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PRODUCTION AND PURIFICATION OF TWO HEMICELLULASES FROM *Cephalosporium sacchari**†

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ABSTRACT

The production of extracellular hemicellulases by the fungus *Cephalosporium sacchari* was studied in the presence of various sources of carbon and at various initial pH values and temperatures. Hemicellulose B and holocellulose from spear grass (*Heteropogon contortus*) were the best sources of carbon, and the optimum temperature was 27°. The initial pH value had little influence on the final yield of hemicellulases. Two hemicellulases (HC-III and HC-IV) were purified by ammonium sulphate precipitation and isoelectric focusing. Their molecular weights were 10,700 and 9,550, and their pI values 9.40 and 6.0, respectively. HC-III hydrolysed hemicellulose B to oligosaccharides without production of monosaccharides.

INTRODUCTION

Hemicellulases are commonly secreted extracellularly by bacteria and fungi, and these and other sources have recently been reviewed[1]. The secretion of such enzymes is usually affected by the source of carbon[2–6] available to the microorganism. Thus, Eriksson and Rzedowski[4] observed that the fungus *Chrysosporium lignorum* produced good yields of cellulase, hemicellulase, mannanase, β-D-glucosidase, and aryl β-D-glucosidase when grown on cellulose as a source of carbon, but only traces or none of the above enzymes when xylan or mannan was used. Similarly, Eriksson and Goodell[5] observed that another fungus (*Polyporus adustus*) produced more cellulase, mannanase, and hemicellulase when cellulose was added to the cultures. Wilson[6] screened several fungi for biochemical and morphological responses to cellobiose, D-glucose, and maltose, and found that the enzymes secreted were determined by the source of carbon. Secretion of extracellular cellulases, hemicellu-

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lases, β-D-xylosidases, and β-D-glucosidases by 10 strains of wood-destroying fungi, with various sources of carbon, revealed that best yields were obtained with willow sawdust as the source of carbon[2].

Earlier observations in this laboratory revealed that the crude, extracellular enzymes produced in relatively low yield by *Cephalosporium sacchari* hydrolyse hemicellulose B mainly to xylose, arabinose, and xylobiose, the ultimate products of hydrolysis being xylose[3]. It seemed likely, therefore, that this enzyme system might contain exo-hemicellulases and/or endo-hemicellulases of types not yet purified in this laboratory. This paper reports efforts to improve the yield of extracellular hemicellulases and their purification. The properties of the enzymes will be detailed in a subsequent paper.

EXPERIMENTAL

Materials. — Cultures of the fungal plant-pathogen *Cephalosporium sacchari* were supplied by Dr. R. F. H. Dekker of this laboratory, the original cultures having been provided by Dr. C. G. Hughes of the Bureau of Sugar Experiment Stations, Brisbane. The cultures were considered to be pure and were maintained on potato-dextrose agar slants. Carrier ampholytes for isoelectric focusing were obtained from LKB Producter AB, Sweden. Horse Cytochrome C, bovine pancreas chymotrypsinogen A, hen albumin, and bovine serum albumin were obtained from Calbiochem, U.S.A., and used as standard proteins for calibration of Bio-gel P-100 columns. Hemicellulose B, an arabinoxylo-4-*O*-methylglucuronoxylan[7] was extracted from delignified spear-grass[8] (*Heteropogon contortus*) with 10% sodium hydroxide[9]. Other polysaccharides used as source of carbon in the culture medium were D-xylose, carboxymethylcellulose (CMC) obtained from BDH Ltd., England, spear-grass holocellulose, and sugar-cane bagasse; the last two materials were coarsely milled.

General methods. — The culture media employed for the growth of the fungal organisms and for the production of hemicellulase(s) were those of Hultin and Nordstrom[10] using 1% (w/v) of polysaccharides for the agar-slant media, and various polysaccharide concentrations in the liquid-culture solutions. Small amounts of fungus were transferred from the potato-dextrose agar slant to agar-plate medium containing CMC, and incubated at 32° under illumination. The resultant isolates were designated CS_1. The isolates obtained by subculturing CS_1 in media containing xylan or hemicellulose B were designated CS_2. The latter were grown in submerged liquid culture in media containing various polysaccharides at various initial pH values and various temperatures, in both stationary and shaking conditions (80–95 r.p.m.), to optimise the production of hemicellulase(s). Small-scale culturing was carried out in 250-ml conical flasks containing 50 ml of the culture solution, and large-scale culturing was carried out in 3-l conical flasks containing 600 ml or 1 litre of culture solution. Culture fluids (5.0 ml) were withdrawn at various intervals and centrifuged at 16,700 *g* for 0.5 h at 5°, to remove mycelia and spores, before being used in the enzyme assays.

The cultures used for purification of enzymes were grown on 2% of holocellulose and, after centrifuging, were stored in the frozen state (a sample showed no loss of hemicellulase activity on freezing and thawing for five cycles).

Paper chromatography (p.c.) for analysis of hemicellulose degradation products was carried out on deionised solutions as described in Part IV.

Assay for hemicellulase activity. — The enzymic digests contained 1.0 ml of 0.5% hemicellulose B in 0.05M sodium acetate buffer (pH 6.0), and 0.02–0.5 ml of enzyme solution (*e.g.*, centrifuged culture fluid). The solutions were incubated at 37° (0.5–4 h), and samples were withdrawn, added to alkaline Nelson-reagent C, and assayed for reducing sugars by the Nelson method[11]. The time periods chosen for the digestion were shown to lie on the linear portion of the graph of reducing power versus time for each type of enzyme solution. In some cases of low activity, where incubation periods of more than 10 h were used, a few drops of toluene were added.

Definition of hemicellulase unit. — One unit of hemicellulase activity is defined as that amount of enzyme which causes the liberation of reducing groups corresponding to 1 μmol of D-xylose per min under the digest condition specified above. Specific activity is then expressed as the units of activity per mg of protein.

Ammonium sulphate precipitation. — Approximately equal amounts of hemicellulase activity were precipitated at each of five equal steps of increasing ammonium sulphate saturation from 20→100%. The recovery of total activity from the precipitates was 67% after the fractions had been dialysed for 48 h against 0.01M sodium acetate buffer (pH 5.5). The 20% ammonium sulphate fraction contained the greatest amount of non-hemicellulase material and was discarded. All subsequent operations were carried out on the precipitate at 40→100% saturation.

Isoelectric focusing. — The activity of the crude enzyme solution (0.2 ml) towards 60% sucrose (1 ml), or towards the hemicellulose B solution (1.0 ml) in the presence of 70% glycerol or ethylene glycol (w/v based on enzyme solution), was assessed by incubating at 37° for 0.5 h followed by determination of reducing power with Nelson's reagent. The sucrose solutions developed reducing power corresponding to 0.05 μmol of glucose.ml^{-1}.min^{-1}, whereas glycerol completely inhibited hemicellulase activity. Ethylene glycol, however, showed no interference with hemicellulase activity and was thus chosen as a density-gradient solute for isoelectric focusing. The precipitate obtained at 40→100% ammonium sulphate saturation (100 mg) was dialysed against 0.01M sodium acetate buffer (pH 5.5, 48 h) followed by dialysis against 1% glycine solution (48 h), and then isoelectric focusing in an LKB 8102 instrument (1% ampholytes, 440 ml, pH 3.5–10 at 5°). The applied voltage was initially 200 volts for 2 h (0.8 watt), followed by 380 volts until the final current and voltage were stable (0.3 watt after 24 h), and then continued at 380 volts for a further 24 h. Fractions (2.5 ml) collected from the column were assayed for hemicellulase activity and absorbance at 280 nm, and their pH values measured (Fig. 1). Fractions 115–129 (HC-III, pI 9.4) and fractions 21–32 (HC-IV, pI 6.0) were separately combined, and polyacrylamide-gel electrophoresis in borate buffer was carried out at pH 8.1 (4 h) and pH 9.1 (2 h) by the method of Richards and Streamer[12]. HC-III

migrated towards the cathode, showing a single protein band at both pH values (2.5 cm, pH 8.1; 0.5 cm, pH 9.1). HC-IV migrated towards the anode, showing two bands at pH 9.1 (1.0 and 1.5 cm). Isoelectric focusing was therefore repeated over a narrower range (pH 5–7) with 100 mg of the same ammonium sulphate precipitate. The applied voltage was initially 200 volts at 4 mamp for 4 h; it was then increased to 400 volts and maintained at this value until the current and voltage were constant (400 volts, 1.3 mamp, 60 h). The focusing was continued for a further 24 h. Five enzymes, designated I_1–I_4 and HC-IV, were resolved (Fig. 2). The purity of HC-IV was again tested by polyacrylamide-gel electrophoresis at pH 9.1, and two bands migrating towards the anode were again obtained. The combined fractions 108–122 (Fig. 2) were next passed through a column (100 × 2.5 cm) of Sephadex G-200 and eluted with 0.03M sodium acetate buffer (pH 5.5, Fig. 3). The enzyme solution from the column (V_E 580–740 ml) was combined, an aliquot (10 ml) was concentrated to ~1.0 ml by adding dry Sephadex G-15 (82 μg of protein/ml), and 0.3 ml was used for polyacrylamide-gel electrophoresis[12] at the two pH values (4 h, pH 8.1; 2 h, pH 9.1). A single band migrating towards the anode was observed in both cases (3.5 cm, pH 8.1; 1.0 cm, pH 9.1). Both HC-III and HC-IV were then separately passed through a column (100 × 2.5 cm) of Bio-gel P-100, as HC-IV had been shown to interact with Sephadex G-200. Both enzymes showed chromatographic homogeneity (Fig. 4). The column was separately calibrated with the standard proteins for determination of molecular weight.

RESULTS AND DISCUSSION

The growth response of *Cephalosporium sacchari* on agar slants was similar to that previously described[3]. Table I shows the effect of temperature on enzyme production in liquid culture with shaking at 80–95 r.p.m. (without shaking, growth was observed but no hemicellulase activity developed). The initial pH of the culture had little effect on the maximum yield of hemicellulase(s). Final activities (100–150 h) of 0.22–0.25 unit per ml were obtained for initial, buffered pH values of 5.6 and 6.6, whereas activities of 0.26–0.27 were obtained for initial pH values of 7.4 and 8.0, with either 1% of hemicellulose B or 4% of holocellulose and phosphate buffers. In the absence of added buffer, the culture pH (initially 5.6) stabilised during growth at ~7.4 after 36 h and gave a final activity of 0.26–0.27 unit. Adjustment of the initial pH to 7.4 with sodium hydroxide did not improve the enzyme yield.

Tables II and III show the dependence of hemicellulase production on the source of carbon and its concentration. The best results were obtained with 1% of hemicellulose B or 4% of spear-grass holocellulose, whereas no hemicellulase activity was observed with D-xylose or CMC as source of carbon. The decrease in activity at high concentrations of bagasse or holocellulose is probably due to the extensive absorption of the culture fluid by these fibrous solids which appeared to delay the growth of the fungus. The volume effect on hemicellulose production (0.28 and 0.09 unit.ml^{-1} for 600 ml and 1 litre of culture fluid, respectively, in 3-l

TABLE I

EFFECT OF TEMPERATURE ON PRODUCTION OF EXTRACELLULAR XYLANASES

Temperature (degrees)	22	27	32	37	42
Hemicellulase activity[a]	0.14	0.17	0.11	0.07	0.0

[a]After growth for 98 h.

TABLE II

EFFECT OF SOURCE OF CARBON ON XYLANASE PRODUCTION

Hemicellulose B (%)	*Other sources* (%)	*Hemicellulase activity*[a] (*unit.ml*$^{-1}$)
0.1	—	0.08
0.5	—	0.17
0.2	CMC (0.8)	0.08
0.2	Xylan (0.8)	0.11
0.2	Xylan (0.8)	0.10
0.2	Holocellulose (2.0)	0.17
—	D-Xylose (2.0)	0.01
—	Holocellulose (2.0)	0.19

[a]After growth for 153 h.

TABLE III

EFFECT OF SUBSTRATE CONCENTRATION ON XYLANASE PRODUCTION

Substrate (%)	*Hemicellulase activity*[a] (*unit.ml*$^{-1}$)
Holocellulose (2.0)	0.19
Holocellulose (3.0)	0.19
Holocellulose (4.0)	0.23
Holocellulose (5.0)	0.21
Sugar-cane bagasse (2.0)	0.17
Sugar-cane bagasse (3.0)	0.16
Sugar cane bagasse (5.0)	0.14
CMC (1.0)	0.00
CMC (1.0)	0.00
Hemicellulose B (1.0)	0.23

[a]After growth for 158 h.

conical flasks) strongly suggests that the hemicellulase yields, and probably the growth of the fungus, are limited by oxygen supply. It is possible that better yields of enzyme would be obtained under forced aeration in a fermentation vessel. The products from hydrolysis of hemicellulose B by the culture fluid were similar to those described earlier[3].

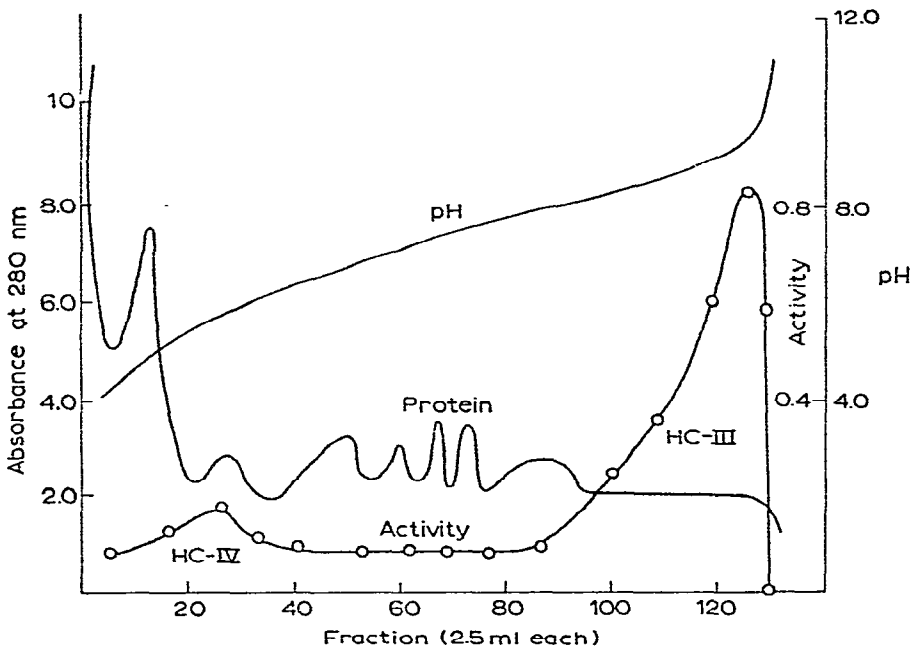

Fig. 1. Isoelectric focusing at pH 3.5–10.

Isoelectric focusing at pH 3.5–10 (Fig. 1) showed major and minor hemicellulase peaks which we have designated as HC-III (pI 9.4) and HC-IV (pI 6.0), respectively. HC-III focused very close to the electrode section of the column and there is no doubt that part of the fraction was denatured as it "spread" into the region of very high pH. However, the combined most-active fractions (115–129, Fig. 1) gave only a single protein band on disc electrophoresis at pH 8.1 and 9.1. These combined fractions therefore appear to contain a single enzyme species. The products of attack by pure HC-III on hemicellulose, as indicated by paper chromatography, are shown in Table VI. The action pattern is clearly different from the previously studied[13] HC-I (which produces arabinose, but little xylose) and from[14] HC-II (which produces xylose, but no arabinose). The detailed action-pattern will be further investigated by degradation of appropriate oligosaccharides. The oligosaccharides indicated in Table VI were compared by paper chromatography in several different solvent systems (Table VIII), with similar products from earlier work.

TABLE IV

PURIFICATION OF HEMICELLULASES HC-III AND HC-IV FROM CELL-FREE CULTURE MEDIUM

Procedure	*Specific activity (unit/mg of protein)*	*Recovery of activity (%)*
Cell-free culture	0.123	100
Ammonium sulphate precipitate (After dialysis in 0.01M sodium acetate buffer)	0.28	61
Isoelectric focusing HC-III	0.38	30
Isoelectric focusing HC-IV	0.09	6
Gel-filtration HC-IV (Sephadex G-200)	0.04	4

TABLE V

MOLECULAR WEIGHT OF SOME GLYCANASES

Enzyme	*Source*	*Molecular weight*	*Ref.*
Acid cellulase	*Aspergillus niger*	46,000	14
Arabinogalactanase	*Bacillus subtilis*	37,000	15
Cellulase	*Penicillium notatum*	34,500	16
Cellulase	*Trichoderma koniugi*	50,000	17
		26,000	17
Cellulase	*Penicillum notatum*	11,000	18
Mannanase	*Bacillus subtilis*	22,000	19
Cellulase	*Myrothecium*	12,600	20
		48,000	20
Cellulase	*Polyporus versicolor*	10,000–11,000	21
Xylanase	*S. sanguinolentum*	21,900–23,000	22

TABLE VI

PRODUCTS[a] OF HEMICELLULOSE HYDROLYSIS BY HC-III AT VARIOUS TIMES

Product	*6 h*	*20 h*	*48 h*	*72 h*	*96 h*
Xylose			6	7	7
Arabinose			6	7	7
AraXyl/Glucose[b]			1	2	3
Xyl_2			9	10	10
$AraXyl_3$			8	9	9
Xyl_3			7	8	8
$AraXyl_4$			8	8	8
Xyl_4	5	4	4	4	

[a]P.c. in solvent *A*. [b]These two possible products could not be resolved in this solvent.

TABLE VII

PRODUCTS FROM HYDROLYSIS OF HEMICELLULOSE B BY ENZYMES I_1–I_4 AND HC-IV

Enzyme	*Xyl*	*Ara*	*AraXyl$_2$*	*Xyl$_2$*	*AraXyl$_3$*	*Xyl$_3$*	*AraXyl$_4$*	*Xyl$_4$*	*Glc*
26 h									
I_1	10	3	—	8	6	5	4	4	10
I_2	10	3	—	8	6	5	4	4	10
I_3	10	3	—	6	5	5	3	3	8
I_4	8	3	—	6	5	5	3	3	8
HC-IV	—	—	—	10	6	5	6	6	—
96 h									
I_1	10	3	—	10	1	1	trace	—	6
I_2	10	3	—	10	1	1	trace	—	6
I_3	10	3	—	10	1	1	trace	—	6
I_4	10	3	—	10	1	1	trace	—	6
HC-IV	1	—	—	10	6	8	6	6	—

TABLE VIII

PRODUCTS FROM HYDROLYSIS OF HEMICELLULOSE B BY HC-III

Oligosaccharide	R_{XYL} (*solvent* A)		R_{XYL} (*solvent* D)		R_{XYL} (*solvent* C)	
	This work	*Lit.*[23]	*This work*	*Lit.*[23]	*This work*	*Lit.*[23]
$AraXyl_2$	0.75	0.78	0.53	—	0.84	0.83
Xyl_2	0.63	0.60	0.49	0.60	0.68	0.68
$AraXyl_3$	0.43	0.39	0.25	0.29	0.56	0.56
Xyl_3	0.32	0.31	0.22	0.28	0.40	0.41
$AraXyl_4$	0.26	0.22	0.11	0.13	0.31	0.32
Xyl_4	0.15	0.15	0.06	—	0.18	0.21

The fractions 21–32 (Fig. 1) containing HC-IV gave two protein bands on disc electrophoresis. An attempt was therefore made to resolve this peak by focusing the original, crude enzyme mixture over a narrow pH range (pH 5–7, Fig. 2). HC-III then migrated into the electrode region of the column and was therefore not recovered. The major HC-IV activity was again obtained at pI 6.0, but a range of hemicellulase activities was also resolved at pI values 4.4, 4.8, 5.1, and 5.4. These four fractions each gave a similar product profile when used to hydrolyse hemicellulose (Table VII). They are tentatively regarded as isoenzymes and have not been further investigated at this stage. Their mode of attack is evidently very different from both HC-IV (Table VII) and HC-III (Table VI), and they appear to be mainly responsible for the production of the relatively high yield of xylose in the hydrolysis of hemicellulose by the culture fluid.

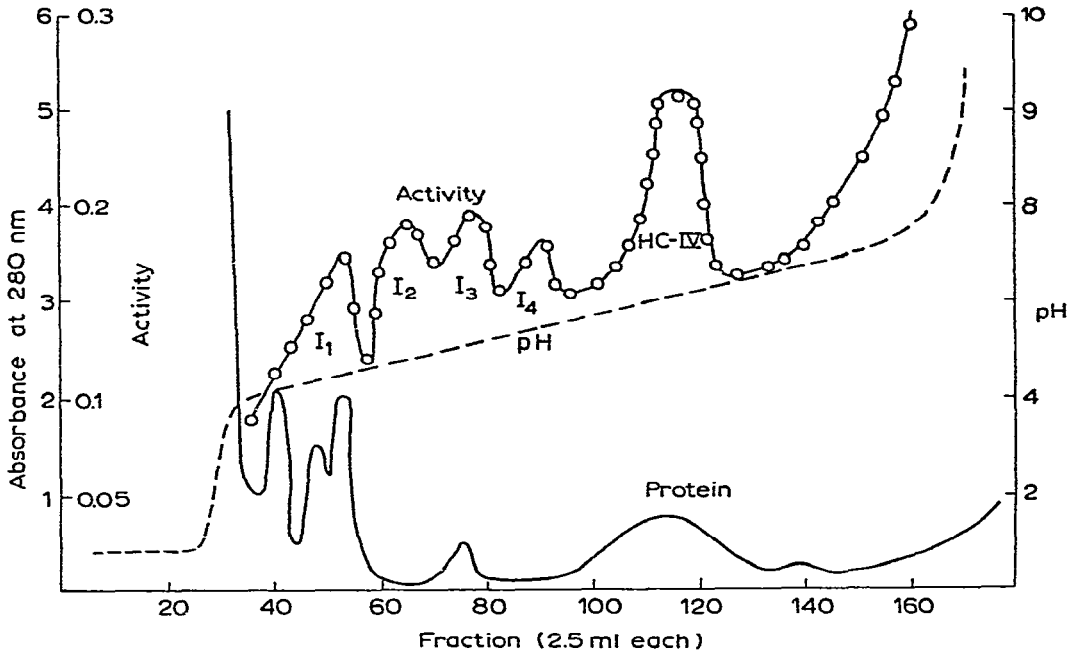

Fig. 2. Isoelectric focusing at pH 5–7.

The HC-IV fraction from narrow-range isoelectric focusing (Fig. 2) again showed a minor protein impurity on disc electrophoresis, so that further purification was necessary. Fractions 108–122 (Fig. 2) were therefore combined and passed through a column of Sephadex G-200. The elution profile (Fig. 3) showed a broad, single hemicellulase peak eluting after the inclusion volume (determined with sodium chloride). The enzyme therefore interacted with the dextran gel, but the eluted material now gave only a single protein band on disc electrophoresis at pH 8.1 and 9.1 and was regarded as pure HC-IV. The minor protein contaminant was apparently lost during the gel filtration without being detected because of its very low concentration.

The molecular weights of HC-III (Fig. 4) and HC-IV (Fig. 4) were determined by gel filtration on Bio-gel P-100 as 10,700 and 9,550, respectively. These hemicellulases are of unusually low molecular weight, and some typical glycan hydrolases of known molecular weight are shown in Table V for comparison. Our values were obtained with very dilute solutions of enzyme, and the molecules are assumed to be fully dissociated. These extremely low molecular weights require confirmation by some other physical method, in case there has been some interaction of the proteins with the Bio-gel, but such interaction between the gel and two different proteins seems rather unlikely.

A generalisation has previously been postulated[15] from work on fungal carbohydrases, that "enzymes hydrolysing high molecular weight substrates are

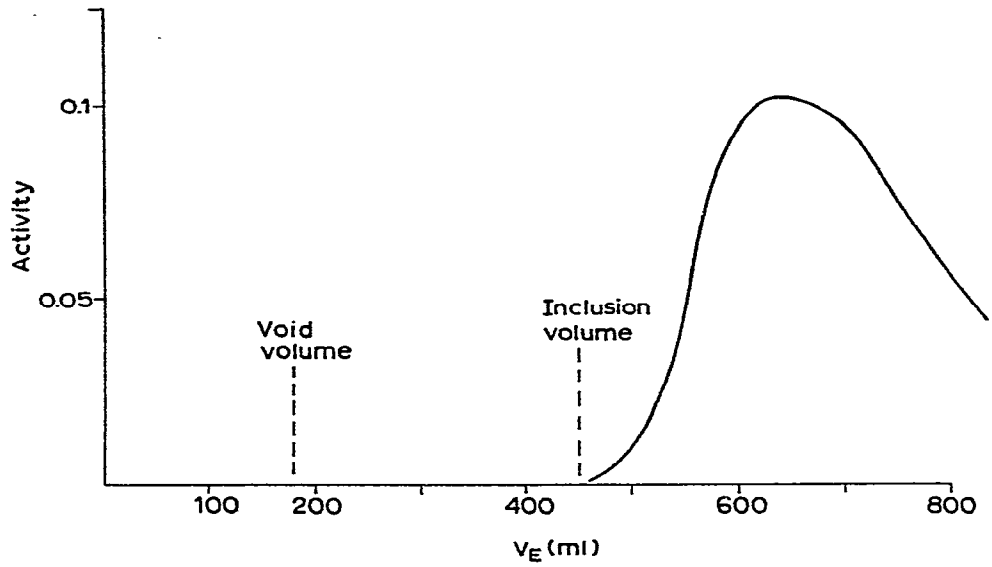

Fig. 3. Gel-filtration of HC-IV on Sephadex G-200.

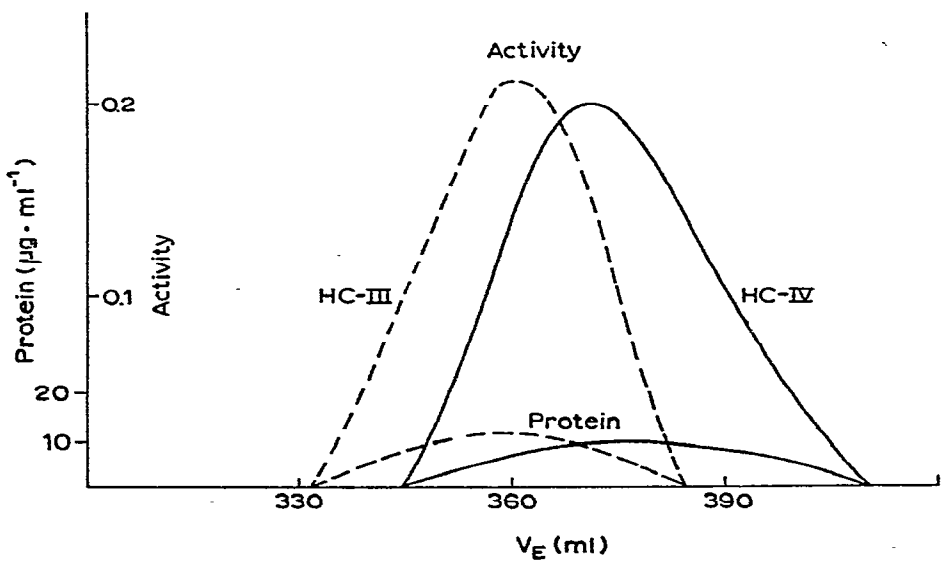

Fig. 4. Gel-filtration of HC-III and HC-IV on Biogel P-100.

smaller molecules than those hydrolysing low molecular weight substrates". Hemicellulases III and IV appear to be extreme examples of this generalisation and, in fact, their molecular weights are among the lowest so far reported for any enzyme. It is possible, however, that the xylanases from a commercial enzyme preparation obtained from the *Aspergillus niger–oryzae* group of fungi, which have been subjected to gel filtration on an uncalibrated Sephadex G-75 column[15], may be of similar molecular weight.

The recovery of enzyme activity from isoelectric focusing was very low, especially for HC-IV (Table IV). This effect has previously been noted elsewhere[16], and in this laboratory we have found a five-fold loss of activity on repeated isoelectric focusing of glucosidases[17]. While the activity decreases during the focusing, the purity of the protein (as indicated, for example, by disc electrophoresis) increases and there is little loss of total protein. The specific enzyme activity therefore decreases during purification of the protein (Table IV). We conclude that the enzyme protein is extensively deactivated, most probably during the several days spent at its isoelectric point. Presumably, this process occurs *via* conformational changes without causing insolubility.

Preliminary inhibition studies with purified HC-III and HC-IV have indicated that both enzymes are inhibited by sulphydryl reagents. The inhibition by *p*-chloromercuribenzoic acid is reversed by addition of an eight-fold excess of cysteine. Disulphide reagents caused inhibition to a smaller extent, and ethylenediaminetetraacetic acid had little effect on the activity.

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