Solubilization of Native and Derived Forms of Cellulose by Cell-Free Microbial Enzymes

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1. Cell-free enzymes from *Myrothecium verrucaria* and *Trichoderma koningii* hydrolyse native undegraded cellulose, as found in cotton fibres, in a random manner to short insoluble fibres and to minor amounts of soluble products. 2. Enzyme preparations from *M. verrucaria* fail to attack the short fibres whereas preparations from *T. koningii* solubilize them completely to sugars at an optimum pH 4·2–4·6. 3. The mode of hydrolysis of cotton cellulose by preparations from *T. koningii* involves from the earliest stages the formation of reducing sugars, followed closely by the appearance of short fibres, until the insoluble and soluble products each constitute about 40–50% of the weight of the initial substrate. After this stage the quantity of sugars increases at the expense of the insoluble short fibres. 4. Depending upon the method of preparation, derived forms of cellulose may be hydrolysed more slowly, much more rapidly, or at the same rate as cotton fibres by enzyme preparations from *T. koningii*.

The ability to metabolize undegraded cellulose in Nature and in the laboratory is limited to relatively few microbial species and these are notoriously reluctant to liberate cellulolytic enzymes into solution. In many instances this may be attributed to absorption of these enzymes on to the insoluble cellulosic substrate immediately they are released from the cell. An exception is found with species of the genus *Trichoderma*, which have been used as a source of enzymes for digesting vegetables and releasing plant contents by attacking cellulose and hemicellulose in cell walls (Toyama, 1963).

Recently, cell-free cellulolytic enzyme preparations from species of *Trichoderma* have been shown to hydrolyse native or natural cellulose as effectively as do some of the most active intact cellulolytic micro-organisms. Cell-free preparations from *Trichoderma viride* or *Trichoderma koningii* produced extensive cellulolysis of the native cellulose of cotton (Mandels & Reese, 1964; Halliwell, 1965*b*) as well as of some derived forms of cellulose (Mandels & Reese, 1964; Halliwell, 1965*a*; Li, Flora & King, 1965). One of these reports followed an earlier suggestion that cellulolytic enzymes might produce physical changes in the substrate without extensive solubilization (Halliwell, 1962) and described conditions controlling one of the primary steps in the enzymic degradation of cellulose, namely the formation of insoluble short fibres (Halliwell, 1965*b*). In the present investigation, cell-free systems from *Myrothecium verrucaria* and *T. koningii* were used to examine the relationship between this random segmenting action of cellulolytic enzymes on native undegraded cellulose and secondary reactions producing more extensive solubilization of cellulose. In addition, partly degraded and highly degraded derived celluloses were tested for their susceptibility to breakdown by the cell-free preparations from *T. koningii*.

MATERIALS AND METHODS

Source of enzymes. Cultures of *M. verrucaria* and *T. koningii* (Halliwell, 1965*b*, 1961) were grown on cotton fibres and rendered cell-free by filtration through a sintered-glass bacteriological filter (Halliwell, 1965*b*). Cell-free enzyme preparations were obtained from mixed rumen bacteria by extracting with butan-1-ol (Halliwell, 1962).

Substrates. De-waxed and non-de-waxed Texas cotton fibres represent native undegraded cellulose, cellulose powder (Whatman) is a partly degraded cellulose and sodium CM-cellulose is a soluble substituted derivative of cellulose (Halliwell, 1961, 1962). Another partly degraded substrate, a hydrocellulose, was prepared from absorbent cotton wool and used as such or after grinding (see below). Highly degraded ground celluloses of this type are widely used as substrates for micro-organisms (see e.g. Hungate, 1950; Sugden, 1953; Abou Akkada, Eadie & Howard, 1963). For the present work, hydrocellulose and ground hydrocellulose were prepared as follows: absorbent cotton wool (22 g.) was immersed in 900 ml. of A.R. 10 N-HCl at 20° for 24 hr. The fine powder so formed was washed with water on a filter

under vacuum until the washings were neutral, and dried in air. A portion (2g. air dry wt., 6% moisture content) of the resultant air-dried hydrocellulose was mixed with water (50ml.) and ground in a pebble-mill for 16hr. at 20°. The ground cellulose was separated and washed with water by centrifuging at 1800*g* for 5min. and finally suspended in water. Dry weights were determined on samples that were rejected.

Determination of protein. The modification described by Halliwell (1961) was used. In terms of crystalline bovine plasma albumin (Armour Laboratories, Eastbourne, Sussex) cell-free preparations from *T. koningii* contained, on average, 0·58mg./ml., from *M. verrucaria* 0·68mg./ml. and from rumen micro-organisms 3mg./ml.

Estimation of the products of enzymic hydrolysis of native cellulose (cotton fibres), chemically derived celluloses and CM-cellulose. In some experiments (e.g. Fig. 2) the residual substrate and soluble and insoluble products of the enzymic degradation of the cellulose of cotton fibres were fractionated before analysis as follows. Insoluble products in the form of short fibres were separated from apparently unattacked native cotton fibres by preparing a 'combined extract of short fibres in water' (Halliwell, 1965*b*). The extract was centrifuged at 1800*g* for 5min. to sediment the short fibres and permit removal of some of the clear supernatant phase containing reducing sugars. Short fibres were separated from the remaining soluble material by filtering and washing on a filter-stick. Glucose, reducing sugars, insoluble short fibres produced from cotton cellulose, and the residual substrate itself were measured by glucose oxidase, potassium ferricyanide or by oxidation with dichromate on filter-sticks (Halliwell, 1965*b*). Cellobiose was determined from the difference between the readings given by the glucose oxidase reagent incubated for 1–17hr. at pH7·0 in 0·2M-sodium acetate–0·2N-acetic acid buffer and in tris hydrochloride buffer, final concn. 0·43M (see Table 1). Glucose and cellobiose were also sought for chromatographically in deionized concentrates of enzymic hydrolysates of cellulose. Paper chromatograms were developed with butan-1-ol–acetic acid–water (4:1:5, by vol.) or butan-1-ol–pyridine–water (10:4:3, by vol.) and sprayed with alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950).

Method of enzyme assay. Non-de-waxed or de-waxed cotton fibres or derived form of cellulose (2mg.) were incubated at 37° in unshaken centrifuge tubes with 4ml. of 0·2M-sodium acetate–0·2N-acetic acid buffer, pH4·8, up to 3ml. of cellulolytic enzyme preparation, and water to give final volume 9ml.

Sterility tests. These were done on enzymic digests of cellulose that had been incubated for prolonged periods. Samples of digest were incubated at 37° with glucose or cellobiose in nutrient broth to detect aerobic organisms and in cooked meat or thioglycollate media for anaerobic micro-organisms. Samples were also tested on cotton fibres at 28° under the conditions used to culture cellulolytic fungi (Halliwell, 1961) and at 37° under the conditions of the standard assay procedure for cell-free cellulolytic enzymes.

RESULTS

Enzymic hydrolysis of the native undegraded cellulose of cotton fibres by cell-free preparations from T. koningii *and* M. verrucaria. De-waxed cotton fibres were attacked somewhat more rapidly than non-de-waxed fibres by cell-free preparations from *T. koningii* but ultimately both forms were virtually completely solubilized with the intermediate formation of short fibres (Fig. 1).

With the sterile reagents and equipment and aseptic technique used here (○ and □ in Fig. 1) 98% solubilization of de-waxed cotton fibres took place in 24 days, measured by oxidation with dichromate of the residual fibres and of the soluble carbohydrates in the filtrate. Reducing sugar as glucose estimated by the ferricyanide or glucose oxidase procedures accounted for 95% of the initial cotton cellulose. Non-de-waxed cotton fibres with their waxy cuticle were more resistant and required about 40% more time than de-waxed fibres for 95%

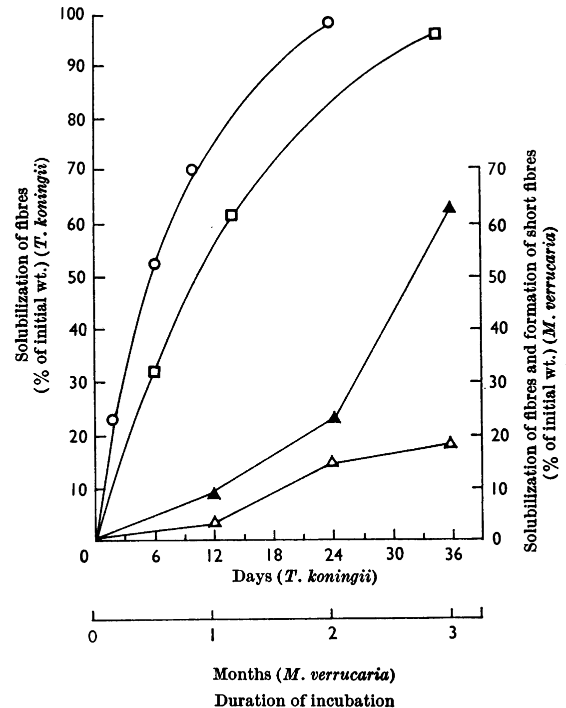

Fig. 1. Enzymic hydrolysis of cotton fibres to soluble products and insoluble short fibres by cell-free preparations from *T. koningii* and *M. verrucaria*. The standard cellulase assay was used to determine (1) the solubilization in 24–34 days of non-de-waxed cotton fibres (□) and of de-waxed cotton fibres (○) by 3ml. of enzyme preparation from *T. koningii*, and (2) the solubilization (△) of, and short fibre formation (▲) from, de-waxed cotton fibres in 3 months by 3ml. of enzyme preparation from *M. verrucaria*. Solubilization = 100% − (short fibres + residual cotton fibres). Left- and right-hand scales refer to *T. koningii* and *M. verrucaria* enzyme preparations respectively.

conversion into soluble products containing reducing sugars equivalent to 92% of the original substrate. Unsterilized cotton fibres (de-waxed and non-de-waxed) incubated in an otherwise completely sterile system as in Fig. 1 (○ and □) were hydrolysed at the same rate as the sterilized fibres (de-waxed and non-de-waxed respectively) of that Figure. Hence for routine assays all reagents except cellulose were sterilized before use. This avoided possible degradation of cellulosic substrates in the autoclave.

In the past, cell-free filtrates from *M. verrucaria* failed to produce the very extensive solubilization of cotton cellulose achieved by the intact microorganism. Filtrates from this organism were therefore re-examined to compare their action with that of cell-free preparations from *T. koningii* under the conditions defined above with cotton fibres. *Trichoderma* filtrates almost completely dissolved de-waxed cotton in 24 days (○ in Fig. 1) whereas cell-free preparations from *M. verrucaria* achieved less than 3% solubilization in the same period and only 18% in 3 months (△ in Fig. 1). *M. verrucaria* preparations were extremely variable in their cellulolytic activity, producing 3–18% dissolution of de-waxed fibres (2mg.) in any time from 0·8 to 5 months. During this period the same enzyme filtrates often produced extensive segmentation of the substrate as in Fig. 1 (▲), where de-waxed cotton fibres were transformed into 62% of short fibres and only 18% of soluble products in 3 months. In other experiments, almost the same degree of solubilization (17%) was accompanied by up to 83% conversion into short fibres, corresponding to complete breakdown of the original fibres. On other occasions, enzyme preparations from *M. verrucaria* dissolved up to 15% of cotton cellulose within 21 days but failed to give more than slight segmentation to short fibres. At no time, however, did cell-free filtrates from *M. verrucaria* solubilize more than 20% of cotton fibres even after all the substrate had been converted into short fibres. Differences in cellulolytic activity between cell-free preparations from *T. koningii* and *M. verrucaria* could not be attributed to the protein content (0·58 and 0·68mg./ml., as albumin, respectively).

Mode of hydrolysis of the native cellulose of cotton fibres by cell-free preparations from T. koningii. Use of a smaller quantity of enzyme than usual reduced cellulolysis and permitted easier fractionation of the products of intermediate stages in the conversion of fibrous cotton into sugars (Fig. 2).

Enzymic degradation of cotton cellulose was associated from the earliest stages with the formation of reducing sugars. These were followed in about 3 days by the first visible signs of breakdown of the cotton as shown by the appearance of short fibres at a stage when approx. 15% of the initial substrate had been hydrolysed to reducing sugars. Thereafter loss of the fibrous structure of the original cotton was rapid and reached completion within 12 days. Formation of soluble and insoluble breakdown products of cellulose proceeded concurrently for about 9 days until the short fibres had reached a maximum value corresponding to approximately half of the 93% total breakdown of the substrate. Subsequently, the short fibres themselves were solubilized enzymically and contributed to the continued rise in reducing sugar values. In the early stages of hydrolysis the amount of anhydroglucose recovered in the soluble and insoluble degradation products was occasionally slightly less than that determined from the loss in weight of the substrate. The difference arose mainly from the escape of small quantities of insoluble non-fibrous cellulose (Halliwell, 1965*b*) through the filter-sticks, as shown by measurements made with the dichromate reagent on the total soluble and insoluble carbohydrates in filtrates from washed short fibres.

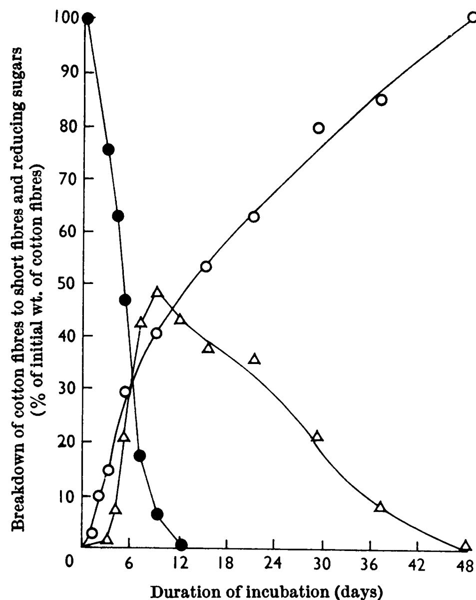

Fig. 2. Enzymic formation and solubilization of short fibres from de-waxed cotton fibres by cell-free preparations from *T. koningii*. The standard cellulase assay was used with 1 ml. of enzyme preparation. ●, Residual cotton fibres; ○, reducing sugars formed; △, short fibres formed. Fractions were separated as described in the Materials and Methods section.

Throughout the incubation period the ferricyanide and glucose oxidase–peroxidase assays usually gave similar values, indicating that the principal reducing sugar was glucose. This was also shown by measuring glucose in the soluble fraction

Table 1. *Estimation of glucose and cellobiose with glucose oxidase (containing cellobiase) and peroxidase*

Glucose or cellobiose (0–100 μg.) was incubated with the glucose oxidase–peroxidase–anisidine reagent at pH 7·0 in acetic acid–acetate buffer (final concn. 0·027 N) or in tris buffer (final concn. 0·43 M) as described in the Materials and Methods section. The coloured reaction product was measured after incubating for 1 hr. at 37° and again after a further 16 hr. at 20°. Readings are given only for the maximum quantity of reducing sugar (100 μg.).

Sugar	Buffer pH 7·0	*E* After 1 hr.	*E* After 17 hr.	*E*(17 hr.)/*E*(1 hr.)
Glucose	Acetate	0·455	0·56	1·23
Cellobiose	Acetate	0·03	0·30	10·0
Glucose	Tris	0·43	0·53	1·23
Cellobiose	Tris	0	0	0

by glucose oxidase and peroxidase in tris buffer or in acetate buffer to inhibit or permit respectively cellobiase activity. Overnight incubation in acetate buffer confirmed the absence of cellobiose (Table 1).

The Table illustrates that both acetate and tris buffers at pH 7·0 are equally suitable for estimating glucose but only tris buffer, final concentration 0·43 M, inhibits cellobiase activity in the glucose oxidase preparation (Dahlqvist, 1961). Simultaneous assays of enzymic hydrolysates of cellulose in acetate and tris buffers at pH 7, together with appropriate calibration curves for glucose and cellobiose, enable mixtures of these two sugars to be determined by difference. For the present work the cellobiase activity of glucose oxidase preparations was increased 50% by using acetate buffer at pH 5·5 instead of pH 7 (Halliwell, 1965*b*).

Effect of pH *on the solubilization of de-waxed cotton fibres by cell-free preparations from* T. koningii. Enzyme preparations from *T. koningii* were most effective at pH 5·0 in segmenting cotton fibres mainly into short fibres with minor solubilization (Halliwell, 1965*b*). Extensive solubilization takes place most readily at pH 4·2–4·6 and appears to be partly dependent upon the formation of short fibres (Fig. 3). Thus after 15 days' incubation at pH 2·5–3·0 few short fibres were present, at pH 3·7–5·7 complete transformation into short fibres and soluble products had occurred, whereas at higher pH values short fibres were still plentiful with much of the original cotton apparently intact.

Enzymic activity, determined by loss of weight of cotton fibres, formation of reducing sugars and of glucose, was confined mainly to pH 3·7–5·8, under which conditions relatively rapid hydrolysis of substrate and intermediate products allowed little

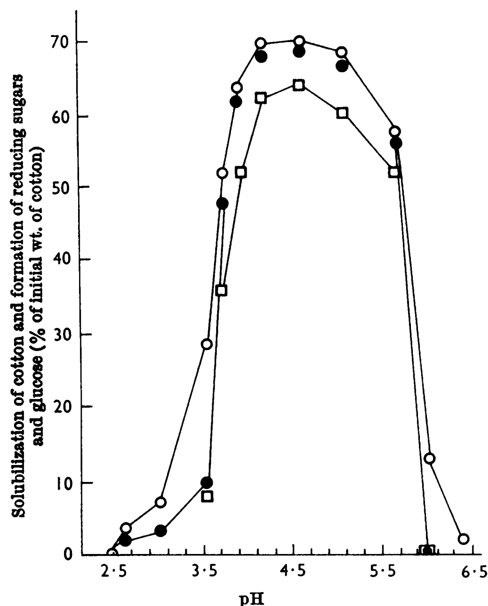

Fig. 3. Effect of pH on the enzymic solubilization of de-waxed cotton fibres and short fibres by cell-free preparations from *T. koningii*. Enzymic activity was measured in the standard cellulase assay with acetic acid–acetate buffer, pH 3·7–6·4, and citric acid–phosphate buffer below pH 4·8. The results were indistinguishable in the two buffers at pH 3·7–4·8. Cotton fibres and enzyme were incubated for 15 days and centrifuged to separate the clear supernatant phase from insoluble short fibres and residual cotton fibres. Both forms of fibres were estimated together as residual cellulose. Cellulose solubilized (○). Reducing sugars (●) and glucose (□) found in the supernatant phase were estimated by ferricyanide and glucose oxidase respectively.

if any accumulation of filter-passing, insoluble, non-fibrous cellulose after 15 days' incubation. Hence the amount of reducing sugars formed and cotton solubilized (insoluble short fibres were included with unattacked substrate) were very similar, indicating that most of the soluble material was glucose. The latter and traces of cellobiose, estimated as in Table 1, accounted for all products in the hydrolysate. At lower pH values the reducing sugars formed constituted a relatively smaller fraction of the total cotton solubilized owing to loss through the filter of non-fibrous cellulose, a characteristic product of the enzymic hydrolysis of cotton fibres at about pH 3·5 (Halliwell, 1965*b*).

Although 0·1 M-citric acid–0·2 M-disodium phosphate was used in Fig. 3 for the region pH 2·5–4·8, other experiments, not illustrated, showed that this buffer and 0·2 N-acetic acid–0·2 M-sodium acetate were equally effective at pH 3·7–4·8 in assisting cell-free preparations from *T. koningii* to solubilize

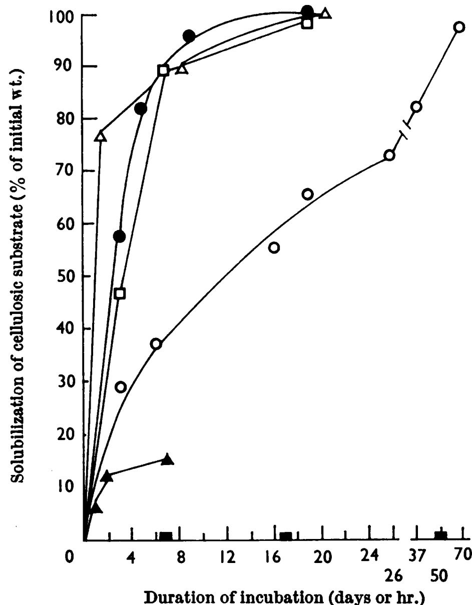

Fig. 4. Hydrolysis of cotton fibres and derived forms of cellulose by enzyme preparations from micro-organisms. The standard cellulase assay was used with cell-free preparations from *T. koningii* to follow the extent of solubilization of de-waxed cotton fibres (●) in 19 days, cellulose powder (Whatman) (□) in 19 days, hydrocellulose (○) in 70 days and ground hydrocellulose (△) in 21 hr. Cell-free preparations from rumen micro-organisms were tested for solubilization of hydrocellulose (■) in 50 days and ground hydrocellulose (▲) in 7 days. Solubilization was measured by loss in weight of the substrate or by the potassium ferricyanide reaction or both.

cotton fibres. At pH 4·8, for example, either buffer enabled the enzyme preparation to achieve 70–73% and 96–99% solubilization of de-waxed cotton (2 mg.) in 5 and 16 days respectively. In acetate buffer, pH 4·8, cell-free preparations from *T. koningii* attained the same degree of hydrolysis of cotton fibres in tubes open to the atmosphere as in completely sealed tubes.

Action of cell-free preparations from T. koningii *on chemically derived forms of cellulose.* Cellulose powder (Whatman) was hydrolysed completely and at the same rate as cotton fibres by cell-free filtrates from *T. koningii* (□ and ● in Fig. 4). Under the stationary conditions of incubation used, hydrocellulose, unlike loosely packed cotton fibres, adopted a compact form on the base of centrifuge tubes and was more resistant to hydrolysis in requiring up to 70 days for complete solubilization. The method of preparation of the hydrocellulose tended to enrich the product in the larger particles and to reject fine material in the washings. As a result the hydrocellulose, like cellulose powder (Whatman), was fully retained when washed with water on a grade 3 sintered-glass filter (nominal pore size 15–40 μ). Hydrocellulose that had been ground for 16 hr. at 20° was a very finely divided powder which dispersed readily in water and mostly passed through a grade 3 filter. The ground hydrocellulose, used unsterilized or after sterilizing in the autoclave, was hydrolysed very much more rapidly than the parent unground material and lost 50% and all of its weight in 1 hr. and 21 hr. respectively in the presence of cell-free preparations from *T. koningii*. Throughout the hydrolysis of ground and unground hydrocellulose the soluble products consisted wholly of glucose. Cellobiose was absent as shown by chromatography and the glucose oxidase procedure of Table 1. In contrast with the *Trichoderma* enzymes, cell-free preparations from mixed rumen micro-organisms were totally ineffective against the hydrocellulose during a period of 50 days and required up to 7 days to attain 15% solubilization of the ground substrate. It appears that butanol extracts of mixed rumen bacteria are reluctant to attack insoluble cellulose, as was found earlier with cellulose powder (Whatman) (10% solubilization) and swollen cellulose powder (unattacked), although the same extracts readily hydrolysed the soluble CM-cellulose (Halliwell, 1957, 1962).

Chromatographic analysis of enzymic hydrolysates of cotton fibres and CM-cellulose. Cell-free enzyme preparations from the three micro-organisms described above were active against CM-cellulose. Enzyme preparations from *T. koningii*, for example, hydrolysed the soluble cellulose derivative mainly to glucose and smaller but appreciable quantities of cellobiose whereas fibrous cotton was converted almost entirely into glucose with occasional traces of cellobiose.

DISCUSSION

Non-de-waxed cotton fibres consist of cellulose in a form that is characteristic of the undegraded polysaccharide as it is encountered in Nature by micro-organisms. Such fibres were virtually completely (95%) dissolved to sugars after incubation for 34 days with cell-free enzyme preparations from *T. koningii*. Careful purification of cotton fibres removes wax and pectins, most of which are located in the cuticle or primary wall of the fibre (Siu, 1951), and permits cellulolytic enzymes to act more effectively. As a result, cell-free preparations from *T. koningii* achieved complete saccharification of de-waxed cotton in about 70% of the time required for non-de-waxed fibres. Otherwise enzymic degradation of de-waxed and non-de-waxed cotton cellulose followed the same pathway of random hydrolysis with the formation of an insoluble

cellulosic intermediate, short fibres, in amounts up to 50% of the weight of the original substrate. Short fibres in turn were finally transformed into glucose by cellulolytic enzyme preparations containing cellobiase.

Under experimental conditions similar to those used with the *Trichoderma* enzymes, but with a fourfold increase in the incubation period, cell-free filtrates from *M. verrucaria* also brought about total breakdown of de-waxed cotton fibres to soluble products (20%) and to short fibres which accumulated to 80% of the weight of the initial substrate. As the protein contents of the cell-free preparations from *T. koningii* and *M. verrucaria* were similar, it appears that enzyme preparations from *M. verrucaria* contained much less of the cellulolytic enzyme system than did preparations from *T. koningii*. Alternatively, that part of the enzyme system of intact *M. verrucaria* which is known to be effective in producing nearly complete solubilization of cotton cellulose may be necessary for solubilizing short fibres, but is virtually absent from cell-free filtrates of this organism. The latter suggestion gains support from the following evidence: first, in this and other Laboratories filtrates from *M. verrucaria* are unable to match the solubilizing action of cell-free filtrates from *T. koningii* on cotton cellulose; secondly, the variation in cellulolytic activity towards cotton fibres of our cell-free preparations from *M. verrucaria* contrasts with preparations from *T. koningii*; thirdly, repeated additions of enzyme preparations from *M. verrucaria* during incubation with cotton fibres failed to imitate the extensive solubilizing activity of cell-free preparations from *T. koningii*. The latter enzyme preparations when used in smaller quantities than that of the standard assay procedure solubilized cotton. Thus 4ml. and 0·8ml. of cell-free filtrate from *T. koningii* transformed de-waxed cotton fibres (2mg.) completely into soluble products (56%) and short fibres in 7 and 10 days respectively.

The derived celluloses varied widely in their rate of hydrolysis, although all were completely solubilized by cell-free enzymes from *T. koningii*. Cellulose powder (Whatman) and the hydrocellulose each contain approximately 16% or more of $\beta+\gamma$ cellulose (Halliwell & Bryant, 1963), but, whereas the former was completely solubilized at the same rate as cotton fibres (19 days), hydrocellulose required almost four times that period. No attempt was made to facilitate the enzymic hydrolysis of the hydrocellulose by selecting or retaining very finely divided material when preparing the substrate. Addition of further quantities of enzyme when 70–80% of the hydrocellulose had been dissolved did not accelerate the reaction. However, complete solubilization to reducing sugars was achieved in 21hr. by the same enzyme preparations acting on the ground hydrocellulose.

The method of preparation, as well as the origin of a cellulosic substrate, evidently plays an important part in its susceptibility to enzymic hydrolysis. This is also apparent in results elsewhere showing that very finely dispersed crystalline hydrocellulose or the smallest particles of a commercial product, Avicel, were much more sensitive to enzymic attack than the normal product (Li *et al.* 1965 and personal communication). Mandels & Reese (1964) found that filter paper was attacked two to three times as rapidly as cotton sliver or cotton duck by the cellulase system from *T. viride*, whereas the same enzyme preparation solubilized the ground cotton products more slowly and more rapidly respectively than the unground material.

The cell-free microbial cellulase systems used in the present work achieve total enzymic degradation of the undegraded cellulose of cotton fibres in a series of reactions. In the first of these, complete breakdown is accomplished by enzyme preparations from *M. verrucaria* and *T. koningii*, which, at the optimum pH 5·0, randomly segment cellulose mainly to insoluble products in the form of short fibres and to minor quantities of soluble products. In subsequent reactions enzyme preparations from *T. koningii*, but not from *M. verrucaria*, solubilize the short fibres to sugars at the optimum pH 4·2–4·6.

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