Colloidal gold labelling of 1,4-β-D-glucan cellobiohydrolase adsorbed on cellulose substrates

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The 1,4-β-D-glucan cellobiohydrolase I (CBHI) from *Trichoderma reesei* was labelled with 4–6 nm gold particles. This CBHI–gold complex still retains 60% of the original CBHI activity and allows good visualization of the enzyme adsorbed at the surface of cellulose mirofibrils or microcrystals. The main features of the adsorption are: rapid and irreversible binding; preference of the crystal edges instead of the crystal surface for the binding; the absence of specificity for the crystal tips where the cellulose chain ends are supposedly located.

Colloidal gold *Enzyme labelling* *1,4-β-D-Glucan cellobiohydrolase* (Trichoderma reesei)
Adsorption *Cellulose* *Electron microscopy*

1. INTRODUCTION

The digestion of semi-crystalline cellulosic materials involves the action of a composite cellulase system where the individual components operate at various rates, depending not only on their ability to bind more or less tightly to the cellulose surface [1,2], but also on the crystallinity and surface area of the substrate [3,4]. For instance, among the various cellulase components produced by *Trichoderma reesei,* one of the 1,4-β-D-glucan cellobiohydrolases (CBH, EC 3.2.1.91), namely CBHI, exhibits a strong affinity for highly crystalline cellulose such as Avicel [5] or *Valonia* cellulose microcrystals [6]. CBHI is even able by itself to digest completely cellulose crystals [6,7] though the digestion progresses at a lower rate than when the other cellulase components are present in the system.

To date, one can only speculate on the mechanism by which an enzyme such as CBHI can break down crystalline material such as cellulose microcrystals. The process must involve a specific adsorption of the enzyme on a given plane or edge of the crystal followed by directional hydrolysis along a given crystallographic direction within the substrate. Here, the visualization of these events was attempted using electron microscopy. For this, the colloidal gold labelling technique [8] was adapted for CBHI and the adsorption of the labelled enzyme on crystalline cellulose substrates was followed.

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2. MATERIALS AND METHODS

CBHI from *T. reesei,* purified from Celluclast™ as in [9] followed by a further purification on Sephacryl S-200, was a gift from Dr M. Schülein (Novo).

Vesicles of *Valonia macrophysa* were slit open and purified as in [10]. Microcrystals of *V. macrophysa* cellulose and microcrystalline bacterial cellulose were obtained after an acido-mechanical treatment as in [11], performed either on the alga cell walls or on the cellulose from the bacteria *Acetobacter xylinum.* Microcrystalline bacterial cellulose was further purified from non-cellulosic contaminants as in [10].

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2.1. Preparation of the Au–CBHI complex

A colloidal gold suspension (particle size 4–6 nm) was prepared as in [12]. Its pH was adjusted to 4.5 by the addition of dilute acetic acid. To 1 ml of 0.2 mg/ml CBHI solution in 0.05 M sodium acetate buffer (pH 4.8), were added 5 ml gold suspension. After mixing, this was followed by the successive addition of 0.5 ml of 5% aqueous NaCl and 0.5 ml of 1% aqueous polyethylene glycol (PEG, M_r 20 000). The mixture was then centrifuged at 15 000 × *g* for 1 h and the red mobile phase was pipetted and diluted into 5 ml citrate–phosphate buffer (CPB, pH 4.8) containing 0.1% PEG. The centrifugation was repeated and the red mobile phase diluted again into 5 ml CPB containing 0.1% PEG.

2.2. Adsorption of the Au–CBHI complex on cellulose

A series of suspensions of cellulose microcrystals were prepared in CPB (pH 4.8) containing 0.1% PEG. The cellulose concentration of these suspensions ranged from 0.02 to 1 mg/ml. The adsorption of the Au–CBHI complex was achieved at room temperature by the addition of 2 ml Au–CBHI complex to 1 ml cellulose suspension. Adsorption of the complex at the cellulose surface took place very rapidly and was estimated to be complete within 30 min. Drops of the complexed microcrystals were then deposited on electron microscope carbon-coated grids and allowed to dry. Blank experiments were also run with PEG-treated Au solutions where CBHI was absent.

Purified cell wall fragments of *V. macrophysa* vesicles were carefully delaminated in small strips and were slowly immersed in a solution of Au–CBHI complex. Within 30 min, the *Valonia* fragments took on the distinct pink color. They were then washed by dripping 3 times into buffer solution and finally deposited on electron microscope grids.

2.3. Hydrolysis of cellulose with the Au–CBHI complex

The residual activity of the complex was estimated with an Au–CBHI complex solution prepared as above except that at the last centrifugation step, the red mobile phase was diluted in 2 ml CPB containing 0.1% PEG. To this concentrated complex solution were added 2 mg microcrystalline bacterial cellulose and the mixture was allowed to react at 45°C for 8 h. The amount of reducing sugars was measured in the supernatant by the dinitrosalicylic acid method [14]. It was compared with a blank experiment where an equivalent amount of uncomplexed CBHI was used instead of the Au–CHBI complex.

2.4. Electron microscopy

A Philips EM 400T electron microscope was used. Commonly, the various specimens were observed without further shadowing or staining and using the minimum dose technique (acceleration voltage 120 kV, reduced beam intensity, reduced time of focusing and fast exposure of Ilfoset plates). Some samples were also observed at 80 kV after shadow-casting with W/Ta alloy.

3. RESULTS

When the Au–CBHI complex is mixed with a suspension of cellulose microcrystals, all the coloured complex readily adsorbs at the cellulose surface. Such a reaction is specific to the complex as illustrated in fig.1: a suspension of microcrystalline bacterial cellulose was mixed with a solution of PEG-treated Au–CBHI complex in fig.1A and with PEG-treated colloidal gold in fig.1B. Clearly,

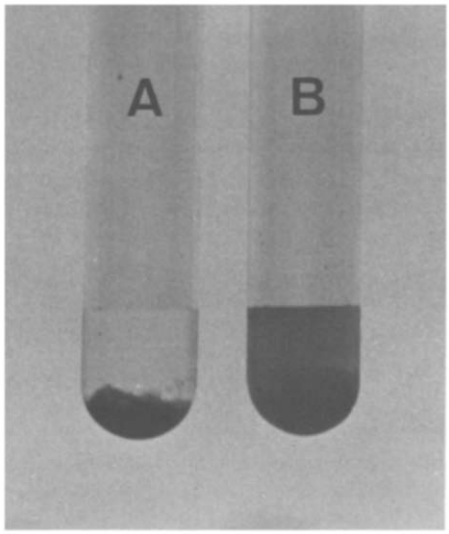

Fig.1. Sedimentation of suspension of bacterial cellulose microcrystals mixed with Au–CBHI complex (A) and only colloidal gold (B). Contact time 30 min.

in fig.1A and after only 30 min, the cellulose microcrystals fix all the gold complex as they settle in the form of a dark-pink sediment leaving a clear supernatant. On the other hand, in fig.1B, no interaction takes place and the supernatant as well as the sediment retain an identical light-pink colour.

The adsorption of the Au–CBHI complex on cellulose can also be visualized at the ultrastructural level by electron microscopy. In the present case, spherical gold particles (~5 nm diameter) are easily resolved and observed at the substrate surface. This is illustrated in fig.2, corresponding to the sorption of Au–CBHI complex on *Valonia* cell wall fragments. This figure reveals that the once smooth and featureless cellulose microfibrils are now outlined by a double string of almost contiguous gold particles. In general, the adsorbed gold particles occur as isolated individuals, in which case they tend to be aligned regularly at the microfibril edges. In several instances however, the particles are aggregated in clusters and in such cases, they cover not only the microfibrils edges but also their surface (several clusters are observed for example in the inset in fig.2).

Fig.3 corresponds to other substrates and different experimental conditions. In fig.3A, the Au–CBHI complex was applied to a concentrated suspension of *Valonia* microcrystals. In this case, the complex is almost cluster-free and occupy only a small percentage of the microcrystals contours. In fig.3B, the cellulose suspension was much more diluted and accordingly, the *Valonia* microcrystal edges are almost totally saturated by the gold-enzyme particles. A similar situation occurs in fig.3C, this time with a bundle of bacterial cellulose microcrystals. In this case, the gold particles outline the individual cellulose crystals within the bundle, with again a gold decoration located preferentially at the edges of the crystals and more seldomly on their surface.

The residual activity of the Au–CBHI complex was estimated with bacterial cellulose as substrate.

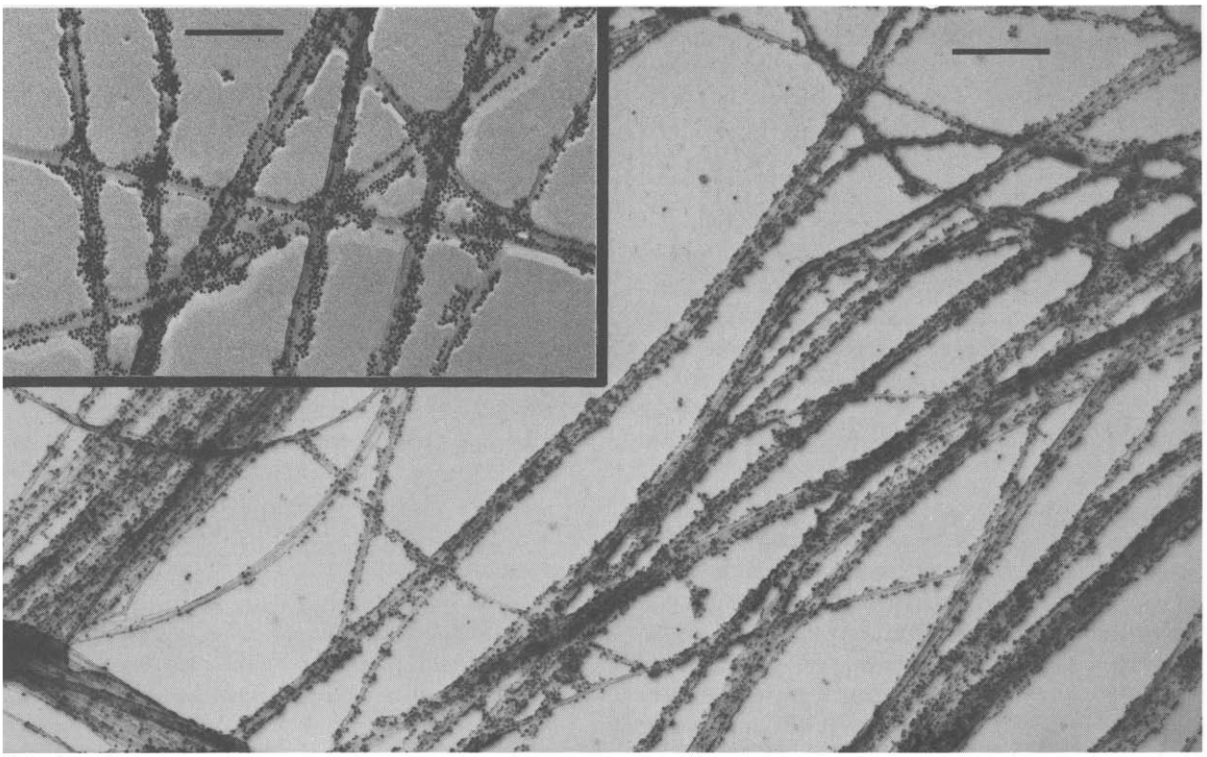

Fig.2. *V. macrophysa* cell wall fragment after immersion for 30 min in a solution of Au–CBHI complex. Unshadowed specimen observed with minimum dose technique (bar = 500 nm). Inset: higher magnification view after shadow-casting with W/Ta (bar = 250 nm).

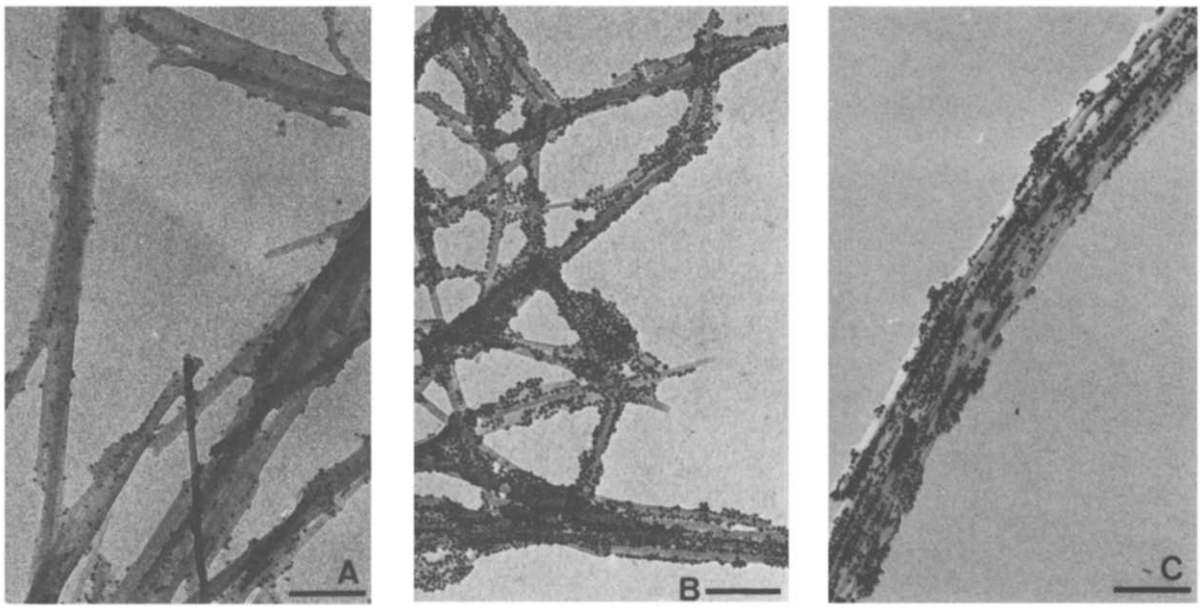

Fig.3. (A) *Valonia* microcrystals after mixing for 30 min with the solution of Au–CBHI complex. Overall cellulose concentration 0.3 mg/ml. Unstained and unshadowed (bar = 200 nm). (B) Identical to A but overall concentration of 6.6×10^{-3} mg/ml. Unstained and unshadowed (bar = 200 nm). (C) Bacterial cellulose microcrystals after mixing for 30 min with the solution of Au–CBHI complex. Overall cellulose concentration 0.15 mg/ml. Shadowed with W/Ta (bar = 200 nm).

It was found to be 60% of that obtained with the uncomplexed CBHI. This finding, together with the above adsorption observations, indicates that the binding ability of the protein is unaffected by its bulky gold associate. On the other hand, the complexation depletes to some extent its catalytic ability.

4. DISCUSSION

Our results provide an illustration of the unusually high sorption characteristics displayed by CBHI from *T. reesei* for its substrate. Our data, obtained with highly crystalline cellulose materials, confirm the sorption kinetics data obtained by the investigators, on either Avicel [5] or cotton fibers [13] (i.e., composite substrates of overall lower crystallinity). Their observations, as well as ours, indicate that CBHI behaves rather differently from classical enzymes: the sorption is extremely rapid and irreversible as long as proper ionic strength and pH are maintained. Furthermore, the enzyme is so tightly bound that drastic hydrogen-bond breakers have to be used to desorb it from the cellulose surface [5].

It has been proposed that variations in sorption characteristics of cellulases may depend on their sugar content [1]. This could account rather well for the high sorption ability of CBHI, as this glycoprotein contains up to 10% of carbohydrates, of which mannose is dominating [14,15]. The high affinity of cellulose crystals for mannose-rich polymers or macromolecules is a well established fact. For instance, with mannan [poly(1,4-β-D-mannose)] of low degree of polymerization, the addition of *Valonia* microfibrils induces nucleation of rows of mannan crystals or mannan precipitate at the cellulose surface, leading to the spectacular shish-kebab morphology [16]. It is remarkable that the adsorption of Au–CBHI on *Valonia* microfibrils (as displayed in fig.2) also gives a shish-kebab-like appearance with rows of gold–enzyme complexes deposited with some regularity at the microfibril surface. This behaviour may find its origin in the strong affinity of cellulose for the mannose-rich part of the glycoprotein. If this is the

case, then the adsorption site of CBHI on its substrate may be different from its catalytic site. A consequence of this hypothesis would be that the enzymes should remain essentially immobilized at the cellulose surface as long as its underlying crystallinity is not totally destroyed.

In fig.2,3 it was shown that most of the Au–CBHI complex had a preference for the edges of *Valonia* microfibrils and microcrystals. Such specificity is expected as the 4 lateral faces of *Valonia* microfibrils are unequivalent, with the (1$\bar{1}$0) (indices refer to the two-chain unit cell defined in [10]) face preferentially flat on the specimen supporting carbon film. The gold–enzyme complex is thus preferentially adsorbed on the (110) face of the microfibril, which may be important for the directionality of the subsequent enzymatic attack.

The present observations raise some questions concerning the enzymatic specificity of CBHI. If, as commonly believed [17], CBHI was attacking the cellulose crystals from the non-reducing chain ends, one should find a high concentration of enzymes at the tips of *Valonia* microcrystals where the non-reducing chain ends should be in abundance. In fact, CBHI do not display any preferential specificity for the microfibrils tips. Thus it is likely that the tightly sorbed enzymes will attack the cellulose microcrystals from their sides, with the implication that the initial hydrolyzing step will be of the *endo*-glucanase type as proposed in [6].

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