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Thermostable carbohydrate binding module increases the thermostability and substrate-binding capacity of *Trichoderma reesei* xylanase 2

Research Paper

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Abstract

To improve the thermostability of *Trichoderma reesei* xylanase 2 (Xyn2), the thermostabilizing domain (A2) from *Thermotoga maritima* XynA were engineered into the N-terminal region of the Xyn2 protein. The xyn2 and hybrid genes were successfully expressed in *Pichia pastoris* using the strong methanol inducible alcohol oxidase 1 (*AOX1*) promoter and the secretion signal sequence from *S. cerevisiae* (α-factor). The transformants expressed the hybrid gene produced clearly increased both the thermostability and substrate-binding capacity compared to the corresponding strains expressed the native Xyn2 gene. The activity of the hybrid enzyme was highest at 65 °C that was 10 °C higher than the native Xyn2. The hybrid enzyme was stable at 60 °C and retained more than 85% of its activity after 30-min incubation at this temperature. The hybrid enzyme was highly specific toward xylan and analysis of the products from birchwood xylan degradation confirmed that the enzyme was an endo-xylanase with xylobiose and xylotriose as the main degradation products. These attributes should make it an attractive applicant for various applications. Our results also suggested that the N-terminal domain A2 is responsible for both the thermostability and substrate-binding capacity of *T. maritima* XynA.

Introduction

Complete degradation of xylans requires the concerted and synergistic action of a variety of enzymes of which endo-β-1, 4-xylanases (EC 3.2.1.8) are the crucial enzymes for depolymerization [1]. The major xylanolytic enzyme has attracted a great deal of research interest in the past decade, particularly owing to its potential in various industrial processes, such as biobleaching, paper making and in food and animal feed industries [2–4]. Xylanases are used in pulp and paper industries to reduce the chlorine consumption in the bleaching process of kraft pulp and thus lower environmental pollution [5]. However, such procedures are usually performed at high temperature and basic pH, which require enzymes exhibiting a high thermostability and activity in a broad pH range. Various microorganisms, such as bacteria, yeasts, and filamentous fungi were found to naturally secreted xylanases. However, most xylanases present an optimum activity around 50–60 °C and a half-life of about 1 h at 50 °C. Currently, two different approaches have been successfully tried to obtain thermostable xylanase: one approach is to discover new enzymes from thermophilic microorganisms and the other is to engineer the presently used mesophilic xylanases to create novel enzymes withstanding the harsh conditions [6].

Thermotoga maritima, the most thermophilic xylan-degrading bacteria currently known, is a strictly anaerobic hyperthermophiles capable of growth at temperatures up to 90 °C. *T. maritima* is able to produce a high-molecular mass xylanase (120 kDa, XynA) and a low-molecular mass xylanase (40 kDa, XynB) [7]. The *T. maritima* XynA is an extremely thermostable modular enzyme with five domains (A1-A2-B-C1-C2). Two related ~170 residue domains (C1-C2) represent cellulose-binding domains (CBDs) that confer upon XynA the ability to reversibly bind to microcrystalline cellulose [8]. The N-terminal domains of XynA

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have been found to be responsible for enzyme stability. And deletion of A-like domains from XynA or other xylanases has resulted in enzyme derivatives that were much more susceptible to thermo-inactivation than the full-length enzymes [9–11]. As a result, these domains are usually called thermostabilizing domains. However, the hypothesis was challenged after the discovery of xylan binding capacity for the domain A2 [11]. It is still argued that polysaccharide binding and not thermostabilization is the main function of A-like domains because of many reasons mentioned by Meissner *et al*. [11].

The endo-1, 4-β-xylanase 2 (Xyn2) secreted by *Trichoderma reesei* is a low-molecular-mass (21 kDa) hemicellulolytic enzyme with an alkaline isoelectric point (pI 9.0) and an activity optimum at pH 4–6. It belongs to the G/11 glycosidase protein family and has a right hand β-sandwich structure [12]. However, the protein has no stabilizing disulfide bridges or any thermostabilizing domain. Xyn2 catalyzes the hydrolysis of xylan by cleaving specifically the endo-1, 4-β-glycosidic bond between the subunits of the xylan polymer chain. But the enzyme rapidly loses its activity when incubated at temperatures over 50 °C [3]. Thus, enhancing thermostability of Xyn2 has been the goal of many studies. The stability of Xyn2 has been successfully improved by the introduction of disulfide bridges, single residue substitution, and alterations in the vicinity of the N-terminal region of the enzyme proteins [13–15]. In addition, the published three-dimensional structure of Xyn2 provides more valuable information about the impact of different structural features on stability of the enzyme [16].

In this work, we have engineered the domain A2 of *T. maritima* XynA into the N-terminal region of Xyn2. Both native Xyn2 and hybrid genes were expressed in *P. pastoris*. Our goal was to improve the performance of the Xyn2 to resist inactivation at high temperatures. In addition, the substrate-binding capacity for the hybrid enzyme and the real functions of the domain A2 were both elucidated.

Materials and methods

Strain and plasmids

The relevant genotypes of the microbial strains and plasmids used in the present study are summarized in Table 1. *P. pastoris* X-33 was cultivated in YPD medium (1% yeast extract, 2% peptone, 2% glucose). *T. reesei* Rut C-30 was cultivated in basal medium (BM) [0.3% oat spelt xylan (Sigma), 0.4% KH_2PO_4, 1% $(NH_4)_2HPO_4$, 1% peptone, 0.3% yeast extract] [17]. Both these organisms were cultured in 1 L flasks containing 100–200 mL of medium at 30 °C on a rotary shaker at 150 rpm. Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5α cultivated at 37 °C in Luria–Bertani liquid medium or Luria–Bertani agar. Ampicillin for selecting and propagating resistant bacteria was added to a final concentration of 100 μg mL^{-1}.

TABLE 1

Microbial strains and plasmids used in this study

Strains or plasmids	Relevant genotype	Source or reference
Strains		
P. pastoris x-33	Wild-type, Mut^+	INVITROGEN
***T. reesei* Rut C-30**	Mutated type	ATCC56765
***E. coli* DH5α**	*F⁻,supE44,*Δ(*argF lacZya*)*U169*, *Φ80lacZΔ15,hsdR17*(*rK⁻,tnK⁻*), *recA1,endA1,gyt96,thi-1,relA1,*λ⁻	TIANGEN CB101
Plasmids		
pPICZαA	3.6 kb, *Zeocin^r^*	INVITROGEN V195-20
pMD18-T Vector	2.7 kb,*Amp^r^,lacZ*α	TAKARA D101A

RNA isolation

One liter of *T. reesei* Rut C-30 culture was prepared in oat spelts basal medium for 48 h at 30 °C. The fungal mycelia were harvested by centrifugation and frozen under liquid nitrogen. The frozen mycelia were ground into a fine powder with a sterile mortar and pestle, and suspended in a mixture of Trizol reagent (Takara D312), and total cellular RNA fraction was isolated as described by the manual.

Gene amplification and sequencing

The first strand cDNA synthesis was carried out with 100 ng of total cellar RNA by using a two-step RT-PCR kit (Takara DRR019A) as specified by the supplier. The DNA fragment encoding the *T. reesei* Rut C-30 Xyn2 was isolated from a first strand cDNA mix by PCR with the two oligonucleotides Up1 (5′-ATAGAATTCCAGACGATTCAGCCCGGCACGGG-3′) and Down1 (5′-TTAGCGGCCGCTTAGCTGACGGTGATGGA AGCAGAGC-3′) supplied with the *Eco* RI and *Not* I restriction sites, respectively. These primers were based on the sequence of the *T. reesei* Xyn2 gene, as published by La Grange *et al* [18]. The PCR reaction was performed in 25-μL reaction mixtures (0.15 μM each primer, 1 μL of template DNA [about 10 ng of first strand cDNA], 12.5 μL PCR premix [Boracker KT201-02]). Denaturation, annealing and polymerization were carried out for 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C, respectively for 35 cycles.

Construction of Xyn2-A2 hybrid gene

The full length of the hybrid Xyn2 gene (Xyn2 fused in frame with the domain A2, 749 bp) supplied with the *Eco* RI and *Not* I restriction sites, was synthesized by TaKaRa Biotechnology (Dalian, China) Co., Ltd. Both the Xyn2 and hybrid Xyn2 gene was directly cloned to the pMD18T Simple Vector (TaKaRa D101A) and sequenced by Invitrogen (Shanghai, China) Co., Ltd. The sequence was analyzed using the software package DNAman 5.0 and the homology was analyzed in GenBank with the BLAST programs.

Construction and transformation of the recombinant plasmid

The *E.coli*/*P. pastoris* shuttle vector, pPICZαA, was used to achieve secreted expression of xylanase. The construction of recombinant plasmids (pPICZαA-Xyn2/pPICZαA-A2-Xyn2) was summarized in Figure 1. The Xyn2 and hybrid gene was gel-purified and digested with *Eco*RI and *Not*I (Takara, Dalian) before cloning into pPICZαA. After transforming into *E. coli* DH5α, recombinant plasmids were selected on Luria–Bertani agar containing 25 μg mL^{-1} zeocin. The selection was checked by restriction analysis and sequencing. For *P. pastoris* integration, about 10 μg of recombinant plasmid was linearized with *sac*I, and transformed in *P. pastoris* by electroporation methods as described by the manufacturer. The transformants were selected at 28 °C on the YPDS agar plates containing

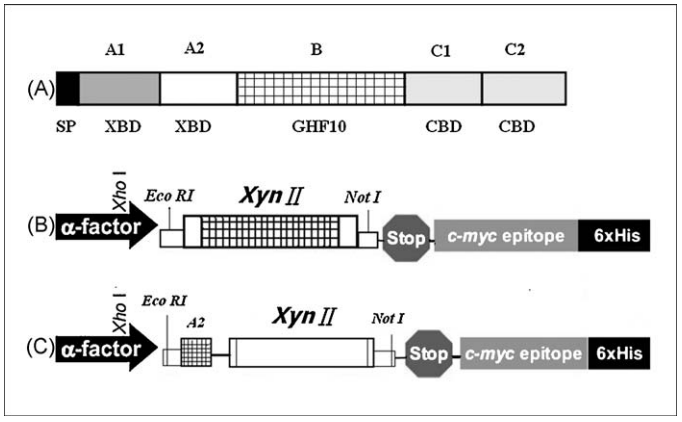

FIGURE 1

Structure of the XynA from *Thermotoga maritima* (A) and schematic summary of the construction of plasmid pPICZαA-Xyn2 (B) and pPICZαA-A2-Xyn2 (C).

100 μg mL^{-1} zeocin. The integration of the Xyn2 gene into the genome of *P. pastoris* was confirmed by PCR using 5′AOX1 and 3′AOX1 primers.

Expression of recombinant xylanase in ***P. pastoris***

P. pastoris transformants were grown in 20 mL of fresh buffered minimal glycerol complex medium, BMGY [1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% YNB, 0.0004% biotin, and 1% glycerol] at 30 °C until an OD_{600} of 5–6 was reached. Then, the cell pellet was harvested and resuspended in 100 mL buffered minimal methanol medium, BMMY [1% yeast extract, 2% peptone, 100 mM potassium phosphatate (pH 6.0), 1.34% YNB, 0.0004% biotin, and 0.5% methanol]. Sufficient supply of oxygen was assured by cultivation of the recombinant *P. pastoris* in 1 L flask (1:10 culture per flask volume ratio) at 250 rpm agitation throughout induction period. Absolute methanol was added every 24 h to a final concentration of 1% to maintain induction. The culture supernatant was collected every day by centrifugation. The supernatant was stored at −80 °C before SDS-PAGE and analysis of its biochemical properties.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide was performed by the method of Laemmli [19]. The protein fractions were boiled for 3 min and applied to the gel. Proteins were visualized by Coomassie brilliant blue R 250 staining. The protein concentration was determined by the Bradford assay using bovine serum albumin as a standard [20].

Enzyme activity assays

For enzyme characterization, all the values presented in graphs and tables are the means of three replications. Xylanase activity was assayed by the method described by Bailey *et al*. [21], with 1% soluble birchwood xylan (Sigma) as the substrate at 50 °C for 10 min. Appropriate dilutions of the recombinant protein (culture supernatant) in 50 mM sodium citrate buffer (pH 5.0) were used as the enzyme source. The amount of released sugar was determined by the dinitrosalicylic acid (DNS) method described by Miller *et al*. [22]. The temperature optimum was measured by performing the xylanase activity assay at temperatures ranging from 20 °C to 90 °C. Assays at different pH values were performed at the optimal temperature over a pH range of 2.0–9.0. The buffers used were 50 mM citrate (pH 2.0), 50 mM citrate phosphate (pH 4.0–7.0), 50 mM phosphate (pH 8.0), and 50 mM Boric Acid-Borax (pH 9.0), respectively.

Determination of thermostability

Thermostability was tested by preincubating the enzyme samples for 30 min at various temperatures (50–75 °C). The residual activity was then measured at 50 °C as described previously.

Substrate-binding studies

The binding capacity for different substrates (cellulose and xylan) was determined by the method described by Tenkanen *et al*. [3]. The recombinant enzyme (30 μg) was incubated with different concentrations of Avicel or birchwood xylan in 50 mM citrate phosphate buffer (pH 5.0), at 4 °C for 1 h with slow shaking. After centrifugation (10000 × *g*, 5 min), the supernatant was collected and tested for its xylanase activity. Unbound enzyme was determined by measuring residual activity in the supernatant.

Influence of temperature on hydrolytic capacity

The hydrolysis of xylan was carried out in 100 mL conical flask containing 50 mL citrate buffer (pH 5.0, 50 mM), 1.0 g birchwood xylan, 5 mg sodium azide and enzyme preparation (1000 U) as described by Adsul *et al* [23]. The hydrolysis was performed for 12 h at different temperatures (55 °C, 60 °C, 65 °C, and 70 °C), with a stirring rate of 150 rpm. The samples were analyzed for the reducing sugars after suitable time intervals.

Analysis of hydrolytic products

The hydrolyzed products of xylan was analyzed by the thin-layer chromatography (TLC) using silica gel Plates 60F 254 (E. Merck, Germany). Aliquots (100 μL) of the samples were collected at different times of the incubation period and 1 μL of the aliquot was spotted on the TCL plates. The plates were subsequently developed with two runs of acetonitrile–water (85:15, v/v) followed by heating for a few minutes at 130 °C in an oven after spraying the plates with a methanol–sulfuric acid mixture (95:5, v/v) [24]. A xylooligosaccharide mixture (Suntory Ltd, Japan) consisting of xylose, xylobiose, and xylotriose was used as the standard.

Results

Expression of the recombinant xylanases in ***P. pastoris***

The construction of the expression plasmids are shown in Figure 1. The two designated restriction enzyme sites (*Eco*RI/*Not*I), allowing the directional cloning of the Xyn2 or hybrid gene into pPICZαA expression vector. The target fragment was integrated into *P. pastoris* X-33 strain by electroporation methods. The transformants were selected on YPDS plate containing zeocin and the integration of target genes into AOX1 location in the genome was further confirmed by PCR. The most desired integrant was chosen for small-scale induction. The sizes of recombinant Xyn2 and hybrid xylanase, determined by SDS-PAGE were 21 kDa and 26 kDa, respectively (Figure 2). The size of recombinant Xyn2 is similar to that of the native xylanase secreted by *T. reesei*. However, the fusion of domain A2 resulted in 52 extra amino acids existing

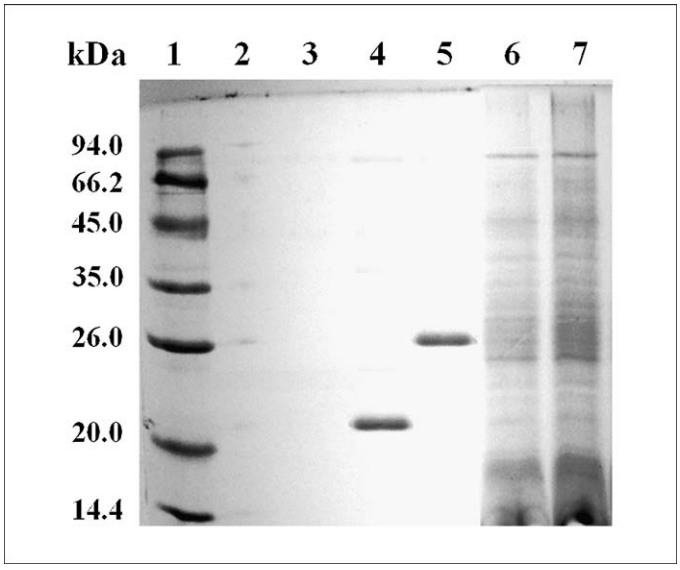

FIGURE 2

SDS-PAGE analysis of the recombinant enzymes secreted by *P. pastoris*: lane1: protein marker; lane2: negative control for Xyn2 (pPICZαA-xyn2); lane3: negative control for hybrid Xyn2 (pPICZαA-A2-xyn2); lane4: Xyn2; lane5: hybrid Xyn2; lane6: cell lysate from Xyn2-secreted *P pastoris*; lane7: cell lysate from hybrid Xyn2-secreted *P. pastoris*.

in N-terminal region of the hybrid protein. Both Xyn2 and hybrid enzyme was the major protein (over 95% of total protein as detected by densitometer) secreted by *P. pastoris* into culture medium. Thus, the procedure for protein purification was not necessary.

Effect of pH and temperature on xylanase activity

Enzymatic assay at different temperatures revealed that the recombinant Xyn2 has an optimal activity at 60 °C (Figure 3A). Activity decreased rapidly with temperature, that is, at 75 °C, activity of the recombinant protein represented only 30% of the optimum. However, the hybrid enzyme has an optimal activity at 65 °C, which was 10 °C higher than native Xyn2 secreted by *T. reesei*. Concerning the effect of the pH, both Xyn2 and hybrid enzyme showed an optimal activity at approximately pH 6.0 (Figure 3B). When the pH was below 3.0 and above 8.0, only 30–40% of the maximum activity was reached.

Thermostability of Xyn2 and hybrid enzyme

Although the highest activity of Xyn2 was measured at 60 °C, the enzyme is not stable at this temperature (only 40% activity retained after 30-min incubation). However, the hybrid enzyme was more stable at 60 °C, and the total activity retained more than 85% after 30 min of incubation at this temperature (Figure 3C). Even incubating at 65 °C, the hybrid enzyme still remained as much as 61% of its total activity.

Substrate-binding analysis

The binding capacity of the recombinant enzyme was determined by incubating the enzyme with Avicel or birchwood xylan. As shown in Figure 4A, neither Xyn2 nor hybrid enzyme had significant binding affinity for Avicel (over 95% enzyme activity still remained in supernatant). However, both of them had the binding affinity for xylan. By contrast, the hybrid enzyme tends to bind more substrate under the same conditions (Figure. 4B).

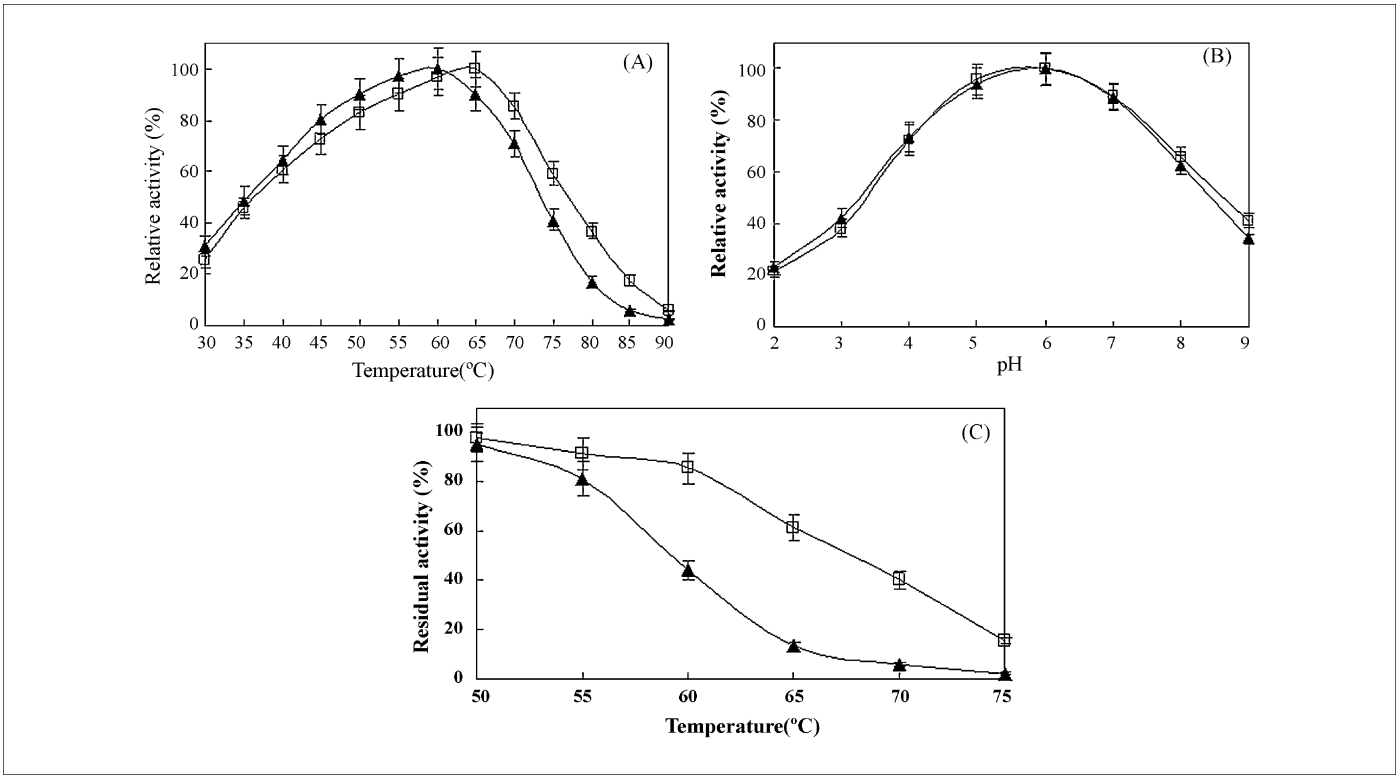

FIGURE 3

Influences of temperature (A) and pH (B) on the activity of Xyn2 (▲) or hybrid enzyme (□); (C) Thermostability of the enzyme was determined after preincubating the enzyme in the absence of the substrate for 30 min at 50, 55, 60, 65, 70, and 75 °C.

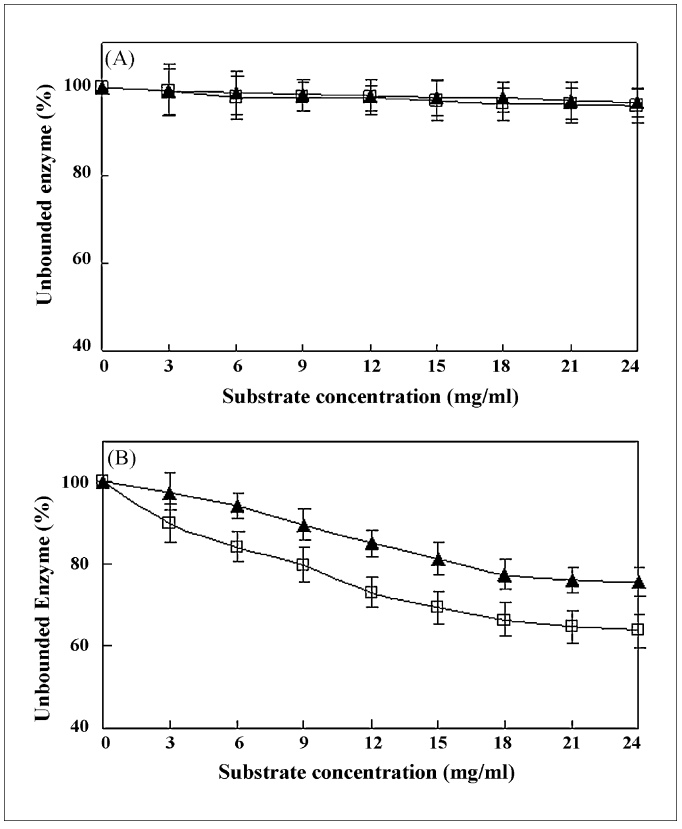

FIGURE 4

Effect of different concentrations of Avicel (A) and birchwood xylan (B) on the binding ability of Xyn2 (▲) and hybrid enzyme (□): xylanases (25 μg) were incubated with 1–24 mg mL^{-1} substrates in 50 mM citrate phosphate (pH 5.0) at 4 °C.

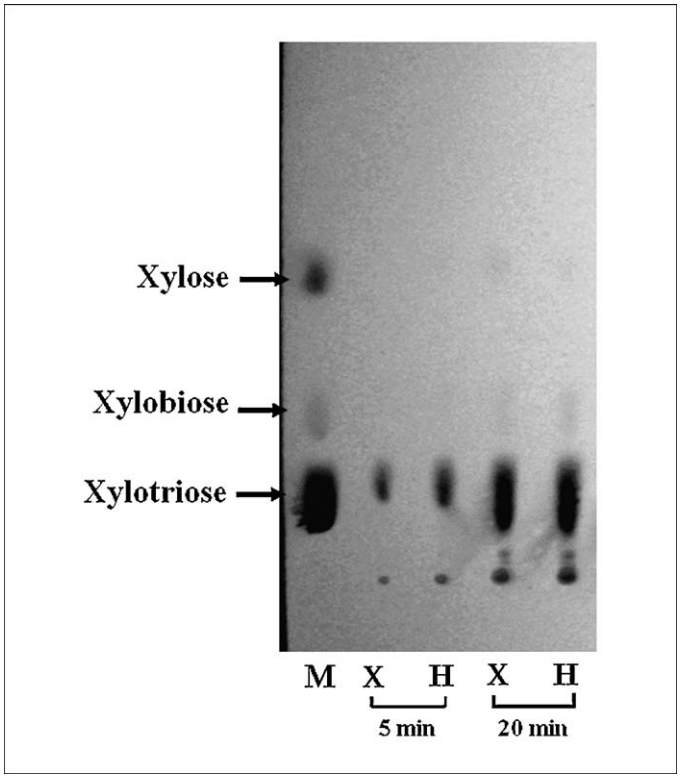

FIGURE 6

Analysis of the hydrolyzed products by the recombinant Xyn2 (X) and hybrid Xyn2 (H) xylan. Birchwood xylan (40 mg) was incubated with 1000 U of the enzyme in 2 mL 50 mM citrate phosphate (pH 5.0) and the reaction was performed at 50 °C, and the hydrolysate was analyzed by TLC.

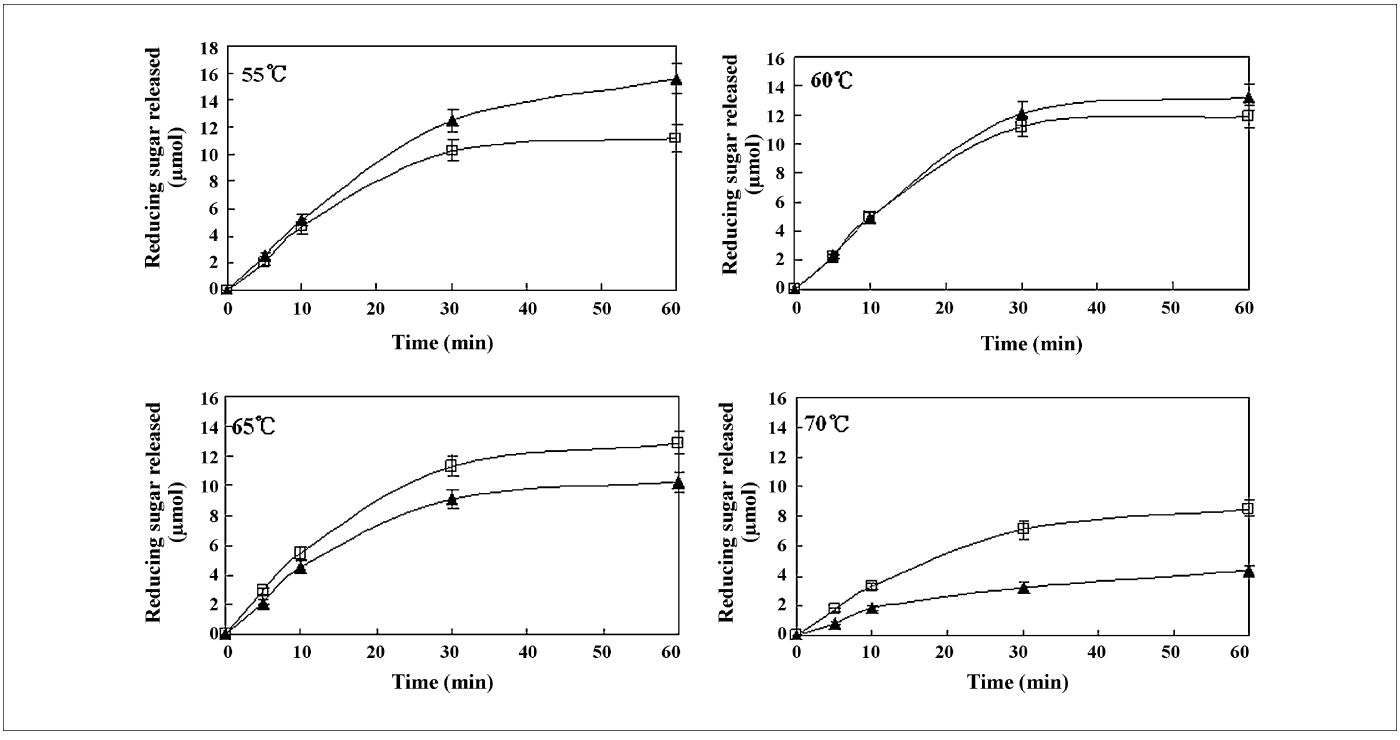

FIGURE 5

Hydrolysis of 2% birchwood xylan by Xyn2 (▲) and hybrid enzyme (□) at different temperatures. Samples were withdrawn at different times after incubation and the amount of released reducing sugar was measured by DNS method.

TABLE 2

Comparison of molecular characteristics of native Xyn2 and recombinant Xyn2

Property	Xyn2 (*T. reesei*)	Xyn2 (*P. pastoris*)	Hybrid Xyn2 (*P. pastoris*)
Mol mass (kDa)	20–21	21	26
Optimum pH	5	6	6
pH stability[a]	4.5–5.5	3.5–8.0	3.5–8.0
Optimum temperature (°C)	55–60[b]	60	65
Temperature stability (30 min, 60 °C)	NA[c]	NA	85[d]

[a] More than 60% of maximal activity retained.
[b] Temperature optimum of β-xylanases produced by *T. reesei*, not only Xyn2.
[c] NA, not available.
[d] Eighty-five percent of total activity retained.

Influence of temperature on enzymatic hydrolysis

To evaluate the influence of temperature on enzymatic hydrolysis, birchwood xylan (2%) was used as the substrate. Both Xyn2 and hybrid enzyme has been used to hydrolyze substrates at elevated temperature. The maximal hydrolyzing ability of Xyn2 and hybrid enzyme were obtained at 55 °C and 65 °C, respectively. The hydrolyzing ability of both proteins was decreased with the increase in temperature. However, the hybrid enzyme more efficiently degraded the substrate than Xyn2 at 70 °C (Figure 5).

Analysis of hydrolytic products

The products of hydrolysis of xylan were analyzed by TLC. The predominant end products of both proteins were xylobiose and xylotriose (Figure 6). The xylotriose was produced within 5 min of the reaction period. As the reaction time increased, both the xylotriose and xylobiose concentration increased. These results confirmed that both Xyn2 and hybrid enzyme were endo-xylanase.

Discussion

Industrial processes require unique properties of enzymes with respect to pH optima, substrate specificity, and thermostability. In this study, the hybrid Xyn2 gene was designed and expressed in *P. pastoris* in order to produce enzyme preparations with enhanced thermostability and substrate-binding capacity compared to the preparations with the native Xyn2 gene.

The fungus *T. reesei* produces two Family 11 xylanases: Xyn1 and Xyn2. Xyn1 has an acidic pI (5.5) and a lower pH optimum. However, Xyn2 has a basic pI (9.0), a more open structure, and a wider pH range [25]. In addition, Xyn2 tends to produce larger obligosaccharides and represent more than 50% of the total xylanolytic activity of this fungus. These attributes render it an attractive applicant for industrial applications, such as in the pulp and paper industry. However, the Xyn2 obtained from *T. reesei* or many recombinant miroorganisms are not thermostable enough and inactivated rapidly above 50 °C [18,25]. Several studies have suggested that the N-terminal region of the protein plays a key role in determining the stability of Family 11 xylanases. For instance, a single residue substitution in the N-terminal region has successfully improved the thermostability of *T. reesei* Xyn2 [14]. Similar results were observed by N-terminus replacement and extension [26]. In addition, increased disulfide bridges and aromatic interactions in the N-terminal region also showed improvement in thermostability of the xylanases [27,28]. In this study, we have engineered the domain A2 of *T. maritima* XynA into the N-terminal region of Xyn2. Both the native Xyn2 and hybrid genes were successfully expressed in *P. pastoris*. The recombinant Xyn2 (21 kDa) obtained from *P. pastoris* showed its temperature optima at 60 °C, which is almost the same as the native Xyn2 obtained from *T. reesei* (Table 2). Both of them are not stable at this temperature. However, the hybrid enzyme (26 kDa) demonstrated increases in both its thermostability and thermophilicity (apparent temperature optimum) (Figure 3). More than 85% of its total activity retained after 30-min incubation at 60 °C (only 44% for Xyn2). The thermostability of the recombinant Xyn2 decreased more quickly than the hybrid enzyme when incubating at a temperature over 60 °C. When incubating at 70 °C, the Xyn2 only retained 5.6% of its total activity (41% for hybrid enzyme). Thus, the hybrid enzyme seems to be more suitable for a higher temperature. Both the recombinant Xyn2 and hybrid enzyme was active over the range of pH 3.5–8.0 with maximum activity at pH 6.0.

The *T. maritima* XynA has previously been proved to be an extremely thermostable modular enzyme with five domains (Figure 1). The intrinsic stability of XynA has been ascribed to the N-terminal domains (A1/A2) [8]. The removal of the N-terminal domain resulted in a decrease in the thermostability of the catalytic domain, whereas the removal of the C-terminal CBDs did not lower the thermostability. Thus, the N-terminal domain are usually called thermostabilizing domain. However, the domain A2 was also designated as a xylan binding domain (XBD) by Meissner *et al*. [11], because domain A2 promoted efficient xylan binding and was not dependent on the presence of A1. Therefore, it is argued that polysaccharide binding, and not thermostabilization, is the main function of A-like domains. In this study, we have successfully proved the thermostabilizing function of domain A2. To confirm whether this domain has the substrate-binding capacity, we incubated the enzyme with different substrates (cellulose or xylan). As shown in Figure 4, both Xyn2 and hybrid enzyme could not significantly bind to Avicel. However, they had the binding affinity for birchwood xylan. As we expected, the hybrid enzyme tends to bind more substrate under the same conditions. Coupled with its enhanced thermostability, the domain A2 may be designated as a multifunction domain that is responsible for both thermostabilization and substrate binding.

As an industrially important enzyme, the hydrolyzing capacity of the recombinant enzyme at different temperatures was also determined. Although the maximal hydrolysis capacity for Xyn2

and hybrid enzyme were obtained at 55 °C and 65 °C, respectively, the substrate hydrolyzing capacity of both proteins decreased as the temperature increased. However, the hybrid enzyme more efficiently degraded the substrate than Xyn2 at a temperature over 65 °C. In addition, the products of hydrolysis of birchwood xylan were predominantly xylobiose and xylotriose and a smaller amount of xylose (Figure 6), which confirmed the endo-acting nature of the enzyme. All these features suggested that the hybrid enzyme may be more useful in various industrial applications.

Conclusion

In this study, we have successfully produced the hybrid Xyn2 in *P. pastoris*. The hybrid enzyme was effective in the degradation of xylan. The introduction of the domain A2 from *T. maritima* XynA has resulted in increases in both the thermostability and substrate-binding capacity for Xyn2. In recent years, the elucidation of the tertiary structure and catalytic mechanisms of the xylanases has enabled a better understanding of what determines the temperature optimum and substrate-binding capacity. Genetic engineering has enhanced stability, but not always with the desired activity at the elevated temperature. However, protein engineering offers an effective way to circumvent these problems, as shown by this work. Further engineering in our laboratory will be focused on the improvement of the enzyme properties for more industrial applications. For the field to progress, the nature of the xylan binding and the details of enzyme interaction with side chains need to be better understood.

Acknowledgements

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