The Action of Cellulolytic Enzymes from *Myrothecium verrucaria*

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Although an extensive literature is available describing cell-free cellulolytic preparations which attack soluble cellulose derivatives, there is a dearth of information on the enzymic breakdown of insoluble cellulose, and virtually nothing is known about the breakdown of undegraded cellulose of the type associated with native cotton fibres [For reviews see Siu (1951), Halliwell (1959)]. Despite the failure of cell-free preparations made from them to attack undegraded cellulose, truly cellulolytic organisms can quantitatively transform all types of cellulose, undegraded or otherwise, to soluble products.

The present report concerns an examination of the breakdown of an acid-swollen cellulose by culture filtrates from *Myrothecium verrucaria*. This substrate is an insoluble although degraded cellulose and was chosen firstly because the relatively mild method of preparation involves only small changes in the degree of polymerization (Walseth, 1952), and secondly only after having shown that our enzyme preparations produced significant solubilization of this substrate. Conditions governing the enzymic breakdown of insoluble cellulose are described below and compared with the enzymic hydrolysis of the soluble cellulose derivative, carboxymethylcellulose.

MATERIALS AND METHODS

Preparation of the culture filtrate

Cultures of *M. verrucaria* (I.M.I. 45541, Commonwealth Mycological Institute, Kew, Surrey), grown on the salt medium of Saunders, Siu & Genest (1948) with cellulose powder (Whatman) or de-waxed cotton fibres as substrate, were aerated at 28° by incubation in a reciprocal shaking machine operating at 60–90 strokes of 38 mm. horizontal movement per minute. After fermentation for 1–3 weeks most of the organisms and all of the residual cellulose were separated off by filtration through a sintered-glass filter (porosity 3). The filtrate was centrifuged at 54 000 g for 30 min. at 1° and subsequently passed through a sintered-glass bacteriological filter (porosity 5/3). In the later work the final filtration was found to be unnecessary by inoculation on cellulose–agar slopes. The cell-free culture supernatant or filtrate fraction was used as the source of cellulolytic enzymes. For enzyme assays the following average volumes were used: for method (a) 0.2 ml. of undiluted filtrate; for method (b) 0.1 ml. of filtrate diluted 50 times.

The solubilization of cellulose by the organism was determined by washing the mixture on the sinter with hydrochloric acid, aq. ammonia solution, Teepol and ethanol, followed by drying and weighing as described elsewhere (Halliwell, 1957a).

Substrates

Native cotton fibres. Texas cotton (kindly supplied by the British Cotton Industry Research Association) was de-waxed by treatment with ethanol, ether and finally with sodium hydroxide under nitrogen (Halliwell, 1957a).

Cellulose powder (Whatman). This was standard-grade ashless powder for chromatography.

Swollen cellulose. This insoluble preparation was made from Texas cotton fibres and also from cellulose powder (Whatman) by soaking in phosphoric acid for 2 hr. at 1° (Halliwell, 1957a).

Sodium carboxymethylcellulose. A soluble cellulose derivative, with a degree of substitution of about 0.5, was generously supplied as Cellofas B by Imperial Chemical Industries Ltd.

Other materials. These were: cellobiose and glucono-1→4-lactone (L. Light and Co. Ltd.); glucose (A.R.; Hopkin and Williams Ltd.).

Analytical procedure

Determination of the degree of hydrolysis of the soluble cellulose derivative carboxymethylcellulose. Hydrolysis of carboxymethylcellulose to 'reducing' sugars was followed by a modification of the colorimetric procedure of Park & Johnson (1949). The latter method was satisfactory with pure aqueous solutions of glucose, but not under assay conditions in the presence of phosphate buffer, owing to precipitation of reagents. This was overcome by substituting phosphoric acid for sulphuric acid in the ferric alum solution as follows: (a) 0.1 g. of ferric ammonium sulphate (A.R.) was dissolved in 1.5 ml. of conc. phosphoric acid (A.R., 90%) and 48.5 ml. of water; (b) to solution (a) was added a mixture containing 0.15 g. of Duponol (Duponol ME, dry, kindly given by E. I. du Pont de Nemours and Co. Inc., Wilmington, Del., U.S.A.) in 1.0 ml. of conc. phosphoric acid and 49 ml. of water.

Modified procedure for determination of 'reducing' sugar. The sample, containing 1–14 µg. of sugar, was neutralized (see below under enzyme assay) and made up to 3 ml. with water. Deproteinizing was unnecessary. After addition of 1 ml. of cyanide–carbonate solution (0.064% of potassium cyanide + 0.52% of sodium carbonate anhydrous) followed by 1 ml. of 0.05% potassium ferricyanide, the mixture was heated in a boiling-water bath for 15 min., transferred to a cold-water bath for 5 min. and treated with 2 ml. of ferric alum–Duponol–phosphoric acid reagent. Colours were

developed at room temperature for 15 min. before being read on a Spekker absorptiometer (Hilger) in a 1 cm. light-path with Ilford orange filters (no. 607, peak transmission 600 $m\mu$).

Glucose and cellobiose gave straight-line calibration curves up to 14 μ g. of sugar when analysed by the procedure above. Absorptiometer readings obtained after 15 min. of colour development increased slightly on further standing, but for periods of colour development up to 75 min. this error was eliminated by including a reagent control whose colour value increased by the same amount.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used on a Spekker absorptiometer in a 1 cm. light-path with Ilford red filters (no. 608, peak transmission 700 $m\mu$). As the copper sulphate-tartrate reagent tends to settle out on standing, tartrate was replaced by trisodium citrate dihydrate (A.R.). Crystalline bovine plasma albumin (Armour Laboratories) provided the standard reference protein. The procedure was used to follow protein formation in culture filtrates and to determine enzyme protein before and after dialysis.

Method of enzyme assay (a) with insoluble substrate, cellulose. As a result of preliminary experiments the following procedure was adopted. To about 26 mg. (or 1 mg. in earlier experiments) of cellulose [cotton fibres, cellulose powder (Whatman) or swollen forms of these celluloses] was added 1.3 ml. of acetate buffer, pH 5.5, (0.2N-acetic acid and 0.2N-sodium acetate), water and cell-free culture filtrate in a final volume of 4 ml., contained in 15-ml. R.B. heat-resistant, centrifuge tubes (110 mm. $\times$ 16 mm.; Measuring and Scientific Equipment Ltd.), or 25-ml. test tubes (125 mm. $\times$ 19 mm.; Griffin and George Ltd.). Test tubes were used when the residual cellulose was estimated after separation from the aqueous phase by means of a filter-stick. Centrifuge tubes were employed when cellulolysis was measured by increase in soluble carbohydrates formed from insoluble cellulose (see below). Cell-free culture filtrate-substrate mixtures were incubated for 1 hr. at 37°, and the enzyme was inactivated by addition of sufficient 0.6N-sulphuric acid (about 0.5 ml.) to lower the pH to 1.90–1.95. Enzyme and substrate controls were included.

Cellulose in the acidified suspensions was determined colorimetrically by two methods: (a) directly, the cellulose in its original tube being filtered, washed on the filter-stick and finally estimated with dichromate (Halliwell, 1958); (b) indirectly, from the amount of soluble carbohydrates present in the aqueous phase: the acidified assay medium containing cellulose in centrifuge tubes was centrifuged at 1300–1800 g for 3 min. at room temperature, and the supernatant passed through a sintered-glass crucible (porosity 3). The carbohydrates in the filtrate were quantitatively determined with dichromate, the values being calculated from a standard calibration curve prepared with glucose (Halliwell, 1960). The dichromate method is preferable to reducing-sugar methods for this purpose because it determines both 'reducing' and 'non-reducing' carbohydrates and thus gives a value much closer to the actual degree of solubilization (cf. Fig. 3).

The preliminary centrifuging was used to deposit the insoluble cellulose firmly, thus facilitating separation of most of the liquid phase. Filtration was essential to remove

fine floating particles of insoluble cellulose, which failed to precipitate even on prolonged centrifuging.

Acidification of cellulose to pH 1.9 produced no solubilization of this substrate during the period required for centrifuging and filtration. Any solubilization that might occur under experimental conditions would be recognized in the substrate controls.

In some enzymic digests of cellulose the buffer solutions and volumes used were different from those given above for the standard assay with acetate. However, when 0.8–1.3 ml. of acetate buffer, pH 5.5, or 1.6 ml. of phosphate buffer (0.067M-disodium hydrogen phosphate + 0.067M-potassium dihydrogen phosphate, pH 5.5) or citrate buffer (0.1M-citric acid + 0.2M-disodium hydrogen phosphate, pH 5.5) were employed, enzymic activities on swollen cellulose powder were identical.

Filtrates obtained from enzymic incubations with cellulose in presence of phosphate or acetate buffers were examined by the indirect dichromate procedure (b) outlined above. As citrate buffer reduces dichromate, cellulolysis in such a medium was determined by direct procedure (a) above. Citrate was used only in measuring the pH optimum of the enzyme on swollen cellulose powder.

Method of enzyme assay (b) with soluble substrate, carboxymethylcellulose. To 0.3 ml. of 1% (w/v) carboxymethylcellulose was added 0.15 ml. of acetate buffer, diluted cell-free culture filtrate and water to 0.6 ml., final pH 5.5. The mixture was incubated at 37° for 1 hr. and the reaction terminated by addition of 0.25 ml. of 0.13% anhydrous sodium carbonate and 1 ml. of cyanide-carbonate (0.064% of potassium cyanide + 0.52% of sodium carbonate, see above). After addition of 2.15 ml. of water (total volume now 4 ml.) subsequent stages, commencing with the addition of 1 ml. of potassium ferricyanide, are identical with those described above (see 'Modified procedure for determination of reducing sugar'). The value, 0.25 ml. of 0.13% sodium carbonate, is experimentally determined as necessary to neutralize the enzyme reaction mixture to about pH 7.0, whereas the cyanide-carbonate adjusts the reaction of the neutralized mixture to about pH 10.7, thus attaining the required alkalinity of the standard glucose curve and simultaneously terminating enzymic activity. The diluted cell-free culture filtrate itself gave a negligible value when oxidized with ferricyanide, thus avoiding the necessity for deproteinization before sugar determination.

RESULTS

In the work described below the term cellulase is used to describe the activity in cell-free preparations attacking insoluble cellulose (swollen cellulose powder, swollen de-waxed fibres, cellulose powder and de-waxed fibres), with the proviso that a true cellulase (as yet not isolated) should produce extensive solubilization of undegraded cellulose (e.g. untreated fibres) in the manner of the parent micro-organism. Enzymic activity on the soluble cellulose derivative, carboxymethylcellulose, is referred to as carboxymethylcellulase.

Decomposition of de-waxed cotton fibres and of cellulose powder by cultures of *Myrothecium verrucaria*. Fig. 1 illustrates the capacity of the organism

to solubilize these less-degraded forms of cellulose. Continuous shaking was beneficial to extensive cellulolysis of both forms of cellulose and was also essential when profitable volumes of medium were required. In stationary cultures growth was much slower, and it was necessary to use smaller quantities of medium to avoid submerging the fungus.

Decomposition of de-waxed cotton fibres and of cellulose powder by enzyme filtrates of Myrothecium verrucaria. The degree of solubilization of these substrates by the cell-free culture filtrate (Table 1) is only a small fraction of that shown on the same substrates by the whole culture (Fig. 1). The initial rate of solubilization of undegraded fibres by the cell-free culture filtrate is comparable with that shown by whole organisms during growth, but the

extent of solubilization by the enzyme preparation becomes constant after 22 hr., whereas with whole organisms, where growth is in progress, it increases to 80 % in 16 days. It is first necessary to subject the cellulose to chemical treatment, such as swelling in acid, before cellulolysis by the culture filtrate becomes significant.

Activity of cellulase on swollen cellulose powder at different pH values. Citrate-phosphate buffer, covering the range pH 2-8 (Fig. 2) was used in mixtures, containing cell-free culture filtrate and

Table 1. Solubilization of different forms of cellulose by cell-free filtrates from *Myrothecium verrucaria*

Swollen cellulose powder (26 mg.) or swollen de-waxed Texas fibres (26 mg.) was incubated at 37° with 0.2 ml. of cell-free filtrate in acetate buffer, pH 5.5, for 1 hr. and 22 hr. Cellulose powder or de-waxed Texas fibres (5 mg.) was examined under the same conditions with 0.5 ml. of cell-free filtrate. Enzymic activity was estimated from the soluble carbohydrates formed. In the determination of carboxymethylcellulase activity by the standard method the cell-free filtrate was used at about one-twentieth of the final concentration of that with the swollen cellulose powder.

Type of cellulose	Degree of solubilization (% of initial wt.) in	
	1 hr.	22 hr.
Cellulose powder	—	3
De-waxed fibres	—	4
Swollen cellulose powder	11	56
Swollen de-waxed fibres	8	45
Carboxymethylcellulose (degree of hydrolysis)	0.4	—

Fig. 1. Solubilization of cellulose by cultures of *Myrothecium verrucaria*. 50-ml. conical flasks containing (a) 50 mg. of cellulose powder (Whatman) in 10 ml. of salts medium, pH 6.6, or (b) 50 mg. of de-waxed Texas cotton fibres in 4.5 ml. of salts medium for shaken cultures (or in 1 ml. for stationary cultures) were inoculated with 1 ml. of a heavy spore suspension of the organism prepared from cultures of *M. verrucaria* grown on filter-paper strips on agar slopes in test tubes. The flasks were aerated by reciprocal shaking at 120 strokes of 50 mm. horizontal movement per minute for cellulose powder (or 60 strokes of 38 mm. for cotton fibres) and at 28° for the periods shown. Flasks were removed at intervals, the mycelium-cellulose residue was filtered on sintered glass, and washed and dried as described under Materials and Methods. Cellulose powder (Whatman) in shaken culture (○), in stationary culture (×); de-waxed cotton fibres in shaken culture (□), in stationary culture (■). Control flasks containing cellulose and medium (no inoculum) at 28° in shaken or stationary incubations showed no loss in weight of cellulose.

Fig. 2. Effect of pH on the activity of cellulase on swollen cellulose powder. Enzymic activity, measured by the standard method but in citric acid-phosphate buffer, was terminated by addition of sulphuric acid followed by filtration and determination of the residual cellulose with dichromate.

substrate, which were checked for constancy of pH during incubation. Optimum activity for the enzyme occurs about pH 5, with some hydrolysis still evident at pH 2.5. As enzymic activity at pH 5.5 is very similar to that at pH 5.0 the former pH was used in the standard assay medium in order to facilitate comparison with results of other workers in the field.

Effect of substrate concentration on enzymic activity. In the original cell-free culture filtrates the relationship between cellulose made soluble and initial weight of substrate indicated that saturation of the enzyme occurred at about 1 mg. of cellulose/4 ml. incubated. Activities were approximately proportional to the concentrations of enzyme used. In later experiments cell-free culture filtrates obtained from subcultures of the original organism provided different curves with saturation occurring at 27 mg. of cellulose/4 ml. of incubation mixture (Fig. 3).

The Figure also illustrates agreement between the two methods used to measure enzymic cellulolysis:

(1) direct loss in weight of cellulose, and (2) production of soluble saccharides.

Carboxymethylcellulase activity in the culture filtrate was examined as in Table 2 with saturation being obtained at about 3 mg. of carboxymethylcellulose in the 0.6 ml. of assay volume.

The K_m values of the culture filtrate on swollen cellulose powder and on carboxymethylcellulose are calculated by the graphical method of Lineweaver & Burk (1934) (Fig. 4), and give values of

Table 2. *Effect of substrate concentration on the production of reducing sugar (as glucose) from carboxymethylcellulose*

Assay of the enzyme was made by the standard procedure. The cell-free culture filtrate was used at about one-fortieth of the concentration of that in Fig. 3 (■).

Carboxymethylcellulose in a total volume of 0.6 ml. (mg.)	Reducing sugar produced (as glucose) (μ g.)
0.11	2.6
0.23	4.1
0.46	5.5
1.17	7.3
2.0	8.3
3.0	8.9
3.5	8.8
4.0	8.1

Fig. 3. Effect of weight of swollen cellulose powder on the amount solubilized by cellulase. Standard conditions of assay except that incubated mixtures, after inactivation with sulphuric acid, were filtered through sintered crucibles to obtain both the filtrate and residual cellulose. The latter was washed and determined gravimetrically. The filtrate was examined for the amount of soluble carbohydrates (see Methods section). Cellulose solubilized as determined from residual weight of cellulose, with culture filtrate A (0.5 ml.) (■), (1 ml.) (□); with another filtrate (0.2 ml.) (○). Cellulose solubilized as determined from soluble carbohydrates in the filtrate, with culture filtrate A (1 ml.) (●). S , Conc. of substrate (mg./4 ml.).

Fig. 4. Determination of the dissociation constant (K_m) of the enzyme-substrate complex for cellulase and carboxymethylcellulase by plotting the reciprocal of substrate concentration (S) against the reciprocal of rate of solubilization (V) of cellulose (for cellulase) or rate of formation of reducing sugar (for carboxymethylcellulase) from data of Fig. 3 (cellulase) and Table 2 (carboxymethylcellulase). ○, Cellulase (C); □, carboxymethylcellulase (CMC).

1 g. of cellulose/l. and 0.5 g. of carboxymethyl-cellulose/l. for the dissociation constant of the enzyme-substrate complex.

Effect of concentration of cell-free culture filtrate on the solubilization of swollen cellulose powder. At high enzyme concentrations the reaction velocity is not proportional to the amount of culture filtrate present (Fig. 5) probably owing in part to preferential enzymic attack on the more susceptible amorphous regions of the swollen cellulose. The effect was not due to the presence of a dialysable inhibitor as the dialysed culture filtrate possessed slightly less activity than the undialysed preparation when equal amounts of filtrate were used, based on protein content. Likewise, possible end products such as glucose or cellobiose produced no marked decrease in cellulolysis (see below). The effect could also indicate the presence of non-dialysable inhibitor in the cell-free culture filtrate.

Solubilization of cellulose as affected by the duration of enzymic activity. The rate of breakdown of swollen cellulose powder is almost linear for the first 45 min. but thereafter decreases progressively

(Fig. 5). This presumably results from the early breakdown of the less refractory amorphous areas in cellulose and from the instability of the enzyme at 37° (see below) together with its increasing degree of unsaturation as the substrate is solubilized.

Effect of agitation on enzymic activity on cellulose and carboxymethylcellulose. Previous paragraphs have indicated that approximately 30-fold more cellulose was required to saturate later culture filtrates than was the case in earlier experiments. In an unsuccessful attempt to explain this difference, the action of cellulase (cell-free preparation) was examined with and without agitation on a Warburg apparatus operating at 107 complete strokes of 50 mm. horizontal movement per minute, when activity was found to be unaffected or increased only slightly by shaking. The same enzyme preparation examined under similar conditions with carboxymethylcellulose as substrate lost 72 % of its activity compared with an unshaken incubation (Table 3).

Activity of cellulase after heat treatment. Cellulase activity in the preliminary experiments (in which the culture filtrate was saturated with 1 mg. of cellulose) was completely terminated by immersing the reaction tubes containing filtrate and substrate in hot water (97°) for 20 min. In presence of the larger amounts of cellulose (27 mg.) required to saturate subsequent filtrates, the same heat treatment was less effective and allowed variable amounts of activity to continue. The effect was further examined by using shorter and longer periods of heating as follows: cell-free culture filtrates-cellulose mixtures incubated for 1 hr. were subjected to the following treatments: (a) immediate filtration (control), (b) immersion at 97° for 10 min. followed by incubation at room temperature (20°) for 30 min., (c) as in (b) but with a 30 min. heating period. In each case the cellulose was finally filtered, washed with hydrochloric acid, water, ethanol and dried at 105° before being weighed, when the degree of solubilization in (b) and (c) were 116 and 97 % respectively of that in (a).

It appears that one or more of the components of the enzymic preparation, possibly one concerned with the shorter but still insoluble chains, is not readily inactivated by brief periods of heat-treatment. Assays were more conveniently terminated by addition of dilute acid as described in the experimental section.

The filtrate was also examined for cellulase and carboxymethylcellulase activity by the standard procedures, after a preliminary incubation under assay conditions in the absence of substrate for 1 hr. and at different temperatures (Fig. 6). The results suggest that carboxymethylcellulase is less stable than cellulase, but in effect this is due to the experimental conditions of the respective assays

Fig. 5. Effect of enzyme concentration and of duration of incubation on the solubilization of swollen cellulose powder. Cellulase activity was measured and terminated by the standard method. Enzyme concentration was expressed as units in 4 ml., each unit corresponding to 0.05 ml. of cell-free culture filtrate. Soluble carbohydrates in the filtrate were determined with dichromate. Cellulose made soluble (0-3 mg.) as affected by concentration of enzyme (O). Cellulose made soluble (0-16 mg.) as influenced by period of incubation (●).

Table 3. *Effect of shaking on the activity of cellulase and carboxymethylcellulase*

Cellulase and carboxymethylcellulase activities were determined by the standard methods on swollen cellulose powder and on carboxymethylcellulose. Procedures for terminating and for estimating enzymic activity are described in the table. Tubes were shaken in a Warburg bath at 107 complete strokes of 50 mm. horizontal movement per minute and held in suitable positions (about 35° to the horizontal for cellulose) to obtain similar degrees of agitation with both enzymes.

degrees of agitation with both enzymes.			Unshaken tubes: cellulose solubilized (% of initial weight)	Shaken tubes: cellulose solubilized (% of that solubilized in unshaken tubes)
Cell-free filtrate	Method of terminating enzymic activity	Method of estimating enzymic activity		
<i>Cellulase activity</i>				
1, 2	Immediate filtration and solvent washing (hydro- chloric acid, water, ethanol and oven-drying)	Gravimetric, on residual cellulose	23	105
2	Addition of sulphuric acid to pH 1.95, rapid filtration under pressure and solvent washing (as above)	As above	21	98
3	Addition of sulphuric acid to pH 1.95, rapid filtration under pressure and solvent washing. Filtrate retained for next experiment	As above	15	106
3	Filtrate from previous experiment	Dichromate-sulphuric acid oxidation of soluble carbo- hydrates in the filtrate	15	108
4	Addition of sulphuric acid to pH 1.95, filtration under gravity, filtrate analysed	As above	15	98
<i>Carboxymethylcellulase activity</i>				
			Unshaken tubes: reducing sugar formed (% of substrate)	Shaken tubes: reducing sugars formed (% of that in unshaken tubes)
3	Addition of carbonate- cyanide to pH 10.7	Ferricyanide determination of reducing sugars formed	0.4	28

rather than to inherent instability of that enzyme. Thus when the cell-free preparation itself was incubated in absence of substrate for 1 hr. (rather than under the respective conditions for the two enzymes) before removal of samples for the standard cellulase and carboxymethylcellulase assays, the latter enzyme appeared to be slightly more stable than cellulase (Fig. 6).

Effect of possible inhibitors on cellulase activity. Glucono-1 → 4-lactone or glucose at a final concentration of 2.5 mM had no effect on the rate of solubilization of cellulose by the cell-free culture filtrate, whereas the same molarity of cellobiose always produced slight inhibition, but never more than 10%. At the high concentration of 25 mM (18 mg. of glucose or 34 mg. of cellobiose in the presence of 26 mg. of cellulose) 15, 10 and 30% inhibition was obtained with gluconolactone, glucose and cellobiose respectively. The reaction medium containing carbohydrates equivalent to 1 mg. of glucose (dichromate method) obtained after incubation of cellulose and enzyme for 1 hr. was transferred to a fresh assay system and pro-

duced only 8% inhibition of cellulolysis, indicating that end products have negligible effect on cellulolysis.

Adsorption of cellulase by cellulose

Immediately after mixing cellulase and its substrate (swollen cellulose powder) the aqueous phase is relatively free from enzyme owing to the latter's adsorption, the extent of which depends on the initial quantity of cellulose present and on subsequent incubation of the mixture (Table 4).

In Table 4 separate samples were used for the 0 and 1 hr. experiments in order to avoid delays in filtration, but substantially similar results were achieved when the original sample of cell-free culture filtrate and cellulose (26 mg.) was successively centrifuged, the supernatant filtered off and the cellulose reincubated with fresh buffer solution. Thus after incubation for 0 and 1 hr., supernatant fractions containing 17 and 20% respectively of the original enzymic activity were obtained by successive centrifugings. The deposited cellulose was then washed once (or three

times) with dilute acetate buffer. On reincubation of the cellulose it was found to retain 49 % of the enzyme originally added. All values are corrected for some adsorption of enzyme by the sinter itself and for the non-linearity of enzyme activity at high enzyme concentrations. It is noteworthy that 86 % of the cellulase has survived 2 hr. incubation with substrate.

DISCUSSION

M. verrucaria produces almost complete solubilization of de-waxed cotton fibres (Fig. 1), whereas cell-free culture filtrates have an insignificant effect

(4 % solubilization) on this substrate. For cellulose powder prolonged incubation, with addition of further quantities of cell-free filtrate, provides a constant maximum of 26 % solubilization achieved in 72 hr., comparable with the values given by our earliest preparations (Halliwell, 1957b). The latter, however, contrasted with present cell-free culture filtrates in being more active (requiring less enzymic material to produce this degree of solubilization) on cellulose powder, but less active on swollen cellulose powder. This activity was associated with a lemon-yellow colour present in the early culture media, but less evident in more recent metabolism fluids. Such apparent variance in chemical properties between cell-free preparations may also account for the different amounts of cellulose required to saturate the various cell-free culture filtrates described above.

After conversion of cotton fibres or cellulose powder into the swollen form, both the rate and extent of breakdown are increased (Table 1) so that almost complete solubilization (90 %) can be achieved in 22 hr. by addition of larger amounts of enzyme.

The results show that the procedure described for the determination of activity on swollen, but insoluble, forms of cellulose powder and fibres is as rapid as it is on the soluble carboxymethylcellulose (Fig. 3, Table 2) under normal conditions of measurement. It thus seems preferable and possible to employ an insoluble cellulosic substrate in studies involving cellulase rather than to substitute carboxymethylcellulose.

Basu & Pal (1956) observed that carboxymethylcellulase preparations from several fungi, including *M. verrucaria*, were denatured (40 %) if agitated during a 3 hr. assay procedure, whereas Myers & Northcote (1959) failed to obtain any inhibition of

Fig. 6. Residual enzymic activity after incubation of the cell-free preparation in absence of substrates at different temperatures. Treatment A: cellulase (○), carboxymethylcellulase (●); the enzyme preparation was incubated in acetate buffer, pH 5.5, under the appropriate assay conditions for cellulase and carboxymethylcellulase (see Materials and Methods), but in the absence of substrate (cellulose or carboxymethylcellulose), at 20°, 37° or 54° for 1 hr. and then transferred to a bath at 37° followed immediately by addition of the respective substrates (cellulose or carboxymethylcellulose) and a further incubation of 1 hr. at 37°. Controls, assayed in the normal manner for 1 hr. at 37°, were taken to represent 100 % enzymic activity (c on abscissa). Treatment B: cellulase (□), carboxymethylcellulase (■); the cell-free preparation was diluted fourfold in acetate buffer, pH 5.5, followed by (a) immediate assay (controls) on swollen cellulose powder or on carboxymethylcellulose, these activities being taken as 100 % (c on abscissa), or (b) incubation at 20°, 37° or 54° for 1 hr. or 97° for 10 min. when samples were removed and assayed on swollen cellulose powder or, after dilution, on carboxymethylcellulose under the usual conditions (see Methods) for 1 hr. at 37°. Cellulase activities were determined from the amount of soluble carbohydrates formed.

Table 4. Adsorption of cellulase by different amounts of cellulose

ES (0) and ES (1) represent enzyme-swollen cellulose powder-buffer mixtures prepared in the standard manner (see Materials and Methods section) with mixing, followed by (a) immediate filtration (no centrifuging), ES (0), or (b) incubation for 1 hr. at 37°, ES (1), before filtration through a sintered-glass crucible. The enzyme in each filtrate was determined by assay on fresh cellulose-buffer as in Materials and Methods and the assay value (solubilization of cellulose) is expressed as a percentage of the enzymic activity of a control filtrate obtained in a similar manner to ES (0), but in absence of cellulose.

Cellulose (mg.)	Enzymic activity in filtrate (% of initial)	
	ES (0)	ES (1)
24	2	27
12	6	28
7	16	36

the same enzymic activity during a 20 min. shaking period with a preparation from snails. In our own work, a 1 hr. period of agitation produced severe loss (72 %) of carboxymethylcellulase, but allowed cellulase activity to continue unimpaired (Table 3). The results achieved by Myers & Northcote (1959) are possibly due to the slower rate of shaking.

Fig. 6 indicates that carboxymethylcellulase loses activity more rapidly under its own particular assay conditions when devoid of substrate than does cellulase. On the other hand cellulase appears to be slightly less stable than carboxymethylcellulase when the cell-free culture filtrate, without additions, other than fourfold dilution in buffer, is incubated at different temperatures (in absence of substrate) before transfer to the two standard assay systems. Under their respective assay conditions but without added substrate the cell-free culture filtrate loses 50 % of carboxymethylcellulase and 10 % of cellulase activity during a 1 hr. incubation at 37°.

The possibility that end products resulting from enzymic activity on cellulose might inhibit further breakdown of this substrate does not exist under the present conditions. Only when glucose or cellobiose was added in very large amounts (18 and 34 mg. respectively in presence of 26 mg. of cellulose) did significant (10–30 %) reduction in activity occur in 1 hr. End products of cellulolytic activity likewise failed to inhibit cellulase when transferred to a fresh enzymic assay on cellulose. Further, Fig. 5 also indicates that the accumulation of end products on prolonged incubation of enzyme with substrate for 22 hr. does not prevent solubilization from rising to at least 60 % breakdown. Larger amounts of cell-free culture filtrate permitted 90 % solubilization of cellulose in the same period. Gilligan & Reese (1954) have reported that relatively high concentrations of cellobiose were required to produce appreciable inhibition of the hydrolysis of hydrochloric acid- and phosphoric acid-treated, ground cotton by filtrates from *Trichoderma viride*.

Table 4 illustrates the high affinity of cellulose for its enzyme and probably accounts for the lack of any beneficial effect of agitation on cellulolysis. Cellulase is strongly adsorbed by its insoluble substrate and is slowly liberated during solubilization of the cellulose.

Cellulase (as examined on acid-swollen cellulose powder) is in fact a relatively stable enzyme, strongly adsorbed by its substrate and thus difficult to recover.

SUMMARY

1. The action of cell-free filtrates from *Myrothecium verrucaria* was examined on various forms

of insoluble cellulose and on the soluble cellulose derivative, carboxymethylcellulose.

2. Unlike the parent organism which completely solubilizes undegraded forms of cellulose (de-waxed cotton fibres), cell-free culture medium has a negligible effect (4 % solubilization) on the same substrate unless this is first swollen with phosphoric acid. After such treatment of cellulose, cellulolytic filtrates produce as much as 90 % solubilization of swollen cellulose powder or of swollen de-waxed cotton fibres. Conditions governing the activity of the cell-free filtrates on swollen cellulose powder are described.

3. The preparation has an optimum activity on swollen cellulose powder at about pH 5.

4. Values of 1 g. of swollen cellulose powder/l. and of 0.5 g. of carboxymethylcellulose/l. were obtained for the Michaelis constants.

5. At pH 5.5 and temperatures between 20° and 54° the cell-free filtrate loses cellulase slightly more rapidly than carboxymethylcellulase activity. In contrast, under the appropriate assay conditions for the two activities, carboxymethylcellulase is denatured more rapidly than cellulase.

6. Agitation of cell-free filtrates in presence of cellulose has no marked effect on the degree of cellulolysis. With carboxymethylcellulose as substrate, however, agitation produces 70 % inhibition.

7. Glucose, cellobiose or reaction products of enzymic cellulolysis fail to retard enzymic solubilization of cellulose unless present in amounts comparable with the initial weight of cellulose.

8. Cellulase in cell-free filtrates is strongly adsorbed on its insoluble substrate and is only slowly released as the cellulose itself is solubilized.

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REFERENCES

- Basu, S. N. & Pal, P. N. (1956). *Nature, Lond.*, **178**, 312.
 Gilligan, W. & Reese, E. T. (1954). *Canad. J. Microbiol.* **1**, 90.
 Halliwell, G. (1957a). *J. gen. Microbiol.* **17**, 153.
 Halliwell, G. (1957b). *J. gen. Microbiol.* **17**, 166.
 Halliwell, G. (1958). *Biochem. J.* **68**, 605.
 Halliwell, G. (1959). *Nutr. Abstr. Rev.* **29**, 747.
 Halliwell, G. (1960). *Biochem. J.* **74**, 457.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Myers, F. L. & Northcote, D. H. (1959). *Biochem. J.* **71**, 749.
 Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
 Saunders, P. R., Siu, R. G. H. & Genest, R. N. (1948). *J. biol. Chem.* **174**, 697.
 Siu, R. G. H. (1951). *Microbial Decomposition of Cellulose*. New York: Reinhold Publishing Corp.
 Walseth, C. S. (1952). *Tech. Pap. Pulp Pap. Ind., N.Y.*, **35**, 228.