Characterization of a Novel Thermostable Chitin-Binding Domain and Its Application in Immobilization of a Multifunctional Hemicellulase

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ABSTRACT: A novel thermostable chitin-binding domain (Tt-ChBD) of chitinase A1 from *Thermoanaerobacterium thermosaccharolyticum* DSM571 was cloned, characterized, and compared for its binding activity with another mesophilic chitin-binding domain (Bc-ChBD). Recombinant protein with Tt-ChBD exhibits stronger affinity to chitin than those with Bc-ChBD at temperatures from 65 °C to at least 75 °C, but not to other polysaccharides including xylan, chitosan, cellulose, and agarose. For repeated production of xylose from arabinoxylan-containing feedstocks, a best-characterized trifunctional chimeric enzyme Xar-L1-Xyn (XX) constructed in our previous work was attempted to be immobilized on chitin efficiently by genetically fusing Tt-ChBD to the N-terminal region of XX (named CXX) and the C-terminal region of XX (named XXC), respectively. The fusing position of Tt-CBD affected the affinity-binding activity to chitin. Recombinant XX, XXC, and CXX were purified to homogeneity and characterized. According to the xylosidase activities, the optimum temperature and pH profiles of the CXX and XXC both in free and immobilized form were the same as those of XX. However, the thermal and pH stabilities of the immobilized XXC and CXX were both greatly improved in the range from 70 to 90 °C and pH 4.2−8.2. The immobilized multifunctional hemicellulase exhibited high stability to producing xylose for at least 19 or 30 times in continuous operation with the achievement of 60% or 80% conversion yield at temperatures up to 65 °C. These results indicate the usefulness of Tt-ChBD as an affinity tag for the simultaneous purification and immobilization of the enzyme on chitin and the great potential applications for thermophilic enzyme immobilization at higher temperatures.

KEYWORDS: thermostable chitin-binding domain, immobilization, multifunctional hemicellulase, xylose production

INTRODUCTION

Enzymes in modern biotechnology are versatile catalysts on an industrial scale because of their specificity, selectivity, efficiency, and sustainability, and improving their immobilization would be beneficial to broadening their applicability.[1] Active immobilization of enzyme on inert supports enables facilitation of segregation of enzyme from the aqueous phase and recovery of enzyme after a bioreaction is completed. Meanwhile, this approach may help to enhance the stability and activity of enzymes in certain cases.[2] Various methods of immobilization have been developed in recent years, and most require chemical modifications of either the matrix or enzyme of interest.[3] However, these modifications, leading to covalent bonding of the enzyme to the matrix, often result in a loss of enzyme activity as well as the retention of toxic organic compounds, which have to be removed completely before the method can be applied to medical or food processes.[4]

The use of genetic engineering approaches to construct a chimeric protein containing a functional catalytic domain together with a binding domain has already proven to be useful, especially in protein immobilization, which allows the objective protein with an affinity tag to be endowed with the ability of specific binding to the affinity cognate supports.[5] This technique has received wide acceptance on the basis of the following merits: (1) direct affinity of enzymes to the support from a crude cell-free extract (hereafter referred to as CFX) and

the simultaneous purification and immobilization of the enzyme;[4] (2) proper orientation of immobilized enzymes to expose their active domains; (3) mild immobilization conditions and the lack of diffusion constraints.[6] Numerous fusion tags have been explored, including the most commonly used, poly-His, FLAG, and glutathione S-transferase. However, the support materials for the binding of these affinity peptides are costly, which limits their widespread application in industrial production.[7]

Recent progress in using a chitin-binding domain (ChBD) as a fusion tag has proven very useful to immobilize enzymes on chitin. The approach of confining enzymes on chitin by fusing target proteins with ChBD is very attractive mainly because of the desirable characteristics of the chitin supports, such as their availability and low cost, high stability to temperature and pH, and resistance to microbial attack.[8] Chung-Jen Chiang et al. have explored the utilization of the mesophilic bacteria *Bacillus circulans* WL-12 ChBD (Bc-ChBD) as an affinity tag to retain *Zymomonas mobiliz* levansucrase on chitin beads, leading to an enhanced production of levan.[9] Jong-Tzer Chern et al. have managed to immobilize D-hydantoinase-Bc-ChBD on chitin for

Table 1. Nucleotide Sequences of Used Primers[a]

primer	nucleotide sequence (5′−3′)
P1	gcatccaggcacataccagcctggtgggttgggaaccgccgaacgtgccggcactgtggcagctgcagTGAGATCCGGCTGCTAAC
P2	atttgtaggtgataccgttgtaggtcaccagatcaccgattttgtagtaggtgttgcttgcccattcCTCGAGTGCGGCCGCAA
P3	gcctgcagccgcataccagcctggcaggttgggaaccgagcaatgttccggcactgtggcagctgcagTGAGATCCGGCTGCTAAC
P4	atttgtaggttttaccgttgtaggtcaccagctgacctgcggtgtatgcggtgttcacctgccatgcCTCGAGTGCGGCCGCAA
P5	CATG**CCATGG**TGCCATTATATTTAGATTCCACTCAATCAGT
P6	CCG**CTCGAG**GCCGCTGCCGCTGCCCG
P7	CCC**TCTAGA**AATAATTTTGTTTAACTTTAAGAAGGAGATATA**CCATGG**AAGCCATTATATTTAGATTCC
P8	CCC**GATATC**TTTATTCTCTACCCTTACTTCC
P9	CCC**TCTAGA**AATAATTTTGTTTAACTTTAAGAAGGAGATATA**CATATG**GAATGGGCA AGCAACAC
P10	CGG**CCATGG**GCCGCTGCCGCTGCCCGCCGCGCTGCTGCCCGCGCTCTGCAGCTGCCACAGT
P11	CGG**CCATGG**CGGAATGGGCAAGCA AC
P12	CGG**CCATGG**CCTGCAGCTGCCACA

[a]Twelve primers for PCR amplification were designed and used for the construction of the chimerical genes. The lower-case nucleotides represent nucleotides of the chitin-binding domain. The restriction enzyme sites are underlined.

continuous production of *N*-carbamoyl-D-hydroxyphenylgly-cine.[10] Jen-You Wang et al. have immobilized *Escherichia coli* with surface-displayed Bc-ChBD on chitin within a wide range of pH (5 to 8) and temperatures (15 to 37 °C).[11] Khatuntseva et al. reported a hybrid protein glutaryl-7-aminocephalosporic acid acylase (GLA)-Bc-CBD stably immobilized on the chitin carrier, displaying physical and chemical stabilities.[12] Yun-Peng Chao et al. reported that evolved *Agrobacterium radiobacter* carbamoylase with Bc-ChBD on chitin beads could be recycled 16 times with the achievement of 100% conversion yield in the efficient production of D-HPG from D,L-HPH.[13] However, there are few reports on thermophilic bacteria ChBD with preferential absorption activity toward chitin used as an attractive affinity tag for thermostable enzyme immobiliza-tion.[14] Of particular importance, the advantages of thermo-stable enzymes in industrial processes include reduced risk of contamination, increased substrate solubility, higher stability of enzymes against denaturing agents and proteolytic enzymes, and reduced cost of external cooling.[15,16] Therefore, in view of the commercial importance of thermostable enzymes−ChBD immobilized with chitin for continuous reutilization at high temperature, there is still a great need for finding a novel thermostable ChBD better than those currently tested. Strong affinity activity and stability of ChBD at high temperature should attract considerable attention as characteristics of an efficient fusion tag in potential industrial applications.

Thermoanaerobacterium thermosaccharolyticum DSM 571 grows optimally at 62 °C, has two different thermostable chitinases, one being chitinase A1, involving a chitin-binding domain (Tt-ChBD), which plays an important part in binding to chitin in a high-temperature environment.[17,18] In our previous study, we constructed a trifunctional enzyme (Xar-linker-XynA, named XX) of *Thermoanaerobacter ethanolicus* xylosidase-arabinosidase (Xar) and *Thermomyces lanuginosus* xylanase (XynA) with an optimal linker, which has an important synergistic effect, leading to an efficient degradation of agricultural byproducts into their monosaccharide constitu-ents.[16] In this study, to make the above production process more industrially appealing, we attempted to construct another hybrid protein fusing Tt-CBD with XX and restrict this engineered trifunctional enzyme on the surface of chitin, allowing our thermostable enzyme to be continuously reutilized without the need of further purification, which is particularly useful for an applied bioprocess in xylose production at 65 °C. The result illustrates the great usefulness of Tt-ChBD for

enzyme immobilization at a higher temperature compared to Bc-ChBD from mesophilic bacteria WL-12, and it clearly opens up a new route in bioprocess engineering for the continued production of xylose with immobilized multifunctional hemi-cellulase.

■ MATERIALS AND METHODS

Materials. In the present study, xyloside, cellulose, oat spelt xylan, agarose, chitosan, chitin, and *p*-nitrophenyl glycoside substrates were purchased from Sigma Chemical (St. Louis, MO, USA). High molecular weight chitosan (HMW-chitosan) and low molecular weight chitin (LMW-chitosan) were provided by ARDC (Agro-Industries Development Corporation, Nanjing, China). Colloidal chitin was prepared from powered chitin by the methods described by Jeuniaux.[19]

All other chemicals used were analytical grade reagents unless otherwise stated. Chemical reagents were purchased from Sigma (St. Louis, MO, USA), and all solutions were made with deionized water. Ex-Taq polymerase, Pyrobest DNA polymerase, T$_4$ DNA ligase, and restriction enzymes were purchased from TAKARA (Dalian, China). Molecular weight markers for native gradient gel or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were pur-chased from Promega (Madison, WI, USA). Qiagen plasmid kit and Qiagen MinElute gel extraction kit were obtained from Gene Company (Qiagen, USA).

Escherichia coli DH10B (Promega) was used as host for gene cloning. *Escherichia coli* JM109 (DE3) (Promega) was used as host for the expression of the fusion gene, via the T$_7$ RNA polymerase expression system with pET-20b plasmids (Novagen).

DNA Manipulation. Routine DNA manipulations were carried out essentially as described.[20] Plasmid DNA and PCR products were purified using Qiagen plasmid kit and PCR purification kit (Qiagen, USA). PCR reactions were performed in a PE Applied Biosystems 9700 thermal cycler (Foster City, CA, USA) using standard reaction conditions. DNA modifying enzymes and polymerases were purchased from TAKARA (Dalian, China). Oligonucleotide primers were synthesized from Sangon (Shanghai, China). DNA sequencing was performed by Sangon.

Construction of Expression Plasmids. In this study, Tt-ChBD from *T. thermosaccharolyticum* DSM 571 was obtained by inverse PCR using four synthetic primers, P1, P2, P3, and P4 (Table 1), respectively. PCR amplification of 30 cycles with Pyrobest DNA polymerase (TAKARA, China) was carried out in a 50 μL reaction containing 0.2 mM dNTPs each, 20−35 pmol of each primers, and 0.2 μg of pET-20b plasmid. Each cycle consisted of heating at 95 °C for 5 min, 94 °C for 40 s, 57 °C for 40 s, and 72 °C for 2 min 30 s. The PCR products were purified using the QIAquick PCR purification kit and ligated by T4-DNA ligase after phosphorylation, resulting in the plasmids pET20b-Tt-ChBD and pET20b-Bc-ChBD.

The Xar gene encoding xylosidase-arabinosidase from *T. ethanolicus* JW200 was amplified from pET-20b-xar-L-xynA (named pETXX) as the template by PCR using four synthetic primers, P5, P6, P7, and P8, respectively. The PCR products were purified using the QIAquick PCR purification kit followed by digestion with Nco I and Xho I . Subsequently, the Xar gene fragments were ligated to corresponding sites of pET20b-Tt-ChBD and pET20b-Bc-ChBD, respectively, resulting in pET20b-Xar-Tt-ChBD and pET20b-Xar-Bc-ChBD.

The Tt-ChBD gene containing the linker SAGSSAAGSGSG was amplified from pET20b-Tt-ChBD as the template by PCR using P7, P8, P9, and P10. It was purified and followed by digestion with Xba I and Nco I, then ligated to corresponding sites of pETXX, resulting in the plasmid pET-20b-Nco I -xar-L-xynA and pET-20b-Tt-ChBD-xar-L-xynA (named pETCXX).

The Tt-ChBD gene containing the linker SAGSSAAGSGSG was amplified from pET20b-Tt-ChBD as the template by PCR using P11 and P12. It was purified, followed by single digestion with Xho I, then ligated to the corresponding site of pET-20b-xar-L-xynA, resulting in the plasmid pET-20b-xar-L-xynA-Tt-ChBD (named pETXXC). All recombinant plasmids were sequenced and characterized by restriction analysis.

Expression of Fused Genes and Purification of Fusion Enzymes. *Escherichia coli* JM109(DE3) harboring various expression plasmids was grown in LB medium containing 100 μg of ampicillin/mL at 37 °C to an OD_{600} of 0.8, followed by induction with 0.8 mM isopropylthio-β-galactoside (IPTG) for 10 h. The cells were harvested by centrifugation, washed twice with water, resuspended in 20 mL of 5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-HCl buffer (pH7.9), and French-pressured three times. The cell extracts were heat-treated (70 °C, 20 min), then cooled in an ice bath, and centrifuged (9600g, 4 °C, 30 min). Because these recombinant enzymes were independently fused with his-tag, the resulting supernatants were purified by Ni-affinity chromatography (Novagen) to homogeneity, as determined by SDS-PAGE using 10% polyacrylamide running gels with 5% polyacrylamide stacking gels. All purified recombinant enzymes were stored at 4 °C.

Protein Determination and Enzyme Assay. Total protein concentration was determined by the Bradford method,[21] using bovine serum albumin (BSA, Sigma) as a standard. Xylosidase activity was determined by assaying the amount of *p*-nitrophenyl (*p*NP) released from the substrate *p*-nitropheny-β-xylopyranoside (*p*NPX) (Sigma, N2132). The reaction was initiated by adding 10 μL of 20 mM *p*NPX in 180 μL of 50 mM phthalate-imidole buffer (PIB), which was preincubated at the respective temperature, followed by addition of 10 μL of diluted purified enzyme. After 5 min, the reaction was stopped, the color was developed by the addition of 0.6 mL of 1 M Na_2CO_3, and the A_{405} was read. A standard curve was prepared by using *p*NP. An enzyme unit was defined as the amount of enzyme producing 1 μmol of *p*NP per min.

Xylanase activity was determined by the 4-hydroxybenzoic acid hydrazide method.[22] Oat spelt xylan (hereafter referred to as OSX, Sigma X0627) was used as the substrate. The reaction mixture containing 100 μL of 0.5% (w/v) OSX in water, 90 μL of 50 mM PIB (pH 6.0), and 10 μL of diluted enzyme was incubated at 65 °C for 10 min. Reducing sugars were assayed by adding 600 μL of 4-hydroxybenzoic acid hydrazide, boiling for 10 min, cooling, and measuring the absorbance at 410 nm. One unit of xylanase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min.

Bioaffinity Adsorption Ability of Tt-ChBD Compared with Bc-ChBD. To compare the bioaffinity adsorption ability of Tt-ChBD toward different polymers as supports, 3 mg of polymers (xylan, swelling chitin, chitosan, cellulose, agarose, and colloidal chitin) was dissolved in 300 μL of 50 mM PIB buffer (pH 6.6) and incubated by adding 3.6 U purified Xar containing Tt-ChBD (named XTC) with 200 rpm continuous stirring at 37 °C for 1 h, respectively.

In order to compare the maximum binding capacity of Tt-ChBD and Bc-ChBD on colloidal chitin, the adsorption reaction was administrated by varying the amount of purified Xar containing Bc-ChBD (named XBC) and XTC in the 50 mM PIB buffer (pH 6.6)

containing 1 mg of colloidal chitin with occasional stirring at 4 °C for 3 h, respectively. For the effect of temperature on the binding capacity toward colloidal chitin, the adsorption reaction was administrated at 55, 65, and 75 °C by adding 0.3 mg of purified fusion enzymes to 0.5 mg of colloidal chitin dissolved in 500 μL of PIB buffer (50 mM, pH 6.6) with occasional stirring for 1 h. For the effect of pH on the binding capacity, the adsorption reaction was administrated at 4 °C with occasional stirring for 1 h by adding 41 μg of purified fusion enzyme to 0.5 mg of colloidal chitin dissolved in 150 μL of 50 mM buffer with pH ranging from 2 to 12. For the effect of NaCl concentration on the binding capacity, the adsorption reaction was carried out at 4 °C with occasional stirring for 1 h by adding 4.2 U purified enzyme to 0.5 mg of colloidal chitin dissolved in 500 μL of PIB buffer (50 mM, pH 6.6) with NaCl concentration ranging from 0 to 3.0 mol/L. Afterward, various supports and colloidal chitin were recovered by centrifugation (8000g, 4 °C, 1 min) and washed twice using deionized water and then once using PIB buffer. Supernatants and washing volumes were pooled after each step, the nonbound activity was determined, and the final immobilized samples were also stored at 4 °C. The efficiency of immobilization was evaluated according to two criteria: immobilized yield (%) = 100 − 100 × (the enzyme amount found in filtrates and washing volumes after immobilization)/the total enzyme amount added to the support in the immobilized reaction; activity yield (%) = 100 × (the enzyme amount detected in the support after immobilization and washing)/(the total enzyme amount added to the support in the immobilization reaction × immobilized yield). The amount of enzyme was calculated through xylosidase activity.

Physiochemical Properties of Fusion Enzymes. The optimum temperature of free and immobilized trifunctional enzyme was determined by carrying out enzyme reactions at different temperatures (60−95 °C) under the conditions used. Thermal stability studies were carried out by incubating free and immobilized enzyme samples at varied temperatures (50−70 °C) for 1 h followed by a xylosidase assay at the respective optimal condition. The highest residual activity was defined as 100%.

The effect of pH on free and immobilized trifunctional enzyme was studied in buffers of different pH (4.2−8.2) using standard conditions, and xylosidase activities were assayed. The pH stability of free and immobilized enzyme was determined by incubating the enzyme in 100 mM buffers at different pH values (4.2−10.0) for 1 h at 37 °C. The residual activity was assayed under optimal conditions. The highest activity was defined as 100%.

Xylose Production from Corncob Xylans by Immobilized Enzyme. For preparation a pentose-rich corncob xylan hydrolysate (CXH), a milliliter of 2% (w/v) corncob xylan (CX) in 50 mM potassium phosphate buffer (PPB) was hydrolyzed at 90 °C for 12 h with purified 0.03 U xylanase B from *Thermotoga maritima* and stopped by autoclaving at 120 °C for 1 h. CX was prepared by submitting destarched wheat bran (provided by ARDC Agro-Industries Development Corporation, Nanjing, China) to 2% (w/v) KOH, as previously described.[15]

Batch hydrolysis of CXH and CX was performed at 65 °C, 50 mM PPB buffer (pH 6.6), as follows: the immobilized XC containing 7.2 U xylosidase activity was incubated with 1 mL of CXH for 2 h; the immobilized XXC containing about 5.4 U xylosidase activity was incubated with 1 mL of CX for 40 min. Reactions were stopped by recovering the immobilized enzyme by centrifugation (1 min at 8 000×g) and boiling the supernatant for 4 min. The immobilized enzyme was collected, washed with distilled water, and resuspended in 1 mL of freshly prepared substrate to start a new run. The supernatant was assayed for reducing sugars (RS) by the DNS (3,5-dinitrosalicylic acid) method with xylose as the standard.[23] The presented results are the average of three replicates of two separate experiments.

■ RESULTS

Comparison of Binding Ability of Tt-ChBD and Bc-ChBD. The Tt-ChBD of chitinase A1 from *T. thermosacchar-olyticum* DSM 571 comprises 45 amino acids and has 72%

amino acid identity with Bc-ChBD from *B. circulans* WL-12 (Figure 1A). As illustrated previously, directed immobilization
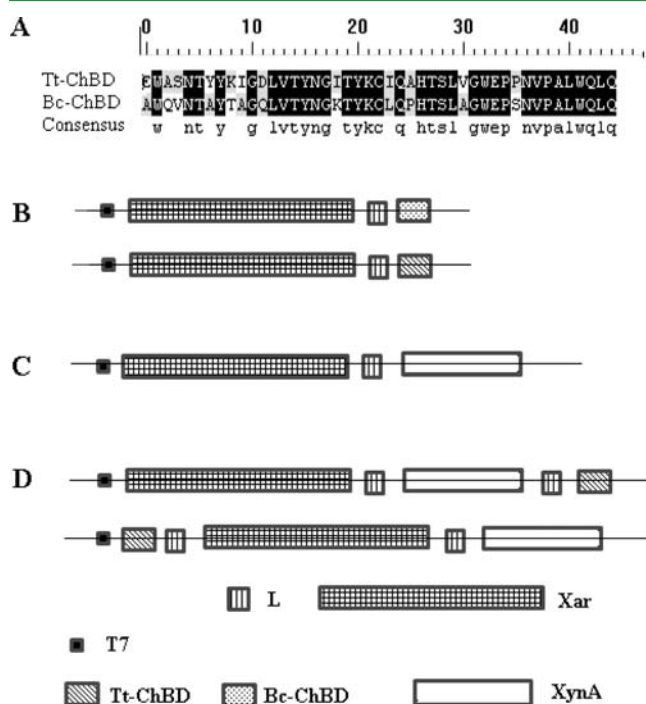

Figure 1. Comparison of amino acid sequences of Tt-ChBD to Bc-ChBD (A) and construction of bi- and trifunctional enzyme—chitin binding domain sequence (ChBD) fusion genes (B, C, D): (B) Fusion genes of bifunctional enzyme containing ChBD; (C) trifunctional enzyme Xra-L-XynA-encoding gene; (D) fusion genes of trifunctional enzyme containing ChBD. T7: T7 promotor sequence, Xar: encoding sequence of *T. ethanolicus* JW200 xylosidase-arabinosidase, XynA: encoding sequence of *T. lanuginosus* xylanase, L: linker-encoding sequence.

of the ChBD-fused proteins on chitin was marked with high efficiency and stability.[10−14] To test the feasibility of this approach, expression plasmid pET20b-Xar-Tt-ChBD containing the fusion of Tt-ChBD to the C-terminus of Xar was then constructed (Figure 1B). The resulting fusion protein XTC was overexpressed in *E. coli* under the control of a T7 promoter in response to IPTG induction and purified to homogeneity (Figure 2A, lanes 1). To evaluate the binding ability of XTC, purified XTC was mixed with different polymers as described in Materials and Methods. As shown in Figure 2B, XTC exhibits remarkably high specificity to insoluble chitin (swelling chitin and colloidal chitin) among various supports but not to other polysaccharides, including xylan, chitosan, cellulose, and agarose. To further test the feasibility of the directed immobilization of the XTC on chitin, CFX was prepared from the IPTG-induced JM109 (DE3) harboring pET20b-Xar-Tt-ChBD strain and mixed with colloidal chitins as described in Materials and Methods. After the adsorption procedure, colloidal chitins were recovered by centrifugation and the supernatant was analyzed by SDS-PAGE (Figure 2C). The results showed that little XTC was left in the filtrates after immobilization and over 98% of XTC was bound on colloidal chitins. No trace of XTC was found in the wash solution (data not shown), indicating the strong binding of the target protein to colloidal chitins.

In order to compare the binding ability of Tt-ChBD and Bc-ChBD, Bc-ChBD was genetically fused to the C-terminus of Xar, and the expression plasmid pET20b-Xar-Bc-ChBD was constructed (Figure 1B). Two fusion proteins (XTC and XBC) were then produced using the recombinant *E. coli* strain and purified by Ni-affinity chromatography. The purified XTC and XBC were established by SDS-PAGE analysis, which produced single bands (Figure 2A, lanes 1, 2). Different concentrations of purified XTC and XBC were mixed with 1 mg (dry weight) of colloidal chitin at 4 °C for 3 h, and the amount of corresponding bound fusion enzyme was evaluated. As shown in Figure 3A, 90% of XTC and XBC proteins could be adsorbed on chitin when 1 mg of chitin was dropped into the standard solutions ranging in fusion protein concentration from 2 to 6 μM, and Tt-ChBD had a slight advantage over Bc-ChBD for protein concentrations from 12 to 24 μM.

Concerning the effect of temperature, there was no significant difference between the two ChBDs at 55 and 65 °C, whereas the binding ratio of Tt-ChBD toward colloidal chitin (74.7%) was obviously higher than that of Bc-ChBD (53.8%) at 75 °C (Figure 3B). The analysis of the effects of pH and NaCl concentration on bioaffinity ability is shown in Figure 3C and D. The two ChBDs had a strong binding ability over
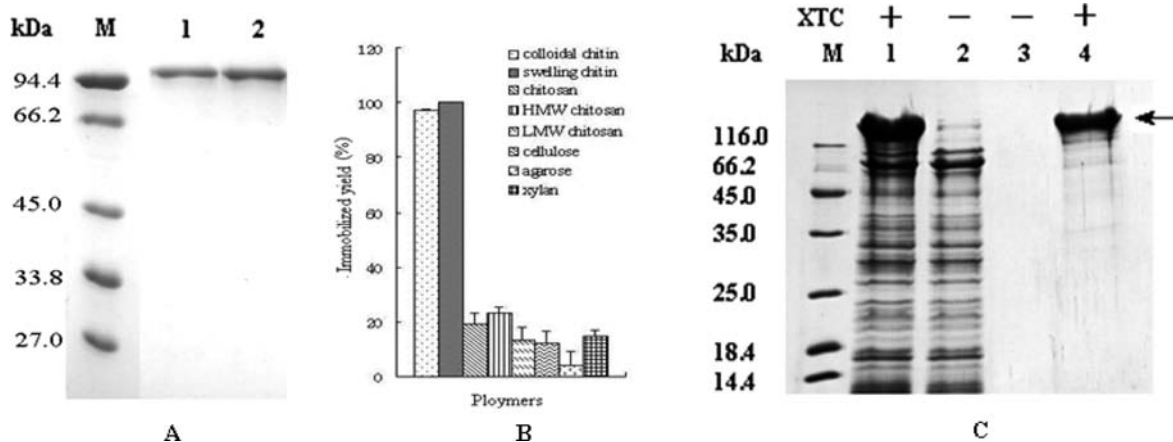

Figure 2. SDS-PAGE of purified XTC and XBC (A) and affinity adsorption of the XTC on chitins (C). (B) Binding specificities of Tt-ChBD. Key: lane M, protein marker; (A) lane 1, purified XTC; lane 2, purified XBC; (C) lane 1, CFX before adsorption; lane 2, CFX after adsorption; lane 3, washing buffer; lane 4, samples from colloidal chitin obtained in 2% SDS solution for 10 min at 100 °C.
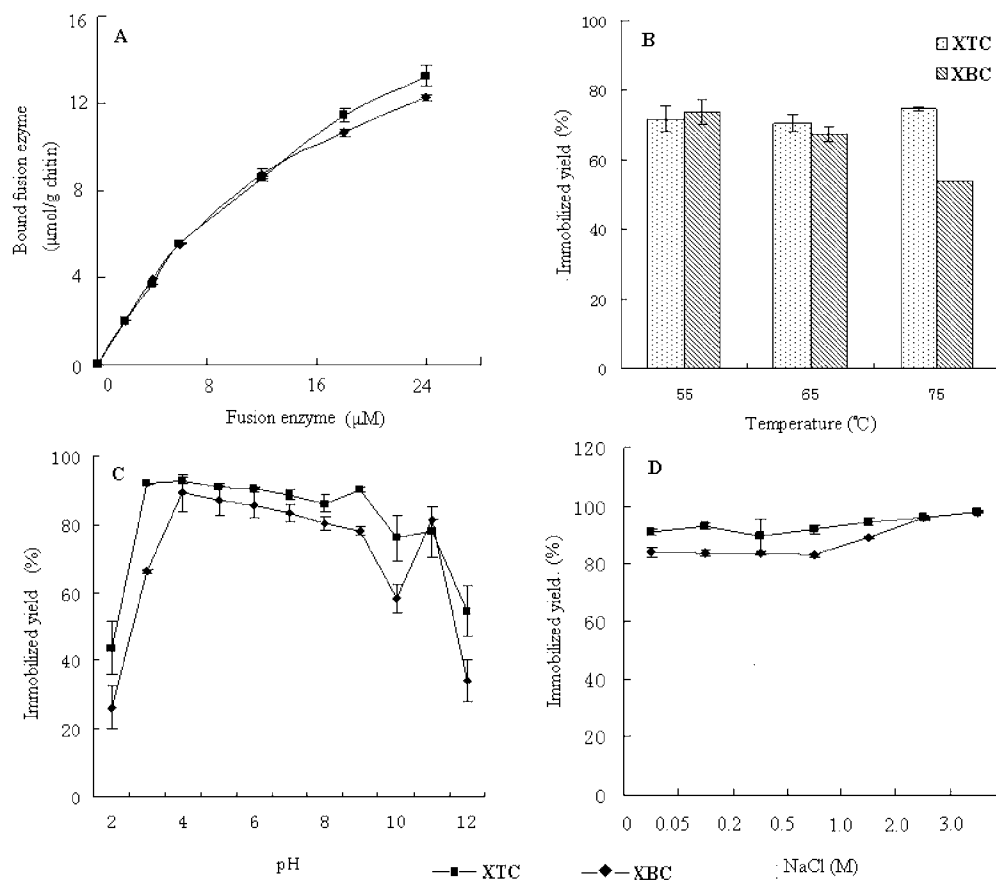
Figure 3. (A) Comparison of binding ability of XTC and XBC. Effect of temperature (B), pH (C), and NaCl (D) on the binding of XTC and XBC to colloidal chitin.

Table 2. Expression of Fusion Enzymes in *Escherichia coli*[a]

	xylosidase		arabinosidase		xylanase		relative activity ratio
	expressed activity (U mg^{-1})	relative level (%)	expressed activity (U mg^{-1})	relative level (%)	expressed activity (U mg^{-1})	relative level (%)	xylosidase/arabinosidase/ xylanase
XX	3.05	100	5.97	100	6.60	100	1:1.96:2.16
CXX	1.21	39.67	2.01	40.20	2.66	40.30	1:1.66:2.20
XXC	1.58	51.80	3.69	61.80	3.11	47.12	1:2.34:1.97

[a]The expressed activity for xylosidase, arabinosidase, and xylanase was detected in CFX in all the transformants. The relative activity ratio represents the proportion of xylosidase, arabinosidase, and xylanase activity for the fusion enzyme in CFX.

the pH range from 4.0 to 9.0 and NaCl concentrations from 0 to 3 mol/L. However, Tt-ChBD had wider binding curves of pH and NaCl concentration than Bc-ChBD, and the maximum binding ability of Tt-ChBD was 90%, while that of Bc-ChBD was 83% under high-salt concentrations.

Expression and Biochemical Characteristics of Fusion Enzymes. The Tt-ChBD was genetically fused to the N- or C-terminal region of XX (Figure 1C and D). In the two enzymes thereby produced, CXX and XXC, Tt-ChBD was fused to the N- and C-terminal region of XX, respectively. All the constructed plasmids were characterized by sequencing and restriction analysis. These chimeric enzymes were expressed in *E. coli*. Three enzyme activities and their activity ratio were detected in the soluble cellular fraction (herein referred to as CFX) in all the transformants. As shown in Table 2, those of pETXXC (1.58, 3.69, 3.11 U/mg total soluble protein of xylosidase-arabinosidase and xylanase, respectively) were higher than those of pETCXX (1.21, 2.01, 2.6 U/mg total soluble

protein of xylosidase-arabinosidase and xylanase, respectively), but lower than those of pETXX (3.05, 5.97, 6.60 U/mg total soluble protein of xylosidase-arabinosidase and xylanase, respectively), and their corresponding activity ratio of xylosidase, arabinosidase, and xylanase showed obvious differences (Table 2).

Biochemical properties of the purified CXX and XXC and their immobilized forms were compared to XX according to the xylosidase activity. The optimal temperature and pH of purified fusion enzymes are shown in Figure 4A and B. The XXC and CXX in free and immobilized forms had similar temperature and pH profiles to XX, their optimal temperature being 90 °C. However, the immobilized XXC and CXX exhibited narrowed temperature−activity curves with sharp optima at 90 °C, and the pH activity curves were wider on the basic sides. Correlations between heat stability and enzyme activity for free and immobilized fusion enzymes are shown in Figure 4C. The thermostability of free XXC and CXX was similar to the
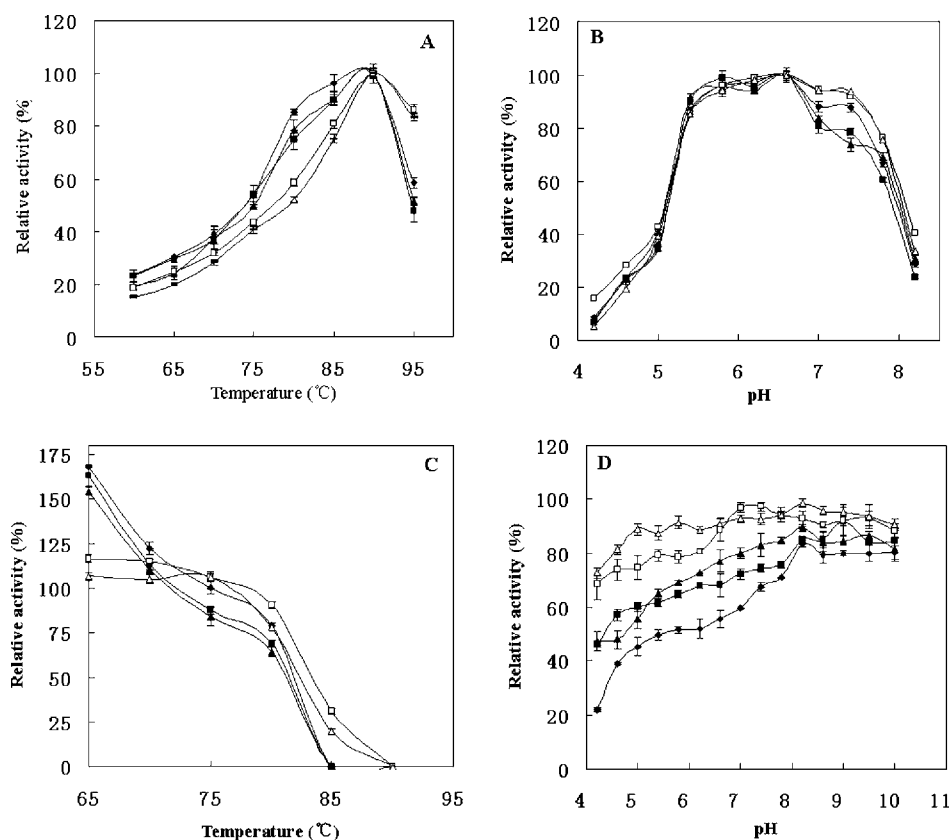
Figure 4. Effects of optimal temperature (A) and pH (B) of recombinant XX (♦), CXX (■), XXC (▲), immobilized CXX (□), and immobilized XXC (△) on xylosidase activity against pNPX as the substrate. The optimal pH was determined in 0.1 M PIB to be from pH 4.2 to 8.2 at 80 °C. Optimal temperatures were determined at the optimal pH values. Aliquots of purified proteins were incubated at various temperatures (60 to 95 °C), and β-xyosidase activities were assayed as described in Materials and Methods. The highest residual activity was defined as 100%. (C) Thermostability profiles: purified enzyme in its optimal pH was preincubated at 65 and 90 °C for different times in the absence of substrate, respectively, and these enzyme activities were then assayed as previously indicated in optimal conditions after cooling in an ice–water bath. (D) Stability at different pH values: the purified enzymes were preincubated in 0.1 M PIB from pH 4.2 to 10.2 for 1 h at 37 °C; then aliquots were transferred in a standard reaction mixture to determine the amount of remaining activity. The activity determined prior to the preincubations was taken as 100%.
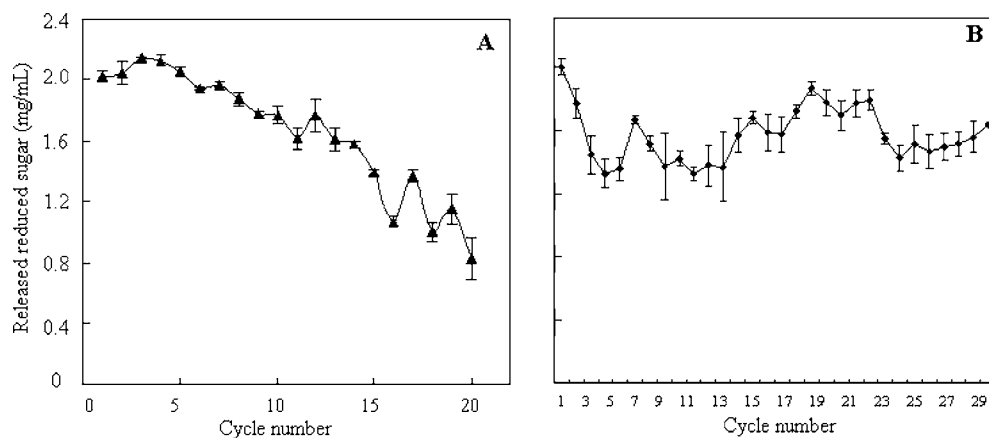

Figure 5. Repeated production of xylose by immobilized XXC (A) and XTC (B).

corresponding XX without Tt-ChBD at a wide range of temperature, 50–85 °C. The free XX, XXC, and CXX exhibited a much greater increase in activity at 65 and 70 °C for 1 h, whereas the immobilized XXC and CXX retained about 100% activity. In the range from 75 to 90 °C for 1 h, the immobilized XXC and CXX were more stable than their free forms. The stability of the fusion enzymes at different pH is shown in Figure 4D. The immobilized XXC and CXX showed more stability (above 70%) than their free forms at pH 4.2 to 8.2, immobilized XXC was more stable than immobilized CXX at the acids side, and the pH stability of the XX fusing Tt-ChBD (free XXC and CXX) was obviously enhanced compared with the XX without Tt-ChBD (free XX).

Xylose Production and Recycling of the Immobilized Enzyme. For repeated production of xylose from arabinoxylan-containing feedstocks, the biotransformation reaction of the immobilized XC and XXC was carried out at 65 °C for 2 h and 40 min, respectively. At the end of the reaction, the immobilized XC and XXC were collected by centrifugation and resuspended in a fresh reaction solution to start a new run. Such a reaction cycle was continued as required. As shown in Figure 5, immobilized XXC had the same RS release amount at the initial reaction for biotransformation capacity, but a significant decrease in RS release amount was observed after the 14th cycle. It exhibited stable activity to producing xylose for at least 19 times in continuous operation with the achievement of 60% RS release efficiency (Figure 5A). The immobilized XC could be reused 30 times to achieve an 80% RS release efficiency (Figure 5B).

■ DISCUSSION

The specific nature of the chitin-binding activity of chitinase has led to the development of immobilization of enzymes on chitin supports, which has been recently employed for a very appealing approach to facilitating product isolation, simultaneous purification, and immobilization of enzymes as well as its reutilization in industrial application. In this study, to get better biocatalysts for continuous production of xylose from arabinoxylan-containing feedstocks, we reported the cloning of a novel thermostable Tt-ChBD from *T. thermosaccharolyticum* DSM571 and comparison of its binding activity with another mesophilic Bc-ChBD. Experimental results showed that target proteins with Tt-ChBD exhibited stronger affinity to colloidal chitin than target proteins with Bc-ChBD at temperatures from 65 °C to at least 75 °C, suggesting that Tt-ChBD has a promising use of new approach by introducing Tt-ChBD to the target protein for immobilization on chitin in engineering applications at high temperature. The reason for low immobilization efficiency of XBC at a higher temperature (more than 65 °C) may be because that Bc-ChBD was cloned from the mesophilic chitinase gene of WL-12.[9−13]

A best-characterized trifunctional chimeric enzyme XX has been constructed to produce xylose.[14] It was immobilized on colloidal chitin efficiently by genetically fusing Tt-ChBD to the N- and C-terminal regions of XX. The resulting fusion enzymes XXC and CXX exhibited not only xylosidase-arabinosidase and xylanase activity but also the strong ability to bind to chitin. However, the expressed activity of cells harboring pETXX was higher than of the others, and that of pETXXC was higher than that of pETCXX (Table 2), which could have been due to the difference in the nucleotide sequence of the 5′-terminal residues of the fusion gene. The nucleotide sequence of 5′-terminal residues often affects the secondary structure of the mRNA of the translational initiation region, and then the transcription and translation rate. Some previous reports demonstrated that the expression rate of the target protein was increased by mutation of its 5′-terminal residues.[24−26] Meanwhile, the relative activity ratio of fusion enzyme exhibited obvious differences (Table 2), suggesting that the presence of Tt-ChBD may lead to folding interference between the fused modules so that the spatial orientation of active sites of the fusion enzyme may be perturbed between fused modules.[14,15] CXX exhibited stronger binding capacity to colloidal chitin than XXC, illustrating that CXX is better than the conformational form XXC due to the influence of the spatial structure of the fusion enzyme by fused modules.[27]

The main physicochemical properties of fusion enzymes were determined. The optimum temperature and pH profiles of CXX and XXC in both free and immobilized form were the same as those of the native XX. However, the thermal and pH stabilities of the immobilized XXC and CXX were both greatly improved in the range from 70 to 90 °C and pH 4.2−8.2, compared to their free counterparts. Possible mechanisms that could contribute to this phenomenon include the possibility of the association of the enzyme within the pores of the carriers (colloidal chitin). The findings we reported herein suggested that the remarkable properties of Tt-ChBD can be applied in the immobilization of enzymes by genetic engineering techniques, especially at a high reaction temperature.

Biocatalyst stability is a major concern in almost all bioprocesses, because it may affect the overall cost of the process. To assess operational stability, the immobilized XXC and XC were exploited for the cycle production of xylose from arabinoxylan-containing feedstocks, and a biotransformation reaction was carried out at 65 °C for 2 h and 40 min. According to our survey, the immobilized XXC exhibited high stability and facile operation in producing xylose for at least 19 times in continuous operation with the achievement of 60% RS release efficiency (Figure 5A), and the immobilized XC could be reused 30 times to achieve 80% RS release efficiency (Figure 5B). Although previous studies have reported the approach of confining enzymes on chitin by fusing target proteins with ChBD, and a conversion reaction by the immobilized enzyme was carried out at 15−45 °C,[9−23] the results that we report herein reveal the great usefulness of Tt-ChBD for enzyme immobilization at temperatures up to 65 °C, indicating that Tt-ChBD is an attractive affinity tag for the simultaneous purification and immobilization of thermostable enzymes in bioprocess engineering of food and medicine.

In conclusion, thermostable enzymes exhibit commercial importance; thus, their immobilization would be beneficial to broadening their applicability. Here, we showed that novel thermostable Tt-ChBD from *T. thermosaccharolyticum* DSM571 exhibits stronger and more stable binding affinity to colloidal chitin at temperatures from 65 °C to at least 75 °C compared to Bc-ChBD. The experimental results demonstrate the success in the utilization of Tt-ChBD for tight retention of the chimeric enzyme on chitins. The engineered multifunctional enzyme immobilized by this approach exhibited high stability and facile operation for at least 19 or 30 times in continuous operation with the achievement of 60% or 80% RS release efficiency, indicating its potential applications for thermophilic enzyme immobilization in a high-temperature environment.

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Notes
The authors declare no competing financial interest.

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dx.doi.org/10.1021/jf3041275 | *J. Agric. Food Chem.* 2013, 61, 3074−3081