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One-step immobilization and purification of genetic engineering CBD fusion EndoS on cellulose for antibodies Fc-glycan remodeling

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ABSTRACT

The endoglycosidase (EndoS and its glycosynthase mutants D233A, D233Q) gene was fused with cellulose binding domain (CBD) using pET-35b vector and the fusion enzymes were successfully expressed in *Escherichia coli*. Then a simplified approach for one-step immobilization and purification of EndoS enzymes using cellulose as matrices were developed and excellent loading efficiency (81–90%) was achieved in optimal condition. The cellulose immobilized CBD-EndoS and the glycosynthase mutants presented high catalytic activity and were successfully applied in a two-step antibody Fc *N*-glycan remodeling, generating a therapeutic antibody with homogeneous glycoform in high efficiency. The cellulose immobilized CBD-EndoS and its mutants (D233A and D233Q) displayed excellent storage stability when stored at 4 degrees for one month. Reusability studies demonstrated that the cellulose immobilized CBD-EndoS and its mutants could be recycled for five times without obvious activity loss.

1. Introduction

Wild type endoglycosidase S (EndoS, EC 3.2.1.96) is an *endo*-β-*N*-acetylglucosaminidase found in *Streptococcus pyogenes*, which can specifically hydrolyze the *N*-glycans on the Fc domain of human IgG antibodies [1]. Recently, two new glycosynthase mutants (EndoS D233A and D233Q) were generated from EndoS by site-directed mutagenesis and demonstrated to have transglycosylation activity capable of transferring predefined complex type *N*-glycan oxazoline to the Fc-deglycosylated IgG antibodies [2,3]. Accordingly, a novel chemoenzymatic strategy, by application of EndoS and the glycosynthase mutants in combination *in vitro*, or along with other glycotransferase, has been developed as an efficient approach to generate a variety of antibodies with homogeneous glycoforms for function and therapeutic studies [4]. For example, the Fc *N*-glycan of therapeutic antibodies, such as Rituximab [2], Herceptin [5] and anti-Her2 [6] antibody, were transformed into a well-defined homogeneous glycoform mediated by EndoS and its mutants to gain enhanced FcγIIIa receptor-binding activity, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Very recently, we developed a chemoenzymatic strategy to prepare glycosite-specific antibody-drug conjugates (ADC) through a one-pot *N*-glycosylation remodeling of IgG catalyzed by these EndoS enzymes [7]. These studies demonstrated that EndoS and the glycosynthase mutants are highly useful biocatalysts with potential applications in the biopharmaceutical industry.

Currently, EndoS and the glycosynthase mutants were *recombinantly* expressed in *E. coli*. However, production of EndoS enzymes remained at low level [2,8]. In addition, free enzymes are frequently used as biocatalysts for chemoenzymatic remodeling *N*-glycan of IgG, which require tedious downstream purification process each step to separate the antibody product and the contamination of residual enzymes. Thus, the inherent drawbacks, such as the limited availability, high cost and difficulty in recovery and recycling, have limited their practical applications in the industrial field. Therefore, developing EndoS immobilization technology will be an effective approach to overcome the aforementioned disadvantages. Recently, Wang's group for the first times developed a site-specific enzymatic method to immobilize the Endo-S2 and its glycosynthase mutant D184M by using microbial transglutaminase (MTG) [9]. In this approach, Endo-S2 and its mutant were genetically engineered with a Q-tag at the C-terminus, and then the enzymes were immobilized onto agarose resin containing a primary amine through MTG catalyzed transglutamination. The immobilized Endos-S2 and its mutant were efficiently applied in IgG antibody Fc-glycan remodeling in streamlining without the need of intermediate

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separation, thus greatly simplifying the glycan remodeling procedures. However, costly pre-purification to obtain the pure enzymes is required before performing the site-specific MTG mediated immobilization. Thus, developing an economical and simple immobilization technology for EndoS and its mutants is highly desirable.

Recently, a novel strategy coupled the enzyme immobilization and purification in one-step has received many attentions in industry [10]. In this strategy, an affinity tag was genetically encoded to the target protein to facilitate the biocatalyst immobilization and purification purpose. The advantages, such as the oriented attachment and the minimization of conformation changes, may enable the immobilized enzyme maintaining the maximum activity and stability in practical application. Among them, genetically encoding the enzyme with a carbohydrate-binding domain (CBD) was particularly attractive because CBD domain has a contiguous amino acid sequence forming flat hydrophobic surfaces, which display a specific and high binding affinity for cellulose material [11]. In addition, using abundant naturally occurring cellulose as solid support for enzyme immobilization and purification may offer other desirable advantages, including a low cost, good biocompatibility and among others [12]. One-step immobilization and purification strategy has been successfully applied in several industrial important enzymes, such as β-glucosidases [13,14], lipase [15], γ-lactamase [16], invertase [17], D-amino acid oxidase [18] and peroxidase [19]. However, to date, these cellulose immobilized biocatalysts were only demonstrated in small molecules synthesis. And to the best of our knowledge, using cellulose immobilized enzymes to mediate biomolecule transformation is largely uninvestigated. In this work, a *cbd* gene from *Closrtiduim cellulovorans* was cloned to the *N*-terminal gene of EndoS and its glycosynthase mutants (D233A and D233Q), respectively. And the CBD-EndoS fusion proteins were successfully recombinantly expressed in *E. coli*. Then a simplified approach for one-step immobilization and purification of EndoS enzymes were achieved and the process parameters were investigated by single factor optimization. The cellulose immobilized EndoS and its glycosynthase mutants (D233A and D233Q) successfully were applied in a two-step antibody Fc *N*-glycan remodeling to obtain antibody with homogeneous glycoforms. And the cellulose immobilized EndoS and its glycosynthase mutants could be recycled and reused for 5 times without significant activity loss.

2. Experiment

2.1. Bacterial strains, plasmids and growth media

Escherichia coli BL21 (DE3) was grown at 37 °C in Luria-Bertani (LB) medium or 20 °C in modified TB medium (10 g/L yeast extract, 25.4 g/L tryptone, 5 g/L NaCl, 4 mL/L glycerol, pH 8.0). The plasmid pET-35b (Novagen) was used as cloning vector and expression vector, encoding the cellulose-binding domain (CBD) from *Closrtiduim cellulovorans*.

2.2. Construction the plasmids of pET35b-CBD-EndoS, pET35b-CBD-EndoS D233A and pET35b-CBD-EndoS D233Q

The EndoS (WT) gene from plasmid pET28a-EndoS was amplified by PCR using primers EndoS-F: 5′-GGTACAGCACCAATGGAAGAGAAGACAGTTCAGGTTCAG-3′ and EndoS-R: 5′-GATCCTCAGTGGTGGTGATGATGATGTTTCTTCAGCAGCTGGCG-3′. The pET-CBD gene from plasmid pET-35b was amplified by PCR using primers CBD-F: 5′- GAAACATCATCATCACCACCACTGAGGATCCGAATTCGAGCTC-3′ and CBD-R: 5′-AACTGTCTTCTCTTCCATTGGTGCTGTACCAAGAACTT-3′. All of the PCR products were digested with T4 DNA polymerase. The underlined sequences represented the sticky ends. After ligation, the recombinant plasmid pET35b-CBD-EndoS was transformed into *E. coli* BL21 (DE3) by heat shock. Plasmid pET35b-CBD-EndoS D233A and pET35b-CBD-EndoS D233Q were constructed in a similar protocol.

2.3. Expression of the fusion enzyme CBD-EndoS and its mutants

The correct *E. coli* BL21 transformant was grown overnight in shake flasks containing LB medium with 100 μg/mL kanamycin at 37 °C. The overnight medium was diluted 50-fold in 1L modified TB medium and cultured at 37 °C until an absorbance of 0.6–0.8 at 600 nm was reached. Then Isopropyl-1-thio-β-D-galactoside (IPTG) was added to the medium to a final concentration of 1 mM. After incubating at 20 °C for 24 h, cells were harvested by centrifugation at 9000 rpm. Pellet fraction was resuspended in 50 mM potassium phosphate buffer (KPB, pH 7.0), and disrupted by ultrasound. After centrifugation at 15,000 rpm for 30 min at 4 °C, the suspension was stored as crude enzyme at 4 °C.

2.4. Immobilization of CBD-EndoS and its mutants onto sigmacell cellulose

The one-step immobilization and purification of CBD-fusions was performed by noncovalent binding between CBD-fusions and cellulose (Sigmacell, type 50). The crude enzyme was mixed with sigmacell cellulose (50 μl per 1 mg) at room temperature under constant shaking at 250 rpm. The mixtures were centrifuged at 15,000 rpm for 5 min and the suspension was discarded. The cellulose was washed with 1 M NaCl in 50 mM KPB once and with 50 mM KPB twice. The resulting cellulose was kept at 4 °C.

To determine the influence of process parameters on immobilization, CBD-EndoS was immobilized on cellulose by varied adsorption time (0, 5, 10, 20, 30, 60, 120 min), different temperature (4, 20, 30 °C), different NaCl concentration (0, 50, 100, 250, 500 mM) and pH value (4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0).

2.5. Characterization of the enzymatic activity of free and the cellulose immobilized CBD-EndoS

The free (0.3 mg/mL) or cellulose immobilized CBD-EndoS (0.3 mg/ml) was incubated with Sialyglycopeptide (SGP, 2 mg/ml) in PBS buffer (100 mM, pH 6.0) at 37 °C for 2 h under constant shaking at 250 rpm. Samples were taken out at proper time (0, 5, 10, 15, 20, 30, 45, 60, 90, 120 min) intervals and were analyzed by HPLC. Kinetic constants of free and immobilized CBD-EndoS were determined by Lineweaver–Burk double reciprocal plots. Various concentrations of SGP (0.5, 1, 1.5, 2, 4, 6 mM) were incubated with enzyme (0.3 mg/ml) in PBS buffer (100 mM, pH 6.0) at 37 °C for 10 min under constant shaking at 250 rpm. The reaction mixture was analyzed by HPLC. HPLC condition: the HPLC was performed on a Hitachi Chromaster liquid chromatograph with a Topsil CX-C18 column (4.6 × 250 mm). The analysis was performed at 25 °C eluting with a linear gradient of 2–20% MeCN containing 0.1% trifluoroacetic acid within 20 min at a flow rate of 1 mL/min. The samples were detected by a UV detector at 214 nm.

2.6. Fc-glycan remodeling of commercial Rituximab by cellulose immobilized CBD-EndoS and its glycosynthase mutant D233A or D233Q

Deglycosylation of Rituximab: The cellulose immobilized CBD-EndoS (30 μg/ml) was incubated with commercial rituximab (10 mg/ml) in PBS buffer (100 mM, pH 6.0) at 37 °C for 1 h under constant shaking at 1250 rpm. Samples were taken out at proper time (0, 1 h) intervals and were analyzed by SDS-PAGE and LC-MS. LC-MS condition: the LC-MS was performed on a Waters Q-TOF mass spectrometer with an ACQUITY UPLC Peotein BEH C4 column (2.1 × 50 mm). The analysis was performed at 80 °C eluting with a linear gradient of 5–90% MeCN containing 0.1% formic acid within 6 min at a flow rate of 0.3 mL/min. The IgG samples were detected by a UV detector at 280 nm.

Transglycosylation of sialoglycan oxazoline to deglycosylated Rituximab by the cellose immobilized CBD-EndoS mutants D233A or D233Q: the immobilized CBD-EndoS D233A or D233Q (0.15 mg/ml) was incubated with (Fucα1,6)GlcNAc – rituximab (5 mg/ml) and

sialoglycan xoazoline (1 mM) in PBS buffer (100 mM, pH 7.2) at 30 °C for 1.5 h under constant shaking at 1250 rpm, respectively. Samples were taken out at proper time (0, 30, 60, 90 min) intervals and were analyzed by SDS-PAGE and LC-MS. LC-MS condition was same as previous described.

2.7. Storage stability and reusability of immobilized CBD-EndoS and its mutants

The storage stability and reusability of immobilized CBD-EndoS was evaluated by measuring the hydrolysis activity for SGP as described before. The cellulose immobilized CBD-EndoS (0.3 mg/ml) was incubated with SGP (2 mg/ml) in PBS buffer (100 mM, pH 6.0) at 37 °C for 1 h under constant shaking at 250 rpm. As for storage stability, the immobilized CBD-EndoS was stored at 4 °C and was carried out for measuring its reactivity at different times (0, 5, 10, 15, 20, 30 days). To evaluate the reusability of immobilized CBD-EndoS, the cellulose immobilized CBD-EndoS was recovered and washed with PBS buffer (100 mM, pH 6.0). Then it was used for a new batch of the reaction.

The storage stability and reusability of immobilized CBD-EndoS D233A and D233Q were evaluated by measuring the transglycosylation activity using (Fucα1, 6)GlcNAc − rituximab as substrate. The cellulose immobilized CBD-EndoS D233A or D233Q (0.15 mg/ml) was incubated with (Fucα1,6)GlcNAc − rituximab (5 mg/ml) and sialoglycan xoazoline (1 mM) in PBS buffer (100 mM, pH 7.2) at 30 °C for 1.5 h under constant shaking at 1250 rpm, respectively. As for storage stability, the immobilized CBD-EndoS D233A and D233Q was stored at 4 °C and was carried out for measuring their transglycosylation activity at different times (0, 15, 30 days). As for the reusability of immobilized CBD-EndoS D233A or D233Q, the cellulose immobilized CBD-EndoS D233A or D233Q was recovered and washed with PBS buffer (100 mM, pH 7.2). Then it was used for a new batch of the reaction.

3. Results and discussion

3.1. Gene cloning and expression of CBD-EndoS and the glycosynthase mutants

Seamless cloning and gene fusion are useful technologies to joint DNA fragments precisely, allowing two or more DNA fragments to be joined without unwanted extraneous sequences based on the homologous ends of insert (DNA fragment) and linearized vector [20]. We followed the seamless cloning protocol and successfully fused the *cbd* gene from *Clostridium cellulovorans* [21] to the N terminus of EndoS gene from *Streptococcus pyogenes* [1]. In addition, a His tag was attached to the C terminus of EndoS protein for purification purpose. Then the three plasmids of pET35b-CBD-EndoS, pET35b-CBD-EndoS (D233A) and pET35b-CBD-EndoS (D233Q) were transformed into *E. coli* BL21 (DE3) cells, respectively. The recombinant cells were cultured in a shaking flask until an OD of 0.6–0.8 was reached. After induction with IPTG, the cells were harvested after 24 h and disrupted to obtain crude enzyme extract. To confirm the successful expression of the EndoS and the mutants, the total cell protein samples were firstly analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). As showed in Fig. 1, three protein bands (lane 1, 2 and 3) with a molecular weight around 128 kDa presented in SDS-PAGE gel, which matched with the predicted molecular weight of CBD-EndoS and the CBD-EndoS mutants (D233A and D233Q). In addition, as the EndoS enzymes were engineered with CBD domain at the protein *N*-terminus, cellulose (Sigmacell, type 50), a commercially available matrices, was incubated with crude enzyme extract to purify the proteins, respectively. Then the corresponding cellulose was washed with high concentration urea (6 M) to release the absorbed proteins, which were further analyzed by SDS-PAGE gel. It was observed that three clean and clear protein bands at 128 kDa appeared on SDS-PAGE (Fig. 1, lane 4, 5 and 6) when the eluents were used as samples. These results further confirmed that CBD-EndoS and the mutants (D233A and D233Q) were successfully expressed in *E. coli*. And the CBD–EndoS proteins displayed a specific binding affinity to cellulose matrices, indicating that cellulose could be used as solid support to immobilize and purify these CBD containing proteins in one step. In another experiment, the crude enzyme extract was purified by Ni-NTA resin *via* his-tag affinity chromatography. In this way, the expression level of CBD-EndoS and the glycosynthase mutants (D233A, D233Q) were determined by the Bradford method using BSA protein as a standard, yielding 50, 40, 50 mg/L of proteins, respectively.

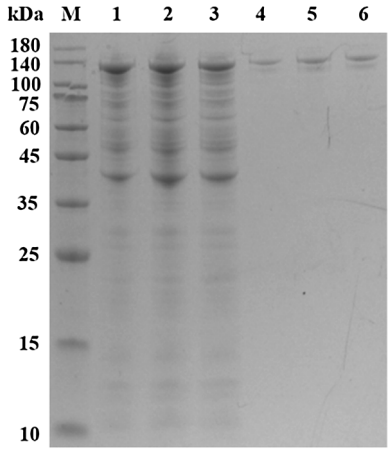


Fig. 1. SDS-PAGE analysis of CBD-fusions Endos enzymes. Lane M, marker; lane 1, total cell protein of CBD-EndoS; lane 2, total cell protein of CBD-EndoS D233A; lane 3, total cell protein of CBD-EndoS D233Q; lane 4, CBD-EndoS eluted from the cellulose matrices; lane 5, CBD-EndoS D233A eluted from the cellulose matrices; lane 6, CBD-EndoS D233Q eluted from the cellulose matrices.

3.2. Process optimization of one-step immobilization and purification of CBD-EndoS and the glycosynthase mutants (D233A, D233Q) onto Sigmacell cellulose

With crude EndoS enzymes in hand, a one-step immobilization and purification protocol for CBD-EndoS were investigated. In optimization experiment, wild type EndoS was used as a model protein. Firstly, the crude CBD-EndoS enzyme was incubated with cellulose under constant shaking at 250 rpm in room temperature. The immobilization course of EndoS onto cellulose was monitored in 2 h. As showed in Fig. 2a, 65%, 72% and 84% of EndoS were immobilized onto cellulose in 5, 10 and 20 min, respectively. And in 30 min, the loading efficiency of CBD-EndoS onto cellulose could reach 88%. After that, a plateau was reached. This result indicated that affinity binding of CBD domain with cellulose is a rapid process, which is consistent with previous reports [13,17]. Therefore, to avoid non-specific protein absorbing onto the cellulose matrices, the incubation time was performed in 30 min in the following experiments.

The influence of temperature to the immobilization efficiency was studied at 4, 20 and 30 °C in 30 min. However, as indicated in Fig. 2b, no significant difference in loading efficiency was observed. Thus, all the proceeding experiments were performed at room temperature.

Since the major binding force between CBD domain and cellulose are the results of hydrophobic interactions [22,23], the effects of salt concentration and solution pH were studied in detail. The results of various concentration of NaCl to EndoS loading efficiency were shown in Fig. 2c. Increasing the NaCl concentration from 0 to 100 mM, the immobilization efficiency was improved by 6%. No significant immobilization enhancement was observed when the NaCl concentration was higher than 250 mM. Therefore, 100 mM NaCl concentration was used as optimal condition. Previous studies have demonstrated that

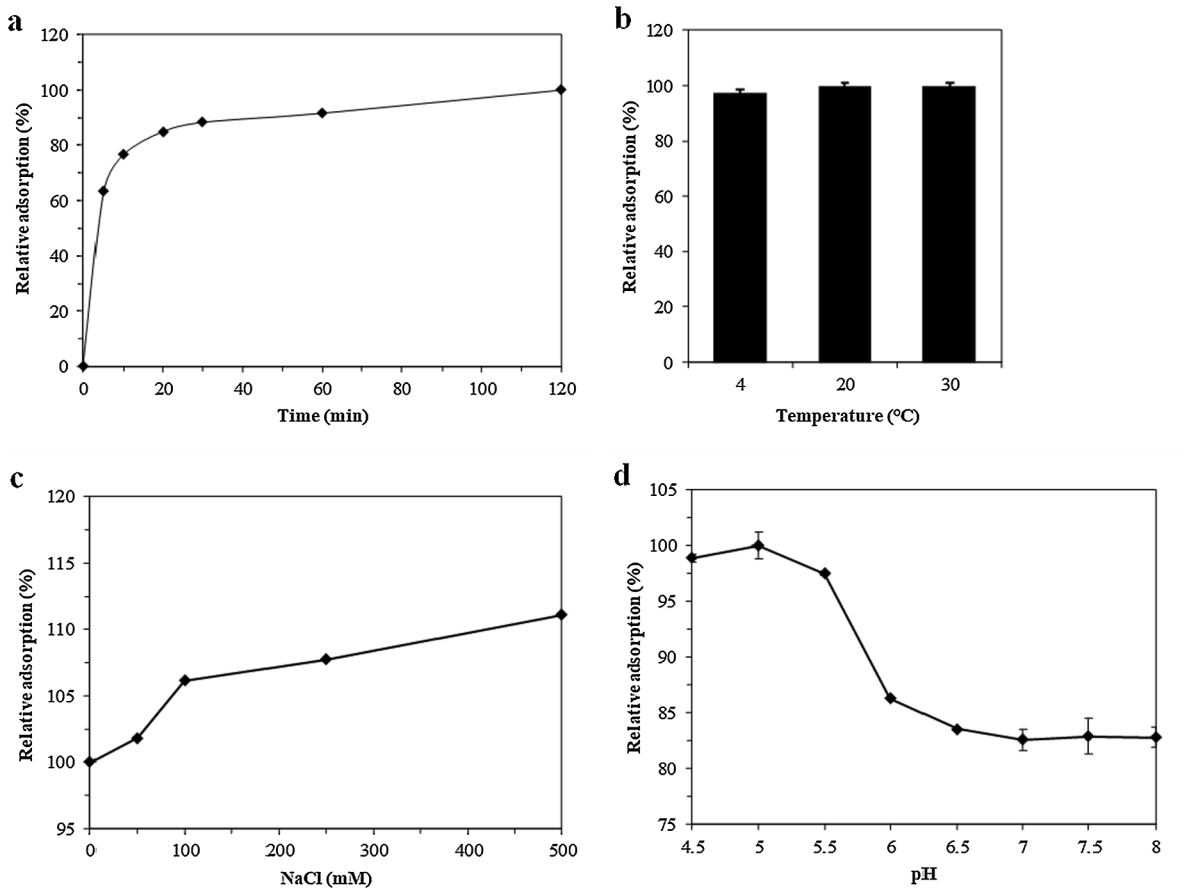


Fig. 2. Effects of time (a), temperature (b), NaCl concentration (c) and pH (d) on the immobilization of the CBD-EndoS onto cellulose.

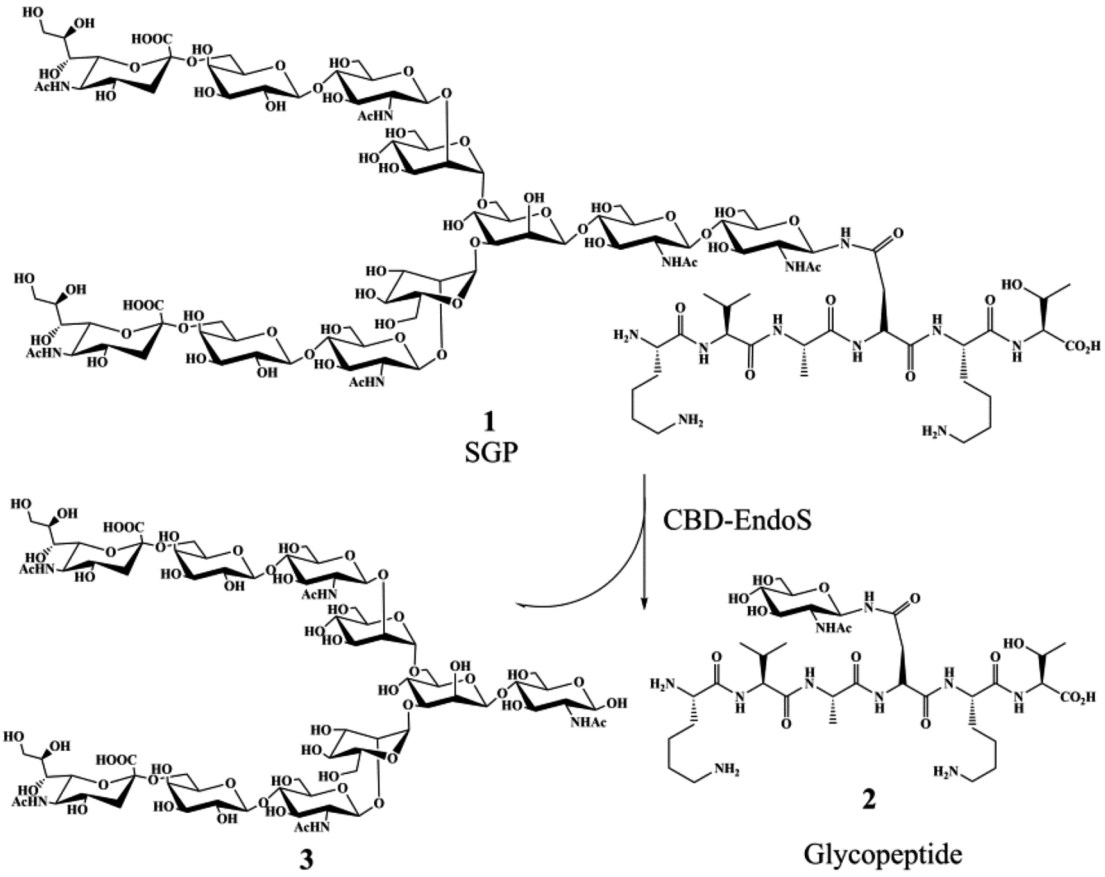
Table 1
Results of one-step immobilization and purification of CBD-EndoS and its mutants (D233A, D233Q) onto cellulose under optimal condition.

Enzymes	Crude enzyme (mg/mL)	Immobilization (mg/g cellulose)	Immobilizationefficiency (%)
CBD-EndoS	0.170	7.30	86
CBD-EndoS (D233A)	0.128	5.20	81
CBD-EndoS (D233Q)	0.191	8.55	90

CBD domain binding with cellulose was significantly affected by pH, because pH change may lead to the conformation change of enzyme and consequently result in different hydrophobic interaction profile. Therefore, crude EndoS extract was incubated with cellulose in a pH range of 4.5 to 8.0. As presented in Fig. 2d, the cellulose could absorb a maximum amount of CBD-EndoS at pH 5.0. Then the binding amount of EndoS onto cellulose gradually decreased with the solution pH increasing to neutral condition. But still more than 80% of EndoS were immobilized onto cellulose at the range of pH 6–8. Considering the preservation of enzyme activity, the optimal adsorption pH was performed at pH 6.0. To determine the immobilization efficiency under optimal conditions, the crude CBD-EndoS and the mutants (D233A and D233Q) were incubated with cellulose in 50 mM KPB buffer (100 mM NaCl, pH 6.0) at room temperature for 30 min, respectively. Then cellulose immobilized with EndoS enzymes was washed with 6 M urea to release the binding proteins followed by desalting and analyzing with BSA assay. As summarized in Table 1, the amounts of immobilized CBD-EndoS and the mutants (D233A and D233Q) onto cellulose were 7.30, 5.20, 8.55 mg/g cellulose, respectively, indicating that the corresponding immobilization efficiency were 81, 86 and 90%.

3.3. Characterization of the enzymatic activities and kinetic profiles of free and immobilized CBD-EndoS

To test the deglycosylation activities of free wild-type CBD-EndoS and the cellulose immobilized CBD-EndoS, sialyglycopeptide **1** (SGP), a native *N*-glycopeptide isolated from eggs [24], was used as a substrate (Scheme 1). The free and immobilized CBD-EndoS were incubated with SGP in PBS buffer (100 mM, pH 6.0) at 37 °C under constant shaking, respectively. The SGP was hydrolyzed to give a biantennary complex type oligosaccharide **3** and a glycopeptide product **2**, and the latter one was analyzed by HPLC and characterized by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) (Fig. S1). The time course of SGP deglycosylation catalyzed by the free wild-type CBD-EndoS and the cellulose immobilized CBD-EndoS were shown in Fig. 3. Using free CBD-EndoS enzyme as biocatalyst, 58 and 86% of SGP were deglycosylated in 10 and 30 min, and the deglycosylation efficiency reached as high as approximately 90% in 45 min. The cellulose immobilized EndoS exhibited comparable deglycosylation efficiency as free CBD-EndoS did, giving a 90% of hydrolysis efficiency in 45 min as well. This result approved that CBD tag engineered onto the *N*-terminus of EndoS retained the full deglycosylation activity, suggesting that this



Scheme 1. Deglycosylation of SGP using free and immobilized CBD-EndoS.

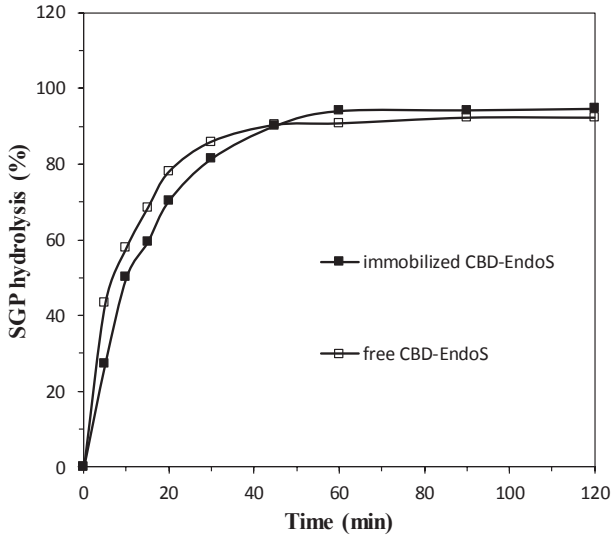


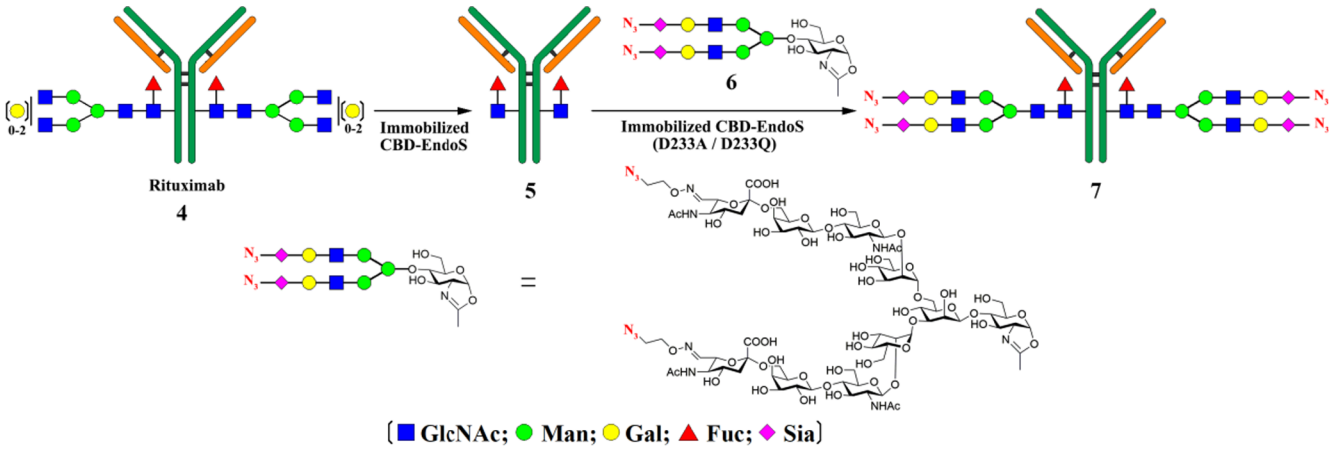
Fig. 3. Time course of SGP deglycosylation catalyzed by free and immobilized CBD-EndoS.

modification did not interfere with the enzymatic activity. In addition, the similar deglycosylation efficiency exhibited by free and the cellulose immobilized EndoS further demonstrated that immobilizing CBD-EndoS onto cellulose did not affect the enzyme activity as well.

To understand the difference of free and cellulose immobilized CBD-EndoS activity, the kinetic behaviors of the enzymes were studied in detail. The V_{max} values of free and immobilized CBD-EndoS for the substrate SGP were 2.89 and 1.99 μM/min, respectively, and the corresponding of K_m values were 0.018 and 0.015 mM. The slightly decreased V_{max} of immobilized CBD-EndoS revealed that the cellulose matrices did not impose obvious diffusion limitation, indicating that the active sites of EndoS are far from the *N*-terminal CBD domain and are not involved in immobilization. The almost unaltered K_m values of free and immobilized CBD-EndoS further confirmed this speculation, suggesting that the enzymes' catalytic centers were equally accessible by SGP under the same condition.

3.4. Glycan remodeling of Rituximab by immobilized CBD-EndoS and the mutants (D233A, D233Q)

EndoS and the glycosynthase mutants (D233A and D233Q) are widely used biocatalysts for antibody Fc *N*-glycan remodeling *in vitro*. To test the deglycosylation activity of cellulose immobilized CBD-EndoS enzymes in antibody Fc *N*-glycan remodeling, Rituximab, a clinical therapeutically monoclonal antibody (mAb) with biantennary complex type oligosaccharides carrying varied (0–2) galactose moieties was used as a model antibody [2] (Scheme 2). The commercial rituximab was incubated with immobilized CBD-EndoS at 37 °C for 1 h under constant shaking. The deglycosylation product was analyzed by SDS-PAGE and further characterized by liquid chromatography–mass spectrometry (LC-MS). As showed in Fig. 4a (Lane 1 and 2), a heavy chain and light chain of Rituximab appeared at approximately 50 and 25 kDa under reducing conditions, respectively. After deglycosylation, the heavy chain band completely shifted to approximately 49 kDa while the light chain band remained the same molecular weight. In LC-MS analysis (Fig. 4b and c), the commercial rituximab **4** displayed multiple glycoforms as named G0F, G1F and G2F, which were completely converted to a uniformed rituximab **5** bearing the fucosylated GlcNAc disaccharide moiety (Fucα1,6 GlcNAc) after treatment with the cellulose immobilized CBD-EndoS. This result clearly demonstrated that cellulose immobilized CBD-EndoS retained the full IgG Fc *N*-glycan hydrolysis



Scheme 2. Glycan remodeling of Rituximab by immobilized CBD-EndoS and the mutants.

activity.

To examine the potential transglycosylation activities of cellulose immobilized glycosynthase mutants (D233A and D233Q), deglycosylation of rituximab **5** was used as an acceptor and a synthetic azide modified sialoglycan oxazoline **6** as the donor [7]. The cellulose immobilized CBD-EndoS D233Q or D233A (0.15 mg/ml) were incubated with (Fucα1, 6)GlcNAc − rituximab **5** (5 mg/ml) and sialoglycan oxazoline **6** (1 mM) in PBS buffer at 30 °C for 1.5 h under constant shaking, respectively. Reaction progresses were monitored by SDS-PAGE and the transglycosylation efficiency was analyzed by densitometric analysis using ImageQuant TL software. As depicted in Fig. 5a (lane 2–4), approximately 64.1% and 79.9% of deglycosylated rituximab **5** were transferred to the fully sialylated *N*-glycans product **7** in 0.5 and 1 h, respectively, when treatment with the cellulose immobilized glycosynthase mutants (D233A). And almost a quantitative conversion yield was reached in 1.5 h. As for the transglycosylation reaction catalyzed by the cellulose immobilized glycosynthase mutant (D233Q) under the same condition, approximately a full conversion was achieved as well in 1.5 h (Fig. 5a, lane 5–7). The final product **7** was further characterized by LC-MS, which gave a single *m/z* mass matched with the calculated MS of Rituximab containing uniformed sialylated Fc *N*-glycans (Fig. 5b). This result demonstrated that both the cellulose immobilized glycosynthase mutant D233A and D233Q maintained the full transglycosylation activities when using deglycosylated antibody as substrate, indicating that immobilizing the enzymes fused with CBD-domain did not affect the enzyme activity.

3.5. The storage stability and reusability of immobilized CBD-EndoS and its mutants (D233A, D233Q)

The storage stability and reusability are important properties of immobilized enzymes for industrial application, which will significantly decrease the overall cost of their use. To examine the storage stability of the cellulose immobilized CBD-EndoS, the enzyme activity was measured in 30 days by incubating with SGP. As indicated in Fig. 6a, 97%, 91%, 89% and 92% of deglycosylation activities were observed in 5, 10, 15 and 20 days when stored in 4 degrees. And more than 90% of enzyme activities remained after storage for 30 days. To access the enzyme activities of cellulose immobilized glycosynthase mutants (D233A and D233Q), they were incubated with deglycosylated Rituximab and sialoglycan oxazoline **6**, and the transglycosylation efficiency was analyzed by SDS-PAGE, respectively. As showed in Fig. 6b, both the cellulose immobilized glycosynthase mutants D233A (lane 2–4) and D233Q (lane 5–7) could transfer sialoglycan oxazoline to the deglycosylated Rituximab in full conversion yield after storage in 4 degree for 10, 20 and 30 days. This result indicated that the cellulose immobilized glycosynthase mutants (D233A and D233Q) displayed excellent storage stability after storage in 4 degrees for one month.

To examine the reusability of the cellulose immobilized CBD-EndoS, SGP was used as substrate. Thus, after the hydrolysis reaction was completed, the cellulose immobilized CBD-EndoS was recovered by simple filtration followed by washing with PBS buffer, then it was used for the next SGP hydrolysis reaction. Following the same procedure, the

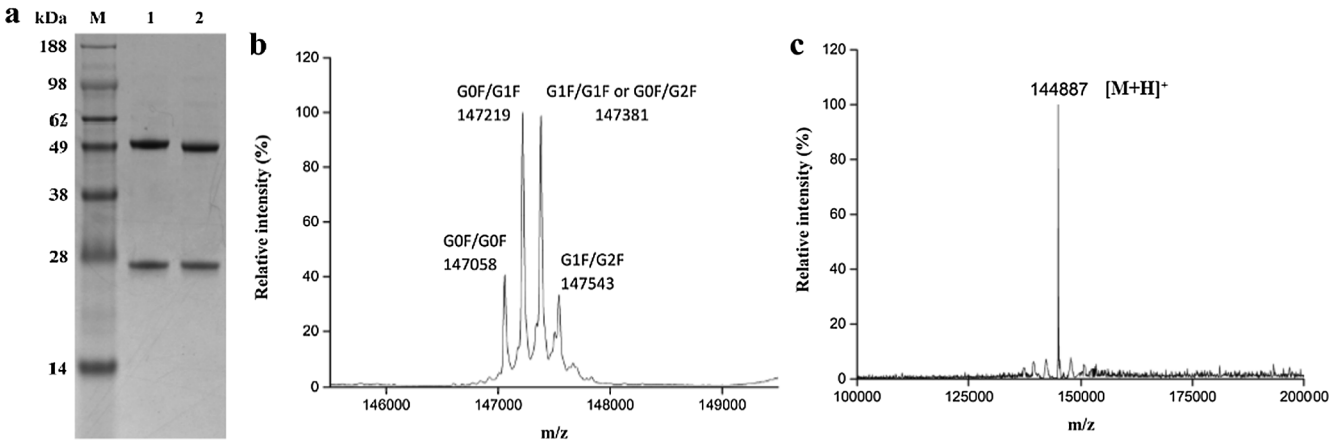


Fig. 4. SDS-PAGE and LC-MS analysis of deglycosylation of rituximab. (a) SDS-PAGE analysis: lane M, marker; lane 1, commercial rituximab; lane 2, deglycosylated Rituximab catalyzed by the cellulose immobilized CBD-EndoS. (b) LC-MS analysis of commercial Rituximab. (c) LC-MS analysis of the deglycosylated Rituximab.

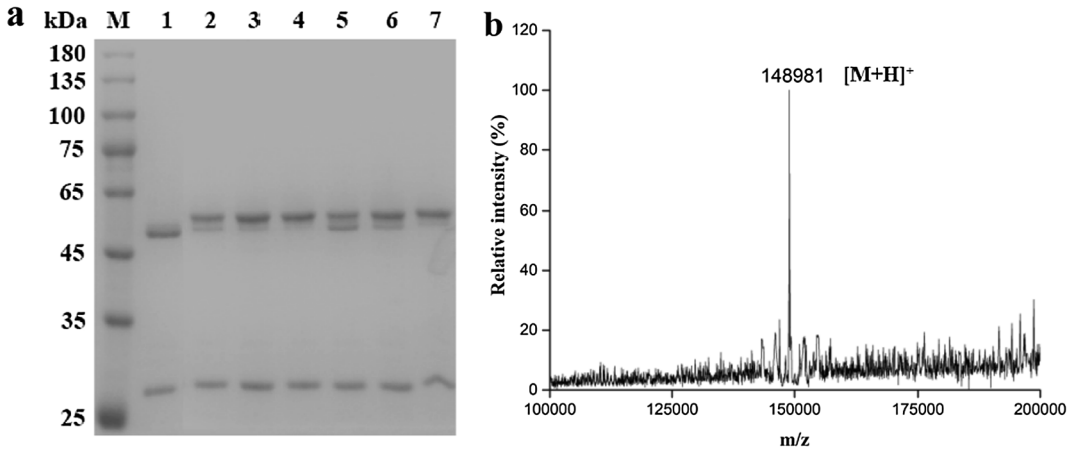


Fig. 5. SDS-PAGE and LC-MS analysis of transglycosylation of Rituximab. (a) SDS-PAGE analysis: lane M, marker; lane 1, deglycosylated-Rituximab; cellulose immobilized EndoS D233A (lane 2–4) and D233Q (lane 5–7) catalyzed the transglycosylation reaction in 30, 60 and 90 min. (b) LC-MS analysis of complete transglycosylation of Rituximab product 7.

cellulose immobilized CBD-EndoS was recovered and recycled for five times. As revealed in Fig. 6c, the immobilized CBD-EndoS maintained stable hydrolysis activity during these processes, and still more than 88% of enzyme activities were retained in the fifth cycle, suggesting that the cellulose immobilized CBD-EndoS displayed excellent stability and reusability in operational process.

Similarly, to access the reusability of the cellulose immobilized glycosynthase mutants (D233A and D233Q), the transglycosylation reactions were performed by incubating immobilized glycosynthase mutants, deglycosylated (Fucα1, 6)GlcNAc – Rituximab 5 and sialoglycan oxazoline 6 in PBS buffer for 1.5 h, respectively. Then the cellulose immobilized enzymes were recovered and recycled following the same protocol as previously described, the transglycosylation efficiency of each cycle was evaluated by SDS-PAGE and relative enzyme

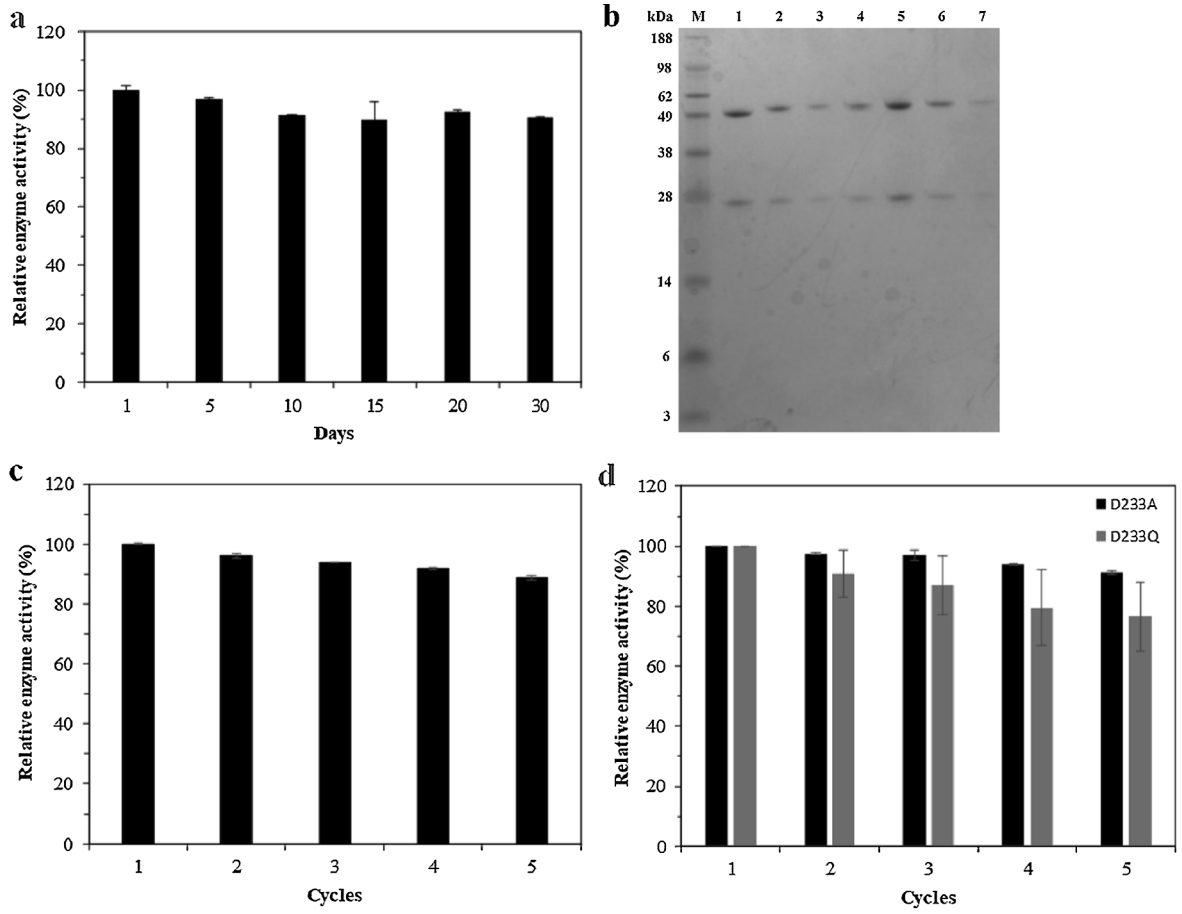


Fig. 6. Storage stability and reusability of the cellulose immobilized CBD-EndoS and the glycosythase mutants. (a) Storage stability of the cellulose immobilized CBD-EndoS in 30 days; (b) SDS-PAGE analysis of the storage stability of the cellulose immobilized glycosythase mutants in 30 days, lane M, maker; lane 1, deglycosylated-Rituximab; storage stability of immobilized CBD-EndoS D233A (lane 2–4) and D233Q (lane 5–7) after 0, 15, 30 days; (c) Reusability of the cellulose immobilized CBD-EndoS; (d) Reusability of the cellulose immobilized glycosynthase mutants.

activity was obtained by compared with that of the first-round reaction. As indicated in Fig. 6d, the relative enzyme activity for the cellulose immobilized EndoS mutants D233A was 100%, 97%, 97%, 93% and 91% in five cycles. While for the cellulose immobilized EndoS mutants D233Q, slightly decreased (76%) relative enzymatic activities were retained in the fifth cycle. These results suggested that the cellulose immobilized EndoS mutants D233A exhibited better recyclability and reusability compared with the other mutants, and it can successfully be recycled and reused for five times without obvious activity loss during these biotransformation reactions.

4. Conclusions

In summary, CBD domain fusion EndoS and the glycosynthase mutants (D233A and D233Q) were recombinantly expressed in *E. coli* in a yield of 40–50 mg/L. Then a one-step immobilization and purification strategy was developed to immobilize these enzymes onto cellulose in high efficiency (81–90%) under optimal condition. The cellulose immobilized CBD-EndoS and the glycosynthase mutants (D233A and D233Q) were successfully applied in a two-step antibody Fc *N*-glycan remodeling, generating a therapeutic antibody with homogeneous glycoform in high efficiency. Moreover, the cellulose immobilized CBD-EndoS and its mutants (D233A and D233Q) displayed excellent storage stability when stored at 4 degree for one month. Reusability studies demonstrated that the cellulose immobilized CBD-EndoS and its mutants could be recycled for five times without obvious activity loss. This strategy provides an alternative approach to immobilize other similar biocatalysts onto cellulose for biomolecule transformation.

Declaration of Competing Interest

The authors declare no competing financial interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103114.

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