releasing protein sulphydryl group(s). In this work, PCMB, a specific sulphydryl reagent, was used to detect the involvement of sulphydryl group(s) at the active site.

The distribution of L-arabinose on the hemicellulose main chain is not uniform[3,4]. The degradation of hemicellulose β-(1→4) main chain by hemicellulases requires at least two unbranched β-D-xylosyl residues in sequence[3]. Dekker and Richards[5] observed that hemicellulase II, obtained from *Ceratocystis paradoxa*, degraded hemicellulose B preferentially in the regions containing more L-arabinose. The regions depleted in L-arabinose precipitated, after those rich in L-arabinose had been degraded. Cellobiose was isolated from the hydrolysates. The cellobiose

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might have resulted from the degradation of a glycan associated[6] with linear hemicellulose B. In the present studies with the enzymes HC III and HC IV, insoluble, degraded hemicellulose B was obtained but no cellobiose was detected. This paper reports the compositional analysis of the insoluble, degraded hemicellulose B obtained from spear grass *(Heteropxyon contortus)* by degradation with HC III and HC IV; and the effect of inorganic, organic, and organometallic compounds on the activity of HC III and HC IV.

EXPERIMENTAL

Materials. — Authentic samples of L-arabinosyl-D-xylose and D-xylose oligosaccharides[7] of degree of polymerisation (d.p.) 3 and 2–5, respectively, were provided by Drs. Dekker and Richards, and authentic L-arabinosyl-D-xylose oligosaccharides[8] of d.p. 4–5 by Drs. Beveridge and Richards[8]. HC III and HC IV were purified as described[9]. Commercial, silica gel t.l.c. plates (0.25 mm thickness) were obtained from E. Merck (Darmstadt, Germany). All chemicals used in this work were analytical grade (A.R.) or were purified before use.

Chromatographies. — Samples to be chromatographed were de-ionised with mixed Amberlite IR-120 (H^+) and IR-45 (OH^-) resins for 30 min, and the solutions evaporated. Qualitative paper chromatography (p.c.) was carried out on Whatman No 1 paper with (*A*) 10:4:3 ethyl acetate–pyridine–water, (*B*) 3:1:1 butan-1-ol–ethanol–water, and (*C*) 12:8:5 butan-1-ol–pyridine–water for mono- and oligo-saccharides; and (*D*) 4:1:5 butan-1-ol–ethanol–water (upper layer) and (*E*) 41:9 butan-2-one–water (all v/v) for methylated sugars. T.l.c. plates were activated for 30 min at 100° and allowed to cool before samples were applied. The solvent systems employed were: (*E*) as in p.c. for methylated sugars; and (*F*) 8:3:1 ethyl acetate–pyridine–water for mono- and oligo-saccharides. On duplicate paper chromatograms, spots were detected by (*a*) the alkaline silver nitrate[10], and (*b*) the *p*-anisidine hydrochloride[11] spray reagents. Mono- and oligo-saccharides were detected, on t.l.c. plates, by spraying with 50% sulphuric acid or saturated ammonium sulphate, followed by heating for 20 min at 100 and 140°, respectively, and methylated sugars with spray (*b*). Migrations are given relative to those of D-xylose and 2,3,4,6-tetra-*O*-methyl-D-glucose (Tmg).

General methods. — Delignification was obtained by the acidified sodium chloride procedure[12], and the hemicellulose extracted, from delignified speargrass, with 10% sodium hydroxide. The oligosaccharide solutions were evaporated at 40°/3 kPa in a rotary evaporator. The concentration of L-arabinosyl-D-xylose and D-xylose oligosaccharides was determined by the phenol–sulphuric acid method[13] with L-arabinose and D-xylose as standards, and that of reducing sugars by the Nelson method[14]. Branched and linear hemicellulose B were prepared by the method of Gaillard[15], in 83.3 and 16.7% yield, respectively.

The compositions of insoluble, degraded products and whole hemicellulose B were determined by total hydrolysis with 72% sulphuric acid[16], followed by quan-

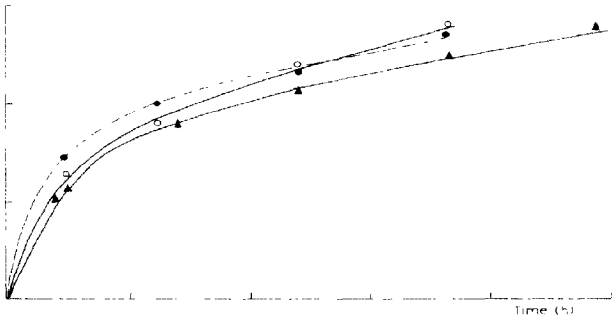

Fig. 1. Comparative rate of attack of HC III on branched, linear, and whole hemicellulose B (○——○) linear hemicellulose B, (●——●) whole hemicellulose B, (▲——▲) branched hemicellulose B

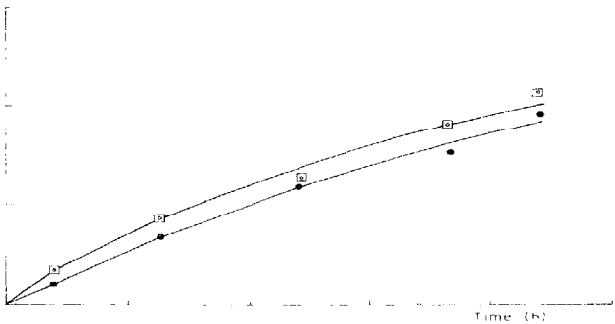

Fig. 2. Comparative rate of attack of HC IV on branched, linear, and whole hemicellulose B (□——□) linear hemicellulose B, (○——○) whole hemicellulose B, and (●——●) branched hemicellulose B. Whole hemicellulose B and linear hemicellulose B showed no difference in rate of hydrolysis with HC IV

titative determination by g.l.c. of the derived alditol acetates (with *myo*-inositol as internal standard) by g.l.c. in an Aerograph 705 gas-liquid chromatograph equipped with a coiled-glass column (146 × 0.5 cm), packed with 5% Lac-4R-886, at temperatures 180, 230, 240° for the column, injector, and detector, respectively; the flow rate of nitrogen carrier gas was 45, and that of hydrogen 25 mL/min. D-Glucuronic acid was essentially determined by the method of Galambos[17] for quantitative estimation of uronic acids.

Comparison of degradations, by HC III and HC IV, of linear, branched, and

whole hemicellulose B. — Solutions (0.5%) of linear, branched, and whole hemicellulose B in 50mM sodium acetate buffer, pH 6.0, were centrifuged at 16 700*g*. To an aliquot (4.0 mL) of these solutions were added toluene (0.02 mL), and either HC III (0.160 mL) or HC IV (2.0 mL). The mixture was incubated at 37°, and samples were withdrawn at various time-intervals for reducing-power assay. After 73 h, the hydrolysates were de-ionised and p.c. performed in solvent *A*. The insoluble, degraded hemicellulose formed was washed (3 × 20 mL) with warm water (60°), and then freeze dried. The rate of hydrolysis is shown in Figs. 1 and 2 for HC III and HC IV, respectively.

Comparison of degradation, by HC IV, of insoluble, degraded hemicellulose B (IDHI) and whole hemicellulose B. — IDHI was dissolved in a minimum amount of M sodium hydroxide, the solution made neutral with M acetic acid, and the concentration of IDHI diluted to 0.5% with 50mM acetate buffer, pH 6.0. The relative rates of attack of HC IV on IDHI and hemicellulose B were compared (see Fig. 3).

Effect of various additives on HC III and HC IV. — Solutions of various inorganic salts (0.04 mL) and organic compounds (10mM) were added to an 0.5% solution of hemicellulose B (2.0 mL) in 50mM acetate buffer, pH 6.0, and a solution of either HC III (0.04 mL) or HC IV (1.0 mL). The final volume was completed to 4.0 mL with acetate buffer, and the mixture incubated at 37°. Samples were withdrawn at 5 min and 1 h for HC III, and at 1 h for HC IV, and the reducing sugar was determined. The 1-h hydrolysates were de-ionised and examined by p.c. in solvent *A*.

Effect of cysteine. — Solutions of *p*-chloromercuribenzoic acid (PCMB, 0.04 mL) and 0.5% hemicellulose B in 50mM acetate buffer (2.0 mL), various volumes of 40mM cysteine, and a solution of either HC III (40 μL) or HC IV (1.0 mL) were combined, the volume was completed to 4.0 mL with acetate buffer, and the mixtures were incubated for 1 h at 37°. Samples were withdrawn and the reducing sugar value was determined.

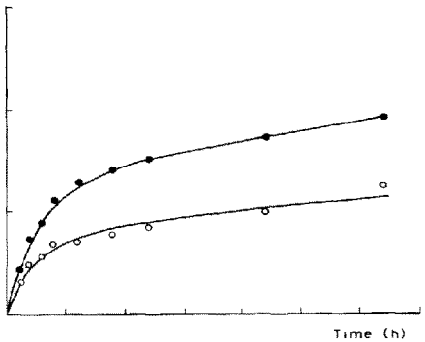

Fig. 3. Comparative rate of attack of HC IV on IDHI and hemicellulose B: (○——○) hemicellulose B and (●——●) IDI (insoluble, degraded hemicellulose B).

TABLE I

SUGAR COMPOSITION OF IDHI, IDHII, AND HEMICELLULOSE[a]

Polysaccharide	*Sugar component (%)*				
	Ara	*Gal*	*Glc*	*Uronic acid*	*Xyl*
IDHI	7.0	0.0	[c]	2.9	90.1
IDHII	7.9	2.1	6.0		84.0
IDHI[18]	9.4	0.0	0.3	3.6	87.2
Hemicellulose	15.9	[b]	5.8	5.1	73.2
Hemicellulose[18]	14.0	2.7	5.0	4.9	73.4

[a]By g.l.c. of alditol acetate derivatives. [b]Trace. [c]Not determined.

Effect of L-*arabinose and* D-*xylose.* — Solutions of 20mM L-arabinose or D-xylose (20 μL) and 0.5% hemicellulose B (1.0 mL), toluene (0.04 mL), and a solution of either HC III (20 μL) or HC IV (1.0 mL) were combined, the final volume was completed to 4.0 mL, and the mixtures were incubated for 24 h at 37°. Samples were withdrawn and the reducing-sugar value was determined.

RESULTS AND DISCUSSION

Effects of composition on enzymic hydrolysis. — The relative rate of attack of HC III and HC IV on branched hemicellulose was slightly less than that on linear or whole hemicellulose B (Figs. 1 and 2). This is not in agreement with the finding that, in rumen digestion, both branched and linear hemicellulose are attacked at the same rate[8]. HC III and HC IV degraded branched, linear, and whole hemicellulose B into IDHI and IDHII, respectively. Formation of IDHI from linear and whole hemicellulose B was observed after 3 h, whereas from branched hemicellulose, only after 8 h. IDHII formation from branched, linear, and whole hemicellulose B was observed after 24 h. From branched, linear, and whole hemicellulose B, IDHI was obtained in 0.13, 10.3, and 10.6% yield, respectively; and IDHII in 0.13, 8.2, and 8.12% yield, respectively, indicating that the highly branched molecules are hydrolysed at a lower rate than the more linear molecules. HC III attacked both hemicellulose and IDHI, which gave similar precipitates after 3-h incubation at 37°. The difference in relative rate of attack is probably not associated with change in substrate concentration during the digestion but rather with structural composition. The analysis of IDHI and IDHII showed that they were depleted in L-arabinose (Table I), suggesting that the regions depleted in L-arabinose are the least hydrolysed. As HC IV does not hydrolyse the α-L-(1→3)-arabinofuranosyl linkage, the results obtained with HC III and HC IV, which degrade linear faster than branched hemicellulose, may be explained only on the basis of the secondary and tertiary structure of hemicellulose. The preferential attack, by

TABLE II

QUALITATIVE ANALYSIS OF HYDROLYSATES OF BRANCHED, LINEAR, WHOLE HEMICELLULOSE B, IDHI, AND IDHII DEGRADED BY HC III AND HC IV[a]

Hemicellulose	*HC Enzyme*	*Components*							
		Ara	*Xyl*	*$AraXyl_2$ or Glc*	*Xyl_2*	*$AraXyl_3$*	*Xyl_3*	*$AraXyl_4$*	*Xyl_4*
Whole	III	7	7	2	10	9	8	8	4
Branched	III	7	7	2	10	9	8	8	4
Linear	III	7	7	2	10	9	8	8	4
Whole	IV		[b]		10	6	8	6	6
Branched	IV		[b]		10	6	8	6	6
Linear	IV		[b]		10	6	8	6	6
IDHI	III	7	7	2	10	9	8	8	4
IDHI	III		[b]		10	6	8	6	6

[a]By p.c. in solvent *A*. The values were scored visually with 10 as maximum. [b]Trace.

TABLE III

EFFECT OF VARIOUS COMPOUNDS[a] ON THE ACTIVITY OF HC III[b]

Compound added	*Incubation time*	
	5 min	*1 h*
$FeCl_3$	117.8	117.8
$MnCl_2$	102.0	102.0
$MgCl_2$	81.6	81.6
$CaCl_2$	90.6	90.6
$HgCl_2$	6.8	6.8
$CuCl_2$	74.8	74.8
p-Chloromercuribenzoic acid	34.0	34.0
Sodium lauryl sulphate	27.2	9.0
N-Bromosuccinimide	00.0	00.0
EDTA (disodium salt)	83.8	83.8
Control	100.0	100.0

[a]As a concentration of 5mM. [b]Percent of activity.

HC III and HC IV, of substituted or branched regions may be due to these regions being in more random polymer configuration than the less branched regions. The slight differences in relative rate of attack, of HC III and HC IV, on branched and linear hemicellulose is probably due to linear hemicellulose having more regions that are accessible to the enzymes than does branched hemicellulose. It is possible that, in the highly branched fractions of the hemicellulose, more and longer side-chains may prevent the approach of the enzymes by simple physical "crowding" or

TABLE IV

EFFECT OF VARIOUS COMPOUNDS[a] ON THE ACTIVITY OF HC IV[b]

Compound added	*Activity (%)*
$FeCl_3$	70.6
$MnCl_2$	70.6
$MgCl_2$	72.4
$MaCl_2$	78.2
$HgCl_2$	73.5
$CuCl_2$	79.4
EDTA (disodium salt)	73.5
Sodium lauryl sulphate	73.5
P-Chloromercuribenzoic acid	50.0

[a]As a concentration of 0.38mM. [b]Incubation for 1 h. [c]Values are the same in the presence of an 8-fold excess of cysteine

steric hindrance. The observation that insoluble, degraded hemicellulose is depleted in L-arabinose and that much less insoluble, degraded hemicellulose was precipitated from the digestion of branched than from that of linear hemicellulose B confirms earlier observations[5] that the branched regions are a solubilising factor. The presence of D-glucose and D-galactose as significant constituents of IDHII, in contrast to IDHI in which they had been removed, suggests that these two sugars are integral components of the original hemicellulose molecule rather than of the mixed β-(1→3)- and D-(1→4)-linked D-glucan and D-galactan impurities that have previously been reported[6]. The observation that cellobiose was not present in the hydrolysates, as compared with other enzymes hydrolysates[7,8], further shows that the glycan impurities were not hydrolysed and could not, therefore, coprecipitate with the insoluble, degraded products. Paper chromatography of the hydrolysates of the degradation products (IDHII or IDHIII) obtained with HC III or HC IV from branched, linear, and whole hemicellulose B gave patterns similar to those obtained for the original hemicelluloses (Table II).

Inhibition of hemicellulases. — As shown in Tables III and IV, sulphydryl reagents inhibit the activity of HC III. Addition of an eight-fold molar excess of cysteine to a specific sulphydryl reagent (PCMB) resulted in complete recovery of the "lost" activity, suggesting that sulphydryl groups are directly involved in the active site. HC III did not appear to be activated by metallic ions. The loss of activity observed with some compounds may be due to denaturation. The addition of D-xylose and L-arabinose did not affect the activity of HC III.

Sulphydryl reagents did not inhibit the activity of HC IV more than did the other reagents, and addition of cysteine to PCMB did not restore the activity. Thus, unlike the activity of HC III, that of HC IV does not depend on sulphydryl groups. None of the additives enhanced the activity of HC IV, and thus, the loss of activity of HC IV due to the presence of additives is probably due to denaturation. The addition of L-arabinose and D-xylose reduced the activity of HC IV to a level of 61.8

or 70.6%, respectively. A similar effect was earlier reported for HC I produced by *Ceratocystis paradoxa*[19], and attributed to product inhibition.

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