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Mechanism of cellobiose inhibition in cellulose hydrolysis by cellobiohydrolase

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Abstract An experimental study of cellobiose inhibition in cellulose hydrolysis by synergism of cellobiohydrolyse I and endoglucanase I is presented. Cellobiose is the structural unit of cellulose molecules and also the main product in enzymatic hydrolysis of cellulose. It has been identified that cellobiose can strongly inhibit hydrolysis reaction of cellulase, whereas it has no effect on the adsorption of cellulase on cellulose surface. The experimental data of FT-IR spectra, fluorescence spectrum and circular dichroism suggested that cellobiose can be combined with tryptophan residue located near the active site of cellobiohydrolase and then form steric hindrance, which prevents cellulose molecule chains from diffusing into active site of cellulase. In addition, the molecular conformation of cellobiohydrolase changes after cellobiose binding, which also causes most of the non-productive adsorption. Under these conditions, microfibrils cannot be separated from cellulose chains, thus further hydrolysis of cellulose can hardly proceed.

Keywords: cellobiohydrolase, cellobiose, inhibition.

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Exo-glucanase (1,4-β-d-glucan cellobiohydrolase I, CBH I) is the major fraction of cellulolytic system, and is a key enzyme component in cellulose biodegradation. Cellobiose is the main final product in hydrolysis process of cellulose both by cellobiohydrolase[1], and by endoglucanase, EG[1,2]. In physiological sense, cellobiose is a stronger inhibitor for cellulase, and its potency of inhibition is greater than that of glucose[3,4]. Thus, the elimination of cellobiose inhibition is necessary for obtaining a complete hydrolysis of cellulolytic materials. In general, adsorption of cellulase to cellulose surface is the first and necessary step in enzymatic hydrolysis, while the presence of cellobiose does not affect the adsorption of cellulase. It is difficult to describe the mechanisms of cellobiose inhibition by using the traditional kinetic methods, because of the insolubility and structural heterogencity of cellulolytic materials and the complexity of cellulase adsorption. Furthermore, we should not take the true results into account when soluble oligose was used as substrate. Although the three-dimensional structure of CBH I has been clearly observed[5], the inhibition mechanism of cellobiose involved is not well understood[3]. This work is a sequel to our previous work[6—12] that was therefore of interest to find out the different characteristics between adsorption of native CBH I and the adsorption of CBH I following binding of cellobiose. A mechanism including change of conformation of CBH I and formation of steric hindrance by non-productive adsorption was proposed and discussed.

1 Experimental methods and materials

1.1 Cellulase

CBH I and EG I were purified from culture filtrate of *Trichoderma pseudokoningii* S-38 as described in refs. [6,7]. A serial and comprehensive investigation of their biochemical properties was carried out in our laboratory[6—12].

1.2 Inhibition experiments

Because CBH I can only effectively hydrolyze microcrystalline cellulose in the presence of EG I, EG I was also added in this reaction system, which contained (*w/v*) 2% microcrystalline cellulose, 1% cellobiose and CBH I and EG I of 2 nmol each. Reducing sugar was determined by the dinitrodsalicylic acid (DNS) method[7].

1.3 Adsorption experiments

The procedure was similar to that reported by Palomen et al.[13], with some modification. Briefly, the initial concentration of CBH I was 250 nmol to 2 μmol. The concentration of microcrystalline cellulose (PH101) suspended in 20 mmol pH 5.0 NaAc buffer was 2 g • L^{-1}. The mixture was centrifuged at 10000 *g* for 15 min after 12 h incubation at 4℃. With *p*-nitrophenyl-cellobiose (*p*NPC) being substrate[6], the binding isotherm data of CBH I were fit in with saturation isotherm.

1.4 Estimation of conformational changes in CBH I molecule and structural change of cellulose

Fluorescence measurements (emission spectra and exciton spectra) were performed on a Hitachi M-850 fluorescence spectrophotometer. Circular dichroism (CD) was measured with a Japan-720 spectropolarimer[11]. The IR spectra were determined on an FT-IR 710 infrared spectrophotometer (Nicolet, Instrument Corp., USA), and recorded using a D-TGS detector in transmission mode at a resolution of 4000 —400 cm^{-1}. Samples were dried for three days in vacuum oven at 40℃ and then dispersed in KBr discs.

Select the 2900 cm^{-1} band (CH stretching) to serve as an internal standard and use OMNIC software package to correctly convert the data of FT-IR spectroscopy, such as using Fourier Sel-deconvolution techniques and by using Gaussian-Lorenzian curve fitting analysis to obtain quantitative information. For each experiment, several measurements were made with a small variation in the static stress. For STM (Scanning tunneling microscope) experiments, the treated cotton fiber was suspended in anhydrous ethanol, dropped on a freshly cleaved and highly oriented pyrolytic garphite (HOPG), and dried in air. STM experiments were conducted with a domestic STM setup CSPM-9100[3,16].

2 Results and discussion

2.1 Inhibition of cellobiose on enzymatic hydrolysis of microcrystalline cellulose

The top curve in fig. 1 presents a typical apparent reaction process illustrating the time course of enzymatic hydrolysis of cellulose under the normal hydrolysis conditions. That also clearly demonstrated the obvious inhibition of cellobiose for enzymatic hydrolysis of microcrystalline cellulose (bottom curve). Fortunately, for the cellulose-cellulase system, the initial hydrolysis rate, either apparent or intrinsic, was difficult to evaluate, because of the complexity of cellulase system as mentioned above. Thus, the methods such as Liveave-Burk or Dixon plots cannot be adopted in predicting the recovery of the inhibition type under this state. By ultrafiltration (Amicon, PM-10 with cut-off 1000 Da) to remove cellobiose from the hydrolysate (aqueous phase), re-hydrolysis was carried out, and the fact that this inhibition phe-

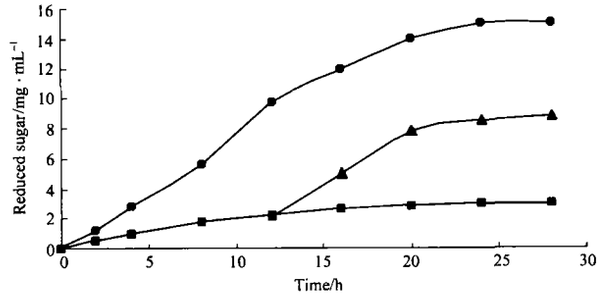

Fig. 1. Inhibition of cellobiose on the enzymatic hydrolysis of microcrystalline cellulose (PH101). ●, Without cellubiose; ■, add cellobiose; ▲, remove cellobiose by ultrafiltration.

nomenon can be eliminated demonstrated that this inhibition type belongs to isosteric inhibition[18,19].

2.2 Effect of cellobiose on the adsorption capacity of CBH I for crystalline cellulose

Experimental data suggested that added cellobiose causes the hydrolysis rates of cellulose to decrease by 90%. However, under these conditions, the adsorbed amount of CBH I only decreased by 10% (figure omitted). This phenomenon differs greatly from those of an ordinary light, thus further research is required.

As mentioned above, adsorption of cellulase to cellulose surface is a necessary step in enzymatic hydrolysis of cellulose[1,2,4], and its hydrolysis rate fundamentally depends on the bounded enzyme[3,17]. Surprisingly, some significantly different phenomena of the effects of cellobiose on adsorption of cellulase and hydrolysis were reported recently. Palomen observed that 1.5 mmol cellobiose can double the adsorptive capacity of catalytic domain of CBH I, but have no effect on intact CBH I[13]. While Herner reported that cellobiose can better the adsorptive capacity for both components[17].

These results imply that the mechanism of cellobiose inhibition should not be directly obtained in deriving the traditional kinetic investigation of cellulose by CBH I.

2.3 Changes in conformation of cellulose molecules after binding CBH I

Fig. 2 shows the IR-spectra of microcrystalline cellulose after binding with CBH I. In fingerprint region 1400—900 cm^{-1}, which is mainly due to the stretching and vibrations of C-C, C-O groups, the position and peak shape of those bands have few or no changes, suggesting that adsorption of CBH I on cellulose has no effect on glycoside bonds.

However, the decrease of the intensity of hydrogen bonds network (around bands of 3600—3200 cm^{-1}) can be clearly observed as the main cause of separation of the microfibrils from cellulose chain (figs. 2 and 3). These structural changes are necessary for further enzymatic hydrolysis of cellulose, and show good agreement with those reports on investigation of EGIII[20,21].

But, an opposite result was obtained in the binding process performed in the presence of 1% (*w/v*) cellobiose. Under these conditions, intensity of hydrogen bond in the region 3600—3200 cm^{-1} was increased (fig. 4), and no separated microfibrils could be observed by STM examination (figure omitted). As mentioned above, the occurrence of microfibril is necessary for enzymatic hydrolysis, so that cellobiose prevents the separation of microfibril would be another reason for its inhibition.

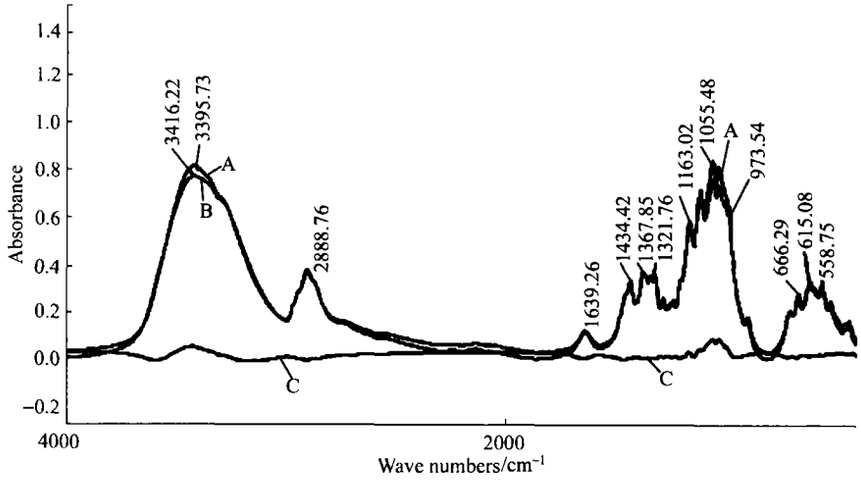

Fig. 2. Differential IR spectra of microcrystalline cellulose after binding CBH I. A, Native microcrystalline cellulose; B, after binding of CBH I; C, differential spectra between A and B.

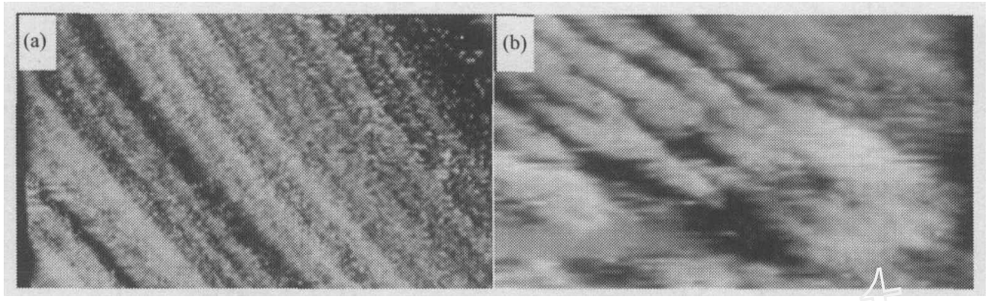

Fig. 3. STM photograph: Changes in super-structure of microcrystalline cellulose before (a) and after binding by CBH I (b). (a) Iref = 0.28 nm Vbias = 420 mV, scan area 32 × 18 nm; (b) Iref = 0.10 nm Vbias = 266 mV, scan area 24 × 14 nm.

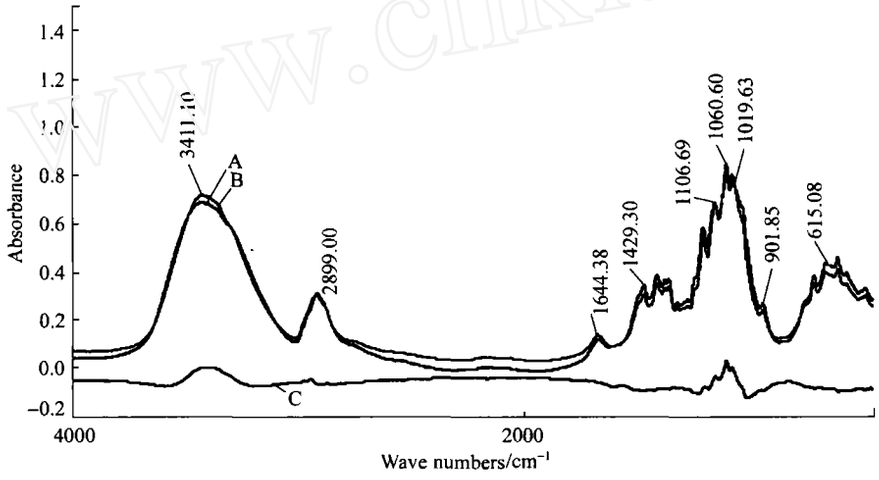

Fig. 4. Differential IR spectra of microcrystalline cellulose before and after binding CBH I with cellobiose. A, Native microcrystalline cellulose; B, after binding CBH I in the presence of cellobiose; C, differential spectra between A and B.

2.4 Conformational changes of CBH I molecule after binding cellobiose

Fig. 5 shows the comparison of the conformation of CBH I before and after binding with cellobiose.

After binding, the intensity of hydrogen bonds in the region 3600—3200 cm^{-1} is significantly increased and that of Amide I (around 1700 cm^{-1}) is also increased, while Amide II and Amide III (1600—1400 cm^{-1} and near 1300 cm^{-1}) have few changes. By using trace CBH I as a perturbation affect cellobiose (molecule ratio : 1 : 100, CBH I : cellobiose), after binding, some obvious changes in IR spectra of cellobiose were observed. Intensity of hydrogen bond significantly decreased, and the peaks 3431 cm^{-1} and 3370 cm^{-1} combined into a broad peak. The position and peak of several bands near 1400—1000 cm^{-1}(O—H) 1300—1000 cm^{-1} (C—O) and in finger-print region (1400—900 cm^{-1}) were both changed (figure omitted). These conformational changes of CBH I and cellobiose molecule indicated that after binding, cellobiose molecule can enter into the active site of CBH I, and the interaction between the hydroxyl group (OH) of cellobiose and the Amide of CBH I occurred, which led to conformational changes of CBH I.

2.5 Changes of fluorescence of CBH I molecules following bound cellobiose

The data of table 1 indicated that the intrinsic fluorescence of CBH I is mainly derived from tryp-

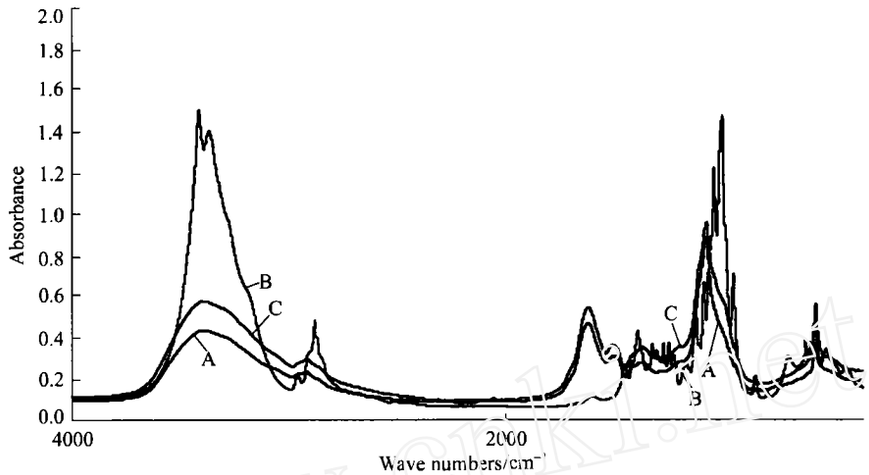

Fig. 5. Differential IR spectra of CBH I without (A) and with cellobiose (B), and differential spectra between A and B (C).

Table 1 Excitation and emission spectra of native CBH I and denatured CBH I by Gu • HCl

Enzyme	Excitation		Emission	
	λ_{ex}	emission	excitation	λ_{em}
Native CBH I	283	310	280	343
	282	360	295	343
Denatured CBH I by Gu·HCl	279	310	280	360
	281	360	295	360

tophan residue. Experimental data (fig. 6) showed that after adding cellobiose (8 mmol • L^{-1}), the intensity of fluorescence of CBH I decreased by 5%—6% and the peak of emission spectrum moved to a higher position of 2 nm.

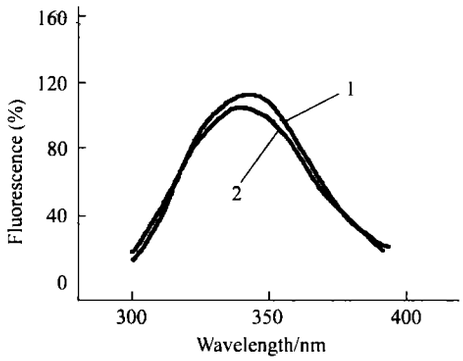

Fig. 6. Fluorescence spectra of CBH I after binding cellobiose. 1, Native CBH I; 2, CBH I in the presence of cellobiose.

According to the effect of binding with cellobiose on intensity of fluorescence of CBH I (figure omitted), its dissociation constant is 2.04 nmol • L^{-1} standard free energy change $\Delta G^0 = -RTIn(KS)^{-1} = -3.67$ Kcal • mol^{-1}. The modification of CBH I with NBS (N-bromosuccinimide and NBS : CBH I = 25 : 1) resulted in complete loss of CBH I activity, while after binding with cellobiose, 92.1% of the activity can be recovered. The modified numbers of tryptophan molecules were decreased from 7.2 to 6.4 following the cellobiose binding (fig. 7).

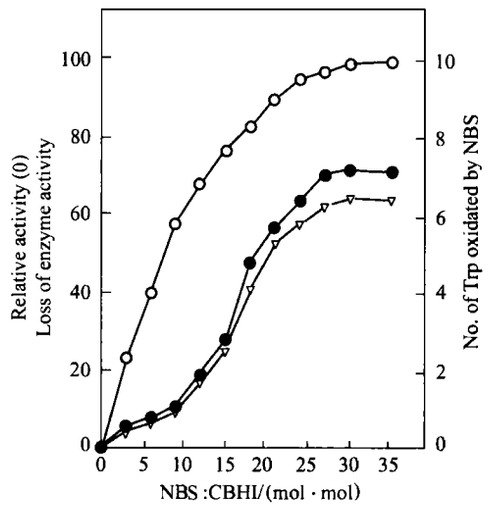

Fig. 7. Effect of cellobiose binding on the fluorescence of CBH I. O, CBH I activity in the presence of cellobiose; ●, number of tryptophan residues by the oxidation of NBS in the presence of cellobiose; ▽, number of tryptophan residues by oxidation of NBS in the absence of cellobiose.

2.6 Circular dichroism measurements

Circular dichroism measurements suggested that CBH I molecule shows a typical β-sheet structure, and

no gross conformational changes resulted from the cellobiose binding, suggesting that one of the tryptophan residues modified by NBS is probably situated on or near the active site of CBH I.

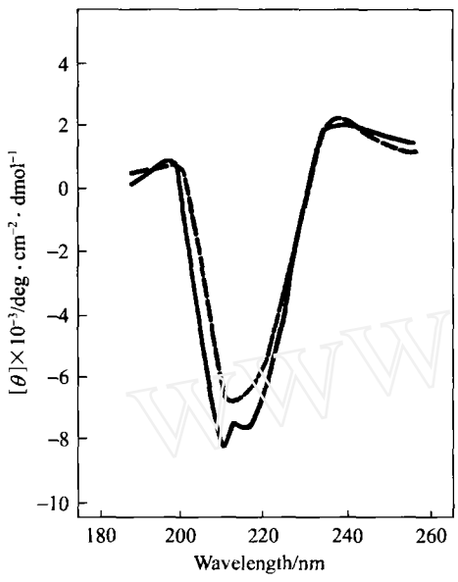

Fig. 8. Effect of oxidation by NBS on the conformation of CBH I.

Taken together, the results suggested that the inhibition of cellobiose towards the hydrolysis of cellulose by CBH I may come from the following two pathways:

1. After CBH I binding with cellobiose, the adsorption became non-productive, which cannot free the microfibrils from cellulose chain. As is well known, the active site of CBH I appears in a tunnel-shape in catalytic domain. It can only accept single microfibril[21], and there are no free microfibrils on the surface of native cellulose chains. Thus, before hydrolysis takes place, the separation of single microfibril is necessary[22,23]. However, the non-productive adsorption by CBH I binding with cellobiose cannot bring about such separation.

2. The combination of cellobiose with tryptophan near active site of CBH I automatically brings steric hindrance on surface of CBH I, which may close the tunnel, so hydrolysis reaction cannot proceed. These inhibitions belong to a reversed type, because when removing cellobiose from the reaction system, hydrolysis could still re-proceed. Recently Lee et al. reported that the inhibition of cellobiose for cellobiose arabone analogue belongs to stronger competitive inhibition[25], and the added β-glucosidase can effectively decrease or even eliminate this inhibition[17,28]. Furthermore, as combined with the biotechnique such as solidified enzyme in hydrolysis process, which can transmit cellobiose and glucose to another product, the enzymatic hydrolysis can continuously proceed.

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References

1. Beguin, P., Aubert, J. P., The biological degradation of cellulose, FEMS Microbiol. Rev., 1994, 13: 25—58.
2. Ven Tilbeurgh, H., Loontiene, F. G., Engelborgs, Y. et al., Studies of the cellulolytic system of *Trichoderma reesei* QM 94014, Eur. J. Biochem., 1989, 184(3): 553—559.
3. Lee, Y. H., Fan, L. T., Kinetic studies of enzymatic hydrolysis insoluble cellulose (II), Biotech. & Bioeng., 1983, 25: 959—966.
4. Tomme, P., Warren, R. A., Gillkes, N., Cellulose hydrolysis by bacteria and fungi, Adv. Microb. Physiol., 1995, 37: 1—8.
5. Divne, C., Stahlberg, J., Reinikaimen, T. et al., The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*, Science, 1994, 65: 524—528.
6. Yan, B. X., Gao, P. J., Purification of two cellobiohydrolase from *Trichoderma pseudokoningii* S-38, Chinese Biochemical Journal (in Chinese), 1997, 13: 362—364.
7. Yan, B. X., Gao, P. J., Purification and intrinsic fluorescence properties of Endoglucanase from *Trichoderma pseudokoningii* S-38, Chinese Biochemical Journal (in Chinese), 1997, 13: 580—585.
8. Zhang, Y. X., Liu, J., Gao, P. J. et al., Structure investigation of cellobiohydrolase I from *Trichoderma pseudokoningii* S-38 with a scanning tunneling microscopy, Applied Physics A, 1998, 67: 483—485.
9. Yan, B. X., Sun, Y. Q., Domain structure and conformation of a cellobiohydrolase from *Trichoderma pseudokoningii*, J. Protein. Chem., 1997, 16: 59—66.
10. Yan, B. X., Sun, Y. S., Gao, P. J., Intrinsic fluorescece of endoglucanase and cellobiohydrolase from *Trichoderma pseudokoningii* S-38, J. Protein Chem., 1997, 7: 681—688.
11. Yan, B. X., Gao, P. J., Role of tryptophan residue in endoglucanase from *Trichoderma pseudokoningii* S-38, Chinese Journal of Biochemistry and Molecular Biology (in Chinese), 1998, 14: 181—185.
12. Wang, D., Qu, Y. B., Gao, P. J., Transglycosylation of extracellular glucosidase of *T. pseudokoningii* S38 and its function in cellulase biosynthesis, J. Gen. Appl. Microbiol., 1996, 42: 363—369.
13. Palomen, H., Tenkanen, M., Linder, M., Dymanic interaction of *Trichoderma reesei cellobiohydrolases* Cella and Ce17 A and Cellulose at equilibrium and during hydrolysis, Appl. Environ. Microbiol., 1999, 65: 5229—5233.
14. Bikales, N. M., Segel, L., Cellulose and Cellulose Derivatives,

New York: Wiley Interscience, 1986, 305—320.
15. Michell, A. J., Second-derivative F. T-I. R spectra of native cellulose, Carbohydrate Research, 1990, 197: 53—60.
16. Zhan, Y. Z., Chen, X. L., Liu, J. et al., Scanning tunneling microscopy of the ultrastructure of cotton fiber, Acta Biochimica et Biophysica Sinica (in Chinese), 2000, 31: 521—523.
17. Herner, M. L., Melnick, M. S., Rabinovich, M. L., Enhancement of the affinity of cellobiohydrolase I and its catalytic domain to cellulose in the presence of the reaction product-cellobiose, Biochemistry (Mosc), 1999, 64: 1012—1029.
18. Chen, H. L., Li, W. J., Molecule Enzymology (in Chinese), Beijing: The public Health Edit, 1983, 233—278.
19. Fersht, A., Stereochemistry of enzymatic reaction, in Enzyme Structure and Mechanism, 2nd ed., New York: W. H. Freeman and Company, 1985, 221.
20 Gao, P. J., Chen, G. J., Wang, T. H. et al., Non-hydrolytic disruption of crystalline structure of cellulose by cellulose-binding domain and linker sequence of cellobiohydrolase I from *Penicillium Janthinellum*, Acta Biochemical et Biophysica Sinica, 2001, 33: 13—18.
21. Xiao, Z. H., Gao, P. J., Qu, Y. B. et al., Cellulose-binding domain of endoglucanase III from *Trichoderma reesei* disrupting the structure of cellulose, Biotech. Letter, 2001, 23: 711—715.
22. Teeri, T. T., Koivula, A., Linder, M. et al., *Trichoderma reesei cellobiohydrolase*: Why so efficient on crystalline cellulose? Biochemical Society Transactions, 1998, 26: 173—178.
23. Sinnott, M. L., The cellobiohydrolase of *Trichoderma reesei*: A review of indirect and direct evidence that their function is not just glycoside bond hydrolysis, Biochem. Soc. Trans., 1998, 26: 160—164.
24. VäLJamäe, D., Sild, V., Pettersson, G. et al., The initial Kinetics of hydrolysis by cellobiohydrolase I and II consistent with a cellulose surface-erosion model, Eur. J. Biochem., 1998, 253: 469—495.
25. Lee, S. B., Park, K. H., Robyt, J. F., Inhibition of β-glycosidases by a carbose analogues containing cellobiose and lactose structures, Carbohydrate Research, 2001, 331: 13—18.
26. Medve, J., Stahlberg, J., Tjerneld, F., Isotherm for adsorption of cellobiohydrolase I and II from *Trichoderma reesei* on microcrystalline cellulose, Appl. Biochem. Biotech., 1997, 66: 39—56.