The action of 1,4-β-D-glucan cellobiohydrolase on *Valonia* cellulose microcrystals

An electron microscopic study

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The interaction between 1,4-β-D-glucan-cellobiohydrolase I (CBHI) from *Trichoderma reesei* and microcrystalline cellulose from *Vallonia macrophysa* was investigated by electron microscopy which allows to visualize the individual enzymes and their strong adsorption at the substrate surface. The microcrystals are slowly degraded and decrystallized by erosion and fibrillation while soluble products are released and characterized by HPLC. CBHI is thus able to break down *Valonia* microcrystals without the help of any endo-β-1,4-glucanase activity.

Cellulose (Valonia macrophysa) *degradation* *Adsorption* *1,4-β-D-glucan cellobiohydrolase* (Trichoderma reesei) *Hydrolysis*

1. INTRODUCTION

A frequently postulated mechanism for the enzymatic degradation of cellulose involves the successive attack on the substrate by an endo-1,4-β-D-glucanase (CMCase, EC 3.2.1.4) followed by a 1,4-β-D-glucan-cellobiohydrolase (CBH, EC 3.2.1.91) [1–4]. Such a widely accepted mechanism, however, does not explain fully why mainly CBH demonstrates affinity for crystalline cellulose such as Avicel [5,6]. This implies that some kind of hydrolytic action has to be initiated by CBH itself, as suggested in [7], in contradiction with the current thinking which attributes this role to an endo-factor.

The present work was devised to clarify this point by direct visualization of the interaction of purified CBH I from *Trichoderma reesei* with *Valonia macrophysa* microcrystalline cellulose. This substrate is the native cellulosic material possessing the highest crystallinity and crystalline perfection [8]. Its breakdown under CBH I exposure was followed by electron microscopy and electron diffraction while the carbohydrates released were analyzed by HPLC.

2. MATERIALS AND METHODS

The 1,4-β-D-glucan-cellobiohydrolase I (CBHI) from *Trichoderma reesei* was purified from Celluclast™ (Novo) according to [9] followed by a further purification on Sephacryl S-200. The purified protein formed only a single precipitate with polyspecific sera against Celluclast and appeared as a single band in SDS gel electrophoresis. The purified CBHI was immunochemically identical with CBHI from [10,11]. Viscosimetric determinations using carboxymethyl-cellulose according to [12] were conducted to detect possible contamination by a CMCase activity. These assays led to the negligible activity of 3.00×10^{-5} μequiv. glycosidic bonds split.s^{-1}.mg protein^{-1}.

Microcrystalline *Valonia macrophysa* cellulose was prepared by acido-mechanical treatment of the

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algae cell wall as in [13].

2.1. *Adsorption of CBHI on* Valonia *cellulose*

Experiments were conducted at 48°C in 0.1 M sodium acetate buffer (pH 4.8) in the presence of a suspension of 0.3 mg *Valonia* microcrystals/ml. Several enzyme concentrations were used from 0.1–2.0 mg/ml. After various contact times, the microcrystals were centrifuged at 3000 rev./min, washed twice in distilled water and finally stored in ethanol.

2.2. *Hydrolysis of* Valonia *cellulose by CBHI*

Degradation of the *Valonia* microcrystals was achieved under the same buffer and temperature conditions as above, using 0.3 mg cellulose/ml and 0.5 mg CBHI/ml, for 5–48 h. At the indicated times, samples were centrifuged, washed successively 5 times in water, once in 0.2% aqueous sodium hydroxide (5 min treatment), twice in distilled water and stored in ethanol. It must be noted that after 48 h of hydrolysis, ~80% of the starting material had disappeared as estimated by the size of the sediment after centrifugation.

2.3. *Electron microscopy*

2.3.1. Specimen preparation

For the visualization of individual CBHI molecules, 10^{-3} mg/ml and 10^{-4} mg/ml solutions of enzyme in 0.1 M sodium acetate buffer (pH 4.8) were used. A drop of the diluted solution was deposited on UV-treated carbon-coated electron microscope grids. After 1 min contact, the drop was drained with filter paper and the grid washed with a drop of distilled water. Shadowing was achieved with W/Ta alloy using either unidirectional or rotative conditions. Blank experiments were conducted under the same experimental conditions but replacing the enzyme solutions with pure buffer. In that case, no spherical particles were detected.

For the visualization of *Valonia* microcrystals before, during or after enzymatic hydrolysis, drops of their ethanolic suspensions were allowed to dry on UV-treated carbon-coated electron microscope grids. These grids were either shadowed with W/Ta alloy or negatively stained with 3% aqueous uranyl acetate.

2.3.2. Observation conditions

A Philips EM 400 T electron microscope was used throughout. Imaging was achieved at 80 kV, while for electron diffraction operation, the accelerating voltage of 120 kV was selected for unshadowed specimens.

2.4. *HPLC analysis*

This was performed on an incubation mixture which included 1 mg/ml *Valonia* microcrystals and 0.5 mg/ml CBHI in 0.1 M pyridine–acetic acid buffer (pH 5.0). After 24 h at 48°C, the mixture was centrifuged and the supernatant freeze dried. Ion-exchange resins (cationic and anionic) were used to remove most of the buffer. Analysis of the products of the reaction was achieved by reversed phase chromatography on Radial pak C-18 μ column (Waters Assoc.) using water as eluent (flow-rate 1 ml/min). Detection of the carbohydrates was performed with a refractive index detector.

3. RESULTS

3.1. *CBHI and its adsorption on* Valonia *microcrystals*

When directly visualized by the electron microscope (fig.1A,B), the CBHI molecules appear as a rather homogenous distribution of spherical particles 7–8 nm diam. A such observed dimension however exceeds slightly the actual dimension of the enzymes as shadowing material has been added to the preparation in order to outline the enzyme contour.

When the microcrystals of *Valonia* cellulose (fig.2A) are incubated with CBHI at < 1 mg/ml, the protein is readily adsorbed and visualized on the cellulosic substrate which becomes decorated by the discrete enzyme (fig.2B). In that case, CBHI is strongly fixed at the cellulose surface since its binding is not affected by the successive washings with distilled water. Furthermore, no enzyme can be detected in the background. When long incubation times are used and with higher concentrations of enzyme, the sorption of CBHI on the substrate is still perfect, but the microcrystals are now encased within a shapeless sticky precipitate in which discrete enzymes can hardly be recognized (fig.2C).

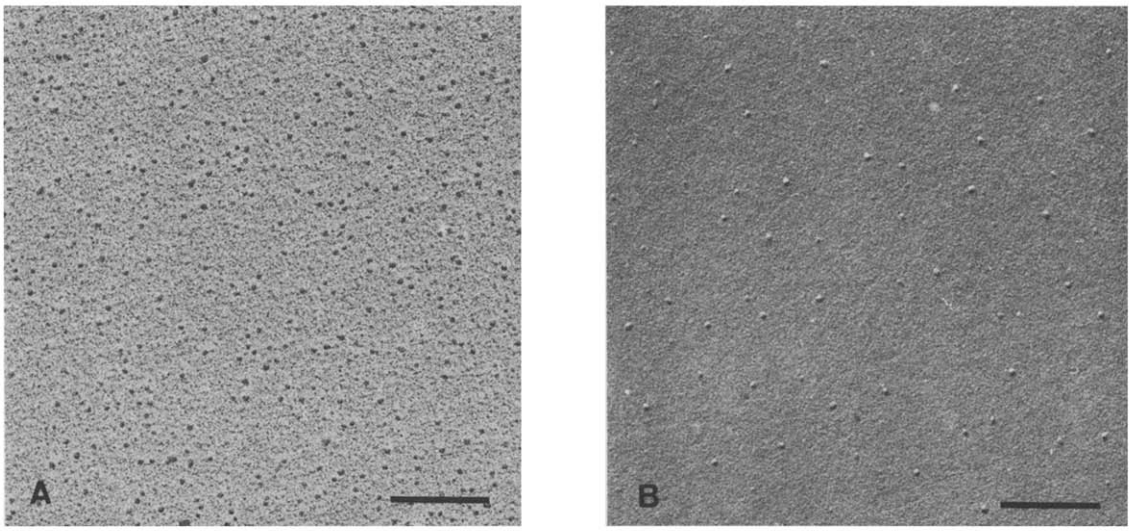


Fig.1. Individualized CBHI molecules (bar = 200 nm): rotating (A) and unidirectional (B) W/Ta shadowing.

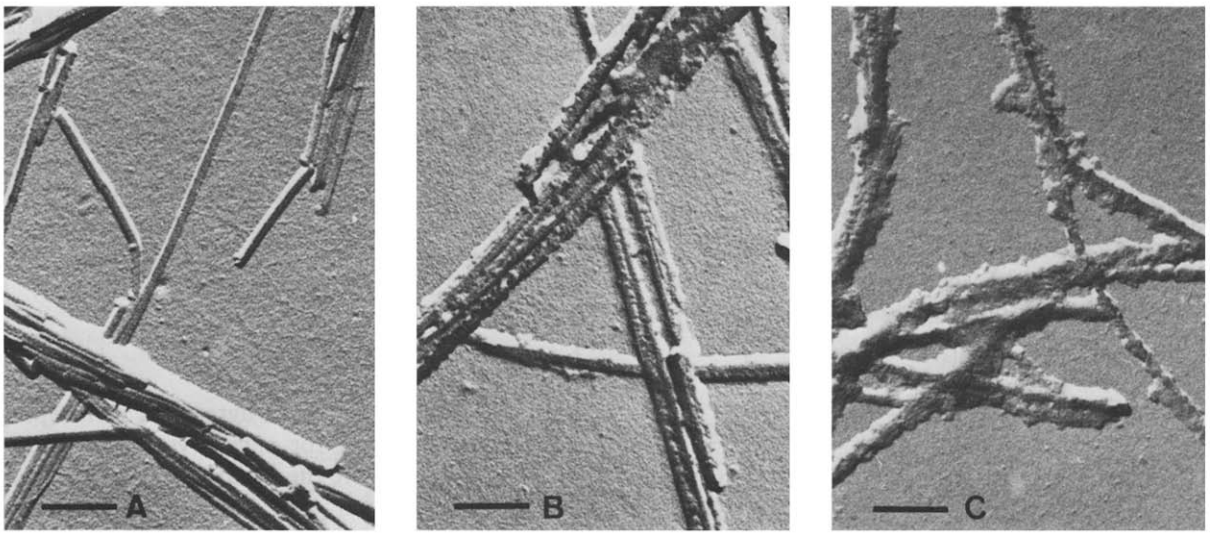


Fig.2. (A) *Valonia* microcrystals; unidirectional W/Ta shadowing; (B) as in (A) but after 15 min contact with 0.1 mg CBHI/ml; (C) as in (A) but after 36 h contact with 2 mg CBHI/ml.

3.2. *Hydrolysis of* Valonia *microcrystals with CBHI*

The coated microcrystals such as in fig.2C can be cleaned readily by dilute aqueous NaOH which has no action on the initial microcrystals (fig.3A), but removes all the adhering precipitate from the digested crystals. This is clearly illustrated in fig.3B which corresponds to the specimen displayed in fig.2C after washing with alkali. The degraded microcrystals are obviously eroded into narrower crystals with lateral dimensions down to a few nanometers. Several microcrystals are subfibrillated into smaller elements while their overall shape is maintained.

The enzymatic degradation of the crystals can also be followed by electron diffraction (fig.4). As the digestion proceeds, the diffraction pattern displays visible modification as the equatorial dif-

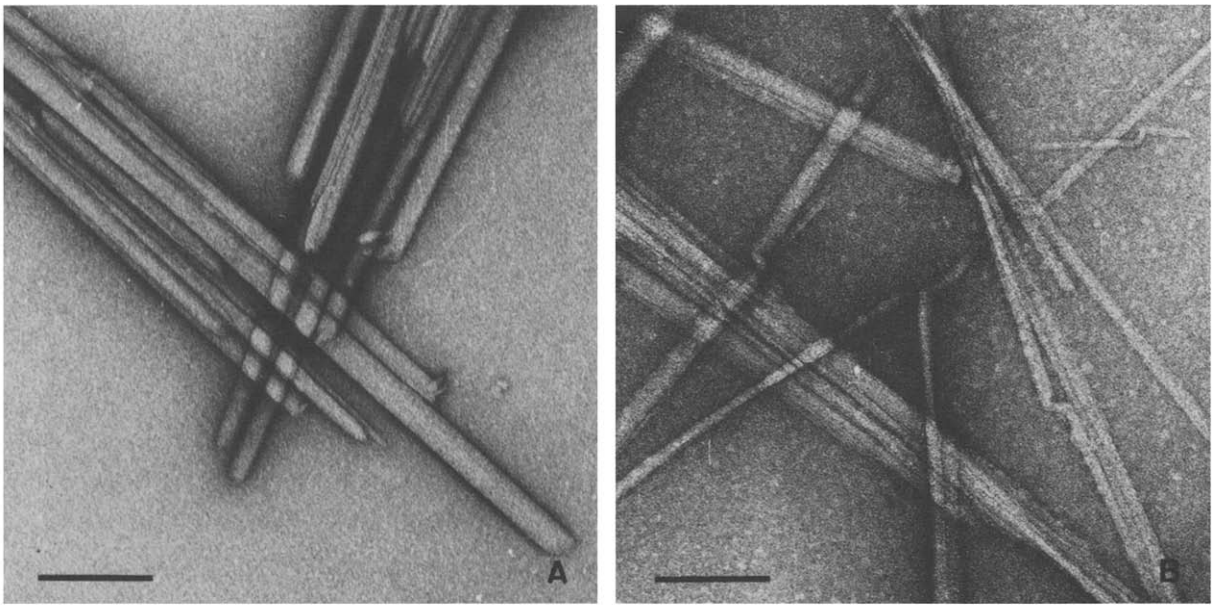
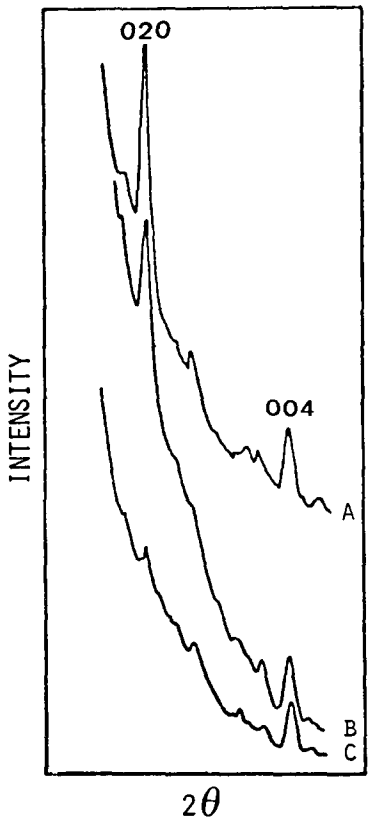


Fig.3. (A) *Valonia* microcrystals, negatively stained with uranyl acetate; (B) as in (A) but after 36 h treatment with 2 mg CBHI/ml and subsequent washing with 0.2% NaOH (bar = 100 nm).



fraction lines (100) and (020)* lose their intensity while the meridional (004) remains unaffected. This is already noticeable after 5 h (fig.4B) of interaction. In the ultimate case, only the meridional diffraction line is still visible while the equatorial lines have almost totally faded away (fig.4C).

4. DISCUSSION

These illustrative results agree well with reports on affinity of CBH for microcrystalline cellulose [5,6]. However, here the *Valonia* microcrystals are close to perfection and display a much larger specific area (~100 m^2/g when based on their geometry) than any Avicel which is rather badly defined at the ultrastructural level [14]. The above electron micrographs show that CBHI is strongly absorbed on *Valonia* microcrystals and remains so

*These indices refer to the two chains unit cell defined in [15].

Fig.4. Radial densitometer tracings of electron diffraction diagrams obtained with: (A) starting *Valonia* microcrystals; (B) *Valonia* microcrystals after 5 h digestion with 2 mg CBHI/ml; (C) as in (B) but after 16 h digestion with 2 mg CBHI/ml.

throughout the washing procedure in distilled water but not in dilute aqueous sodium hydroxide. This behavior confirms the results in [6].

Our results unambiguously suggest that CBHI from *Trichoderma reesei* alone is able to degrade highly crystalline cellulose, yielding a quantity of insoluble product which remains adsorbed on the core of crystalline cellulose together with the enzyme molecules. When the total cellulase complex is used on *Valonia* cellulose, the digestion occurs much more rapidly than this case owing to the synergistic action of the various enzymes present [13]. Here, the initial attack is more likely due to CBH, as proposed in [16] than to the classical endo-glucanase as suggested in [1]. CBHI was so far classified as an exoglucanase. However, in [11] it lacked specificity against cellodextrins. Our results suggest that with crystalline, CBHI displays an endoglucanase mode of action. Its recognition site is most likely connected with unsubstituted cellobiose; this would explain its low activity against CMC.

The similarity between the degraded microcrystals presented here with those after digestion with the total cellulase complex [13] is striking and suggests that the same component (CBH) is responsible for the disruption of the cellulose crystals. In both cases, the crystals break down by the splitting of lateral hydrogen bonds, thus yielding narrower and subfibrillated crystalline elements which nevertheless keep their initial length.

The present disruption, obtained with true cellulose crystals, can be correlated to the observed splaying of the composite bacterial cellulose subjected to cellulase degradation [17].

During the digestion of *Valonia* microcrystals by CBHI, soluble sugars are released. They consist of a majority of cellobiose, but with significant amounts of glucose as seen by the HPLC analysis (fig.5). This finding fits well with those obtained earlier with the same enzyme [10,11] but different substrates. It is likely that a quantity of insoluble cellodextrins are also produced and constitute a great part of the coating which decorates the microcrystals during their digestion (fig.2C). The analysis of the coating and its reactivity towards the various cellulase components should provide valuable information on the known synergism of these components during cellulose degradation.

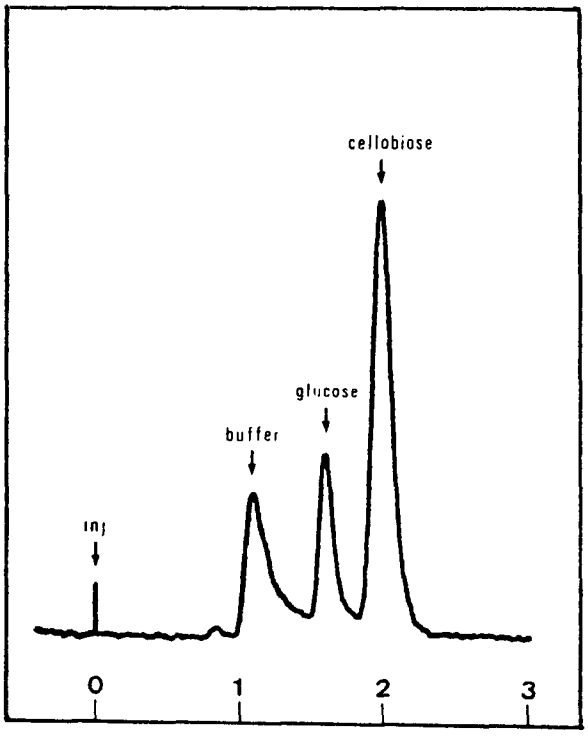


Fig.5. HPLC analysis of the soluble carbohydrates released after 24 h digestion of *Valonia* microcrystals with 0.5 mg CBHI/ml.

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