Biochem. J. (1982) **205**, 129–137
Printed in Great Britain

Preparation of the cellulase from the cellulolytic anaerobic rumen bacterium *Ruminococcus albus* and its release from the bacterial cell wall

Thomas M. WOOD, Catriona A. WILSON and Colin S. STEWART
Department of Microbial Biochemistry, Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K.

(*Received 14 December 1981/Accepted 17 March 1982*)

1. Most of the cellulase (CM-cellulase) elaborated by the rumen bacterium *Ruminococcus albus* strain SY3, which was isolated from a sheep, was cell-wall-bound. 2. The enzyme could be released readily by washing either with phosphate buffer or with water. 3. The amount of enzyme released was affected by the pH and ionic strength of the phosphate buffer. 4. The cell-wall-bound enzyme was of very high molecular weight ($\geqslant 1.5 \times 10^6$) as judged by its chromatographic behaviour on Sephacryl S-300. 5. The molecular weight of the extracellular enzyme was variable and depended on the culture conditions. 6. When cellobiose was used as the energy source and the medium contained rumen fluid (30%), the extracellular enzyme was, in the main, of high molecular weight. 7. When cellulose replaced the cellobiose, the cell-free culture filtrate contained only low-molecular-weight enzyme (M_r approx. 30000) in late-stationary-phase cultures (7 days). 8. Cultures that did not contain rumen fluid contained mainly low-molecular-weight enzyme. 9. Under some conditions the high-molecular-weight enzyme could be broken down to some extent into low-molecular-weight enzyme by treatment with dissociating agents. 10. Cell-free and cell-wall-bound enzymes showed the same relationship when the change in fluidity effected by them on a solution of CM-cellulose was plotted against the corresponding increase in reducing sugars, suggesting that the enzymes were the same. 11. It is possible that *R. albus* cellulase exists as an aggregate of low-molecular-weight cellulase components on the bacterial cell wall and in solution under certain conditions.

Ruminococcus albus is one of the most important cellulolytic bacteria found in the rumen. It occurs along with the cellulolytic bacteria *Ruminococcus flavefaciens* and *Bacteroides succinogenes* in proportions that vary according to the diet (Mackie *et al.*, 1978; Akin, 1980). With good-quality diets ruminococci are predominant; it is assumed that *B. succinogenes* proliferates with fodders that are difficult to digest. *R. flavefaciens* and *B. succinogenes* can ferment the most highly ordered substrates, such as the archetypal cotton fibre (Stewart *et al.*, 1981); most strains of *R. albus*, in contrast, can utilize only substrates in which the cellulose is present in a more disordered form. The particular strain of *R. albus* (SY3) used in the present study showed no action on cotton but rather surprisingly fermented the highly crystalline Avicel.

Of the cellulolytic bacteria found in the rumen, only *R. albus* can be cultured on a regular basis in synthetic media to yield reasonably large amounts of extracellular cellulase. Some strains of *R. flavefaciens* are reported to produce extracellular cellulase in quantity (Pettipher & Latham, 1979), but none of the strains isolated in this Institute do so. *B. succinogenes* never produces large amounts of extracellular cellulase *in vitro*.

Cell-free cellulases from *R. albus* have been reported (Leatherwood, 1965; Smith *et al.*, 1973) to be able to degrade the soluble derivative of cellulose, or cellulose that has been partially disordered by physical or chemical treatment, but highly ordered cellulose is not hydrolysed to any significant extent: the cellulase of *R. albus* SY3 is similar in all of these respects.

Much of the cellulase of *R. albus* SY3 is cell-wall-bound, but it is readily released by suitable manipulation of the cell. In the present paper we discuss the preparation of the extracellular and cell-bound cellulases and the effect of the composition of the media on the molecular weights of the enzymes.

Experimental

Materials

Ruminococcus albus strain SY3 was isolated in March 1976 from a clearing in an anaerobic

cellulose roll-tube (van Gylswyk, 1970) that had been inoculated with a 10^6-fold dilution of rumen contents from a sheep fed on a roughage diet. H_3PO_4-swollen cellulose was prepared from absorbent cotton wool by the method of Walseth (1952) or as described by Wood (1971). Powdered filter paper was prepared from Whatman no. 1 filter paper (Whatman, Maidstone, Kent, U.K.) by grinding (dry) in a rotary porcelain ball mill (Pascall Engineering Co., Crawley, Sussex, U.K.) for 7 days. Cotton fibres (slivers) were purchased from the Shirley Institute (Didsbury, Manchester, U.K.), and were dewaxed by a modification of the method of Corbett (1963). Cellofas B (sodium CM-cellulose) was a gift from I.C.I. Nobel Division (Stevenston, Ayrshire, Scotland, U.K.). Sephacryl was purchased from Pharmacia (London W.5, U.K.).

Bacto Casitone and Bacto yeast extract were supplied by Difco Laboratories (Detroit, MI, U.S.A.). DL-2-Methylbutyric acid was from the Eastman Kodak Co. (Rochester, NY, U.S.A.). MN-300 cellulose powder was purchased from Macherey Nagel and Co. (Düren, W. Germany), and Avicel was from Honeywill and Stein (Wallington, Surrey, U.K.). All other chemicals were purchased either from Sigma Chemical Co. (Kingston-upon-Thames, Surrey, U.K.) or from BDH Chemicals (Poole, Dorset, U.K.). BDH chemicals were of analytical-reagent grade, except for the volatile fatty acids and K_2HPO_4, which were of laboratory grade. Sigma chemicals were of the purest grade available. Molecular-weight markers were obtained from the sources indicated (Wood & McCrae, 1978).

Anaerobic culture methods

Media for stock cultures and for growth experiments were prepared and maintained in culture tubes and flasks (see below) under 100% CO_2 by using the strictly anaerobic technique of Bryant (1972). Larger cultures (800ml) for enzyme preparations were prepared under CO_2 by the method of Allison *et al.* (1962).

Culture maintenance

Between the date of isolation and December 1977, the organism was maintained by transfer of stock cultures at approximately 1-monthly intervals. Stock cultures were 10ml broths of HS medium (see below) containing 0.5% (w/v) cellobiose in anaerobic Hungate tubes with butyl-rubber-septum stoppers (Bellco, Vineland, NJ, U.S.A.). From December 1977, cultures were preserved by freeze-drying them (Lapage *et al.*, 1970) in an Edwards B5A Freeze-drier (Edwards High Vacuum, Crawley, Sussex, U.K.) with a vacuum during secondary drying of between 7 and 3Pa (0.05 and 0.02 Torr).

Composition of culture media

Both semi-defined (SD) and habitat-simulating (HS) media were used. The semi-defined medium contained cellobiose or cellulose at the concentration specified in the text and (per litre) 3.0g of Bacto-Casitone, 0.45g each of KH_2PO_4 and K_2HPO_4, 0.9g each of NaCl and $(NH_4)_2SO_4$, 0.09g each of $CaCl_2$ and $MgSO_4$, 0.4g of $NaHCO_3$, 66mg of isobutyric acid, and 80mg each of n-valeric acid, isovaleric acid and 2-methylbutyric acid. Cysteine hydrochloride (reducing agent) resazurin (oxidation–reduction indicator) and vitamins were also added at the concentrations used by Scott & Dehority (1965). The HS medium was a modification of medium 2 described by Hobson (1969), with cellobiose or cellulose at the concentration specified in the text in place of the carbohydrates in the original formulation. Clarified rumen fluid, prepared from rumen contents by filtration through surgical gauze and centrifuging at 75000*g* for 1h, was added to the HS medium to a final concentration of 30% (v/v). Strain SY3 did not grow well on medium SD when maintained continuously on this medium, and so the SD medium was inoculated with cultures grown on the HS medium. The carry-over of nutrients from the inoculum would therefore have been an important factor in the growth of strain SY3 on medium SD. The use of SD medium in this way allowed the growth of strain SY3 in the presence of minimal quantities of rumen fluid.

Analytical methods

Microbial protein was determined by the Folin–Ciocalteu method and DNA by the diphenylamine method as described by Herbert *et al.* (1971). Cellulose was determined by the method of Updegraff (1969). All other assay methods apart from those mentioned below were as detailed elsewhere (Wood & McCrae, 1978).

Measurement of enzyme actions on various cellulosic substrates

Cellofas B CM-cellulose was used to measure CM-cellulase activity (Wood & McCrae, 1977) unless stated otherwise. Sigma CM-cellulose was the low-viscosity grade. The activity on insoluble celluloses was determined by incubating (at 37°C for 7 days) 50mg of substrate, 5ml of 0.1M-KH_2PO_4/NaOH buffer, pH6.68, 0.2ml of 50mM-NaN_3 and enzyme and water to give a total volume of 10ml. The supernatants were assayed for reducing sugar, glucose and total sugar by using the methods described elsewhere (Wood *et al.*, 1980).

Preparation of enzyme

Cultures were centrifuged (at 76000*g* for 20min) and the enzyme was precipitated immediately by the

addition of solid $(NH_4)_2SO_4$ at 1°C until the solution was 85% saturated. The precipitate was collected by centrifugation (at 76000*g* for 20min), dissolved in 0.1M-KH_2PO_4/NaOH buffer, pH6.5, containing 0.02% NaN_3, to give a 5–20-fold concentrate, and stored at −18°C.

The $(NH_4)_2SO_4$ precipitation had to be performed as soon as possible after removal of the cells as the crude enzyme was relatively unstable, 20% of its activity towards CM-cellulose being lost after 18h storage at 7°C and 40% being lost after 6 days. Concentrated partially purified [by $(NH_4)_2SO_4$ precipitation] enzyme was much more stable and could be stored at 10°C for several weeks without significant loss of activity. At −18°C there was no detectable decrease in activity in 6 months. Approx. 85% of the original cell-free cellulase was recovered by precipitation with $(NH_4)_2SO_4$; freeze-drying was equally effective.

The cellulase activity of the concentrated partially purified enzyme preparation was not altered by centrifugation at 100000*g* for 2h, suggesting that cell wall fragments and large membrane structures associated with cellulase activity were not present.

Enzymes from four different media were prepared. These are designated cellobiose–HS medium, cellobiose–SD medium, cellulose–HS medium and cellulose–SD medium in the Results section. HS and SD are the abbreviations for habitat-simulating and semi-defined media respectively, as already defined above.

Release of cell-bound cellulose with buffer or water

The cells from 800ml cultures incorporating cellobiose as an energy source were isolated by centrifugation (at 76000*g* for 20min), suspended in 50ml of KH_2PO_4/NaOH buffer, pH6.67 and *I* 0.1, or water, and kept at room temperature for 30min before centrifugation (as above). The supernatants were assayed for CM-cellulase activity as indicated above.

Effects of pH and ionic strength on the release of cell-bound enzyme

After isolation of the cells as described above, the pellets were dispersed in 55ml of KH_2PO_4/NaOH buffer, pH6.67 and *I* 0.1, and a 10ml sample was pipetted into each of five 15ml centrifuge tubes. After centrifuging (at 76000*g* for 20min) the supernatants were removed and the cells were suspended in 10ml of KH_2PO_4/NaOH buffer, *I* 0.1, of different pH values (5.86, 6.47, 6.89, 7.5 or 7.92) to test the effect of pH on the release of enzyme.

The effect of ionic strength on the release of enzyme was determined by suspending the cells in 10ml of KH_2PO_4/NaOH buffer, pH6.67, of various ionic strengths from 0.005 to 0.5.

Transmission electron microscopy

Bacterial cells were treated with Ruthenium Red and prepared and examined with the electron microscope by a minor modification of the method of Dinsdale *et al.* (1978).

Results

Cellulolysis by R. albus

Table 1 shows the loss in weight (solubilization) of different forms of cellulose on incubation with strain SY3 for up to 7 days, together with an estimate (DNA accumulation) of the growth of the bacterium. The less-highly ordered substrates H_3PO_4-swollen cellulose and ground filter paper were more rapidly and extensively degraded and supported more growth than did the relatively highly ordered substrates Avicel and MN-300 cellulose powder. The data in Table 1 are the averages of four experiments performed approximately 3 years after

Table 1. *Solubilization of cellulose and accumulation of DNA from R. albus strain SY3*

Known amounts of cellulose (approx. 125mg) were weighed or, for suspensions in water of H_3PO_4-swollen cellulose and powdered filter paper, pipetted into anaerobic flasks of 70ml nominal capacity. HS medium (without sugars) was added to the flasks under strictly anaerobic conditions to a final volume of 50ml. After being autoclaved (at 121°C for 15min) the flasks were cooled and inoculated with 2ml of an overnight culture of strain SY3. After incubation at 39°C for the times indicated the contents of the flasks were harvested and analysed. The methods of analysis, origins of the celluloses, HS medium and the anaerobic flasks and methods are described in the Experimental section.

	Cellulose* fermented (%)			DNA content (μg/ml of culture)		
Substrate — Incubation period (days) ...	1	3	7	1	3	7
H_3PO_4-swollen cellulose	50.0	77.5	97.4	11.0	10.0	8.0
Ground filter paper	10.0	55.0	63.0	16.0	10.2	6.0
Avicel	0.0	15.3	21.8	2.8	4.4	2.8
MN-300 cellulose	0.0	19.4	37.6	3.8	4.0	2.8
Dewaxed cotton	0.0	0.0	0.0	Not studied		

* Initial amount 125mg in 50ml of medium.

the first isolation of strain SY3. It was clear that by this stage the bacterium had lost some of its original activity, since when studied 18 months before the experiments summarized in Table 1 strain SY3 was able to cause more extensive solubilization. Thus in these (two) earlier experiments the solubilization that occurred on incubation for 7 days was as follows: H_3PO_4-swollen cellulose, 95.3%; ground filter paper, 87.8%; Avicel, 72.2%; MN-300 cellulose powder, 56%. Although strain SY3 was not able to cause detectable solubilization of dewaxed cotton fibres, it did retain the ability to cause losses in tensile strength of dewaxed cotton yarn for at least 2 years. When dewaxed cotton yarn (J. and P. Coates, Paisley, Scotland, U.K.) replaced cellobiose in HS medium, incubation in the presence of strain SY3 decreased the tensile strength of the yarn by 30% in 8 days. Continued incubation for up to 28 days resulted in a further 10% loss in the tensile strength of yarn. The ability to cause losses in tensile strength of cotton yarn was eventually lost.

Electron microscopy of bacterial cells

When strain SY3 was viewed by transmission electron microscopy (Plate 1) it was noted that the cells varied in size from about 600 to 1500nm in diameter. On first isolation the cells possessed a thick polyanionic extracellular coat or capsule (Plate 1*a*). This coat was much thinner in cells from more recent cultures (Plate 1*b–e*). Cells of strain SY3 grown in HS medium differed from these grown on SD medium (Plate 1*c*) in that the latter tended to show more extensive formation of cross-walls without separation of the resultant cells. Further differences were seen after the cells were washed to extract the cellulase, when the walls of cells grown on HS medium became irregular (Plate 1*d*) whereas the cells grown on SD medium maintained a smooth configuration of the wall but showed the development of electron-dense regions in the centre of the cells (Plate 1*e*).

Production of enzyme

Of the energy sources tested, cellobiose and H_3PO_4-swollen cellulose were the most effective for the production of extracellular enzyme. HS medium was better than SD medium (Table 2).

Enzyme production continued long after the end of the exponential growth phase, which was 6h for cellobiose cultures and 12h for cellulose (H_3PO_4-swollen) cultures. In some cultures in which cellulose was the energy source nearly 10 times as much enzyme was produced in the stationary phase as in the exponential phase (Table 2).

Release of cell-bound cellulase

Most of the cellulase (CM-cellulase) found in various cultures was cell-wall-bound. In cellobiose–HS-medium cultures the cell-bound component was about 90–94%: in cellobiose–SD-medium cultures it was lower (85–90%) as a rule. Much of the enzyme (80–88%) could be released simply by repeated washing of the isolated cells with buffer. Water was also capable of releasing enzyme, but it was less efficient than buffer, seven consecutive treatments releasing 40–45% of the cell-bound enzyme. Of the cell-wall-bound enzyme remaining after seven treatments with buffer (12–20%) or water (55–60%), 90% could be released by a 5min ultrasonic treatment.

The pH and ionic strength of the buffer used to wash the bacterial cells had a profound effect on the amount of enzyme released. It was necessary, however, to go beyond the physiological range of pH and ionic strength to achieve optimal release. With the cells cultured in cellobiose–HS medium, 33% more enzyme was released at pH7.5 than at pH5.86; with the cells found in the cellobiose–SD medium the increase in enzyme released was 70%. At pH7.5 and at room temperature twice as much enzyme appeared in solution when the ionic strength of the buffer was raised from 0.1 to 0.5.

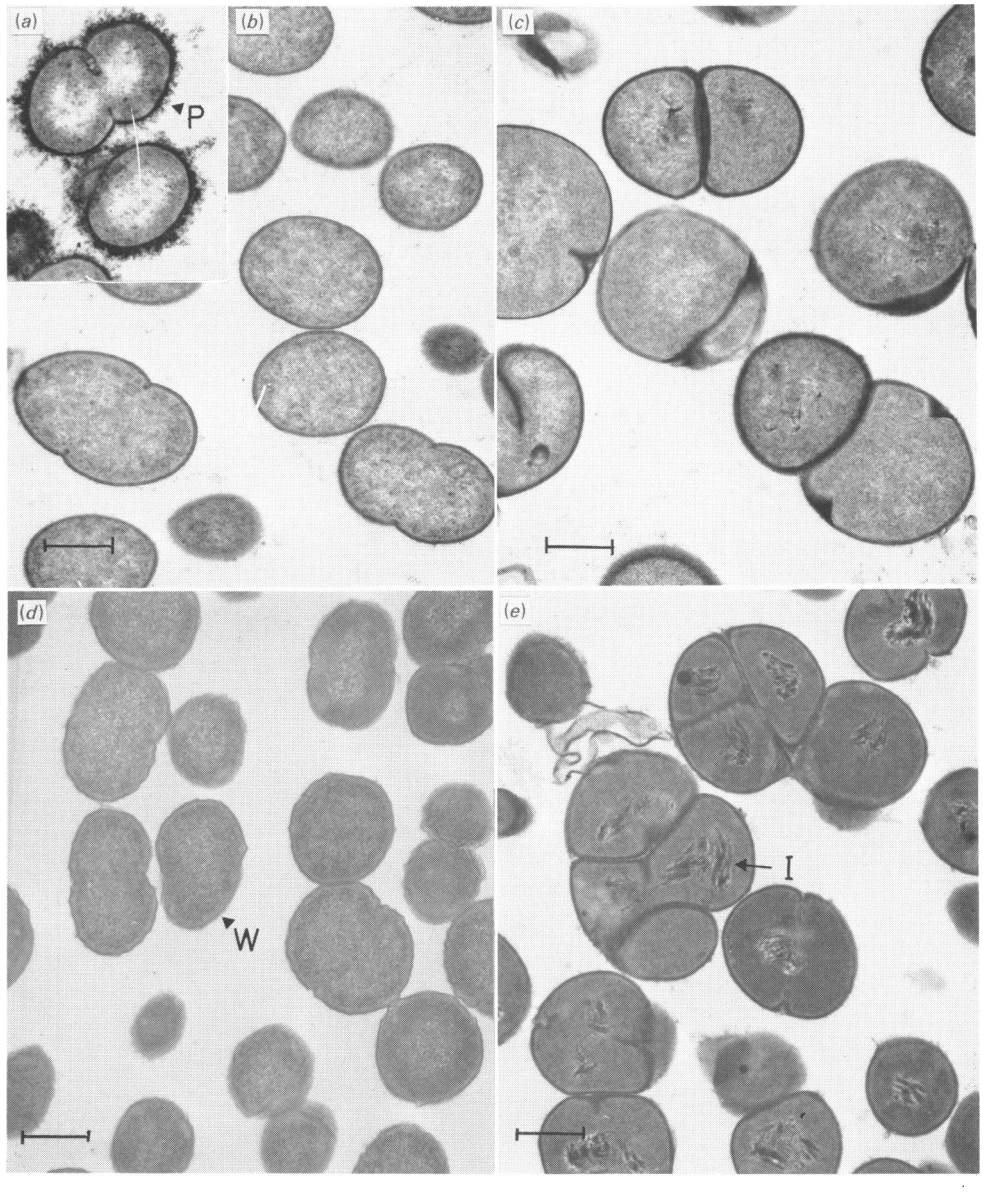
Gel filtration of the cell-free and cell-wall-bound cellulases

Extracellular cellulase from all four types of culture, as well as cell-wall-bound enzymes from washed cells and cell-wall-bound enzymes released from sonicated cells, were compared on a column of Sephacryl S-300. Fig. 1 shows an elution pattern

Table 2. *Comparison of amounts of extracellular enzyme produced by R. albus strain SY3 in different media*
Cultures were prepared as described in the Experimental section. The exponential phase ended after 6h for cellobiose cultures and after 12h for the cellulose cultures. Cellulase activity was measured as described in the text.

	Cellulase (CM-cellulase) activity (units/ml)	
	At end of exponential phase	During stationary phase*
Cellobiose–SD medium	17	22
Cellobiose–HS medium	25	40
H_3PO_4-swollen cellulose–SD medium	15	147
H_3PO_4-swollen cellulose–HS medium	31	382

* 24h for cellobiose cultures; 7 days for cellulose cultures.



EXPLANATION OF PLATE 1

Thin sections of R. albus strain SY3 cells stained with Ruthenium Red and examined by transmission electron microscopy after growth for 24 h in batch cultures containing 0.5% (w/v) cellobiose

(*a*) Cells grown in HS medium approx. 1 year after isolation, showing extracellular polyanionic capsule (P). (*b*) Cells grown in HS medium approx. 4 years after initial isolation. (*c*) Cells grown in SD medium approx. 4 years after initial isolation. (*d*) Cells from (*b*) after washing with phosphate buffer to extract cellulase, showing irregular outer wall (W). (*e*) Cells from (*c*) after washing with phosphate buffer showing intracellular electron-dense regions (I). The preparation of HS and SD media is given in the Experimental section. Bars indicate 500 nm.

typical of that obtained from the gel filtration of the extracellular cellulase found in either cellulose–SD-medium cultures or cellobiose–SD-medium cultures that had reached the stationary phase (48h). The major CM-cellulase component in each case had the same mobility on the column as a protein of mol.wt. approx. 30000–35000; the minor component (tubes 59–61 in Fig. 1) was excluded from the gel (exclusion limit mol.wt. 1.5×10^6). In one extracellular enzyme preparation from another cellobiose–SD-medium culture, which had been frozen before precipitation with $(NH_4)_2SO_4$, a major component of mol.wt. 70000 was separated from the enzyme of mol.wt. 35000 on a column of Ultrogel AcA-44; this enzyme was present as a minor component in several other culture filtrates, but in the main it was absent.

The high-molecular-weight component (excluded from Sephacryl S-300), which was an insignificant feature of the extracellular enzyme found in cultures incorporating SD medium, was a major enzyme component in stationary-phase (48h) cultures in which HS medium was used (Fig. 2). The relative proportions of the high-molecular-weight and low-molecular-weight cellulase components varied, however, according to the energy source when HS medium was used. Whereas in those cultures in which cellobiose was used the high-molecular-weight enzyme was predominant, the use of cellulose resulted in the formation of an enzyme system in which the low-molecular-weight component was the major enzyme.

The enzyme released from the cells by extraction with buffer consisted almost entirely of the high-molecular-weight component: this was true of cells isolated either from a cellobiose–HS-medium culture or a cellobiose–SD-medium culture. There was no diminution in the activity of the cellulase in the buffer extract after centrifugation at 100000*g* for 2h, suggesting that particulate matter was absent.

Changes in composition of extracellular cellulase during culture and induced by chemical treatment

When the extracellular enzyme from cellobiose–HS-medium cultures was treated (at 40°C for 18h) with urea or guanidinium chloride under conditions

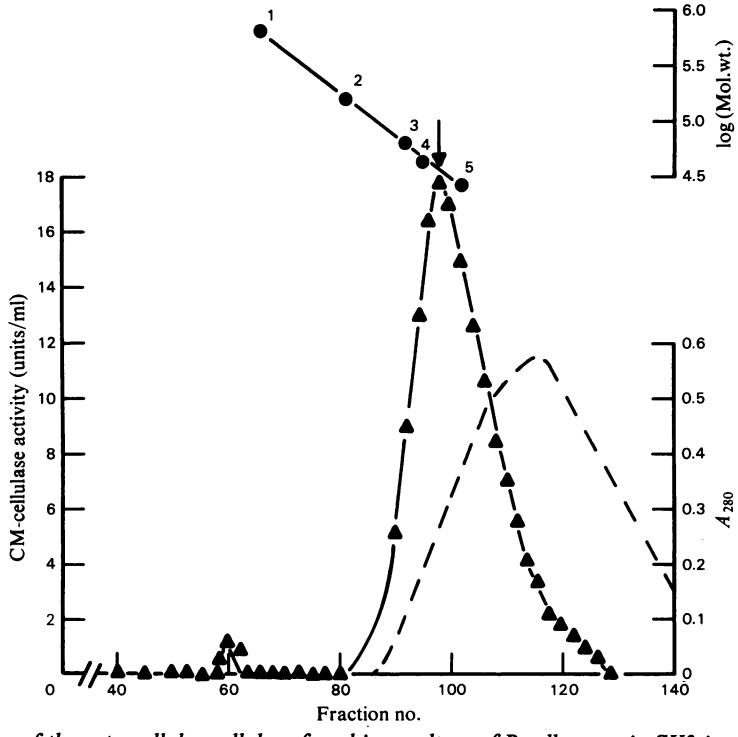


Fig. 1. *Gel filtration of the extracellular cellulase found in a culture of R. albus strain SY3 in semi-defined medium with cellulose as an energy source*

The enzyme used was a concentrated (20-fold) sample of cellulase from a cell-free culture filtrate of *R. albus* grown for 48h (stationary phase) in SD medium containing cellulose as an energy source. The sample (0.5ml) was applied to a column (85cm × 1.5 cm) of Sephacryl S-300. Fractions (1ml) were assayed for CM-cellulase activity (▲). The column was calibrated by using the molecular-weight markers thyroglobulin (mol.wt. 670000) (1), γ-globulin (mol.wt. 160000) (2), bovine serum albumin (mol.wt. 67000) (3), ovalbumin (mol.wt. 45000) (4) and chymotrypsinogen (mol.wt. 25000) (5). ——, Protein (A_{280}).

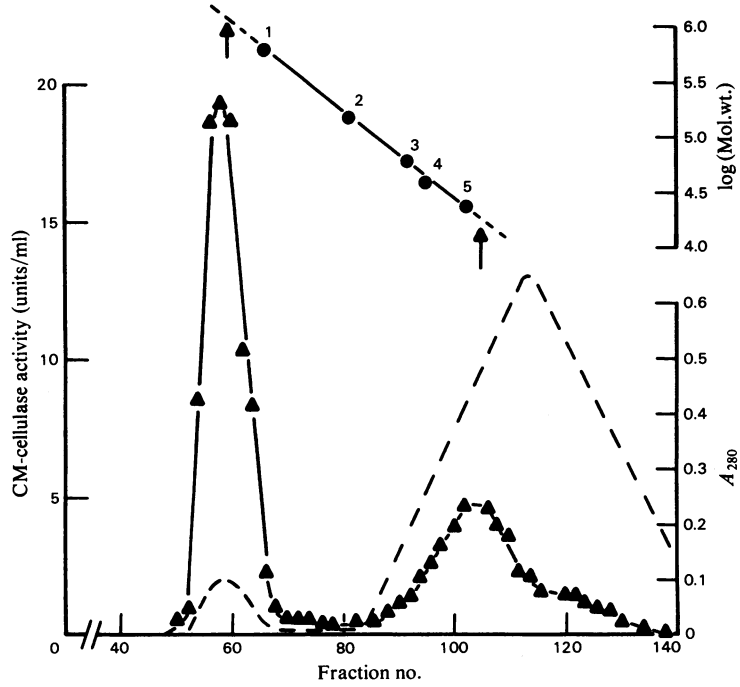


Fig. 2. *Gel filtration of the extracellular cellulase found in a culture of R. albus strain SY3 in HS medium with cellobiose as an energy source*

A sample (1ml) of concentrated (20-fold) cell-free enzyme was applied to a column (86.1cm × 1.5cm) of Sephacryl S-300 equilibrated with KH_2PO_4/NaOH buffer, pH6.68 and *I* 0.1, and eluted at 10ml/h with the starting buffer. Fractions (1ml) were assayed for CM-cellulase (▲) by the method indicated in the Experimental section. The enzyme was isolated from cultures that had reached the stationary phase (48h). The column was calibrated (●) with proteins of known molecular weight (see the legend to Fig. 1). ——, Protein (A_{280}).

favouring dissociation (4M), a small but significant increase (19%) in the amount of the low-molecular-weight component was observed after gel filtration on the Sephacryl S-300 column described in Fig. 2. However, although the change was observed with the enzyme that had been isolated from early-stationary-phase cultures (8h), remarkably these dissociating agents seemed to have no effect on enzymes obtained from later-stationary-phase (24h) cultures. But the high-molecular-weight enzyme found in the later cultures is not stable under all conditions, for during preliminary attempts to purify the high-molecular-weight enzyme by various chromatographic procedures (hydrophobic interaction, ion-exchange), a marked tendency for it to disaggregate into lower-molecular-weight fragments ranging from mol.wt. approx. 70000 to 30000 has been observed in buffers of pH values 4.0–5.0.

The ratio of the high-molecular-weight to the low-molecular-weight extracellular CM-cellulase components was also followed throughout the growth cycle in both cellobiose–HS-medium and cellulose–HS-medium cultures. During the exponential growth phase (6h) the ratio of the two components in the cultures containing cellobiose was approx. 1:1, but at 12h, at which time the culture had entered the stationary phase, the ratio had changed to approx. 4:1. No further alteration to this ratio was detected in 24h and 48h (as in Fig. 2) cultures. When cellulose replaced cellobiose in the culture medium the low-molecular-weight enzyme was the major component in the cellulase found in both late-exponential-phase (12–24h) and early-stationary-phase (48h) cultures. However, with more prolonged fermentation (120h) the proportion of the high-molecular-weight enzyme increased significantly. After 7 days the culture filtrate contained only one peak of CM-cellulase activity, but the activity was disperse, covering a wide range of molecular weights. The peak of activity had the same mobility as a protein of mol.wt. 29000.

No proteinase was detected in any of the cultures, with casein as substrate.

Relationship between fluidity and reducing power during the hydrolysis of CM-cellulose shown by the various cellulase preparations

The various extracellular cellulases obtained in different ways and the cell-wall-bound cellulase

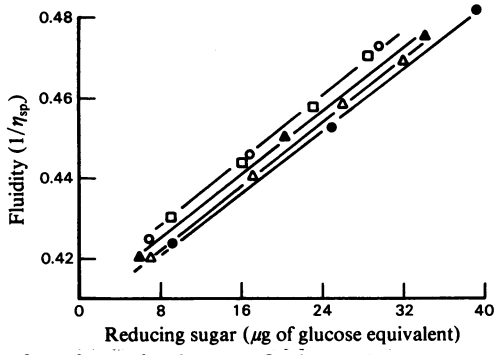


Fig. 3. *Relationship between fluidity and reducing power during the hydrolysis of CM-cellulose by the cellulases produced from R. albus strain SY3 in different ways*

●, Cellulase from a culture in HS medium containing cellobiose; △, cellulase from a culture in HS medium containing cellulose; □, cellulase isolated from bacterial cells by extraction with phosphate buffer. ▲, Cellulase from a culture in SD medium containing cellobiose; ○, cellulase from a culture in SD medium containing cellulose. See the text for details of preparation and concentration of the cellulases from the various cell-free culture filtrates and from the bacterial cells. The assay methods are indicated in the Experimental section.

extracted with phosphate buffer from cells found in a cellobiose–HS-medium culture effected the same fall in the viscosity of a solution of CM-cellulose per unit increase in reducing power: Fig. 3 shows identical slopes when fluidity was plotted against reducing power.

Adsorption of enzyme on H_3PO_4-swollen cellulose

There was no detectable difference in the adsorption of the cellulase produced in cellobiose–HS and cellulose–HS media when tested in the pH range 5.9–7.1. Since the enzymes used were from cultures that had reached the stationary phase, the enzyme isolated from the cellobiose–HS-medium culture would consist, in the main, of high-molecular-weight enzyme (Fig. 2), and the enzyme from the cellulose–HS-medium culture of low-molecular-weight fragments.

Discussion

Changes in activity of the bacterium

Changes in activity of strain SY3 after isolation have presented some problems. The changes that occurred in the cellulolytic activity are comparable with changes reported by Smith *et al.* (1973) in the cellulolytic activity of another strain of *R. albus*. In the present study the partial loss of the ability of strain SY3 to attack cellulose, especially the more-highly ordered substrates Avicel and MN-300 powder, occurred during a period in which the extracellular polyanionic coat was greatly diminished. It is noteworthy that the rumen bacteria that are capable of degrading the more-highly ordered forms of cellulose tend to adhere closely to the substrate (Latham *et al.*, 1978; Stewart *et al.*, 1979). If, as is generally assumed, the polyanionic coat of *R. albus* serves as a medium for adhesion of the bacteria to their substrate, the diminution of the coat might decrease the ability of the cells to degrade the more-highly ordered substrates.

Enzymes

Enzymes that attack CM-cellulose are found as a rule in multiple forms in cultures of many cellulolytic micro-organisms. Thus the cell-free supernatant from the cultures of the fungus *Trichoderma koningii* (Wood & McCrae, 1978) has been found to contain six CM-cellulase components, and those from the fungi *Trichoderma viride* (Gum & Brown, 1977) and *Sporotrichum pulverulentum* (Eriksson & Pettersson, 1975) have been found to contain four and five respectively. In these cases the enzymes have been separated, purified, characterized and shown to differ in their mode of action of CM-cellulose. Each enzyme has, for example, been found to produce changes in the degree of polymerization of CM-cellulose such that, when the fluidity (a parameter related to chain length) is plotted against the reducing power, a characteristic slope is obtained. On the basis of the gel-filtration data it would appear that *R. albus*, like other cellulolytic bacteria and the cellulolytic fungi, produces multiple cellulases, but the fact that the relationship obtained by plotting fluidity against reducing power is identical regardless of the composition of the cellulase enzyme system produced is not consistent with this interpretation. Indeed, if the fluidity/reducing power relationship is an acceptable property for the characterization of cellulases (CM-cellulases), and it has been well tested, one would have to conclude that *R. albus* produces only one cellulase. To explain, then, the enzyme of various molecular weights observed in the gel-filtration studies one could suggest that the enzyme exists in various degrees of aggregation: a comparison of the gel-filtration profile of cellulase treated with dissociating reagents such as urea and guanidine with that of untreated cellulase supports such a speculation, as do the observations made during attempts to purify the high-molecular-weight enzyme.

If the cellulase of *R. albus* can be discussed in terms of complex-formation between enzymes, it would appear that the degree of aggregation and/or the stability of the complex varies according to culture conditions. Alterations to the composition of

the medium and also duration of culture produce variations in the relative proportions of high-molecular-weight and low-molecular-weight enzyme. Some rationalization is possible.

The enzyme may exist on the bacterial cell wall as high-molecular-weight enzyme. It can be removed from the cell wall in this form by mild treatment with water or phosphate buffer, and little or no breakdown into low-molecular-weight fragments is encountered during gel-filtration studies. Under some cultural conditions it would appear that the high-molecular-weight material may dissociate into fragments of mol.wt. 25000–30000. For example, when the cells are cultured in semi-defined medium with cellulose as the energy source, very little high-molecular-weight material is detected at any stage during the fermentation. However, when the more easily metabolized cellobiose is substituted for cellulose, small quantities of the high-molecular-weight component appear in solution along with the low-molecular-weight enzyme. Under these culture conditions high-molecular-weight enzyme is always a very minor constituent, which disappears on more prolonged incubation.

When rumen fluid is present in the medium (HS medium) the stability of the high-molecular-weight material appears to be enhanced. Indeed, in those cultures where cellobiose was the energy source the high-molecular-weight enzyme was the major component in late-stationary-phase cultures. During the fermentation the proportion of high-molecular-weight enzyme actually increased, presumably as a result of autolysis of the cells. A similar increase in the proportion of high-molecular-weight enzyme was observed during the fermentation of cellulose, but, in contrast with the observations made with the cellobiose cultures, in the very late stationary phase the high-molecular-weight enzyme had disappeared, leaving a much smaller enzyme that was polydisperse.

Aggregation of cellulase enzymes has been observed in both fungal (Björndal & Eriksson, 1968) and rumen cellulases (Pettipher & Latham, 1979) and is of particular interest in the context of the mechanism of cellulase action. Enzyme–enzyme complex-formation has been suggested to be an essential prerequisite of hydrolysis of crystalline cellulose by some fungal cellulase systems (Wood & McCrae, 1979) and some rumen cellulase systems (Leatherwood, 1965). In the latter case an affinity factor of high molecular weight and a hydrolytic factor of low molecular weight were postulated to be essential features of an enzyme system with the capability of degrading insoluble cellulose. In the present enzyme preparation we can, however, find no evidence to support this speculation, since both high-molecular-weight and low-molecular-weight components were capable of hydrolysing H_3PO_4-swollen cellulose to a similar extent, and there were no apparent differences in the adsorption of the two types of enzyme.

The suggestion that enzyme aggregation or complex-formation can account for the various observations recorded is clearly speculative. Much more work to substantiate the hypothesis is required, and this would ideally involve purification of the various molecular species and comparison of their mobilities after electrophoresis in sodium dodecyl sulphate/polyacrylamide gels. However, because of the apparent unpredictable tendency of the various components, and the high-molecular-weight component in particular, to aggregate and disaggregate into components of various molecular weight during the manipulations involved in preparing samples for chromatography, it has proved difficult to obtain the various molecular species in homogeneous forms.

References

Akin, D. E. (1980) *Appl. Environ. Microbiol.* **39**, 242–252

Allison, M. J., Bryant, M. P., Katz, I. & Keeney, M. (1962) *J. Bacteriol.* **83**, 1084–1093

Björndal, H. & Eriksson, K.-E. (1968) *Arch. Biochem. Biophys.* **124**, 135–141

Bryant, M. P. (1972) *Am. J. Clin. Nutr.* **25**, 1324–1328

Corbett, W. M. (1963) *Methods Carbohydr. Chem.* **3**, 3–4

Dinsdale, D., Morris, E. J. & Bacon, J. S. D. (1978) *Appl. Environ. Microbiol.* **36**, 160–168

Eriksson, K.-E. & Pettersson, B. (1975) *Eur. J. Biochem.* **51**, 193–206

Gum, E. K. & Brown, R. D. (1977) *Biochim. Biophys. Acta* **492**, 225–231

Herbert, D., Phipps, P. J. & Strange, R. E. (1971) *Methods Microbiol.* **5B**, 209–344

Hobson, P. N. (1969) *Methods Microbiol.* **5B**

Lapage, S. P., Shelton, J. E., Mitchell, T. G. & MacKenzie, A. R. (1970) *Methods Microbiol.* **3A**, 135–228

Latham, M. J., Brooker, B. E., Pettipher, G. L. & Harris, P. J. (1978) *Appl. Environ. Microbiol.* **35**, 156–165

Leatherwood, J. M. (1965) *Adv. Chem. Ser.* **95**, 53–59

Mackie, R. I., Gilchrist, F. M., Robberts, A. M., Hannah, P. E. & Schwartz, H. M. (1978) *J. Agric. Sci.* **90**, 241–254

Pettipher, G. L. & Latham, M. J. (1979) *J. Gen. Microbiol.* **110**, 21–27

Scott, H. W. & Dehority, B. (1965) *J. Bacteriol.* **89**, 1169–1175

Smith, W. R., Yu, I. & Hungate, R. E. (1973) *J. Bacteriol.* **115**, 729–737

Stewart, C. S., Dinsdale, D., Cheng, K.-J. & Paniagua, C. (1979) in *Straw Decay and its Effects on Disposal and Utilisation* (Grossbard, E., ed.), pp. 123–130, John Wiley and Sons, New York

Stewart, C. S., Paniagua, C., Dinsdale, D., Cheng, K.-J. & Garrow, S. H. (1981) *Appl. Environ. Microbiol.* **41**, 504–510

Updegraff, D. M. (1969) *Anal. Biochem.* **32**, 420–424
van Gylswyk, N. O. (1970) *J. Agric. Sci.* **74**, 169–180
Walseth, C. S. (1952) *Tappi* **35**, 228–233
Wood, T. M. (1971) *Biochem. J.* **121**, 353–362
Wood, T. M. & McCrae, S. I. (1977) *Carbohydr. Res.* **57**, 117–133
Wood, T. M. & McCrae, S. I. (1978) *Biochem. J.* **171**, 61–72
Wood, T. M. & McCrae, S. I. (1979) *Adv. Chem. Ser.* **181**, 181–209
Wood, T. M., McCrae, S. I. & McFarlane, C. M. (1980) *Biochem. J.* **189**, 51–65