Fusing a Carbohydrate-Binding Module into the *Aspergillus usamii* β-Mannanase to Improve Its Thermostability and Cellulose-Binding Capacity by *In Silico* Design

Cun-Duo Tang1*, Jian-Fang Li2*, Xi-Huan Wei2, Rou Min3, Shu-Juan Gao3, Jun-Qing Wang1, Xin Yin1, Min-Chen Wu4*

1 School of Biotechnology and Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, Wuxi, Jiangsu, China, 2 School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, China, 3 School of Pharmaceutical Science, Jiangnan University, Wuxi, Jiangsu, China, 4 Wuxi Medical School, Jiangnan University, Wuxi, Jiangsu, China

Abstract

The AuMan5A, an acidophilic glycoside hydrolase (GH) family 5 β-mannanase derived from *Aspergillus usamii* YL-01-78, consists of an only catalytic domain (CD). To perfect enzymatic properties of the AuMan5A, a family 1 carbohydrate-binding module (CBM) of the *Trichoderma reesei* cellobiohydrolase I (TrCBH I), having the lowest binding free energy with cellobiose, was selected by *in silico* design, and fused into its C-terminus forming a fusion β-mannanase, designated as AuMan5A-CBM. Then, its encoding gene, *Auman5A-cbm*, was constructed as it was designed theoretically, and expressed in *Pichia pastoris* GS115. SDS-PAGE analysis displayed that both recombinant AuMan5A-CBM (reAuMan5A-CBM) and AuMan5A (reAuMan5A) were secreted into the cultured media with apparent molecular masses of 57.3 and 49.8 kDa, respectively. The temperature optimum of the reAuMan5A-CBM was 75°C, being 5°C higher than that of the reAuMan5A. They were stable at temperatures of 68 and 60°C, respectively. Compared with reAuMan5A, the reAuMan5A-CBM showed an obvious decrease in *Km*, and a slight alteration in *Vmax*. In addition, the fusion of a CBM of the TrCBH I into the AuMan5A contributed to its cellulose-binding capacity.

Citation: Tang C-D, Li J-F, Wei X-H, Min R, Gao S-J, et al. (2013) Fusing a Carbohydrate-Binding Module into the *Aspergillus usamii* β-Mannanase to Improve Its Thermostability and Cellulose-Binding Capacity by *In Silico* Design. PLoS ONE 8(5): e64766. doi:10.1371/journal.pone.0064766

Editor: Jose M. Sanchez-Ruiz, Universidad de Granada, Spain

Received January 16, 2013; Accepted April 18, 2013; Published May 31, 2013

Copyright: © 2013 Tang et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by National Nature Science Foundation of China (grant No. 31271811) (http://www.nsfc.gov.cn/); Doctoral Research Funds of Jiangnan University, China (grant No. JUDCF11011) (http://yjsb.jiangnan.edu.cn/); and Postgraduate Innovation Training Project of Jiangsu, China (grant No. CXZZ11_0480) (http://www.ec.js.edu.cn/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: biowmc@126.com

† These authors contributed equally to this work.

Introduction

β-Mannanases (EC 3.2.1.78), abbreviated from β-1,4-D-mannan mannohydrolases, can hydrolyze the internal β-1,4-D-mannosidic linkages of mannans. They could be applied in industrial processes, such as bleaching pulps, depolymerizing antimicrobial saccharides, and extracting oils from leguminous seeds [1]. To date, many researches have been performed on exploiting novel β-mannanases with good properties, improving their catalytic activities by mutating enzyme-producing strains and optimizing fermentation conditions, as well as producing β-mannanases on an industrial scale [2,3]. However, the applicability of β-mannanases, exemplified by preparing feedstuffs and bleaching pulps, was limited by their low stability at the high temperature and/or extreme pH. To meet the increasing needs for β-mannanases, more interests are being focused on modifying their molecular structures by means of genetic engineering [4].

Based on the sequence alignment, 25,580 CBMs in the CAZY database [July, 2012] were classified into 64 families. The recognized function of CBMs was to bring enzymes into close vicinity to their substrates by binding carbohydrates [5]. Some studies also demonstrated that the fusion of CBMs into enzymes improved the catalytic activity and/or thermostability. For instance, delignification efficiency of the *Pycnoporus cinnabarinus* laccase for softwood kraft pulp bleaching was improved by fusing a family 1 CBM of the *A. niger* cellobiohydrolase B into the laccase [6]. After two CBMs of the *Thermohibida fusca* and *Cellulomonas fimbi* cellulases were fused into the *T. fusca* cutinase, respectively, both fusion cutinases exhibited a dramatic increase of up to 3-fold in the amount of fatty acids released from cotton fiber [7].

Almost all β-mannanases reported have been classified into GH families 5, 26 and 113 [http://www.cazy.org/fam/acc_GH.html] [8]. The family 5 β-mannanases either contain an only CD [9] or, besides a CD, carry an additional CBM located at the C-terminus such as *T. reesei* β-mannanase [10] or at the N-terminus such as *Phanerochaete chrysosporium* β-mannanase [11]. In our previous work,
Improved Properties of AuMan5A by In Silico Design

Materials and Methods

Strains, Vectors and Media

A. amanii YL-01-78 and T. reesei LW-22, isolated from the soil in China, were used as donors of the Auman5A and CBH I-encoding gene (Trcbh), respectively. E. coli JM109 and pUCm-T (Sangon, Shanghai, China) were used for gene cloning and DNA sequencing. Two recombinant T-vectors, pUCm-T-Auman5A and pUCm-T-Trcbh, were constructed according to the sequences of the Auman5A (GenBank accession: HQ839659) and Trcbh (GL985084). E. coli DH5α and pPIC9K (Invitrogen, San Diego, CA) were used for the construction of recombinant expression vectors. E. coli JM109 and DH5α were cultured in a LB medium.

P. pastoris GS115 was cultured in a yeast extract peptone dextrose (YPD) medium, and its transformant culture and induced in following media that were prepared as described in the manual of Multi-Copy Pichia Expression Kit (Invitrogen): minimal dextrose (BMGY) and buffered methanol-complex (BMMY).

Bioinformatics Analysis of the β-mannanase and CBM Sequences

The multiple protein sequence alignment of the CBMs was performed using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The putative N-glycosylation site of the β-mannanase was located using the NetNGlyc program (http://www.cbs.dtu.dk/services/NetNGlyc/). The ProtParam program (http://au.expasy.org/tools/protparam.html) was used for predicting the β-mannanase physicochemical properties. The phylogenetic tree of the CBMs was constructed using both the ClustalW2 program and MEGA 4.0 software. The three-dimensional (3-D) structures of the CBMs were predicted by homology modeling using the MODELLER 9.9 program (http://salilab.org/modeller/).

In silico Design of the Fusion β-mannanase

The candidate CBMs were chosen from the phylogenetic tree, and their 3-D structures were modeled based on a CBM crystal one of the TrCBH I (PDB code: 1CBH). While the 3-D structural information of cellobiose, used as the ligand, was handled using the GlycoBioChem PRODRG (http://davacp1.bioch.dundee.ac.uk/prodrg/submit.html). Then, the interaction between the candidate CBM and cellobiose was predicted by molecular docking (MD) simulation. The binding free energy of the CBM with cellobiose, contrary to their affinity [13], was calculated using the AutoDock 4.2 program (http://autodock.scripps.edu), which combines a rapid binding free energy evaluation through precalculated grids of affinity potentials with a variety of search algorithms to find the most suitable binding position for a ligand on a given macromolecule [14]. Finally, a CBM having the lowest binding free energy was selected, and fused into the Auman5A forming an AuMan5A-CBM.

Construction of the Fusion β-mannanase Gene

The Auman5A-cbm was constructed by fusing the 3′-end region (lcbm) of the Trcbh, which encodes both a Ser/Thr/Pro-rich linker and a CBM, into 3′-end of the Auman5A by overlapping PCR. The Auman5A was amplified from the pUCm-T-Auman5A with primers F1 (5′-GAATTCTCCCTGGCCAGACCTC-3′ with an EcoRI site, underlined) and R1 (5′-GAGGGTGTCGGGCACATAT-CAATAGCAGC-3′). The lcbm was amplified from the pUCm-T-Trcbh with primers F2 (5′-TGATATGTCGGGC-CAACCCTCCCGGCG-3′) and R2 (5′-GCGGCACCTTAA-CAGGCACCTAGGAGTAG-3′, with a Not I site, underlined). Using the Auman5A and lcbm as primers and templates, the first-round overlapping PCR for the Auman5A-cbm was performed as follows: a denaturation at 94°C for 4 min; 10 cycles of at 94°C for 30 s, 52°C for 30 s, 72°C for 75 s; an elongation at 72°C for 10 min. Then, F1 and R2 were added to the above PCR system to run the second-round PCR under the same conditions, except 30 cycles and an annealing temperature of 56°C. The amplified target band was cloned into pUCm-T, and transformed into E. coli JM109. The recombinant T-vector, pUCm-T-Auman5A-cbm, was confirmed by DNA sequencing.

Transformation of the Recombinant Expression Vectors

The Auman5A-cbm and Auman5A were excised from the pUCm-T-Auman5A-cbm and pUCm-T-Auman5A by digestion with EcoRI and Not I, and inserted into pPIC9K, followed by transforming them into E. coli DH5α, respectively. Recombinant expression vectors, pPIC9K-Auman5A-cbm and pPIC9K-Auman5A, were confirmed by sequencing. The resulting recombinant vectors were separately linearized with Sac I, and transformed into P. pastoris GS115 using a Gene Pulser Apparatus (Bio-Rad, Hercules, CA).

Screening and Expression of the P. pastoris Transformants

All P. pastoris transformants were primarily screened based on their ability to grow on a MD plate, then inoculated successively on G418-containing YPD plates at increasing concentrations of 1.0, 2.0 and 4.0 mg/mL to screen multiple copies of the Auman5A-cbm and Auman5A, respectively. P. pastoris GS115 transformed with pPIC9K vector without any insert was used as the negative control. Expression of the gene Auman5A-cbm or Auman5A in P. pastoris GS115 was performed according to the instruction of Multi-Copy Pichia Expression Kit (Invitrogen) with some modifications [15].

Enzyme Activity and Protein Assays

β-Mannanase activity was determined by measuring the amount of reducing sugars from locust bean gum (Sigma, St. Louis, MA), using the 3,5-dinitrosalicylic acid (DNS) method as reported previously [15]. One unit (U) of β-mannanase activity was defined as the amount of enzyme liberating 1.0 μmol of reducing sugar equivalent per min under the standard assay conditions (pH 3.6 and 60°C for 10 min). The protein concentration was measured using the BCA-200 Protein Assay Kit (Pierce, Rockford, IL). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 12.5% gel using the reported method [16], and the isolated proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma).

Purification of the Recombinant β-mannanases

After 96-h induction, the culture medium of the transformant was centrifuged to remove cells. A total of 60 mL of supernatant
was brought to 75% saturation by adding solid ammonium sulfate. The resulting precipitate was collected, dissolved in 6.0 mL of 20 mM Na2HPO4-NaH2PO4 buffer (pH 7.0), and dialyzed against the same buffer overnight. The dialyzed solution was concentrated to 1.5 mL by ultrafiltration using a 30-kDa cutoff membrane (Millipore, Billerica, MA), and then loaded onto a glass column (0.8 × 10 cm), followed by elution with the same buffer at a flow rate of 0.3 mL/min. Aliquots of 1.5 mL eluent only containing the reAuMan5A-CBM or reAuMan5A were pooled and concentrated for further studies.

Temperature Optimum and Stability Assays

The temperature optima of the reAuMan5A-CBM and reAuMan5A were determined under the standard assay conditions, except temperatures ranging from 50 to 80°C. To estimate the thermostability, the reAuMan5A-CBM or reAuMan5A was incubated at pH 3.6 and various temperatures (45–75°C) for 1.0 h, then the residual enzyme activity was measured under the standard assay conditions. The thermostability here was defined as a temperature, at or below which the residual reAuMan5A-CBM or reAuMan5A activity retained more than 85% of its original activity.

Enzymatic Kinetic Parameter Assays

Reaction rate (U/mg) of the reAuMan5A-CBM or reAuMan5A was measured under the standard assay conditions, except locust bean gum concentrations ranging from 1.0 to 10 mg/mL. The reaction rate versus the substrate concentration was plotted to confirm whether the catalyzing mode of the reAuMan5A-CBM or reAuMan5A conforms to the Michaelis-Menten equation. The enzymatic kinetic parameters, $k_m$ and $V_{max}$ values, were graphically determined from the Lineweaver-Burk plotting.

Cellulose-binding Capacity Assay

The cellulose-binding test was performed according to the method [17] with some modifications. The cultured supernatant of the transformant was dialyzed against the same buffer overnight. The dialyzed solution was filled, at a flow rate of 0.2 mL/min, followed by washing with 20 mM Na2HPO4-NaH2PO4 buffer (pH 7.0), and dialyzed against the same buffer overnight. The dialyzed solution was concentrated to 1.5 mL by ultrafiltration using a 30-kDa cutoff membrane (Millipore, Billerica, MA), and then loaded onto a glass column (0.8 × 80 cm), followed by elution with the same buffer at a flow rate of 0.3 mL/min. Aliquots of 1.5 mL eluent only containing the reAuMan5A-CBM or reAuMan5A were pooled and concentrated for further studies.

Construction of the Fusion β-mannanase Gene

About 1050- and 210-bp bands of the Auman5A and lcbm were amplified from the pUCm-T-Auman5A and pUCm-T-Trcbh, respectively. Then, they were subjected to the overlapping PCR to construct the fusion gene. As a result, an about 1250-bp band of the Auman5A-cbm was amplified and inserted into pUCm-T. DNA sequencing result verified that the cloned Auman5A-cbm is 1241 bp in length (containing EboR 1 and Not I sites), encoding an AuMan5A-CBM of 408 amino acid residues with a theoretical molecular mass of 43,798 Da and a pl of 4.22. The AuMan5A-CBM consists of a 345-aa CD from the AuMan5A, and both a 27-aa linker and a 36-aa CBM from the TrCBH I (Fig. 5).

Screening and Expression of the P. pastoris Transformsants

A total of 30 P. pastoris transformsants with Auman5A respectively resistant to 1.0, 2.0 and 4.0 mg/mL of geneticin G418, numbered as P. pastoris GSAuM1-1 to GSAuM1-10, GSAuM2-1 to GSAuM2-10 and GSAuM4-1 to GSAuM4-10, and 30 transformsants with Auman5A-cbm, numbered as P. pastoris GSAuMC1-1 to GSAuMC1-10, GSAuMC2-1 to GSAuMC2-10 and GSAuMC4-1 to GSAuMC4-10, were picked out for flask expression tests. P. pastoris GS115 transformed with pPIC9K, numbered as P. pastoris GSC, was used as the negative control. After induction by adding 1.0% (v/v) methanol at 24 h intervals for 96 h, the cultured supernatants were used for β-mannanase activity assay. As a result, two transformsants with the highest reAuMan5A-CBM and reAuMan5A activities of 40.6 and 44.1 U/mL, numbered as P. pastoris GSAuMC4-5 and GSAuMC4-8, were selected, respectively. No β-mannanase activity of P. pastoris GSC was detected under the same expression conditions.

Purification of the Recombinant β-mannanases

The amount of the reAuMan5A-CBM or reAuMan5A in the cultured supernatant of the GSAuMC4-5 or GSAuMC4-8 accounted for more than 85% of that of the total protein. So they were purified to homogeneity only by a simple combination of ammonium sulfate precipitation, ultrafiltration and Sephadex G-75 gel filtration (Fig. 4). Specific activities of the purified reAuMan5A-CBM and reAuMan5A, towards locust bean gum under the standard assay conditions, were 312.3 and 341.3 U/mg, respectively.

SDS-PAGE analysis of the purified reAuMan5A-CBM and reAuMan5A showed two single protein bands with apparent molecular masses of 57.3 and 49.8 kDa, respectively (Fig. 4), which were much larger than their respective theoretical ones (43,798 and 37,614 Da). P. pastoris enables some post-translational modifications, including the assembly of disulfide bond, exclusion of the signal peptide, and N-glycosylation, etc. Analysis showed that there are two putative N-glycosylation sites (N156-S157-S158 and N225-F226-T227) in the AuMan5A-CBM or AuMan5A sequence (Fig. 3). To verify whether the difference between apparent and theoretical molecular masses was resulted from N-glycosylation, The N-deglycosylation assay was performed using an endoglycosidase H (New England Biolabs, Ipswich, MA) according to the manufacturer’s instruction. As a result, molecular sizes of the N-deglycosylated reAuMan3A-CBM and reAuMan3A on SDS-PAGE (data not shown) were good agreement with their respective theoretical ones. So the larger apparent molecular masses may be caused by N-glycosylation. Carbohydrate contents of the purified reAuMan5A-CBM and reAuMan5A were determined to be 19.8 and 21.3%, respectively, using the phenolsulfuric acid method [18].

Results

In silico Design of the Fusion β-mannanase

From the CAZY database, we randomly selected 23 family 1 CBMs. Then, the CBM phylogenetic tree was constructed (Fig. 1). Based on the topology of the tree, those CBMs were further grouped into 4 subfamilies. One to three CBMs from each subfamily were chosen as the candidate CBMs. The interaction between 3-D structures of the candidate CBM and cellulose was predicted by MD simulation, and then the binding free energy was calculated using the AutoDock 4.2 program (Table 1). As a result, a CBM of the TrCBH I having the lowest binding free energy of -2.27 kcal/mol was selected, and fused into the C-terminus of AuMan5A. The molecule-docked conformation of the CBM with cellulose was illustrated (Fig. 2).
Enzymatic Properties of the Recombinant Enzymes

The temperature optima of the reAuMan5A-CBM and reAuMan5A were 75 and 70 °C (measured at pH 3.6), respectively (Fig. 5A). They were highly stable at temperatures of 68 and 60 °C, respectively, but over which their residual activities declined.

Figure 1. The phylogenetic tree showing the evolutionary relativity and homology among family 1 CBMs. The CBM-containing hydrolases are as follows: Pma EG, P. marneffei endoglucanase (XP_002152969); Tvi GH62, T. vires GH family 62 enzyme (EHK19840); Tre CBH I, T. reesei cellobiohydrolase I (EGR44817); Pde Xyn10, P. decumbens family 10 xylanase (ADX86896); Ate EG I, A. terreus endoglucanase I (XP_001217291); Ate EG, A. terreus endoglucanase (AAW68436); Tte GH11, T. terrestris GH family 10 enzyme (XP_003653793); Mth GH7, M. thermophila GH family 7 enzyme (XP_003663441); Ath CB, A. thermophilum cellobiosidase (CAM98445); Cth CBH6, C. thermophilum family 6 cellobiohydrolase (AAY88915); Pin Est, P. indica acetylxylan esterase (CCA73570); Pin EGc, P. indica endoglucanase c (CCAA7649); Hje Man, H. jecorina mannanase (AAA34208); Tlo Man, T. longibrachiatum mannanase (ADN93457); Tvi Man, T. viride mannanase (AFP95336); Hru EG V, H. rufa endoglucanase V (AAQ21385); Pin EG IV, P. indica endoglucanase IV (CCA70703); Tat GH11, T. atroviride GH family 11 enzyme (EHK46770); Shi XynB, S. hirsutum xylanase B (EIM91441); Pox XynB, P. oxalicum xylanase B (ADV31286); Npa Xyn, N. patriciarum xylanase (ABW04217); Tte GH11, T. terrestris GH family 11 enzyme (XP_003649436); Tte Est5, T. terrestris family 5 carbohydrate esterase (XP_003653797).

doi:10.1371/journal.pone.0064766.g001

Table 1. The binding free energy of the candidate CBM combining with cellobiose.

| CBM-containing hydrolase | GenBank accession no. | Length of CBM (aa) | Aromatic residues in the groove surface | Apolar area/energy of CBM (Å²) | Binding free energy (kcal/mol) |
|--------------------------|-----------------------|--------------------|----------------------------------------|-----------------------------|-----------------------------|
| Pma EG                   | XP_002152969          | 34                 | Trp¹, Tyr2, Tyr30                      | 1775.44                    | –1.56                       |
| Tre CBH I                | EGR44817             | 36                 | Trp³, Tyr5, Tyr13, Tyr31, Tyr32        | 1644.23                    | –2.27                       |
| Ate EG                   | AAW68436             | 33                 | Trp10, Tyr20, Trp20, Trp29             | 1780.38                    | –1.21                       |
| Pin Est                  | CCA73570             | 33                 | Trp2, Tyr12, Trp23, Trp27, Trp28       | 1753.77                    | –1.64                       |
| Tvi Man                  | AAF95336             | 34                 | Trp2, Tyr1, Trp23, Trp28, Trp29        | 1776.76                    | –1.98                       |
| Hru EG V                 | AAQ21385             | 33                 | Tyr7, Trp3, Trp10, Trp20, Trp29        | 1727.44                    | –1.65                       |
| Pox XynB                 | ADV31286             | 30                 | Trp10, Tyr16                           | 1275.21                    | –1.78                       |
| Tte GH11                 | XP_003649436         | 33                 | Trp3, Trp1, Trp20, Trp29               | 1725.45                    | –1.16                       |

The apolar area/energy of CBM were calculated by the GETAREA server (http://curie.utmb.edu/getarea.html).

doi:10.1371/journal.pone.0064766.g001
rapidly and only retained 62.3 and 36.8% of the original ones at 75 °C, respectively (Fig. 5B).

The $K_m$ and $V_{max}$ values of the reAuMan5A-CBM were determined to be 0.66 mg/mL and 389.1 U/mg, respectively, and those of the reAuMan5A were 1.36 mg/mL and 415.8 U/mg, respectively. The reAuMan5A-CBM displayed an obvious decrease in $K_m$ but a slight alteration in $V_{max}$, as compared with the reAuMan5A.

Cellulose-binding Capacity

A total of 20 mL of dialyzed cultured superntant, in which the reAuMan5A-CBM or reAuMan5A activity was adjusted to 20 U/mL, was loaded onto the cellulose column. The test results showed that cellulose-binding capacity of the reAuMan5A-CBM with Avicel PH-101 was up to 92.3%, whereas that of the reAuMan5A was not detected.

Discussion

Mannans, the major hemicelluloses in plant cell walls and the specific substrates for $\beta$-mannanases, are always cross-linked with various kinds of carbohydrates, such as celluloses, xylans and arabinans [19]. CBMs often existed in some hydrolases decomposing plant cell walls, such as $\beta$-mannanases, xylanases and arabinofuranosidases. Being natively in enzymes or by fusing CBMs into the N- or C-termini of other enzymes, the CBMs could obviously enhance the catalytic efficiency of enzymes by increasing their local concentrations around the polysaccharide substrates, and/or improve the thermostability [17]. Because the AuMan5A consists of an only CD, it is reasonable to perfect its enzymatic properties, such as thermostability and cellulose-binding capacity, by fusing a CBM into the AuMan5A. However, there are a total of 25,580 CBMs classified into 64 families in the CAZY database, so the choice of a suitable CBM from so numerous CBMs is time-consuming and laborious. It was reported that the family 1 CBMs are mainly cellulose-binding modules, while celluloses are often covalently and non-covalently attached to mannans [5]. Therefore, our present work was focused on the family 1 CBMs.

The MD simulation can rapidly predict the most suitable binding position for a ligand on a given macromolecule and calculate the binding free energy, which was used as a powerful tool in indirectly reflecting the affinity between ligand and receptor, such as the design of human phospholipase A2 or HIV-1 protease inhibitors [20,21]. In this work, we selected 23 family 1 CBMs to construct the phylogenetic tree, from which 8 candidate CBMs were chosen. And their 3-D structures were modeled based on a CBM crystal one of the TrCBH I. Next, the interaction between 3-D structures of the candidate CBM and cellulobiose was predicted, and the binding free energy was calculated. And finally, a CBM having the lowest binding free energy was selected, and then the Auman5A-cbm was constructed and expressed in P. pastoris. As can be seen from Fig. 2, several aromatic amino acids are exposed to the groove surface of a CBM of the TrCBH I. In recent years, the 3-D structures of representative members from 22 CBM families have been resolved. Data from these 3-D structures speculated that these aromatic amino acids may play a key role in binding cellulobiose or carbohydrates [22], which needs to be proved by our further studies.

$P.\ pastoris$ transformant that can resist a higher concentration of G418 might have multiple copies of integration of a heterologous gene into $P.\ pastoris$ genome, which could potentially result in a high expression level of a heterologous protein as explained in the manual of Multi-Copy Pichia Expression Kit (Invitrogen). However, the expression level was not directly proportional to the concentration of G418 [9,23]. Due to those reasons, in this work, a total of 30 transformants with Auman5A-cbm resistant to different concentrations of G418 and 30 transformants with Auman5A were picked out for flask expression tests. This screening procedure has been applied to conduct the over-expression of other recombinant proteins or enzymes in $P.\ pastoris$ [24,25].

$P.\ pastoris$ expression system has many advantages, one of which is that the purity of expressed recombinant proteins or enzymes is high. It was reported that the purity of recombinant A. sulphurus $\beta$-mannanase expressed in $P.\ pastoris$ X-33 was 97% [9]. In this work, the purity of reAuMan5A-CBM or reAuMan5A was more than 85%. So they were purified to homogeneity only by a simple combination of ammonium sulfate precipitation, ultrafiltration and Sephadex G-75 gel filtration (Fig. 4). The purified reAuMan5A-CBM and reAuMan5A were N-glycosylated pro-

![Figure 2. The molecule-docked conformation between 3-D structures of a CBM of the TrCBH I and cellulobiose. (A) a cartoon model and (B) a surface model.](doi:10.1371/journal.pone.0064766.g002)
Figure 3. Nucleotide sequence of the Auman5A-cbm and its deduced amino acid sequence of the AuMan5A-CBM. The amino acid residues of the AuMan5A are marked in grayed background. A linker of the T. reesei CBH I is underlined and its CBM is boxed. Two triangles below the boxed letters indicate the catalytic residues (E168 and E276) and five active site residues (R52, N167, H241, Y243, and W306) are located in grayed boxes. Two putative N-glycosylation sites of the AuMan5A-CBM are underlined in grayed background. The bold arrows above the letters represent the PCR primers.

doi:10.1371/journal.pone.0064766.g003
teins, which were confirmed by N-deglycosylation and carbohydrate content assays.

The temperature optimum and stability of the reAuMan5A-CBM were 75 and 68°C, which were 5 and 8°C higher than those of the reAuMan5A, respectively, indicating that the fusion of a CBM of the TrCBH I conferred the elevated tolerance to high temperature on the AuMan5A. The $K_m$ value (0.66 mg/mL) of the reAuMan5A-CBM was much lower than that (1.36 mg/mL) of the reAuMan5A, implying that the former has a higher affinity towards locust bean gum. The cellulose-binding capacity of the reAuMan5A-CBM with Avicel PH-101 was up to 92.3%, suggesting that it can be gathered around the natural substrate (that is, mannan cross-linked with cellulose, xylan and arabinan) by exclusively binding cellulose [26].

Conclusions
In this work, the fusion β-mannanase gene, Auman5A-cbm, was constructed as it was designed theoretically, and functionally expressed in P. pastoris GS115. The fusion of a family 1 CBM of the TrCBH I into the C-terminus of AuMan5A obviously improved its thermostability and cellulose-binding capacity, which are beneficial for industrial applications. The superior properties of the reAuMan5A-CBM make it a good candidate in industrial processes. To our knowledge, this work first established a novel strategy for molecular modification of enzymes by in silico design.

Acknowledgments
We are grateful to Prof. Xianzhang Wu (School of Biotechnology, Jiangnan University) for providing technical assistance.

Author Contributions
Conceived and designed the experiments: MCW JFL CDT. Performed the experiments: CDT XHW. Analyzed the data: RM SJG JQW. Contributed reagents/materials/analysis tools: XY. Wrote the paper: CDT MCW.

References
1. van Zyl WH, Rose SH, Trollope K, Gorgens JF (2010) Fungal β-mannanases: Mannan hydrolysis, heterologous production and biotechnological applications. Proc Biochem 45: 1203–1213.
2. Heck JX, de Barros Soares LH, Ayub MAZ (2005) Optimization of xylanase and mannanase production by Bacillus circulans strain BL53 on solid-state cultivation. Enzyme Microb Technol 37: 417–423.
10. Stalbrand H, Saloheimo A, Vehmaanpera J, Henrissat B, Penttila M (1995) Cloning and functional expression of an Aspergillus aculeatus endo-1,4-β-mannanase in Pichia pastoris. Mol Biotechnol 43: 112–120.

11. Benech RO, Li XM, Patton D, Powlowski J, Storms R, et al. (2007) Cloning, functional expression, biochemical properties and novel applications. Microbiol Mol Biol Rev 70: 836–852.

12. Tang CD, Guo J, Wu MC, Zhao SG, Shi HL, et al. (2011) Cloning and molecular dynamics and docking simulations of the cytidine deaminase from Mycobacterium tuberculosis H37Rv. J Mol Model 18: 467–479.

13. Timmers LF, Ducati RG, Sanchez-Quian J, Basso LA, Santos DS, et al. (2012) Combining molecular dynamics and docking simulations of the cytidine deaminase from Mycobacterium tuberculosis H37Rv. J Mol Model 18: 467–479.

14. Phusiripong N, Ungwityatorn J (2010) Molecular docking study on anticancer activity of plant-derived natural products. Med Chem Res 19: 817–835.

15. Li JF, Zhao SG, Tang CD, Wang JQ, Wu MC (2012) Cloning and functional expression of an acidic 1,4-β-mannanase (AnMan5A) from Aspergillus niger in Pichia pastoris. J Agric Food Chem 60: 763–773.

16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

17. Thongkawkar J, Ieda H, Inui H (2012) Increases thermal stability and cellulase-binding capacity of Cryptococcus sp. N-2 lipase by fusion of cellulase binding domain derived from Trichoderma reesei. Biochem Biophys Res Commun 420: 183–187.

18. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28: 350–356.

19. Moreira LRS, Filho EXF (2008) An overview of mannan structure and mannan-degrading enzymes. Appl Microbiol Biotechnol 79: 165–178.

20. Ortiz AR, Piaibarro MT, Gago F, Wade RC (1995) Prediction of drug binding affinities by comparative binding energy analysis. J Med Chem 38: 2681–2691.

21. Perez C, Pastor M, Ortiz AR, Gago F (1998) Comparative binding energy analysis of HIV-1 protease inhibitors: incorporation of solvent effects and validation as a powerful tool in receptor-based drug design. J Med Chem 41: 836–852.

22. Shoeboyov O, Shani Z, Levy I (2006) Carbohydrate binding modules: biochemical properties and novel applications. Microbiol Mol Biol Rev 70: 283–295.

23. Li JF, Tang CD, Shi HL, Wu MC (2011) Cloning and optimized expression of a neutral endoglucanase gene (cel5A) from Volvariella volvacea WX52 in Pichia pastoris. J Biosci Bioeng 111: 537–540.

24. Tan ZB, Li JF, Wu MC, Tang CD, Zhang HM, et al. (2011) High-level heterologous expression of an alkaline lipase gene from Phanerochaete chrysosporium PG37 in Pichia pastoris. World J Microbiol Biotechnol 27: 2767–2774.

25. Shi HL, Yin X, Wu MC, Tang CD, Zhang HM, et al. (2012) Cloning and bioinformatics analysis of an endoglucanase gene (Auel124) from Aspergillus usamii and its functional expression in Pichia pastoris. J Ind Microbiol Biotechnol 39: 347–357.

26. Ye XH, Zhou ZG, Zhang CM, Zhang YHP (2011) Fusion of a family 9 cellulose-binding module improves catalytic potential of Clostridium thermocellum cellulodextrin phosphorylase on insoluble cellulose. Appl Microbiol Biotechnol 92: 551–560.