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Effects of dockerin domains on Neocallimastix frontalis xylanases

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Abstract

Two xylanase genes were cloned from the anaerobic fungus *Neocallimastix frontalis*. Xyn11A had a modular structure of two catalytic domains and two dockerin domains, while Xyn11B had one catalytic domain and two dockerin domains. The characteristics of the xylanases with and without dockerin domains were investigated. The deletion of dockerin domains had little influence on the optimal pH of xylanases, while it significantly affected the optimal temperatures. The optimal temperatures increased from 55 to 60 °C for Xyn11A and 60 to 65 °C for Xyn11B after the deletion of dockerin domains. The increase of optimal temperatures was attributed to the lower stability of the second structure in full length xylanase than that in the truncated one as evidenced by the circular dichroism spectroscopy. The specific activity of Xyn11A and Xyn11B increased about 64% and 330%, respectively, after the deletion of the dockerin domains. The removal of dockerin domains appeared to increase the overall efficiency of Xyn11A' (1.2-) and Xyn11B' (2.9-) fold with oat spelts xylan as reflected by the values of k_{cat}/K_m . The results suggest that the dockerin domain might play an important role in the characteristics of xylanases from anaerobic fungi.

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1. Introduction

Xylanases have received tremendous attention due to their application potential in many industries such as pulp and paper, animal feed, baking, brewing, and biofuel, etc [1]. Many microorganisms, including bacteria, actinomyces, yeasts as well as aerobic and anaerobic fungi have been screened for xylanase activity [2]. Among the sources, the anaerobic fungi have exhibited much higher xylanase activity than other microorganisms. Gilbert et al. [3] reported that anaerobic fungus *Neocallimastix patriciarum* XylA expressed in *Esche*-

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richia coli with a specific activity about 5980 U mg^{-1} crude protein in comparison with those from other microorganisms which ranged between 0.01 and 28.5 U mg^{-1} .

Xylanases usually have a modular structure containing one or two catalytic domains joined to one or more ancillary domains with different functions [4]. The ancillary domains of anaerobic fungal xylanases comprised non-catalytic domain of two dockerin domains or a cellulose binding domain. For example, the xylanase cDNA *xyn3*, isolated from *N. frontalis*, contained a single open reading frame of 1821 bp which encoded a 607amino-acid xylanase, XYN3. The predicted structure of XYN3 included two large reiterated catalytic domains (XYN3A and XYN3B) of 223 amino acids each with 88.3% identity and two dockerin domains with 85%

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identity. Each isolated catalytic domain of Xyn3 expressed in *E. coli* retained activity against xylan and produced similar hydrolysis products [5]. The role of the dockerin domain was thought to bind to the high molecular polypeptides associated with the anaerobic fungal cellulosome [6,7]. However, the effects of dockerin domains on xylanase activity of catalytic domains remained unclear.

In this study, two different xylanase genes from *N*. *frontalis* were cloned. One contained two catalytic domains at the N termini and two dockerin domains at the C termini, while the other contained one catalytic domain and two dockerin domains. The effects of dockerin domains on xylanase activity were investigated by constructing recombinant proteins with deletion of the two dockerin domains. The optimal temperature/pH, specific activity, hydrolyzed products, and thermodynamic stability of recombinant xylanases were also analyzed.

2. Materials and methods

2.1. Strains and cultivation

The anaerobic fungus *N. frontalis* SK1 was isolated from the rumens of the Formosan Sika Deer (*Cervus Nippon* taiwanus) as described by [8]. *N. frontalis* was cultured in a rumen fluid-containing medium supplemented with 0.5% (w/v) glucose or 0.5% rice straw for the cDNA library construction under anaerobic conditions at 39 °C for 48 h.

E. coli DH5 α (GIBCO BRL, Grand Island, NY), grown on LB medium (Difco, Detroit, MI), was used as the host for the various plasmid constructions. The vector pET21a (Novagen, Madison, WI) was used for construction of each xylanase expression vector. The resultant vectors were transformed into *E. coli* BL21 (Amersham Bioscience, Uppsala, Sweden) for the production of recombinant proteins.

2.2. Cloning of xylanase gene

Total RNA was isolated by the TRizol reagent (Invitrogen, Carlsbad, CA), and mRNAs were purified using the PolyATract mRNA Isolation System Kit (Promega, Medison, WI). The *N. frontalis* cDNA library was constructed using the SMARTTM cDNA Library Construction Kit (BD Bioscience, Palo Alto, CA) and packaged with the Gigapack III plus Packing Extract kit (Stratagene, San Diego, CA), following the manufacturers' protocols. The cDNA library was screened for xylanase activity using the results of Congo red staining [9].

The total DNA of *N. frontalis* SK1 was extracted as described by Hseu et al. [10]. The full-length of xylanase gene was amplified from *N. frontalis* chromosomal

DNA by polymerase chain reaction (PCR) using the following primers: forward primer 5'-ACTGTTGCTAA-GGCCCAATGGGGT-3' and reverse primer 5'-ACC-CCATTTACCATCGTCATCAGTG-3'. To construct each expression vector, the full-length genes of xylanases were amplified using the above primer set with addition of restriction sites (5' BamHI and 3' EcoRI). Deletion of dockerin domain fragments were amplified using the same forward primer as in the amplification of the full-length genes, and the reverse primers were replaced by the primer: 5'-GAATTCGCTGGACTA(A/G)-AACCCT-3'. The underlined sequence is the restriction enzyme cutting site of *Eco*RI. Each xylanase gene was amplified, purified, digested, and ligated into pET21a. The resultant plasmids were transformed into E. coli to express recombinant proteins. All recombinant proteins had the His₆-tag at each protein C-termini.

2.3. Recombinant proteins expression and purification

For the xylanase production in *E. coli*, the positive clone was grown in 500 ml LB to an OD₆₀₀ of 0.6–0.9 before 0.5 mM of isopropyl-thio- β -D-galactopyranoside (IPTG, Sigma) was added for the induction. After 16 h of induction at 20 °C, the cells were harvested by centrifugation (3000g, 15 min) for recombinant protein purification. Fusion protein purification was performed using a nickel affinity column (Ni NTA-agarose, Qiagen) and the recombinant protein was eluted with 250 mM imidazol. Protein concentrations were determined using a Micro BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL).

2.4. Circular dichroism spectroscopy

The far-UV CD spectra (250–190 nm) were recorded at 25 °C using the Aviv 202 spectrometer (Piscataway, NJ) with a 1-mm-pathlength quartz cuvette. Data were collected in 1-nm increments with a 10-s averaging time. The effects of temperature on protein secondary structure was monitored at 218 nm with 5 °C increments from 25 to 85 °C, a 3-min equilibration time between successive temperature points, and 10 s averaging time. Protein samples were dissolved in 25 mM sodium phosphate buffer (pH 7.0) at a concentration of 20–25 μ M.

2.5. Enzyme assay

The xylanase activity was determined as described by Georis et al. [11] with modifications. Reducing sugar that resulted from the enzymatic reaction was determined by measuring the absorbance at 540 nm [12] using xylose as the standard after the reaction mixture cooled down. One unit (U) is defined as the amount of enzyme that releases 1 µmol of reducing sugar per min.

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Enzyme was tested for its ability to hydrolyze the variety of substrates, including 2% oat spelts xylan (Sigma), 0.5% rye arabinoxylan (Megazyme, Wicklow, Ireland), 0.5% wheat arabinoxylan (Megazyme), 0.5% carboxymethylcellulose (Sigma) and 0.2% avicel (Sigma) and 0.2% β -glucan (Megazyme). For the effect of pH and temperatures, the enzyme activity was assayed in 25 mM Brittion and Robinson's universal buffer [13] at different pH (pH 3–10) values or under different temperatures (40–90 °C) conditions.

The kinetic parameters of the xylanase were determined at each of the enzyme's optimal temperature in 25 mM phosphate buffer by non-linear regression using the Michaelis–Menten equation of the initial rates determined between 0 and 20 mg/ml oat spelts xylan.

3. Results

3.1. Cloning and characterization of xyn11A and xyn11B

The xylanase cDNA isolated from the constructed cDNA library of *N. frontalis*, designated as *xyn11A*, contained a complete open reading frame (ORF) of 2132 bp with 5' and 3' untranslated regions of 199 and 109 bp, respectively. The untranslated region of *xyn11A* cDNA had an A/T-content of approximately 87.3%, excluding the poly(A) tail. The coding region contained approximately 43% G + C which was similar to those of other anaerobic fungus hemicellulase and cellulase cDNAs.

According to the DNA and deduced amino acid sequence analysis, *xyn11A* exhibited a high level of homology when compared with *N. patriciarum xylA* [3] and *N. frontalis xyn3* [5]. Particularly, *xyn11A* had the identities of 97.8% in DNA sequence and 97% in amino acid sequence compared with *xyn3*, which was also isolated from *N. frontalis*. The primer set was designed from the signal peptide sequence and the second dockerin domain of *xyn11A*. The PCR product with 1.8 and 1 kb were both amplified from *N. frontalis* genomic DNA. After comparing with cDNA sequence, no intron was found in the 1.8 kb-size genomic PCR product of *xyn11A*. This observation is consistent with other ruminal fungal xylanase genes [3,5,7,14]. By blasting in the National Center for Biotechnology Information (NCBI, NIH, Bethesda, MD) [15], the 1 kb-size PCR product was also found to be an intron-less xylanase gene and named *xyn11B*. The gene *xyn11B* appeared homologous to *Orpinomyces* sp. PC-2 (*xynA*) with 88.3% of the identities of DNA sequence. The Accession Nos. of *xyn11A* and *xyn11B* are AY601669 and AY131336.

3.2. Dockerin domain analysis of Xyn11A and Xyn11B

The 607-amino-acid-long polypeptide Xyn11A, which was encoded by xyn11A, had a modular structure of two catalytic domains and two dockerin domains, while Xyn11B, which was encoded by xyn11B, had one catalytic domain and two dockerin domains in the polypeptide of 337 amino acids. Xylanases with similar modular structures were also found in other anaerobic fungal xylanases [3]. Both dockerin domains of Xyn11A and Xyn11B were composed of 40 amino acids and were rich in residues of cysteine. Fig. 1 illustrates the alignment of the first dockerin domain of Xyn11A and Xyn11B with dockerin domains of other xylanases. Based on the number and position of the cysteine residues, the dockerin domain of Xyn11A and Xyn11B were classified as the type I subfamily within anaerobic fungal dockerin domains (noncatalytic docking domains, NCDD) [16].

3.3. Temperature and pH profiles

To investigate the effects of the dockerin domains of Xyn11A and Xyn11B on xylanase activity, the dockerin domains were deleted, and the resultant xylanases were designated as Xyn11A' and Xyn11B', respectively. The SDS–PAGE analysis of full-length and truncated xylanases is shown in Fig. 2. The molecular weights of Xyn11A, Xyn11A', Xyn11B and Xyn11B' were consistent with those estimated. The optimal pH for Xyn11A, Xyn11B, Xyn11A' and Xyn11B' was pH 7 regardless of the presence of dockerin domains (data not shown).

The effects of dockerin domains on the optimal temperatures of Xyn11A and Xyn11B are illustrated in Fig. 3. Xyn11A and Xyn11A' had the optimal tempera-

	*	**	*		* *
NF-Xvn11A	CSARITAQGY	KCCSDP	- NCVVYY	YTDEDGTWGVENND	WCGCG
NF-Xvn11B	CSARITAQGY	KCCSDP	- NCVVYY	YTDDDGTWGVENNE	WRGCG
NF-Xyn3	CSARITAQGY	KCCSDP	- NCVVYY	YTDEDGTWGVENND	WCGCG
NP-XvlA	CSARITAQGY	KCCSDP	- NCVVYY	YTDEDGTWGVENND	WCGCG
Orp-XynA	CSAKITAQGY	KCCSDP	- NCVVYY	YTDEDGTWGVENNQ	WCGCG
Pir-XylA	CPSTITSQGY	KCCSS-	- NCDIIY	YRDQSGDWGVENDE	WCGCG
Pir-Cel45A	CWAQ SQGY	INCCNN	SSTKVEY	Y TDA S GQWGVQNGQ	WCG

Fig. 1. Alignment of dockerin domains of *N. frontalis* SK1 Xyn11A and Xyn11B with other dockerin domains of the xylanases from *N. frontalis*, *N. patriciarum*, *Orpinomyces* sp. PC-2, *Piromyces* sp. NF-Xyn3: *N. frontalis* Xyn3; NP-XylA: *N. patriciarum* XylA; Orp-XynA: *Orpinomyces* sp. PC-2 XynA and Pir-XylA: *Piromyces* sp. XylA. Cysteine residues are marked by asterisks. Gaps (dashes) were introduced to maximize the regions of sequence alignment.



Fig. 2. The SDS–PAGE analysis of the purified Xyn11A, Xyn11A', Xyn11B and Xyn11B' expressed in *E. coli*. Electrophoresis was conducted in 12.5% polyacrylamide gels and stained with coomassie brilliant blue. Lane 1: Xyn11A; lane 2: Xyn11A'; lane 3: Xyn11B; lane 4: Xyn11B'; lane M: molecular weight marker (in kDa).



Fig. 3. Effect of temperature on activity of Xyn11A and Xyn11A' (a), and Xyn11B and Xyn11B'(b). Xyn11A (\bullet), Xyn11A' (\bigcirc), Xyn11B' (\triangle) and Xyn11B' (\triangle) activities against 2% oat spelts xylan were determined at pH 7 in 25 mM phosphate buffer. The specific activity of full length Xyn11A at 55 °C represents 100%.

tures at 55 and 60 °C, respectively. Xylanase activity of Xyn11A' was higher than that of Xyn11A below 65 °C. No significant differences were found between Xyn11A

and Xyn11A' when the temperatures were over 65 °C. The optimal temperatures of Xyn11B and Xyn11B' were 60 and 65 °C, respectively. Removal of the dockerin domain from Xyn11B resulted in an increment of the xylanase activity across all temperatures tested in this study.

3.4. Circular dichroism spectroscopy

The thermodynamic stability of Xyn11A, Xyn11B, Xyn11A' and Xyn11B' were determined by thermal denaturation monitored using circular dichroism spectroscopy. Fig. 4 shows the typical temperature-induced unfolding curves of the recombinant xylanases. The midpoint temperature of the transition state (T_m) shifted from 53.8 °C (Xyn11A) to 60.2 °C (Xyn11A') and 60 °C (Xyn11B) to 63.9 °C (Xyn11B') after deletion of the dockerin domains.

3.5. Substrate specificity and hydrolysis pattern

Table 1 summarizes the substrate specificity of recombinant Xyn11A, Xyn11A', Xyn11B and Xyn11B' against different polysaccharide substrates. Xyn11A, Xyn11A' and Xyn11B had the highest specific activity



Fig. 4. Temperature-induced unfolding of Xyn11A and Xyn11A' (a) and Xyn11B and Xyn11B' (b) at pH 7 measured by CD spectroscopy. The unfolding curves were measured at 218 nm with $5 \,^{\circ}$ C increment.

Table 1 Substrate specificities and kinetic parameters of Xyn11A, Xyn11A', Xyn11B and Xyn11B' expressed in *E. coli*

Substrates	Xyn11A	Xyn11A'	Xyn11B	Xyn11B'
Oat spelts xylan				
Specific activity	3964.2 ± 220.9 (100)	6517.1 ± 366.7 (100)	3756.6 ± 55.3 (99)	$16309.0 \pm 236.2 (100)$
K _m	6.1	7.4	6.4	7.2
K _{cat}	4229.9	5927.7	2421.5	7899.1
$k_{\rm cat}/K_{\rm m}$	693.4	801.0	378.3	1094.3
Wheat arabinoxylan	2185.3 ± 147.8 (54)	3583.3 ± 800.1 (55)	2914.7 ± 25.6 (76)	7459.5 ± 112.0 (46)
Rye arabinoxylan	3088.2 ± 155.7 (78)	5021.4 ± 446.5 (78)	3794.5 ± 53.0 (100)	11437.1 ± 145.4 (70)
CMC	ND	ND	ND	ND
Avicel	ND	ND	ND	ND
β-glucan	ND	ND	ND	ND

Values in parentheses are percentages of the maximum activity for each enzyme.

The unit value of each enzyme is U/mg protein.

ND: not detectable.

Kinetic parameters for all xylanases catalyzed hydrolysis of oat spelts xylan at each enzyme's optimal temperature in 25 mM phosphate buffer, pH 7.0. The units of the kinetic parameters: K_m (oat spelts xylan mg ml⁻¹), k_{cat} (s⁻¹) and k_{cat}/K_m (ml mg⁻¹ s⁻¹).

against oat spelts xylan, while Xyn11B' gave the highest specific activity against rye arabinoxylan. All xylanases were inactive against β -glucan, CMC and avicel. After the dockerin domain deletion, the specific activity of Xyn11A' increased approximately 60% compared with that of Xyn11A, while Xyn11B' increased by 330% compared with that of Xyn11B. The kinetic parameters for the hydrolysis of oat spelts xylan by each xylanase are also provided in Table 1. The overall catalytic efficiency of Xyn11A' and Xyn11B' as reflected by k_{cat}/K_m values, were approximately one and three folds greater than Xyn11A and Xyn11B, respectively. After 2 h of hydrolysis by xylanases with 1% soluble oat spelts xylan as the substrate, the main products of TLC analysis were xylobiose, xylotriose and xylotetraose and no xylose, which indicated that the recombinant enzymes were endoxylanases (data not shown).

4. Discussion

Xylanases often exhibit a modular structure composed of catalytic domains linked to one or more noncatalytic domains, such as cellulose binding domains (CBDs), thermostabilizing domains (TSDs), S-layer-like domains, and dockerin domains [17]. Dockerin domains were regarded as connectors to anchor enzymes on the scaffoldin of the cellulosome and were independent of enzyme activity and specificity [3,18,19]. Anaerobic fungal enzymes contain one, two, or three copies of the dockerin sequences in tandem within the catalytic polypeptides. The role of the dockerin domain in xylanases was to bind to the high molecular polypeptides associated with the anaerobic fungal cellulosome [6,7].

In this study, no differences were found in hydrolysis products between xylanases with or without dockerin domains. *N. frontalis* XYN3 (wild-type) and XYN3A (catalytic domain only) released the same hydrolysis products [5]. This indicates that dockerin domains had little influence on hydrolysis patterns. The deletion of dockerin domains did not affect the optimal pH, but it significantly increased the optimal temperatures of xylanases from *N. frontalis*. The increase of optimal temperatures was attributed to the less stability of the second structure in the full length xylanase than that in the truncated one as evidenced by the circular dichroism spectroscopy.

After deletion of dockerin domains, Xyn11A' and Xyn11B' had higher specific activities and values of k_{cat}/K_m than Xyn11A and Xyn11B, respectively. Removal of the dockerin domain was led to an enhancement in the catalytic activity, as evidenced by the increase in k_{cat}/K_m , which suggests that interdomain interactions may influence the catalytic activity of Xyn11A and Xyn11B. The increase in catalytic efficiency after the dockerin domain deletion was consistent with the results reported by Marrone et al. [20]. They found that the deletion of the dockerin domain of anaerobic *Fibrobacter succinogenes* S85 XynC significantly increased the value of k_{cat}/K_m on birch wood xylan or xylopentaose.

The deletion of the dockerin domains had more influence in Xyn11B than in Xyn11A. The dockerin domains deletion raised the specific activity of Xyn11B' up to 4.3 times while only a 1.6-fold change was observed in Xyn11A'. Based on the kinetic analysis, it was apparent that Xyn11A' and Xyn11B' were much more efficient as enzymes than their wild-type counterparts, Xyn11A and Xyn11B. The different influences of dockerin domains on enzyme characterization of Xyn11A and Xyn11B might be the result of the variations in amino acid sequences or the number of catalytic domains. Xyn11A had two homologous catalytic domains with 91% amino acid sequence identity and 96% similarity and both exhibited xylanase activity well (data not shown). The catalytic domain of Xyn11B had relatively high amino acid sequence identity (94%) and similarity (94%) compare with the first catalytic domain of Xyn11A. Similar high levels of amino acid sequence identities were also observed between the catalytic domain of Xyn11B and the second catalytic domain of Xyn11A with 94%. The results presented in this study suggest that the dockerin domain might play an important role in the characteristics of xylanases from anaerobic fungi. The detailed mechanism of dockerin domains on xylanase properties requires further investigation.

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