

Action modes of recombinant endocellulase, EGA, and its domains on cotton fabrics

Meilan Yu · Yaqin Qiu · Wei Chen ·
Fukun Zhao · Jianzhong Shao

Received: 29 January 2015 / Accepted: 2 April 2015 / Published online: 15 May 2015
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Abstract

Objectives The action modes of an endocellulase, EGA, and its domains (CD9 and CBM3) during enzymatic treatment of cotton fabrics were investigated.

Results EGA, CD9 and CBM3 had the binding capacity to cellulose substrates, of which the filter paper was the substrate with the strongest binding capacity. Analyses of scanning electronic microscopy indicated that EGA and its catalytic domain CD9 etched the surface of cotton fabrics and broke the fibers of long chains. On the other hand, the binding domain CBM3 only resulted in swelling of cotton fibers. Both EGA and its catalytic domain CD9 had minimal effect on the weight loss of cotton fabrics, whereas the effect of EGA and CD9 on the degree of polymerization and breaking strength was significant. After 12 h enzymatic action, the values of weight loss ratio for EGA and CD9 were 2.07 and 2.21 %, respectively, meanwhile the reductions in fabric strength were 27.04 % for EGA and 17.23 % for CD9.

Conclusions In contrast to the action of EGA and CD9, CBM3 showed no significant changes in terms of the weight loss ratio, degree of polymerization, and fabric strength.

Keywords Carbohydrate-binding module · Catalytic domain · Cotton fiber · Degree of polymerization · Endocellulase · Endoglucanase · Fabric strength

Introduction

Cellulases have been increasingly used in the textile industry toward modification of cotton fabric and color, such as stonewashing of denim garments and defuzzing or depilling of fabrics (Azevedo et al. 2000). Cellulases refer to a class of enzymes that act on the β -1,4-glycosidic bond, thereby degrading cellulose (Manning 1983). Cellulases are classified as endo- β -1,4-glucanase (EC 3.2.1.4), exo-1,4- β -D-glucanase (EC 3.2.1.91), and β -1,4-glucosidase (EC 3.2.1.21) on the basis of their modes of action (Eveleigh et al. 2009). The majority of endoglucanases are tadpole-shaped molecules composed of a catalytic head [catalytic domain, (CD)] and one or more wedge-shaped ancillary module. The most common type of ancillary module is the carbohydrate-binding module (CBM) (Gilkes et al. 1988). CBM is located at the carboxyl-terminal end or

M. Yu · Y. Qiu · W. Chen · F. Zhao
College of Life Sciences, Zhejiang Sci-Tech University,
Hangzhou 310018, China

M. Yu · J. Shao (✉)
Engineering Research Center for Eco-Dyeing & Finishing
of Textiles, Ministry of Education, Zhejiang Sci-Tech
University, Hangzhou 310018, Zhejiang, China
e-mail: jshao@zstu.edu.cn

amino-terminal end of cellulases, which is linked to CD through a segment of highly glycosylated linker domain. Flexible linker sequence connects the two domains, ensuring that the two domains are efficiently positioned for enzymatic action (Tomme et al. 1988; Srisodsuk et al. 1993). The linker sequence generally ranges between 6 and 59 amino acids in length; these amino acids are rich in serine and threonine, which are the sensitive sites for proteolytic hydrolysis. CBMs are responsible for attaching enzyme to the insoluble polysaccharide surface on substrates, thereby improved thermal stability and ligand complexes (Sandgren et al. 2005; Notenboom et al. 2001), whereas CDs perform the hydrolysis of substrates. By restriction cleavage of the protease or recombination expressed relatively independent and complete CD and binding module can be isolated and obtained (Ståhlberg et al. 1988; Shirai et al. 2001). It has been shown that that these two modules function independently and that CD is catalytically active even in the absence of CBM.

Cellulases are produced by a wide range of sources, including microorganisms, plants, insects, and even higher animals. Most animal endogenous cellulases obtained till date belong to endocellulases, a group of enzymes important for textile processing. Cellulase-encoding genes have been cloned and obtained from higher animals such as mussels and abalone (Xu et al. 2001; Suzuki et al. 2003). We previously isolated and purified a novel endocellulase (=endoglucanase, EGA) from the cellulose-degrading bacterium *Bacillus* sp. strain AC-1, which exhibits high enzymatic activity and has the potential for modifying cellulose fibers (Yin et al. 2011; Li et al. 2006). The gene was 1980 bp long, encoding 659 amino acids. The gene product was further identified to contain CD9 at the *N*-terminal end belonging to GH-9 and CBM belonging to CBM-3 located at the *C*-terminal end (Zhang et al. 2007). Although both recombinant full length EGA and the truncated enzyme with CD9 domain were enzymatically active, the role of the CD and the CBMs in the hydrolysis of cellulose has not been studied thoroughly. In the present study, the endocellulase, EGA, and its two isolated domains (CD9 and CBM3) were used in the enzymatic treatment of cotton fabrics and their effect on the cotton fabric were thoroughly investigated in terms of binding capacity, surface morphology, and fabric strength.

Experimental

Materials and methods

White pure cotton fabric was purchased from Testfabrics, Inc. (West Pittston, PA, USA). Absorbent cotton was purchased from Qingdao Hiking Medical co., Ltd. (China). CMC-Na (medium viscosity) and was from Sigma. Avicel pH101 was purchased from Fluka (Switzerland). KOD-Plus-Neo DNA polymerase was from Toyobo Co., Ltd. (Osaka, Japan). Taq DNA polymerase and gel extraction kit were purchased from Tiangen Bioengineering Co., Ltd. (Shanghai, China). Restriction endonucleases, T₄ DNA ligase, and PCR extraction kit were purchased from TaKaRa (Dalian, China). Plasmid extraction kit was purchased from Bodataike Biotechnology Co., Ltd. (Shanghai, China). *Escherichia coli* strain BL21(DE3) was used for protein expression.

Expression and purification of recombinant EGA, CD9, and CBM3

For expression of the recombinant EGA, CD9, and CBM3, pET28a-based plasmids were constructed according to Zhang et al. (2007). Each plasmid was transformed into *E. coli* BL21 (DE3). The resulting recombinant cells were cultured in the LB medium containing 30 µg kanamycin/ml at 37 °C. Protein expression was induced with 1 mM IPTG at OD₆₀₀ of 0.8 and the cultures were incubated for additional 5 h at 37 °C (for EGA and CBM3) or 16 h at 16 °C (for CD9). The cells were harvested by centrifugation. The cell pellets were washed twice with 20 mM Tris/HCl buffer (pH 8), resuspended with the lysis buffer (20 mM Tris/HCl, 100 mM NaCl, 0.5 mM PMSF, pH 8.0), and disrupted through ultrasonication in an ice bath for 30 min. After centrifugation at ~10,000×*g* for 30 min, the supernatant was loaded onto a Ni-NTA chelating affinity column equilibrated with the binding buffer (500 mM NaCl in 20 mM Tris/HCl, pH 8). The bound enzymes were eluted by applying a stepwise gradient of imidazole from 40 to 200 mM (Zhao et al. 2008). Fractions containing the targeted enzymes were eluted with 200 mM imidazole, desalted with 20 mM Tris/HCl (pH 8) by ultrafiltration, and stored at -20 °C for further studies. Purity of the proteins was determined on 12 % SDS-PAGE followed by staining with Coomassie Brilliant Blue

R250. The protein concentration of the purified enzymes was measured at 650 nm by the Lowry method with bovine serum albumin as the protein standard.

Binding assay

To investigate the binding affinity of cellulose to recombinant EGA, CD9, and CBM3, the defined amount of protein was incubated with 10 mg insoluble microcrystalline cellulose Avicel in 1 ml 100 mM acetic acid/sodium acetate buffer, pH 5.2. Samples were shaken on a 4 °C, and the supernatants were collected after centrifugation at specific times. The precipitate after the last centrifugation was washed several times with the acetate buffer solution. The washed precipitate was resuspended with SDS loading buffer, boiled in water bath for 2–3 min, and analyzed together with the previously collected supernatants using 12 % SDS-PAGE (Li et al. 2006; Zhang et al. 2007).

Pre-treatment of cotton fabrics

The cotton fabric samples used in the test were cut to a defined size (5 cm × 24 cm) and washed 3 times with warm water. The washed samples were dried in oven, left overnight at room temperature, and weighed as the mass before drying. Samples were further dried at 105 °C for 8 h, and weighed again as the mass after drying. The dampening rate was calculated using the following equation:

$$\text{Dampening rate (\%)} = \frac{(\text{M before drying} - \text{M after drying})}{\text{M before drying}} \times 100 \%$$

where M denotes mass of the sample.

Enzymatic treatment of cotton fabrics

For the enzymatic treatment of the cotton fabric, the liquor ratio and the amount of enzyme were set as 1:20 and 1 % of mass of the cotton fabric, respectively. The treatment was performed at pH 6.5, 35 °C, and 100 rpm on a shaker. The treated samples were washed three times with water and dried. Each test was performed in triplicates and the samples were withdrawn at time intervals of 2, 4, 8, and 12 h.

Determination of weight loss ratio of cotton fabrics after treatment with EGA and its domains

The samples after enzymatic treatment were boiled in water for 5 min, rinsed with water for 2 min, and air dried at room temperature. The samples were further dried at 105 °C to obtain constant weight. The weight loss ratio was calculated using the following equation:

$$\text{Weight loss ratio (\%)} = \frac{(\text{M before treatment} - \text{M after treatment})}{\text{M before treatment}} \times 100 \%$$

where M before treatment and M after treatment were the masses of sample dried to constant weight before and after enzyme treatment, respectively.

Surface morphology of cotton fabrics after treatment with EGA and its domains

Surface morphology was investigated using a scanning electron microscope (SEM). During nonconductive specimen preparations in SEM, the samples and the corresponding controls were placed onto conductive adhesives using an ion sputter coating method. The dried samples were sprayed for 80 s at low 0.1–0.01 atm, in a vacuum-sputtering coat, and the resulting materials were observed under the scanning tunneling microscope.

Fabric breaking strength test

The breaking strength test of the sample was performed using Instron 2365 tester (Instron Corporation, England) according to the single rip method. The fabric sample was strengthened at constant velocity until rupture. The sample should have a width of 50 ± 5 mm and a length of 200 mm. Three assays were performed for each sample test in radial direction.

Determination of the degree of polymerization (DP)

To determine the fluidity of cuprammonium solutions of cellulose, the solution is usually prepared in the viscometer. The fluidity of cuprammonium solutions with/without cellulose was determined at 20 °C using a 0.6–0.7 Ubbelohde viscometer and each test was run

in triplicates. The degree of polymerization was calculated using a previously described formula (Isogai et al. 2008):

$$C = 0.11 \times (1 - \varphi) = 0.106$$

$$\eta_{sp} = (t_1 - t_0)/t_0[\eta] = \eta_{sp}/C$$

$$DP = 200 \cdot [\eta].$$

Results and discussion

Expression and characterization of EGA, CD9, and CBM3

Recombinant EGA, CD9, and CBM3 were expressed in *E. coli* with hexa histidine tag and purified by metal affinity chromatography. The purity of the proteins was determined by SDS-PAGE and is shown in Fig. 1. The molecular weights of EGA, CD9, and CBM3 were 72, 54.4, and 21.3 kDa, respectively (Fig. 1).

Binding affinity of EGA, CD9, and CBM3 to cellulose substrates

The purified EGA and its CD CD9 were active. To investigate the binding affinity of EGA, CD9, and CBM3, a group of cellulose substrates with different

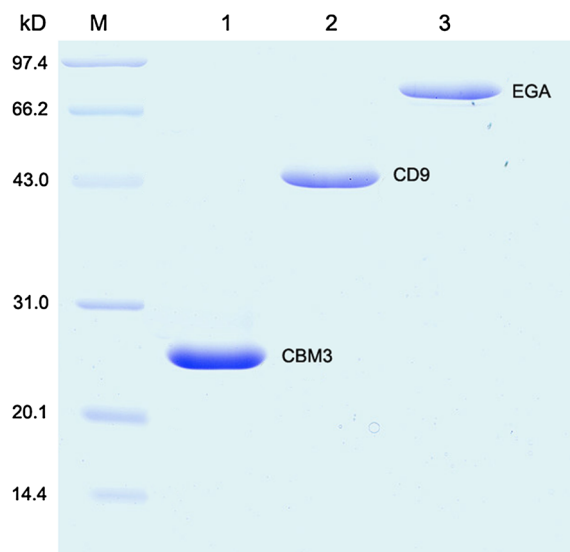


Fig. 1 SDS-PAGE of the recombinant proteins. Lanes 1–3: EGA, CD9, and CBM3; M: Protein molecular weight standard

degrees of crystallinity, such as microcrystalline cellulose Avicel, absorbent cotton, and filter paper, were tested. The adsorption of each cellulose substrate to EGA, CD9, and CBM3 was determined based on the time course experiment and visualized by SDS-PAGE analysis. For all the three cellulose substrates, the quantity of unbound enzyme has reduced with incubation time (Fig. 2). On the other hand, the bound enzyme on cellulose substrates after 2 h incubation was significant. These results indicated that EGA, CD9, and CBM3 have varying affinities toward substrates of different degrees of crystallinity. Among the tested cellulose substrates, filter paper showed highest and rapid binding to the all three proteins whereas Avicel was the poorest substrate. In absence of the binding domain, the CD CD9 still showed a certain substrate binding capacity.

Surface morphology of cotton fibers after enzymatic treatment of recombinant EGA and its domains

The surface of natural cotton fiber is relatively smooth and its fibers have a natural helical twist (Fig. 3a). The treatment with cellulase has an etching effect on the surface of cotton fiber, which makes the surface of the cotton fiber more fragile (Miettinen et al. 2001). In comparison with the control (Fig. 3b), in EGA- and CD9-treated samples the cotton fibers were swollen, the primary cell walls were partially eroded with uneven cracks along the fiber axis leading to rough fiber surfaces. After 2 h of EGA and CD9 treatments, the cotton fabric was swollen, the fiber surfaces were no longer smooth, and some “wrinkles” appeared (Fig. 3e, f). The longer treatment times resulted in more severe erosion on the surface of the cotton fibers. During the 12 h of enzymatic treatment, swelling of the cotton fibers and surface abrasion (Fig. 3e, g) occurred initially followed by cracking (Fig. 3h). The SEM images demonstrated even breaking of cotton fibers after 12 h of enzymatic treatment (Fig. 3h). In CBM3-treated cotton fabric samples even though the fibers were swollen and significantly thicker than the fibers of the control group, they did not show signs of obvious damage and erosion (Fig. 3d), indicating that CBM3 alone cannot hydrolyze cellulose. Although CBM3 cannot damage the surface morphology of cotton fibers, CBM3 has ability to break cotton fibers to short fragments and defiber the crystalline cellulose

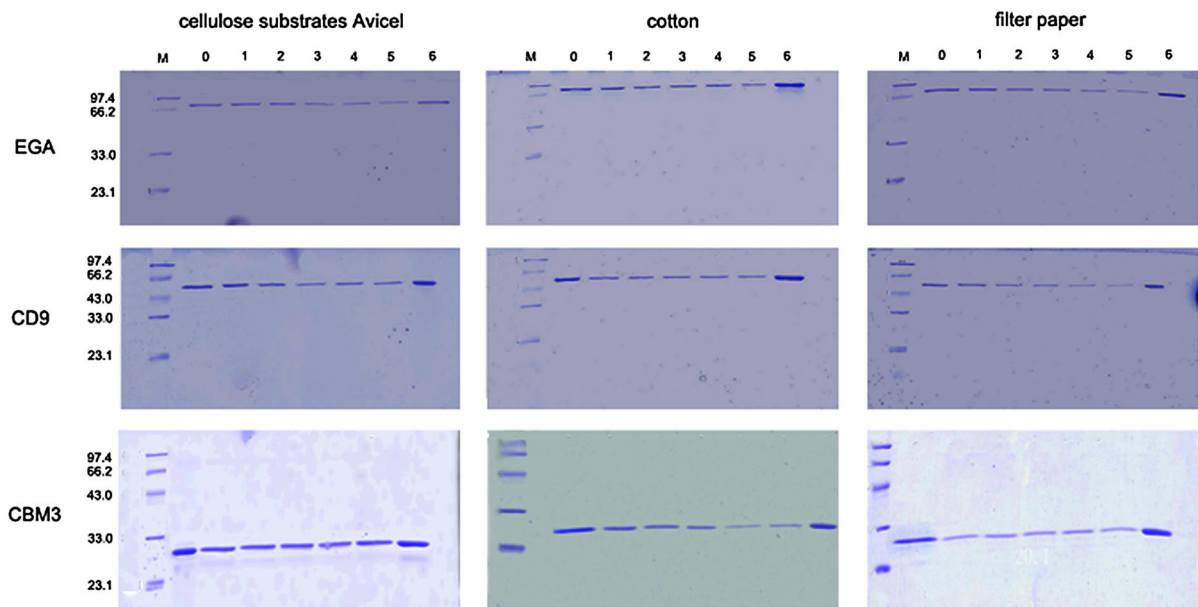


Fig. 2 SDS-PAGE analyses of the binding assays of EGA, CD9, and CBM3 to the cellulose substrates Avicel, cotton, and filter paper. Lanes 0–5 correspond to the protein in the

supernatant at the time points 0, 5, 10, 30, 60, and 120 min, lane 6 corresponds to the protein bound to cellulose substrate

and enhance water absorption capacity resulting in swelling of fibers, thereby destroying the cellulose structure (Knott et al. 2014).

The performance of cotton fabrics after treatment with EGA and its domains

It is logical to speculate that the “erosive” effect of EGA and its domains on surface morphology of cotton fibers would lead to corresponding changes in the weight loss ratio and the breaking strength of the cotton fabrics. In presence of EGA and CD9, the weight loss ratio has gradually increased (Fig. 4a), along with a decrease in the fabric breaking strength and degree of polymerization (Fig. 4b, c). The decline in cotton fabric strength roughly corresponds to the increase in weight loss ratio, indicating an intrinsic connection between the weight loss and strength reduction. After 8 h treatment of EGA and CD9, the weight loss ratio of the cotton fabric was 1.8 and 1.9 %, respectively, with a 14.4 and 12.6 %, respective reduction in fabric strength. After 12 h of enzymatic action, there was a 27 and 17.2 %, reduction in fabric strength with only slight increase in weight loss ratio (EGA, 2.1 %; CD9, 2.2 %), suggesting involvement of alternate factors, such as loss of

cellulose macromolecules, thinning of fibers, and related defects (Zhang et al. 2005). Intact enzyme, EGA is connected to CBM3 and CD9 domains through a linker. CBM3 is responsible for linking enzyme to insoluble polysaccharide surfaces, while CD9 catalyzes hydrolysis of polymer fiber chain (Ståhlberg et al. 1988). CBM3 thus strengthens the binding of enzyme to cellulose surface, resulting in cavities and corrosion cracks, ultimately leading to the loss of fiber strength. Even though the CD9 domain without CBM3 is efficient in catalytic hydrolysis, the binding of CD9 to the cellulose substrates can be nonspecific resulting in much broader and weaker effects on the surface of the fiber.

EGA and CD9 significantly decreased the degrees of polymerization (DP) of the cotton fabric (DP of untreated, EGA- and CD9-treated cotton fabric: 2226, 1414, and 1452, respectively). CBM3 treatment did not cause any changes in DP. As an endocellulase, EGA randomly cleaves the intermediate portion of amorphous region of cellulose chain producing varying lengths of cellulose oligosaccharide chain segments, thus the degree of polymerization of cotton fibers decreased significantly (Zhang et al. 2006). However, the cleaved cellulose fragments still remained in the fabric surface and were not completely

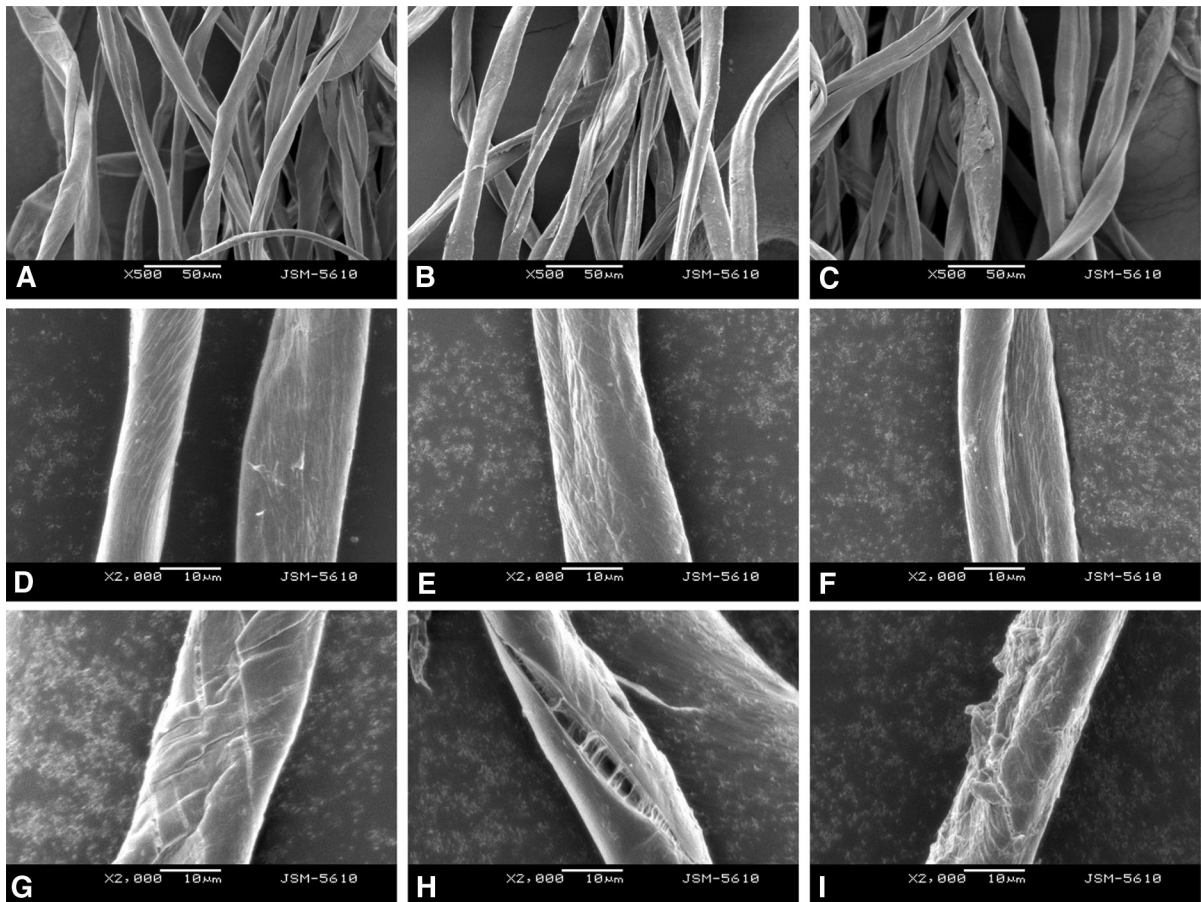


Fig. 3 SEM images of cotton fibers before and after EGA, CD9, and CBM3 treatments. **a.** Cotton fiber from the control group $\times 500$; **b.** cotton fiber after 12 h of EGA treatment $\times 500$; **c.** cotton fiber after 12 h of CD9 treatment $\times 500$; **d.** comparison of cotton fiber after 12 h of CBM3 treatment (right) with the

control group (left) $\times 2000$; **e.** cotton fiber after 2 h of EGA treatment $\times 2000$; **f.** cotton fiber after 2 h of CD9 treatment $\times 2000$; **g.** cotton fiber after 4 h of EGA treatment $\times 2000$; **h.** cotton fiber after 8 h of EGA treatment $\times 2000$; **i.** cotton fiber after 12 h of EGA treatment $\times 2000$

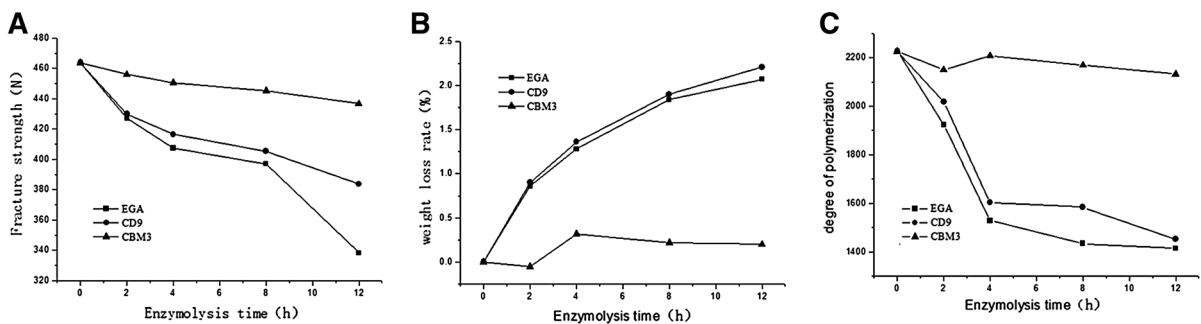


Fig. 4 The performance of cotton fabrics after treatment with EGA and its domains. **a.** Weight loss ratio of cotton fabrics; **b.** Breaking strength of cotton fabrics; **c.** Degree of polymerization of cotton

degraded, therefore no significant changes were observed on the weight loss ratio in enzyme-treated samples. It should be noted that the changes in weight

loss ratio, breaking strength, and degree of polymerization after treatment with CBM3 were not as obvious as those after treatment with EGA or CD9 (Figs. 3, 4),

which supports that CBM3 has no ability to hydrolyze cellulose.

Conclusion

The recombinant endocellulase/endoglucanase (EGA) and its two domains differ in their binding capacity to three cellulose substrates with different degrees of crystallinity. The strongest binding capacity was toward S filter paper consisting of amorphous regions, indicating that the endocellulase belongs to family-3 CBM that primarily acts on amorphous regions of cellulose. EGA and CD9 treatment caused a moderate swelling, peeling and breaking of cotton fibers along with an increase in the weight loss ratio and decrease in the breaking strength and degree of polymerization. In comparison with the action of complex enzymes, the treatment of a single endocellulase can cause obvious change to the degree of polymerization of cotton fabrics but little changes to the weight loss ratio and relatively small damage to the fabric strength as well. The treatment of CBM3 led to the swelling of cotton fabrics but without any obvious changes in weight loss, fabric strength, or degree of polymerization. The action of full length EGA on cotton fabrics is different from that of the individual CD, CD9, indicating that the presence of the CBM3 enhances the hydrolytic activity and guillotine action of the enzyme. This probably occurs by increasing the amount of enzyme attached to the substrate and by improved enzyme stability, though there is some evidence for non-hydrolytic disruption of cellulose by CBM.

Acknowledgments This work was financially supported by The National Natural fund of China (21405140), Research Foundation of Education Bureau of Zhejiang Province (Y201122330), the Commonwealth analysis test project of Zhejiang Province (2012C37056), Zhejiang Province Natural science Foundation of China (LQ14B050003), Supported by Zhejiang Provincial Top Key Discipline of Biology and Zhejiang Provincial Top Key Academic Discipline of Chemical Engineering and Technology.

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