Carbohydrate Polymers 3 (1983) 161–173

Electron Microscopy Study of the Enzymic Hydrolysis of *Valonia* Cellulose

H. Chanzy & B. Henrissat

Centre de Recherches sur les Macromolécules Végétales (CNRS),*
53X – 38041 Grenoble Cédex, France

(Received: 14 May 1982)

SUMMARY

The enzymic degradation of cellulose from Valonia macrophysa *was observed by electron microscopy and evaluated by electron diffraction. Two types of* Valonia *samples were subjected to digestion; firstly, intact fragments cut from cell wall material and, secondly, cellulose microcrystals resulting from the acid hydrolysis of entire vesicles. These two substrates, when subjected to the action of crude cellulase complexes either from* Trichoderma reesei *or* Schizophyllum commune *were readily degraded. During the degradation, each microfibril or microcrystal became fibrillated into longitudinal crystalline sub-elements having widths ranging from below 2 nm to the full size of the initial* Valonia *microfibrillar width. These observations are evaluated in term of current theories concerning the topological action of cellulases.*

INTRODUCTION

The enzymic hydrolysis of cellulose has been the subject of numerous reports and review articles during the last two decades (Jurasek *et al.*, 1967; Hajny & Reese, 1969; Whitaker, 1971; Emert *et al.*, 1974; Kanda *et al.*, 1979; Gritzali & Brown, 1979; Ryu & Mandels, 1980). As noted by most investigators in this field, the hydrolysis proceeds through a heterogeneous mechanism where the accessibility and the crystallinity

* Laboratoire propre du CNRS, associé à l'Université Scientifique et Médicale de Grenoble.

Carbohydrate Polymers 0144-8617/83/$03.00

of the cellulosic substrate are the principal factors which control the kinetics of degradation. The various enzymes constituting the cellulase complex have diameters of 2–7 nm (Hofsten, 1975; White & Brown, 1981). For this reason, the most efficient hydrolysis is obtained when channels and pores of at least this size are generated throughout the substrate which then becomes entirely accessible to the enzymes. This explains why spectacular enhancements of the cellulolytic action are observed when mechanical or physico-chemical pretreatments are used to increase the initial surface area of the substrate (Millett *et al.*, 1976; Fan *et al.*, 1980; Chang *et al.*, 1981).

Even, if it is understood why the accessibility of the cellulose substrate is a prerequisite for an efficient cellulase hydrolysis, it is still necessary to explain how the various enzymes constituting the cellulase complex solubilize and digest the crystals of cellulose. In its native state cellulose occurs in the form of endless microfibrils of various sizes depending on the species considered (Colvin, 1972). The cellulose microfibrils are crystalline and according to current thinking, they are believed to consist of extended chain crystals (Stockmann, 1972; Sarko, 1978; Blackwell *et al.*, 1978) whose widths have dimensions comparable to those of the microfibrils. In the case of *Valonia* cellulose which is considered to be the most perfect native cellulose material, it was even shown that each microfibril was an individual crystal of cellulose (Bourret *et al.*, 1972; Chanzy, 1975; Revol, 1982).

Several mechanisms have been proposed, but not demonstrated, to describe the digestion of cellulose microfibrils by a cellulase complex. Some authors (Halliwell & Griffin, 1973; Emert *et al.*, 1974; Wood & McCrae, 1979) favour a scheme where the enzymic degradation will proceed through a synergistic attack of endo- and exocellulases directly at the cellulose microfibril surface. For other workers (Reese, 1977), the first step in the enzymic hydrolysis is the creation of a region of disorder in the highly ordered cellulose crystal which will then swell and become susceptible to a series of cellulolytic events: endoglucanase, cellobiohydrolase, and β-glucosidase action.

There is every reason to believe that a better understanding of the topological action of the cellulase complex on cellulose microfibrils should be obtained by the techniques of electron microscopy. Indeed, as shown recently by White & Brown (1981), the various enzymes can be seen directly in transmission electron microscopy with negative staining. Remarkably, the initial adsorption of these enzymes on bac-

terial cellulose microfibrils could also be visually demonstrated. In similar vein, this paper deals with the electron microscopy of the enzymic hydrolysis of *Valonia* cellulose. This substrate was selected because it is not only adapted to conventional electron microscopy but also to electron diffraction. These two techniques were used to follow the enzymic digestion of *Valonia* cellulose, starting with either intact cell wall fragments or microcrystalline cellulose resulting from acid treatment of the *Valonia* cell wall. Two cellulasic systems were used in this work. They included a crude extract from *Trichoderma reesei* (QM 9414) where all the enzymes involved in cellulose degradation were present: endo-1,4-β-D-glucanase (EC 3.2.1.4), 1,4-β-D-glucan-cellobiohydrolase (EC 3.2.1.91) and β-D-glucosidase (EC 3.2.1.21). The other system investigated was an enzymic complex from *Schizophyllum commune*. This complex was rather poor in 1,4-β-D-glucan-cellobiohydrolase but contained the other cellulolytic enzymes. The present results are compared with those obtained in a previous electron microscopical study of the biodegradation of *Valonia* cellulose by helicase (Wardrop & Jutte, 1968).

MATERIALS AND METHODS

Cellulose substrates

Vesicles of *Valonia macrophysa* were purified following the procedure of Gardner & Blackwell (1974) and stored in ethanol.

Microcrystals from *Valonia* were prepared from purified vesicles. Cellulose (200 mg) from *Valonia* was suspended in 100 ml of dry methanol and cooled to 0°C. Acetyl chloride (5 ml) was then added slowly to generate hydrogen chloride *in situ*. The flask was tightly stoppered and shaken vigorously for 24 h at room temperature. *Valonia* cell wall broke down to microcrystalline *Valonia* which was washed thoroughly by successive centrifugations in methanol until neutrality was achieved. The microcrystals in suspension were stored in methanol.

Enzymes

The crude, lyophilised cellulase system of *Trichoderma reesei* was a gift from Dr M. Mandels (US Army Natick Research and Development

Laboratories, Natick, Massachusetts 01760, USA). The crude, lyophilised preparation of *Schizophyllum commune* was kindly provided by Dr M. Desrochers (Pulp and Paper Research Institute of Canada, Pointe Claire, Québec, Canada H9R 3J9).

Enzymic hydrolysis

Valonia vesicles or microcrystals were carefully exchanged against sodium acetate buffer (pH 4·8).

The lyophilised enzymes were dissolved in the same buffer and centrifuged to eliminate insoluble material. The treatment with cellulase from *Trichoderma reesei* was achieved at 48°C. For the intact vesicles, 1 cm^2 of *Valonia* cell wall was suspended for 24 h in 5 ml of buffer containing 10 mg of enzymes. For the microcrystals, a suspension containing 2 mg/ml substrate and 2 mg/ml enzymes was used and allowed to digest for 6 h only.

The hydrolysis with the cellulase from *Schizophyllum commune* was performed at 30°C. For the intact vesicles, 1 cm^2 of *Valonia* cell wall was suspended for 48 h in 5 ml buffer containing 50 mg of enzymes. For the microcrystals, a suspension containing 2 mg/ml substrate and 10 mg/ml enzymes was used and allowed to digest for 24 h.

At the indicated times of hydrolysis, the samples were washed thoroughly with buffer, water and were stored in ethanol.

Electron microscopy

Fragments of *Valonia* cell wall were delaminated with sharp needles and mounted on carbon coated electron microscope grids. For *Valonia* microcrystals, drops of their suspension were deposited on carbon coated grids and allowed to dry.

Negative staining was achieved in conventional fashion with 3% uranyl acetate on a specimen mounted on UV-treated carbon coated grids.

Electron microscopy was performed with a Philips EM 400 T electron microscope, operating at 80 kV for imaging purposes and 120 kV for electron diffraction. The negatively stained specimens were examined at a plate magnification of 36 000. The electron diffraction powder diagram on *Valonia* was recorded while translating slowly the specimen

under the beam. This gave diagrams with a homogeneous distribution of the diffraction circles.

Microdensitometer traces of the electron diffraction patterns were recorded with a Joyce Loebl apparatus.

RESULTS

When delaminated, *Valonia* cell wall material breaks down into arrays of smooth and endless cellulose microfibrils having lateral dimensions of the order of 20 nm. A negatively stained preparation of such a specimen is shown in Fig. 1 which corresponds to similar pictures already described by previous workers (Franke & Falk, 1968).

The enzymic treatment of such cell wall material induces drastic morphological changes. At a gross level, the well formed vesicles break down into a number of small fragments displaying shredded contours when observed with the optical microscope. At the ultrastructural level, the morphological changes are even more pronounced. This is illustrated in Fig. 2 which corresponds to a fragment remaining in suspension after a 48 h digestion of *Valonia* cell wall by the cellulase from *Schizophyllum commune*. In Fig. 2, the typical microfibrillar array of *Valonia* is still clearly visible. On the other hand, most of the microfibrils have lost their integrity as they are now sub-fibrillated into

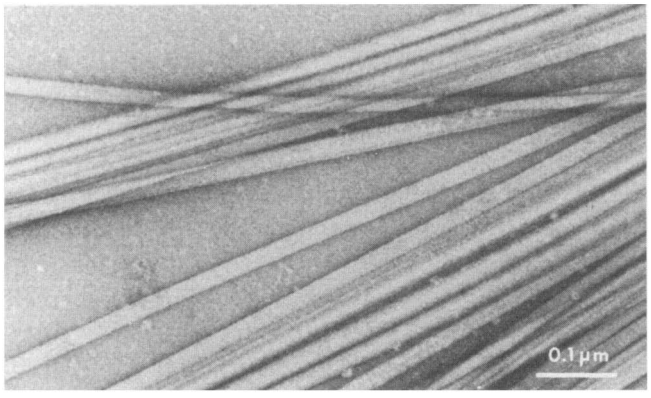

Fig. 1. Cellulose microfibrils from *Valonia macrophysa*, negatively stained with uranyl acetate.

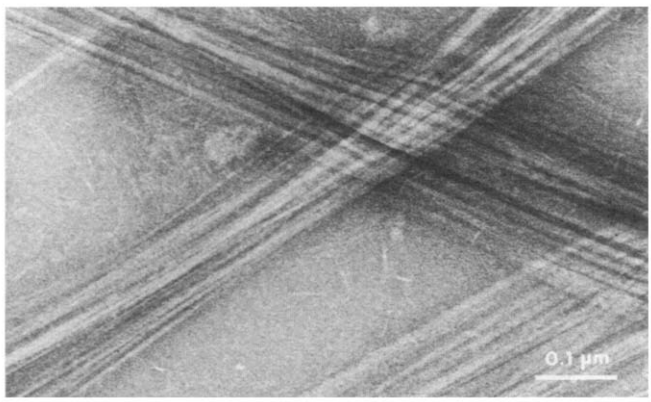

Fig. 2. Same as in Fig. 1 but after 24 h digestion by a cellulase complex from *Schizophyllum commune*.

smaller elements with a distribution of width ranging from 2 nm (most likely the resolution of the technique) to around 10 nm. In the background of Fig. 2, one can distinguish a dispersion of small cellulose splinters corresponding most probably to a more advanced state of hydrolysis where the long fibrillated elements are cut longitudinally.

Experiments with enzymes from *Trichoderma reesei* yielded images (not shown) of similar appearance; here the enzymic system was acting at a higher temperature and a much faster rate.

When followed by electron diffraction techniques, the drastic changes in morphology occurring between Fig. 1 and Fig. 2 can be confirmed at the crystalline level. This is illustrated in Figs 3A and 3B which correspond respectively to an unstained specimen, such as in Figs 1 and 2. Figure 3A is a typical well resolved electron diffraction fibre diagram of the initial native *Valonia* cellulose. As reported by Honjo & Watanabe (1958) this diagram contains several hundreds of independent diffraction spots. Its resolution goes beyond 0·09 nm^{-1} along the fibre axis and around 0·2 nm^{-1} in a direction perpendicular to it. Figure 3B corresponds to a specimen which was digested for 6 h by the cellulase of *Trichoderma reesei* and has the typical appearance shown in Fig. 2. The pattern in Fig. 3B is dramatically different from that in Fig. 3A as it consists of less than 10 independent diffraction spots while an amorphous halo has also developed. As in the case of native *Valonia*, the pattern in Fig. 3B does not have the same resolution along and

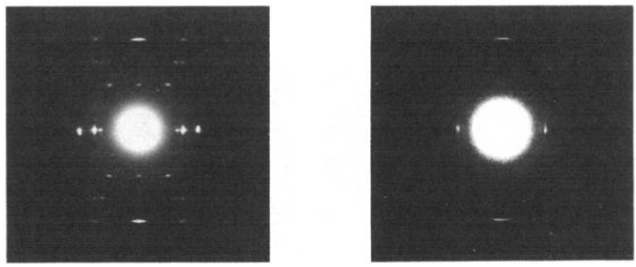

Fig. 3. A, Electron diffraction diagram of an unstained bundle of cellulose microfibrils from *Valonia macrophysa*. The cellulose fibril's axis is vertical. B, Same as in 3A but after 24 h digestion by a cellulase complex from *Trichoderma reesei*.

perpendicular to the fibre axis. Along the fibre axis, the 8th meridional reflection (0·12 nm^{-1}) is clearly resolved on the original negative while on the equator, the diffraction diagram does not expand beyond the diffraction spot 020* (0·39 nm^{-1}). Remarkably, this very intense spot in the initial diffraction diagram has now only a weak intensity. On other diagrams recorded with the same sample, this spot is even sometimes totally absent.

Microcrystalline *Valonia* cellulose in suspension is far more accessible to the enzymes than the compact *Valonia* cell wall. This explains why the enzymic hydrolysis of microcrystalline *Valonia* proceeds at a much faster rate than that of intact cell wall material. For instance, it was found that after 6 h hydrolysis with the cellulase complex of *Trichoderma reesei*, 90% of the cellulose microcrystals were totally solubilized. In the case of the complex of *Schizophyllum commune*, the microcrystals of cellulose were also digested rapidly but at a somewhat lower rate as it took 24 h to attain the same degree of hydrolysis. As with the entire vesicles, the ultrastructural changes occurring during the enzymic hydrolysis of the *Valonia* microcrystals are clearly visible. This is illustrated in Figs 4 and 5. Figure 4 corresponds to the initial microcrystals; they display the classical rod-like appearance of such material (Kai *et al.*, 1974). The crystals have a smooth appearance with lateral dimen-

* Throughout this paper, the indexing of *Valonia* cellulose crystals refers to the 2 chains unit cell as proposed by Gardner & Blackwell (1974) with *c* as fibre axis.

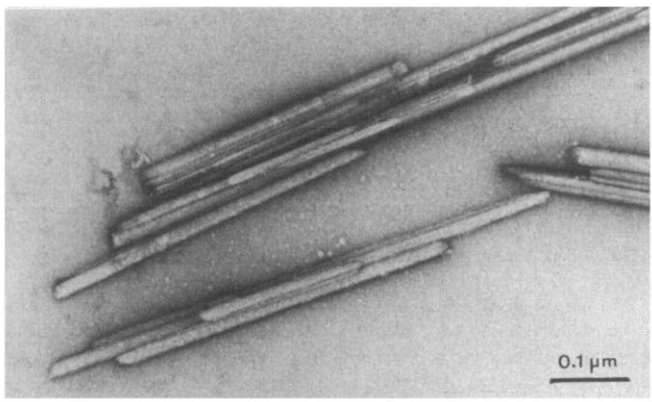

Fig. 4. Cellulose microcrystals obtained by acid treatment of cell wall material from *Valonia macrophysa*. The specimen was negatively stained with uranyl acetate.

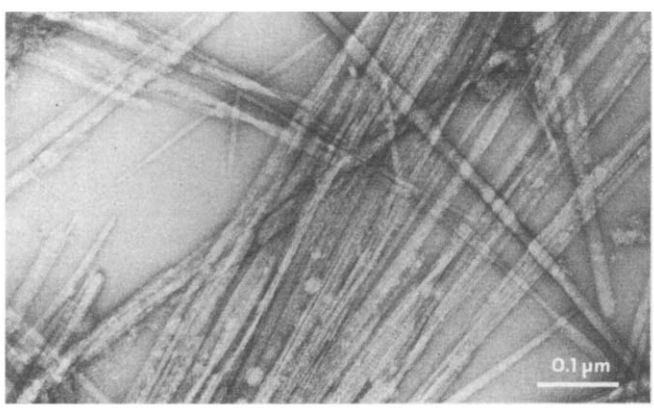

Fig. 5. Same as in Fig. 4 but after 6 h digestion by a cellulase complex from *Trichoderma reesei*.

sions corresponding to that of the initial micro-fibrils and longitudinal dimensions comprised between 200 nm and several µm. The crystal ends do not have a specific shape but are either cut very sharply or rounded. In particular, it is not possible to decide whether these crystal ends follow a crystallographic plane. Also, the existence of a reducing and a non-reducing end in the crystals cannot be determined. The enzymic hydrolysis yields a fibrillation of the crystals parallel to their largest dimension. This is clearly seen in Fig. 5 which displays also a

series of small crystalline splinters of various lengths and widths. These smaller elements correspond to fragments of the initial crystals which went loose during the hydrolysis.

When the microcrystals are studied by electron diffraction, the enzymic degradation brings sharp modifications in the initial cellulose diffraction pattern. This is illustrated in Figs 6 and 7. Figure 6A is a powder diffraction diagram of the initial crystals while Fig. 6B is recorded on a digested sample such as in Fig. 5. Both patterns are scanned with a microdensitometer and their intensity tracings reproduced in Fig. 7. It is remarkable that, in going from curve A to curve B, the meridional spacing 004 keeps its intensity and sharpness during the sample digestion while the equatorial spots – especially the strong 110 – have almost disappeared. In fact, as can be seen in Fig. 7, the electron diffraction intensity of the reflection 110 is about three times stronger than that of 004 in the initial sample. In the digested sample the situation is completely reversed, as 004 is now by far the predominant diffraction line.

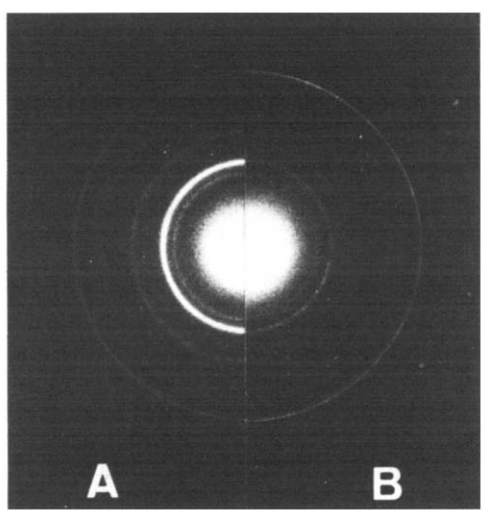

Fig. 6. A, Electron diffraction diagram of an unstained dispersion of cellulose microcrystals of *Valonia*. B, Electron diffraction diagram of an unstained dispersion of cellulose microcrystals of *Valonia* after 6 h of enzymic digestion by a cellulase complex from *Trichoderma reesei*.

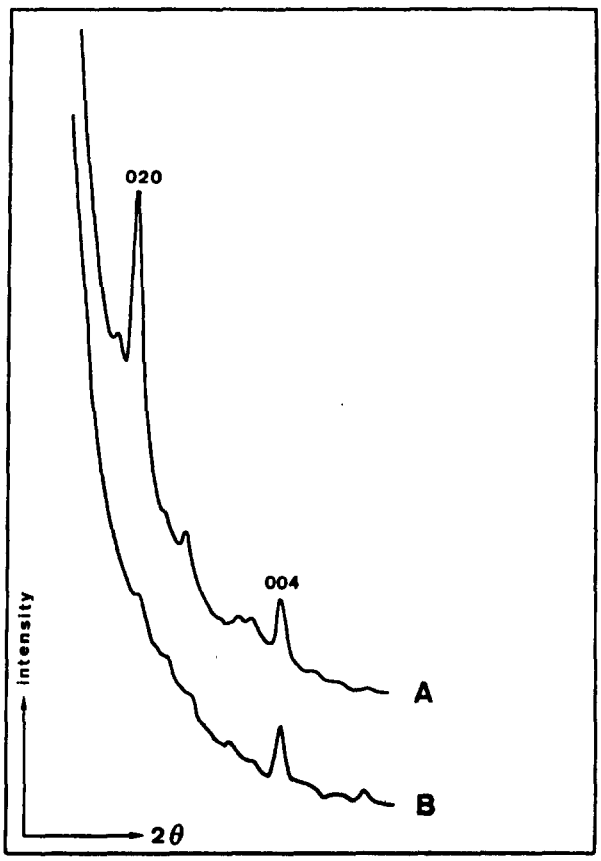

Fig. 7. Radial densitometer tracings of the electron diffraction diagrams in Fig. 6 (intensities in arbitrary units).

DISCUSSION

The results obtained in this study present some instructive observations on the mode of degradation of highly crystalline *Valonia* cellulose by cellulase complexes. The enzymic degradation seems to proceed in two successive steps. At first, cellulose microcrystals are fibrillated into sub-elements having the initial material length but much smaller width. The fibrillated material shown in Figs 2 and 5 consists of quasi-unidimensional cellulose crystals having almost perfect coherence along the fibril axis.

Owing to their small width, their lateral organisation is best described in terms of paracrystalline rather than crystalline order. This step is followed by a second event where the 'sub-fibrils' or the 'sub-crystals' are cut down and hydrolysed.

Another explanation for the fibrillated appearance of the enzymatically treated samples has also to be considered: each bundle of sub-fibrils may just be constituted of single eroded microfibrils that have coalesced together upon drying. Such mechanism may occur to some extent. However, in the material such as that shown in Fig. 5, one can clearly recognise the overall shape of several initial microcrystals. In that case, the fibrillation of each crystal appears real and must be accounted for to describe the enzymic digestion.

Our data confirm the electron diffraction findings of Betrabet & Paralikar (1977, 1978) on the enzymic hydrolysis of various cellulosic substrates with a cellulase complex from *Penicillium funiculosum*. In that case, a reduction of the width of the cellulose crystals was noticed while their length was maintained. The authors explain the hydrolysis of their cellulose by a specific action of the enzymes along the 020 and $1\bar{1}0$ planes of the cellulose lattice. In the present case, a similar conclusion could be drawn as the corresponding reflections, especially the 020, are sharply reduced in intensity after bio-degradation of the specimen. However, the possibility of a specific uniplanar orientation of the hydrolysed specimen on the supporting carbon cannot be ruled out. This situation is quite common when cellulose is studied by electron diffraction (Claffey & Blackwell, 1976) and can lead to misleading conclusions.

In contrast to the findings of Wardrop & Jutte (1968) in their early study of the degradation of *Valonia* by helicase, our degraded microfibrils do not display sub-units having lateral dimension of 4 or 8 nm in width. In the present case, one obtains rather a distribution of fibrils whose widths are ranging from below 2 nm to the full width of the initial *Valonia* microfibrils. Wardrop & Jutte (1968) had also noticed that eroded *Valonia* microfibrils presented pointed tips with oblique ends making specific angles with respect to the microfibril axis. This observation is not confirmed here and the fibrillation is the dominant morphological event resulting from cellulose digestion.

The present findings confirm the recent observations of White & Brown (1981) on the enzymic hydrolysis of bacterial cellulose. These

authors have shown that the cellulose degradation begins with an initial splitting of the cellulose ribbons along their axis leading to fibrillation. With bacterial cellulose, however, it is not clear whether the cellulose crystals themselves are split longitudinally during hydrolysis. The bacterial cellulose ribbons are composed of parallel aggregates of 50–80 cellulose microfibrils. In that case, the initial action of the cellulolytic enzymes may just be the separation of the microfibrils from one another as the resolution of the electron microscopy technique is not sufficient to reveal any sub-fibrillation of the narrow bacterial cellulose microfibrils. When working with *Valonia* microfibrils, one deals with much wider microfibrils, each having a monocrystalline character. Their evident fibrillation during the enzymic hydrolysis proves that the enzymes are not only able to release the loose hydrogen bonds holding the cellulose microfibrils together, but also the strongest ones linking the cellulose chains within the crystals.

In the present work, the *Valonia* cellulose degradation was obtained with complete cellulase systems. The observed fibrillation of the substrate may be the result of several synergistic events as it is always suggested when discussing cellulase degradation mechanism. Another explanation would be that only one enzyme is responsible for the fibrillation. This would confirm the hypothesis of Reese (1977) of an initial enzymic swelling of the crystalline cellulose and is consistent with the observations of White & Brown (1981) who noticed a splaying of cellulose microfibrils bundles after incubation with purified endoglucanase. The interaction of *Valonia* cellulose microfibrils and microcrystals with purified cellulase components is thus the logical next step to this study. It is presently being performed and will be reported in the future.

REFERENCES

Betrabet, S. M. & Paralikar, K. M. (1977). *Cellulose Chem. Technol.* **11**, 615.

Betrabet, S. M. & Paralikar, K. M. (1978). *Cellulose Chem. Technol.* **12**, 241.

Blackwell, J., Kolpak, F. J. & Gardner, K. H. (1978). *Tappi* **61**, 71.

Bourret, A., Chanzy, H. & Lazaro, R. (1972). *Biopolymers* **11**, 893.

Chang, M. M., Chou, T. Y. C. & Tsao, G. T. (1981). *Adv. Biochem. Eng.* **20**, 15.

Chanzy, H. (1975). In *Structure of fibrous polymers*, eds. E. D. T. Atkins and A. Keller, 'Colston Papers', No. 26, Butterworths, London, p. 417.

Claffey, W. & Blackwell, J. (1976). *Biopolymers* **15**, 1903.
Colvin, J. R. (1972). *CRC, Criticals Reviews in Macromolecular Sci.* **1**, 47.
Emert, G. H., Gum, E. K., Liu, T. H. & Brown, R. D. Jr. (1974). In *Food related enzymes*, ed. J. R. Whitaker, 'ACS Symposium Series', Vol. 136, Washington, DC, ACS, p. 79.
Fan, L. T., Lee, Y. H. & Beardmore, D. H. (1980). *Biotechnol. Bioeng.* **27**, 177.
Franke, W. W. & Falk, A. (1968). *Z. Naturforsch.* **23B**, 272.
Gardner, K. H. & Blackwell, J. (1974). *Biopolymers* **13**, 1975.
Gritzali, M. & Brown, R. D. Jr. (1979). In *Hydrolysis of cellulose: Mechanism of enzymatic and acid catalysis*, eds. R. D. Brown Jr. and L. Jurasek, 'ACS Symposium Series', Vol. 181, Washington, DC, ACS, p. 237.
Hajny, G. A. & Reese, E. T. (eds) (1969). In *Cellulases and their applications*, 'ACS Symposium Series', Vol. 95, Washington, DC, ACS, p. 7.
Halliwell, G. & Griffin, M. (1973). *Biochem. J.* **135**, 587.
Hofsten, B. V. (1975). In *Symposium on enzymatic hydrolysis of cellulase*, eds M. Bailey, T. M. Enari and M. Lindo, Helsinki, The Finnish National Fund for Research and Development (SITRA), p. 281.
Honjo, G. & Watanabe, M. (1958). *Nature* **181**, 326.
Kai, A., Kaino, T. & Kobayashi, Y. (1974). *Sen I Gakkaishi* **30**, T. 203.
Kanda, T., Nakakubo, S., Wakabayashi, K. & Nisizawa, K. (1979). In *Hydrolysis of cellulose: Mechanism of enzymatic and acid catalysis*, eds. R. D. Brown Jr. and L. Jurasek, 'ACS Symposium Series', Vol. 181, Washington, DC, ACS, p. 211.
Jurasek, L., Colvin, J. R. & Whitaker, D. R. (1967). *Advances in Applied Microbiology* **9**, 131.
Millett, M. A., Baker, A. J. & Satter, L. D. (1976). *Biotechnol. and Bioeng. Symp.* **6**, 125.
Reese, E. T. (1977). *Recent Adv. Phytochem.* **7**, 311.
Revol, J. F. (1982). *Carbohydrate Polymers* **2**, 123.
Ryu, D. D. Y. & Mandels, M. (1980). *Enzyme Microb. Technol.* **2**, 91.
Sarko, A. (1978). *Tappi* **61**, 59.
Stockmann (1972). *Biopolymers* **11**, 251.
Wardrop, A. B. & Jutte, J. M. (1968). *Wood Science and Technology* **2**, 105.
Whitaker, D. R. (1971). In *The enzymes*, ed. P. D. Boyer, Vol. 5, 3rd edition, New York, Academic Press, p. 273.
White, A. R. & Brown, M. R. Jr. (1981). *Proc. Natl. Acad. Sci.* **78**, 1047.
Wood, T. M. & McCrae, S. (1979). In *Hydrolysis of cellulose: Mechanism of enzymatic and acid catalysis*, eds. R. D. Brown Jr. and L. Jurasek, 'ACS Symposium Series', Vol. 181, Washington, DC, ACS, p. 181.