# Undirectional degradation of *Valonia* cellulose microcrystals subjected to cellulase action

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Cellulose microcrystals from the alga *Valonia macrophysa* have been subjected to enzymatic degradation with purified cellulase components from the fungus *Trichoderma reesei*. Electron microscopy reveals that 1,4-β-D-glucan cellobiohydrolase II erodes only one of the two tips of the microcrystals. This demonstrates not only the exo-action pattern of the enzyme, but also the parallel packing of the cellulose chains within *Valonia* cellulose microcrystals. When 1,4-β-D-glucan cellobiohydrolase II is mixed with 1,4-β-D-glucan glucanohydrolase II, several sites of attack are observed, located at defects along the microcrystals. In that case again, the biodegradation is persistently unidirectional and visual evidence is given for the endo-exo co-operativity between the two enzymes.

*Cellulose degradation* *1,4-β-D-Glucan cellobiohydrolase* *1,4-β-D-Glucan glucanohydrolase* Trichoderma reesei Valonia *Endo-exo cooperation* *Cellulose chain polarity*

## 1. INTRODUCTION

The mechanism by which crystalline cellulose is degraded by cellulases is still poorly understood, perhaps because of the features of the substrate which is an insoluble, fibrous, semi-crystalline macromolecule. Nevertheless, according to a widely accepted hypothesis[1–4], 1,4-β-D-glucan glucanohydrolase (EC 3.2.1.4) is believed to catalyze hydrolysis of amorphous domains in cellulose. This would be followed by the recurrent removal of cellobiosyl units from the non-reducing ends of cellulose chains, promoted by 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91). Although these proposals provide a logical explanation for the observed synergism between the two types of enzyme, no direct evidence for such a mechanistic action has yet been found to support these hypotheses.

We have begun a study of the enzymatic breakdown of cellulose by using the cellulose microcrystals from the alga *Valonia macrophysa* as a model substrate for visualizing the action pattern of purified cellulases by transmission electron microscopy [5,6]. *Valonia* cellulose is considered to be one of the most perfect native cellulose materials [7], and each microfibril consists of an individual crystal of cellulose [8,9]. Here we examine the behavior of these cellulose crystals upon degradation by purified 1,4-β-D-glucan cellobiohydrolase II (CBHII) and 1,4-β-D-glucan glucanohydrolase II (EGII) from the fungus *Trichoderma reesei*.

## 2. MATERIALS AND METHODS

Microcrystals from *V. macrophysa* cellulose were prepared as in [10]. CBHII and EGII from *T. reesei* were purified from Celluclast™ as described [11]. Enzymatic hydrolyses were conducted with 2 mg·ml$^{-1}$ cellulose and 200 μg·ml$^{-1}$ enzyme in 50 mM sodium acetate buffer at 45°C for 16 h. Samples were centrifuged (3000 × *g*) and washed thoroughly with water. Drops of their aqueous suspensions were deposited onto carbon-coated

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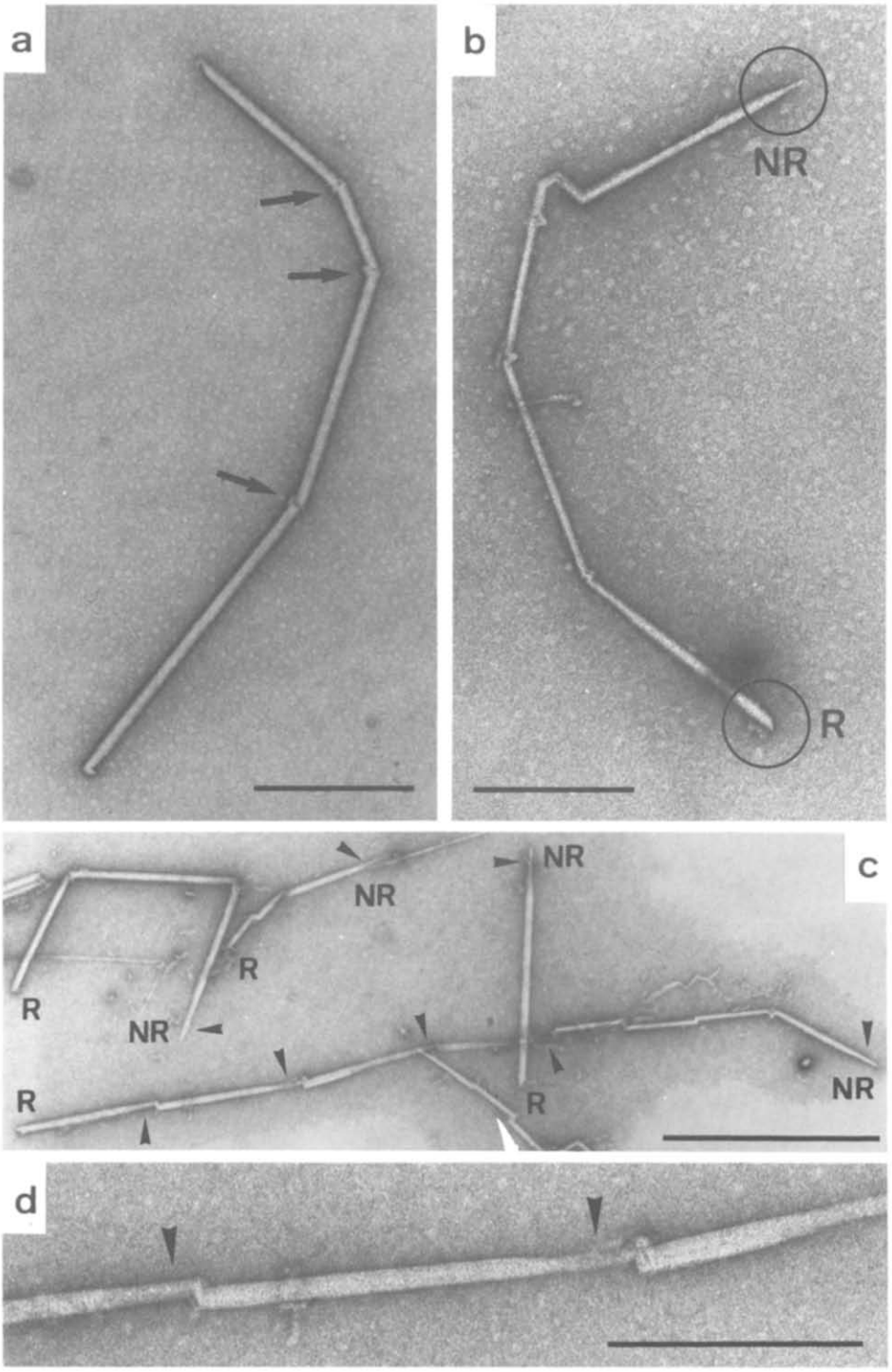


Fig.1. (a) Electron micrograph of a typical microcrystal of cellulose from *Valonia macrophysa*. The arrows indicate the kinked defects of the microcrystal (scale bar, 250 nm). (b) Identical to (a), but after 16 h of digestion with CBHII. Circled areas: R, reducing end of the microcrystal; NR, non-reducing end (scale bar, 250 nm). (c) Identical to (b), but after 16 h of digestion with a 60:40 mixture of CBHII and EGII. R, reducing ends of the microcrystals; NR, non-reducing ends. The arrowheads denote the areas of CBHII attack (scale bar, 500 nm). (d) Enlargement of an area of (c) (scale bar, 200 nm).

electron microscope grids and negative staining was achieved with 1.5% uranyl acetate.

Electron microscopy was performed with a Philips EM 400T instrument operating at 80 kV. Images were recorded at a plate magnification of 28000 on Kodak 4489 electron microscope film.

## 3. RESULTS AND DISCUSSION

The microcrystals of cellulose from *V. macrophysa* [10] exist as segments of the initial microfibrils (fig.1a). They have the same width (15–20 nm) and perfection as the original microfibrils, but are of variable length, ranging from 500 nm to several micrometers. In addition, along a given microcrystal, a number of kinks can be observed (arrows in fig.1a). These kinks presumably correspond to areas attacked by acid, but not fully disrupted by the mechanical agitation. The tips of the microcrystals have irregular shapes ranging from very sharp oblique cuts to blunt extremities sometimes carrying microfibrillar debris. In no case is it possible to correlate such features with either the polarity of the cellulose chains within the microcrystals or with any crystallographic plane of cellulose.

Upon digestion by CBHII, each cellulose microcrystal becomes eroded at only one of its two tips whereas the other tip remains unaffected. This is illustrated in fig.1b. Typically, the eroded tips display an elongated pointed shape, with the point roughly centered along the long axis of the crystal.

When the microcrystals are digested with a mixture of CBHII and EGII, the erosion occurs not only at one of the tips of the microcrystals, but also at the kinks which become also sites of unidirectional attack. Such a phenomenon is illustrated in fig.1c. It corresponds to a sample treated as in fig.1b, the only difference being that the enzymatic solution contained a 60:40 mixture of CBHII and EGII. In that case, the digested specimen appears as a string of pointed tip fragments, each point being oriented towards the pointed tip of the partially digested crystal.

Our results bear several implications concerning the structure of crystalline native cellulose, as well as the mechanistic action of cellulases. As observed above, CBHII digests the cellulose microcrystals only from one of their ends. Since exo-cellulases hydrolyze cellulose chains from their non-reducing end [12], this proves that, within a given *Valonia* microcrystal, all the non-reducing ends point towards the same direction. These findings substantiate the parallel packing for cellulose chains, in such cellulose I crystals, in agreement with X-ray analysis data [13,14] and specific staining experiments [15]. Consequently, our results rule out the possibilities of chain folding [16] or antiparallel packing of the cellulose molecules within the microcrystals [17].

The occurrence of pointed tips in the *Valonia* microcrystals digested by CBHII indicates that the sequential attack presumably starts by peeling off the accessible surface of non-reducing end. Underlying chains are thus uncovered and become degraded in a subsequent step. These successive attacks yield a pointed tip as schematically described in fig.2.

The synergism between endo-cellulase such as EGII and exo-cellulase such as CBHII is clearly demonstrated in fig.1c. This corroborates the scheme previously suggested for the enzymatic hydrolysis of cellulose [1–4]. When acting on the almost perfect microcrystals of *Valonia* cellulose, EGII is only able to hydrolyze the kinked areas of the microcrystals in a typical endo fashion. Nevertheless, this limited action creates a number of newly accessible non-reducing chain ends. In the

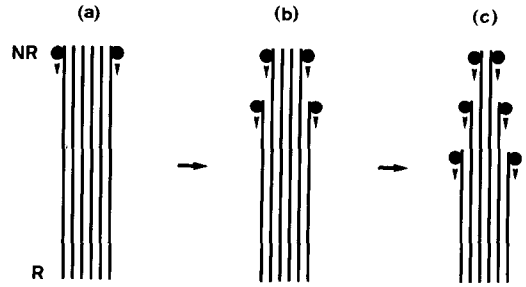


Fig.2. Schematic representation of the exo-attack giving rise to sharpened microcrystal tips. In (a) the exo-enzyme (●) attacks accessible non-reducing chain-ends (NR) at the crystal surface. In (b) degradation of the outer layer exposes a new surface of non-reducing chain-ends to the enzymes. As this progresses (c), a sharpened tip morphology appears at the non-reducing end of the microcrystal.

mixed enzyme mode, the hydrolytic production of CBHII is consequently multiplied by the number of kinks present in the average *Valonia* microcrystal.

Our results can be compared to those reported on the enzymatic degradation of bacterial cellulose ribbons by 1,4-β-D-glucan cellobiohydrolase I (CBHI) and 1,4-β-D-glucan glucanohydrolase IV from *T. reesei* [18]. In that case, however, no unidirectional degradation was found, presumably because of the endo character of CBHI [5,6,19].

## 4. CONCLUSION

The present study of the topological degradation pattern of CBHII demonstrates not only the exo-activity of the enzyme, but also the parallel packing of the cellulose chains within *Valonia* cellulose microcrystals. It is worth mentioning that the topological action of CBHII is definitely different from that reported for CBHI, the latter being shown to degrade the cellulose microcrystals laterally [5,6]. It is therefore apparent from our results that CBHI and CBHII operate on the crystal of cellulose in orthogonal directions. This feature may be related to the observed, but yet uninterpreted co-operativity found between these two enzymes [20].

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