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Structure investigation of Cellobiohydrolase I from *Trichoderma pseudokoningii S38* with a scanning tunneling microscope

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Abstract. Cellobiohydrolase I (CBH I) was isolated from a cellulolytic fungal strain *Trichoderma pseudokoningii S38*, and its ultrastructure was investigated with a scanning tunneling microscope (STM). The STM images showed that the shape of intact CBH I was tadpole-like, consisting of a big head and a long tail. It could be deduced that the head domain was the core protein for the catalytic function, and the long tail was the cellulose binding domain for substrate binding. Thus, for this enzyme molecule, functional differentiation is reflected in the structure peculiarities. This is the first direct observation of the three-dimensional structure of intact CBH I from real space at nanometer scale. The functional mechanism is also discussed.

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Cellulose is the major polysaccharide component of a plant cell wall, and is the most abundant organic material on the planet. A number of bacterial and fungal organisms can use cellulose as a carbon source, possessing a cellulase system (cellobiohydrolases, endoglucanases, β-glucosidase, etc.) that can catalyze the hydrolysis of β-(1,4) glycosidic bonds. Cellobiohydrolases (CBH) are the most abundant components in the cellulase family, especially CBH I (E.C.9.2.1.94.), which plays an important role in the hydrolysis of crystalline cellulose [1]. It cleaves the β-(1,4) glycosidic bonds to release cellobiose and has strong synergism with endo-glucanases [2, 3]. So far, the mechanism of cellulose biodegradation can not be completely explained, because of the complicated structures of native cellulose substrates and cellulases. For the protein of CBH I, it is difficult to be crystallized and analyzed by the traditional method – X-ray diffraction. However, the whole protein, which has been sequenced, could be cleaved into two parts by protease catalysis, that is, core protein (CP) and cellulose binding domain (CBD) [4]. The three-dimensional structure of the catalytic domain of Cellobiohydrolase I from the potent cellulolytic fungus *Trichoderma reesei* has been determined by X-ray diffraction [5]. Schmuck et al. (1986) [6] put forward the tadpole-like structure model of intact CBH I molecules from *Trichoderma reesei* according to the small-angle X-ray scattering (SAXS) data. But to date, the three-dimensional structure of intact CBH I molecule was not much better resolved. At the same time, such structural information was not enough for us to understand the behavior of enzymes on the substrates and the interaction between them. Here, we report the STM images of intact CBH I molecule from *Trichoderma pseudokoningii S38*, and compare them with the model structure of CBH I from *Trichoderma reesei*. Finally, the functional mechanism of CBH I is discussed.

1 Materials and methods

1.1 Separation of CBH I

CBH I was isolated from the solid-fermentation products [7] of *Trichoderma pseudokoningii S38*, a fungal strain with high cellulolytic ability isolated in our lab [8].

It was purified with ultrafiltration and a series of chromatographic methods, including columns of DEAE Sephadex A-25 and Sephadex G-100. There was only a single band in SDS-PAGE [9]. The activity of CBH I was determined as described in [10].

1.2 Observation of CBH I by STM

The CBH I component was diluted to the concentration of 5 μg/ml with distilled water. 5 μl of the CBH I solution was dropped on a freshly cleaved HOPG (highly oriented pyrolytic graphite) surface, and then adsorbed on it for 30–60 s in air at room temperature. The excess solution was removed with filter papers.

STM experiments were carried out in ambient environment with a domestic STM setup CSPM-930a (manufactured by Institute of Chemistry, Academia Sinica). STM measurements were performed with normal STM constant-current mode, using tungsten tips made by electrochemical etching. Tunneling current and bias voltage are indicated in the relevant legends of photographs. All STM images presented here are raw data images without any smoothing or filtering.

2 Results and discussions

STM can only observe the surface structure of objectives with electric conductivity. In general, macrobiomolecules have relatively poor electric conductivity. However, CBH I is a kind of water-soluble protein, a lot of hydrophilic groups are distributed on the molecular surface. Therefore, when the CBH I was dissolved in the buffer solution, water molecules and ions in the buffer adsorbed on the molecular surface, which will enhance the electric conductivity. So STM can be used to investigate the structure of CBH I molecules.

Figure 1a,b is the STM image of intact CBH I molecules on graphite surface. The picture clearly shows that CBH I molecules are evenly distributed on the HOPG surface. As the molecules are randomly adsorbed on the HOPG surface, every molecule has a different orientation. Therefore, there should be a little difference in the molecules' shapes, which shows that the STM images are true. Though, we can also see that all the molecules were similar in shape, which was like a tadpole.

With higher resolution, the clearer STM images of intact CBH I were obtained and these are shown in Fig. 2a,b. From these images, we can see more clearly that the CBH I molecule has an unusual shape like a tadpole. The intact molecule consists of an isotropic head and a long tail. The maximum length of the whole molecule was 29 ± 2 nm, and the maximum width was about 9 ± 1.0 nm. According to the reported results, it could be deduced that the isotropic head coincided with the core protein (CP), and the flexible tail part

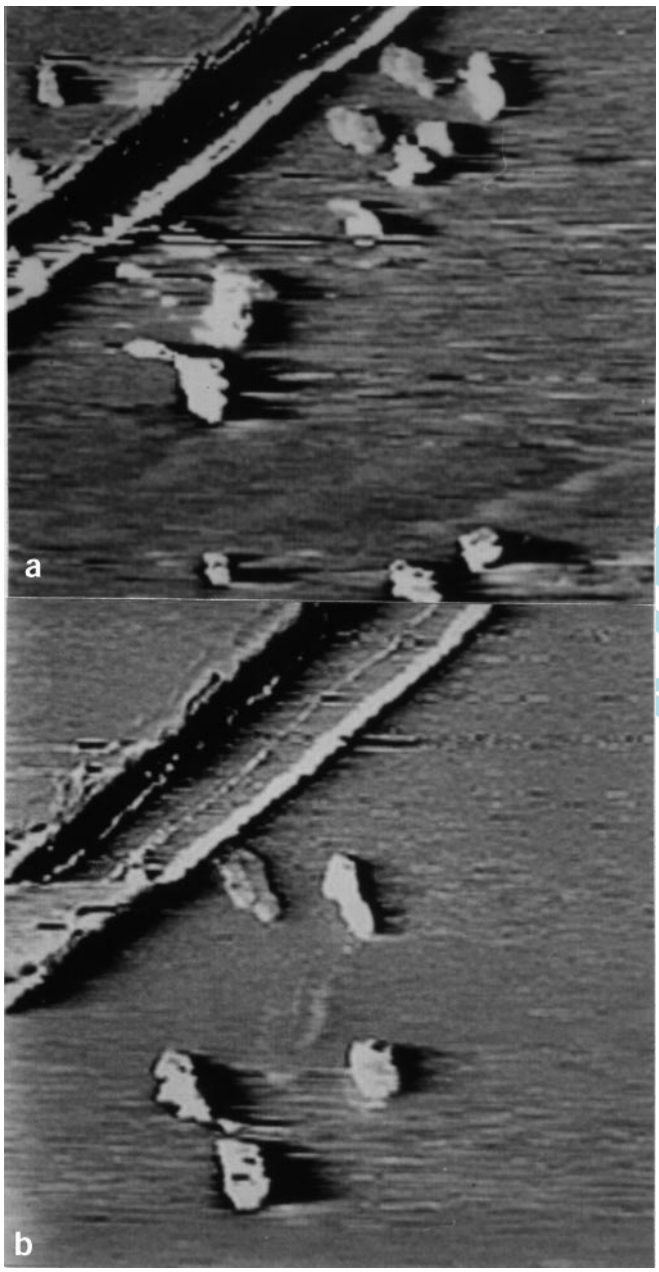

Fig. 1a,b. STM images of CBH I from *Trichoderma pseudokoningii S38*. $I_t = 0.35$ nA, $V_{bias} = 534$ mV. Scan area: **a** 160 nm × 160 nm, **b** 160 nm × 160 nm

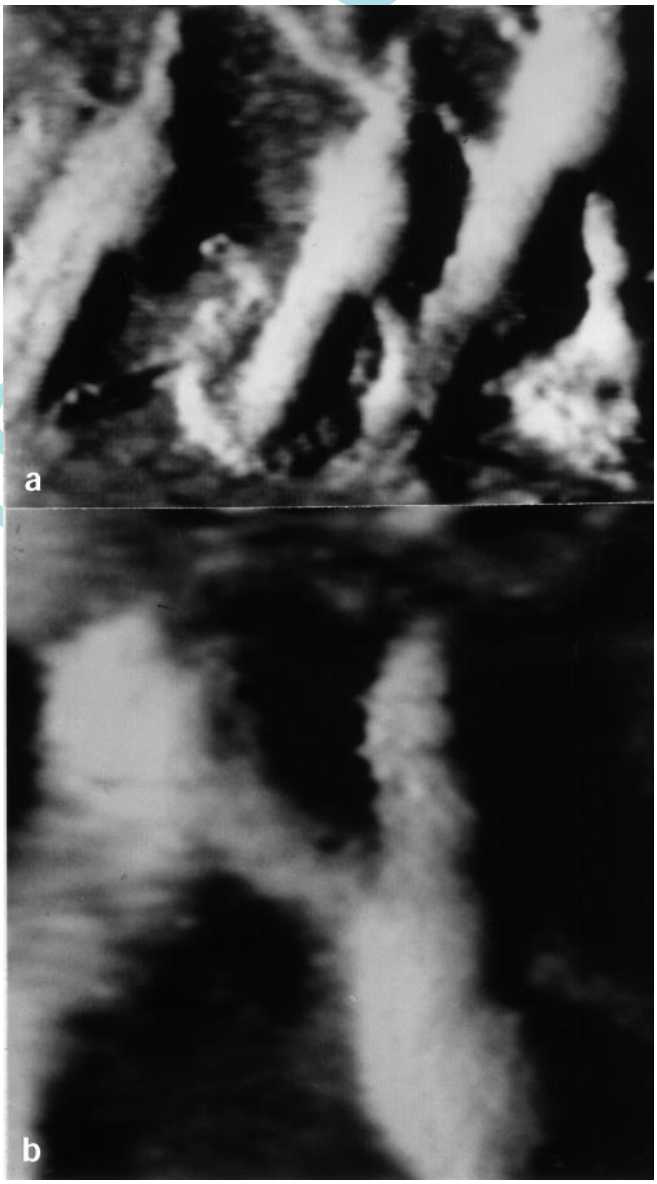

Fig. 2a,b. STM images of CBH I from *Trichoderma pseudokoningii S38* with higher resolution. $I_t = 0.19$ nA, $V_{bias} = 180$ mV. Scan area: **a** 36 nm × 30 nm, **b** 32 nm × 32 nm

should be identified with the C-terminal glycopeptide, i.e. the cellulose binding domain (CBD). The CP domain had a maximum length of 10 ± 1.2 nm, its largest width was up to 9 ± 1.0 nm, and the maximum height was up to 6 ± 0.8 nm. It was responsible for the catalytic function. While the CBD was responsible for enzyme molecules binding on the surface of the substrate – cellulose, its maximum length was 19 ± 2.0 nm, and the maximum width and height were approximately 7 ± 1 nm and 4.5 ± 0.6 nm, respectively. Schmuck et al. (1986) put forward the structure model of CBH I from *Trichoderma reesei* according to small-angle X-ray scattering (SAXS) data which is shown in Fig. 3. In the model structure of CBH I, the length and the diameter of CP was 6.7 nm and 4.4 nm, respectively, the length and the maximum diameter of CBD was 12.9 nm and 3.2 nm, respectively. We can see that the shape of CBH I from *Trichoderma pseudokoningii S38* was very similar to the model structure of CBH I from *Trichoderma reesei*. We can also see that the ratio of head and tail of CBH I from *Trichoderma pseudokoningii S38* by STM was very similar to that from *Trichoderma reesei* by SAXS. In general, the hydrolase was globular proteins. CBH I was a special one in shape, which was like a tadpole. According to the SAXS results, the CBH I molecule was cylindrical. In the STM images, the width was much larger than the height, which was due to the error induced by the curvature radius of the STM tip [11].

We have successfully observed the ultrastructure of the native cellulose and crystalline cellulose [12, 13]. The results suggested that elementary fibril was the smallest structural unit of the crystalline cellulose. The internal diameter of elementary fibril was about 3 nm, whereas the diameter of microfibril, which was composed of elementary fibrils, was 20–30 nm. The elementary fibrils piled up in parallel [13]. For biomaterials, the structure is closely connected with its function. Such kind of structure of CBH I makes it more easy for the enzyme molecule to act on the surface of cellulose. At first, the CBD binds on the surface of cellulose, then, the CP domain moves back and forth on the cellulose surface to degrade the cellulose molecule. As for the CBD, it might play its function in two possible ways [14]: (i) adsorbing on the surface of the substrate to help the CP domain with the hydrolytic function; (ii) cleaving the hydrogen bond network between cellulose molecules and separating the cellulose clusters so as to increase the susceptibility of the substrate and improve the degrading efficiency. The CBH I structure revealed from the STM images proved the hypothesis of functional mechanism of CBH I. In addition, the maximum length and diameter of CBD was 19 nm and 7 nm, respectively. Therefore, CBD should adsorb on the elementary fibrils.

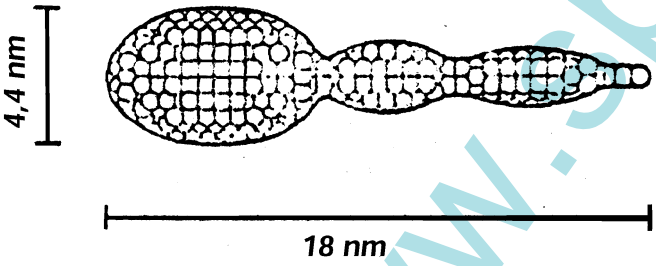

Fig. 3. The model structure of CBH I in *Trichoderma reesei* deduced from small-angle X-ray scattering data (Schmuch et al. 1986)

It is very difficult to obtain the crystal of cellulase because its structure was not symmetrical and it was highly glycosylated. So far there is no crystal structure for the whole molecule of CBH I. This is the first time to observe directly the shape of intact enzyme molecules from real space at nanometer scale, which will help to elucidate the enzymatic degradation mechanism of native cellulose. Later, STM will be used to observe directly the adsorption of cellulase on the surface of the cellulose, the structure of cellulose–cellulase complex, the changes of CBH I conformation during degradation, and the dynamic hydrolytic process of cellulose by cellulases.

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