International Journal of Agricultural Technology 2025 Vol. 21(4):1503-1520
DOI: https://doi.org/10.63369/ijat.2025.21.4.1503-1520
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

The family 36 carbohydrate-binding module of *Paenibacillus xylaniclasticus* TW1 xylanase: Characterization and recognition in epidermal tissue of sweet potato roots

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Tangthirasunun, N., Sornyotha, S. and Jatupornpipat, M. (2025). The family 36 carbohydrate-binding module of *Paenibacillus xylaniclasticus* TW1 xylanase: Characterization and recognition in epidermal tissue of sweet potato roots. International Journal of Agricultural Technology 21(4):1503-1520.

Abstract *Paenibacillus xylaniclasticus* TW1 was able to aerobically produce a multienzyme complex. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the crude enzyme preparation revealed at least 12 proteins that were bound to insoluble cellulose. Only the band representing cellulose-bound protein 12 (CBP12) from SDS-PAGE was identified as xylanase family 11 with a carbohydrate-binding module family 36 (CBM36) using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS). In this report, CBM36 of *P. xylaniclasticus* TW1 (*Px*TW1CBM36) was cloned, expressed, purified and studied for binding characteristic. The results found that *Px*TW1CBM36 displayed broad binding ability to polysaccharides which high affinity for xylan and insoluble cellulose. Interestingly, this is the first report indicated that CBM36 had an affinity for insoluble cellulose. Although the amino acid residues involved in *Px*TW1CBM36 binding were conserved, the binding capacity of *Px*TW1CBM36 do not perturb by the addition of ethylenediaminetetraacetic acid (EDTA). It is possible that *Px*TW1CBM36 had different binding mechanisms with other CBM36. In addition, the binding characteristic of CBM36 on polysaccharides embedded within plant cell walls was also elucidated. It displayed the strong recognition for ligands located in epidermal tissue of sweet potato roots. Therefore, this study might provide a new tool for targeting enzymes to surface of plant.

Keywords: Carbohydrate-binding module family 36, MALDI-TOF/TOF MS, *Paenibacillus xylaniclasticus* TW1, Sweet potato roots, Xylanase family 11

Introduction

The most abundant and renewable resource is found in plant cell walls, which provide valuable bioproducts, including fermentable sugars, chemicals, and biofuels (Carpita and McCann, 2020). Primarily, these walls consist of cellulose, hemicelluloses (mainly xylan), lignin, and various other elements.

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Together, these constituents are intricately linked and intertwined through covalent bonds, hydrogen bonds, and van der Waals interactions, forming a highly complex and recalcitrant structure (Himmel *et al*., 2007). Efficient and complete hydrolysis of plant cell wall polysaccharides require the synergistic action of cellulolytic enzymes (Hasunuma *et al*., 2013) and xylanolytic enzymes (Gírio *et al*., 2010).

The previous reports indicated that a few microorganisms could produce multienzyme complexes under aerobic conditions (Jiang *et al*., 2005; van Dyk *et al*., 2009; Pason *et al*., 2010; Tachaapaikoon *et al*., 2012a). Among these, *Paenibacillus xylaniclasticus* TW1 has been identified as a true cellulolytic/xylanolytic organism, capable of utilizing xylan, filter paper, or agricultural residues as its sole carbon source. Remarkably, it can aerobically synthesize a xylanolytic-cellulolytic multienzyme complex (Tachaapaikoon *et al*., 2012a, b). These multienzyme complexes are crucial for deconstructing plant cell wall components. Their ability to bind insoluble cellulose and/or xylan, facilitated by carbohydrate-binding modules (CBMs) like cellulose-binding or xylan-binding modules, enables this function (Bayer *et al*., 1994). Non-catalytic CBMs improve the efficiency of enzymatic hydrolysis. They do this by leading their attached catalytic modules to target substrates within the plant cell wall (Shoseyov *et al*., 2006). In the CAZy database, 106 identified families categorize CBMs according to their amino acid sequence similarity. Some earlier-designated families (e.g., CBM7 and CBM33) have since been reclassified and no longer contain active entries (Henrissat *et al*., 2024; see http://www.cazy.org/Carbohydrate-Binding-Modules.html). CBMs are categorized into three functional types, in addition to sequence-based classification, determined by their ligand-binding topography (Boraston *et al*., 2004). These types are: Type A, which recognizes crystalline polysaccharide surfaces; Type B, which attaches to interior portions of glycan chains; and Type C, which typically recognizes short oligosaccharides at chain ends. Consequently, their role in the efficient degradation of plant cell wall components is well-recognized (Ohmiya *et al*., 1997). Intriguingly, no information on the CBMs appearing in the xylanolytic-cellulolytic multienzyme complex from *P. xylaniclasticus* TW1 is reported. Therefore, in this study, we sought to identify the secreted enzymes that appear to contain CBMs by using affinity purification and applying MALDI-TOF/TOF MS. Based on our strategies, we have found CBM36 with the xylanase family 11 produced from the strain TW1. To date, only CBM36 from *P. polymyxa* ATCC 842 xylanase 43A was characterized (Jamal-Talabani *et al*., 2004), while CBM36 from the strain TW1 showed low amino acid sequence similarities (50% identities) to CBM36 from the strain ATCC 842. Thus, a CBM36 from the strain TW1 was interesting to be further studied.

In this report, the r*Px*TW1CBM36-CFP was constructed. The binding characteristics and specificities of CBM36 from the strain TW1 against the isolated carbohydrates and the recognition of carbohydrates located within plant cell walls as the substrate available for the associated catalytic domain were investigated. Moreover, the target region of CBM36 in plants was currently unknown and the fluorescent tag was used for monitoring CBM36 binding in plants.

Materials and methods

Enzyme preparation

Berg's mineral salt medium (pH 7.0) (Berg *et al*., 1972) was used to grow *P. xylaniclasticus* TW1*,* a strain that produces multienzyme complexes (Tachaapaikoon *et al*., 2012b). For three days, the mixture was shaken at 200 rpm, and the medium was provided with 0.5% (w/v) corn hull as the only carbon source. The experiment was conducted at 37°C. Following incubation, the culture was centrifuged for 20 minutes at 4°C with 8,000 × *g*. The crude enzyme extract was obtained by collecting the clear supernatant.

Isolation of cellulose-bound proteins

To isolate cellulose-bound protein, 250 µl of protein solution (25 mg/ml) was combined with 15 mg of ball-milled cellulose, KC flock (Nippon Paper Chemicals, Tokyo, Japan), suspended in 10 mM potassium phosphate buffer (KPB, pH 7.0) in 1.5 ml microcentrifuge tubes at 4 °C for 30 minutes. Following incubation, the samples were centrifuged, and the resulting precipitate was rinsed off five times with the same buffer. The precipitate (cellulose-bound fraction) and supernatant (unbound fraction) were then evaluated using SDS-PAGE.

Gel electrophoresis

An 8% (w/v) polyacrylamide gel was used for SDS-PAGE, as stated by Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue R-250 (Nacalai Tesque, Kyoto, Japan) to visualize them following electrophoresis. A calibration kit (GE Healthcare, Tokyo, Japan) provided the molecular mass standards.

Protein identification using MALDI-TOF/TOF MS

In accordance with Hanna *et al*. (2000), twelve protein bands were taken out of an SDS-PAGE gel and digested in-gel using trypsin. A 4800 Plus MALDI-TOF/TOF Analyzer (Applied Biosystems, Tokyo, Japan) equipped with a 200 Hz Nd:YAG laser (337 nm) was used to identify proteins. The 20 most energetic ions (signal-to-noise > 25) for each MALDI spot were chosen for 100 laser pulses in 1 kV mode for MS/MS analysis. The colliding gas was air. The 4700 Mass Standard Kit was used for data acquisition and processing, and ProteinPilot with MASCOT was used to search MS/MS spectra against the NCBInr database. The search parameters were variable oxidation (Met), fixed carbamidomethylation (Cys), one missed cleavage, and 200 ppm mass tolerance.

Polymerase chain reaction (PCR) amplification and sequencing for CBP12

PCR primers were made using MALDI-TOF/TOF MS data and BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) to target a gene that is very similar to the xylanase gene (*xyn11A*) from *Paenibacillus curdlanolyticus* B-6 (GenBank accession no. gi|283970946). Two sets of synthetic oligonucleotide primers were created for this gene: Pbxyn11F (5′-ACCTTGTTGTTAGCTGGGACAAACGT-3′) and Pbxyn11R (5′-TTGTTCGATGTGAAGCCGGGCA-3′). The Takara PERSONAL Thermal Cycler was utilized for PCR amplification. PCR products were separated on a gel made of 0.8% agarose in TAE buffer, stained with ethidium bromide (0.5 μg/ml), and viewed under UV light. Before cloning, the specific DNA piece was cut out from the gel and cleaned up using a TA Cloning Kit. The introduced gene was later purified and sequenced utilizing an ABI 3130X Genetic Analyzer (Applied Biosystems). The Compute pI/Mw tool on ExPASy (http://web.expasy.org/compute_pi/) was utilized to estimate the molecular weight and isoelectric point (pI) of the *cbp12* gene product.

Cloning, expression and purification of rPxTW1CBM36-CFP

To construct a plasmid encoding a CFP-fused r*Px*TW1CBM36 protein, the coding region of the *PxTW1cbm36* gene was amplified from the genomic DNA of *P. xylaniclasticus* TW1 using PCR. Primers were designed from the GenBank sequence (accession no. JX069975), incorporating *Bam*HI and *Eco*RI sites at the 5′ ends of the forward and reverse primers, respectively. *Px*TW1cbm36F (5′-**GGATCC**GGTAATACAGGTGGAAC-3′) served as the forward primer, and *Px*TW1cbm36R (5′-**GAATTC**AGGAGTCGAATTGATTTCC-3′) as the reverse primer. Restriction sites are indicated by the underlined sequences. Initially, the PCR product was cloned into the pCR2.1 vector (Invitrogen). The

insert was subsequently sequenced to verify the lack of mutations. After digestion with *Bam*HI and *Eco*RI, the verified insert was ligated into the pRSET/CFP expression vector (Invitrogen) which was similarly digested, resulting in plasmid pRSET/CFP-*Px*TW1CBM36. This construction encodes a recombinant protein with a 6×His-tagged N-terminus.

The pRSET/CFP-*Px*TW1CBM36 plasmid-carrying recombinant *Escherichia coli* BL21(DE3) cells (Toyobo, Kyoto, Japan) were cultivated aerobically at 37°C in 1L of Luria-Bertani (LB) broth supplemented with ampicillin (50 μg/ml). When the culture's optical density at 600 nm (OD_{600}) reached about 0.4, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to enhance protein expression. The culture was then incubated for an additional 3 hours. After centrifugation at 8,000 × *g* for 20 minutes, cells were washed and sonicated for disruption. The supernatant was then purified using His-tag affinity purification with Ni-NTA agarose resin (Qiagen, Hilden, Germany), as per the manufacturer's instructions. SDS-PAGE was used to evaluate protein purity. The purified r*Px*TW1CBM36-CFP's concentration was then calculated from its absorbance at 280 nm, utilizing a molar extinction coefficient of 47,960 M^{-1} cm^{-1} (Mach *et al*., 1992).

Ligand-binding assays with affinity gel electrophoresis

To evaluate the binding specificity of r*Px*TW1CBM36-CFP for various soluble carbohydrates, affinity gel electrophoresis was employed. Native polyacrylamide gels (10% (w/v) acrylamide) were prepared under non-denaturing conditions, without the inclusion of β-mercaptoethanol, SDS, or heat. Before polymerization, soluble carbohydrates were added to the gel matrix to a final concentration of 0.1% (w/v). Each lane was loaded with approximately 5 μg of either the recombinant protein or bovine serum albumin (BSA; Sigma-Aldrich), which acted as a non-binding negative control. At room temperature, electrophoresis was performed, and protein bands were visualized using Coomassie Brilliant Blue R-250 staining.

Effect of EDTA on the binding of rPxTW1CBM36-CFP to insoluble carbohydrates

For 30 minutes on ice, r*Px*TW1CBM36-CFP (40 μg) was bound to insoluble carbohydrates (15 mg) in a 250 μl volume of 50 mM KPB (pH 7.0), with or without EDTA. Following this, the mixtures were centrifuged for 5 minutes at 8,000 × *g*. After washing the pellets five times with the corresponding buffer, we analyzed both the bound (pellet) and unbound (supernatant) protein

fractions via SDS-PAGE. Oat-spelt xylan insoluble residue, following the methods outlined by Zhang *et al*. (2012) and ball-milled cellulose were served as the insoluble carbohydrate substrates.

Preparation of plant material and CBM-labeling

This investigation adhered to the guidelines established by Araki *et al*. (2010) and McCartney *et al*. (2004). Hydroponically grown sweet potato (*Ipomoea batatas* cv. Beni Kokei) roots were preserved in 50 mM sodium phosphate buffer (pH 7.2) with 50 mM NaCl and 3% (w/v) paraformaldehyde. This was accomplished by incubating on ice for four hours after five minutes of vacuum infiltration. After being dehydrated using an ethanol series, the samples were embedded in paraffin and cut into transverse sections that were 6 µm thick. Following deparaffinization and mounting on poly-L-lysine-coated slides, these sections were incubated for 30 minutes with 50 µg/ml of r*Px*TW1CBM36-CFP in 20 mM KPB (pH 7.0). The slices were seen using an Axioskop 2 Plus microscope (Zeiss) equipped with an AxioCam HRC camera following washing with 50 mM KPB (pH 7.0).

Results

Isolation and identification of cellulose-bound proteins

To characterize the features of carbohydrate-binding modules (CBMs), we isolated cellulose-bound proteins from the supernatant of *P. xylaniclasticus* TW1 cultures grown aerobically on corn hulls until the stationary phase. Affinity purification using ball-milled cellulose was employed to selectively isolate proteins with cellulose-binding capacity from the crude enzyme preparation. The crude enzyme was incubated with ball-milled cellulose at 4 °C to facilitate binding of cellulose-affinitive proteins. Bound proteins were separated from unbound components by centrifugation, followed by a series of washes. To elute the bound proteins, sample application buffer was added, and the mixture was heated in a boiling water bath for 5 minutes. SDS-PAGE analysis (data not shown) revealed that at least twelve distinct proteins were present among the cellulose-bound fraction, designated CBP1 through CBP12. MALDI-TOF/TOF mass spectrometry was subsequently used to identify these proteins.

All twelve cellulose-bound protein bands resolved by SDS-PAGE were tryptic-digested. MALDI-TOF/TOF mass spectrometry was employed for the subsequent analysis of the peptide mixtures. The peptide mass fingerprints of the unknown components from SDS-PAGE were almost identical to the unknown

function proteins (Table 1). Only 2 CBPs could be identified in this study. As shown in Table 1, three of the peptides from the CBP5 protein (#2, 8, 18) were obtained showing similarity to published glycoside hydrolase family 31 sequences from *P. curdlanolyticus* YK9 (GenBank no. gi|304405232). However, no information on the CBM appearing in the glycoside hydrolase family 31 from *P. curdlanolyticus* YK9 was presented. Besides, the band of CBP12 from SDS-PAGE gel could be identified as xylanase. An NCBI BLAST search of the amino acid sequence of CBP12 (KGTITVDGGTYDIYETTRV) shows similarity to *P. curdlanolyticus* B-6 xylanase belonging to glycoside hydrolase family 11 with a CBM family 36 (GenBank no. gi|283970946; http://www.ncbi.nlm.nih.gov/BLAST/). Therefore, the gene encoding protein CBP12 of *P. xylaniclasticus* TW1 was further identified by cloning and sequencing.

The amino acid sequence KGTITVDGGTYDIYETTRV revealed high similarity to xylanase 11A (Xyn11A) from *P. curdlanolyticus* B-6 when subjected to a BLAST search. Specific primers were designed to clone the CBP12 gene from *P. xylaniclasticus* TW1. Their design was based on the amino acid and nucleotide sequences of the *xyn11A* gene from strain B-6. The PCR product was then cloned into a TA vector and sequenced, following the procedures outlined in the Methods section. At 1,155 bp, the *cbp12 gene* encodes a 385-amino acid protein (Figure 1A) with an estimated molecular weight of 42 kDa and a pI of 7.7. Sequence analysis of the predicted protein indicated that CBP12 contains two distinct functional domains: a glycoside hydrolase family 11 catalytic domain and a family 36 carbohydrate-binding module (CBM). These domains are separated by a linker region enriched in glycine, asparagine, and threonine residues (Figure 1B).

Ligand specificity of CBM36 from P. xylaniclasticus TW1

To study the binding characteristics of CBM36 from *P. xylaniclasticus* TW1, r*Px*TW1CBM36 was ligated in pRSET/CFP for construction of CFP fusion r*Px*TW1CBM36 (r*Px*TW1CBM36-CFP), expressed under the induction of IPTG and purified for subsequent analysis. After SDS-PAGE analysis, the purified r*Px*TW1CBM36-CFP was dialyzed against 10 mM KPB (pH 7.0) to prepare it for further investigations.

Table 1. Peptide sequences identified from digested samples of carbohydrates-binding proteins

Protein	Peptide no. and sequence	BLAST identification
CBP1	#10: GKQLADDSSSAGR #12: WLANIMLNGDPR #18: EQRVLDSDGNPLPSEPR	Unknown protein
CBP2	#6: YDMYDKYFQK #7: IKASVLGVSAAYVR #13: IKQYPFQLSGGLCQR	Unknown protein
CBP3	#15: QISNLQQSISDAEQR #16: NARTVFSELSQATSNK #18: FVEFYGSGLSNISLADR	Unknown protein
CBP4	#7: GWPSTIGFLVDTSR #9: SVPKLFSWIR #13: IKQYPFQLSGGLCQR	Unknown protein
CBP5	#18: KGYGVFVNQPELVSFEVGSEKV	Glycoside hydrolase family 31 (*Paenibacillus curdlanolyticus* YK9)
CBP6	#1: DDELLSAYR #3: LVIENPEKPR #14: IVGVNMGDSIPQVDIPRLIK	Unknown protein
CBP7	#1: FYLVGSKMR #3: MMDTIESIR #5: EQFGGVKDTAGR	Unknown protein
CBP8	#2: ASEQPTQIR #3: QVVNPEDGSR #6: EEFLGVIACIR	Unknown protein
CBP9	#3: EIAAAQDGIAR #4: ENNIQFVDFR #13: DTLCVCCMAASNVTGR	Unknown protein
CBP10	#10: QVASDFKSLSGVGSSQR #11: NGFFAMGMAEKANSASR #15: SLADAEGYETFVIPDDVGGR	Unknown protein
CBP11	#4: GPLTTPIGGGIR #14: DLLNEYEFPGDDTPIVR #16: YQLVQPQTIIVILEKIR	Unknown protein
CBP12	#15: KGTITVDGGTYDIYETTRV	Xylanase (*Paenibacillus curdlanolyticus* B6)

In vitro binding studies using semi-quantitative affinity gel electrophoresis showed that the migration of purified r*Px*TW1CBM36-CFP through native polyacrylamide gels loaded with soluble birchwood and oat-spelt xylans was slower than that of BSA (control) (Figure 2). Moreover, r*Px*TW1CBM36-CFP was able to bind tightly to ball-milled cellulose and both soluble birchwood and oat-spelt xylans, moderately to sugar beet arabinan (α-linked arabinan), and weakly to laminarin (β-1,3-glucan), tamarind seed xyloglucan, methylcellulose, and lichenan. However, r*Px*TW1CBM36-CFP was unable to bind to carboxymethylcellulose, barley β-glucan, galactomannan (guar gum), pectin, or starch (Table 2).

(A)

```
   1  atgacaacgaaggaggaagatgtgatgaaaccaagaaaaatgaaatggatgctggccatgctgctcagcttcaca
   1  M  T  T  K  E  E  D  V  M  K  P  R  K  M  K  W  M  L  A  M  L  L  S  F  T

  76  ctcgcattgccagcaggcgtcgcgaacgccgtaacgattacgaataatgagtccggcacccatgacggctactat
  26  L  A  L  P  A  G  V  A  N  A  V  T  I  T  N  N  E  S  G  T  H  D  G  Y  Y

 151  tatgagctatggaaggattccgggaacacaagcatgacgcttaacagcggcggcaccttcagtgcgacgtggagc
  51  Y  E  L  W  K  D  S  G  N  T  S  M  T  L  N  S  G  G  T  F  S  A  T  W  S

 226  aacattaacaatgcgttattccgtaaaggcaaaaaattcaatgaaacacagacccaccagcaaatcggcaacatc
  76  N  I  N  N  A  L  F  R  K  G  K  K  F  N  E  T  Q  T  H  Q  Q  I  G  N  I

 301  tcggtcaattacagcgctaactattctccgaacggcaactcttatctctgcatctatggatggtcggttgacccg
 101  S  V  N  Y  S  A  N  Y  S  P  N  G  N  S  Y  L  C  I  Y  G  W  S  V  D  P

 376  ctcgtcgagtattatatcgtcgacagctggggcagctggcgtcctcctggcgcctcgtccaaaggtacgatcacc
 126  L  V  E  Y  Y  I  V  D  S  W  G  S  W  R  P  P  G  A  S  S  K  G  T  I  T

 451  gttgacggcggcacctacgacatttatgagacgactcgtgtgaaccagccttccattaaaggcacggcaacgttc
 151  V  D  G  G  T  Y  D  I  Y  E  T  T  R  V  N  Q  P  S  I  K  G  T  A  T  F

 526  cagcagtattggagcgtccggacgtcgaagcgtacgagcggcacaatctcggtaaccgatcacttcaatgcttgg
 176  Q  Q  Y  W  S  V  R  T  S  K  R  T  S  G  T  I  S  V  T  D  H  F  N  A  W

 601  gcaagcagaggcatgcagctgggcaaaatgtacgaagtatcgctcacggtagaaggctaccaaagcagcggaagc
 201  A  S  R  G  M  Q  L  G  K  M  Y  E  V  S  L  T  V  E  G  Y  Q  S  S  G  S

 676  gcgacaattacaagcaatacgattacaatcggcggcaacggcggcggcggtaatacgggcggtggcaataacggc
 226  A  T  I  T  S  N  T  I  T  I  G  G  N  G  G  G  G  N  T  G  G  G  N  N  G

 751  ggcggcaacaatggaggaggcaacaatggcggcggtaatacaggtggaacgacgagaattgaagccgagagcatg
 251  G  G  N  N  G  G  G  N  N  G  G  G  N  T  G  G  T  T  R  I  E  A  E  S  M

 826  acgaagagcggccaatacactggcaatatctactctccgttcaatggtgtagcactatacgctaacaacgactcg
 276  T  K  S  G  Q  Y  T  G  N  I  Y  S  P  F  N  G  V  A  L  Y  A  N  N  D  S

 901  gtgaaattcacgcataactttacgaaaagcacgaacaacttctcgctccgcggcgcttcgaacaactcccaaatg
 301  V  K  F  T  H  N  F  T  K  S  T  N  N  F  S  L  R  G  A  S  N  N  S  Q  M

 976  gctagagtcgatctgaaggtcggcggcgtgacgaagggcaccttctattacggcggcagctatcctgcagtctat
 326  A  R  V  D  L  K  V  G  G  V  T  K  G  T  F  Y  Y  G  G  S  Y  P  A  V  Y

1051  acgataaacaacgtcaatacgggaaccggaaatcaacaggtcgagctcattcttacagccgataacgggcaatgg
 351  T  I  N  N  V  N  T  G  T  G  N  Q  Q  V  E  L  I  L  T  A  D  N  G  Q  W

1126  gatgcgttcctcgattatttggaaatcaattag
 376  D  A  F  L  D  Y  L  E  I  N  *
```

(B)

Figure 1. The predicted amino acid sequence of the protein product and the nucleotide sequence of the *cbp12* gene(A). Schematic diagram of the CBP12 protein domain architecture. Amino acid residue numbers above the schematic indicate the domain boundaries(B). GH11 represents the glycoside hydrolase family 11 catalytic domain; CBM36 denotes the family 36 carbohydrate-binding module

Binding of CBM36 from P. xylaniclasticus TW1 to insoluble carbohydrates in the presence of EDTA

To evaluate ligand recognition, the binding affinity of r*Px*TW1CBM36-CFP toward insoluble carbohydrates was assessed in the presence of 10 mM EDTA. To separate bound and unbound proteins, the recombinant CBM and insoluble carbohydrate substrates were incubated together, then centrifuged. The resulting supernatant (unbound) and pellet (bound) fractions were both analyzed

via SDS-PAGE. Binding ability was inferred by comparing the relative amounts of protein present in the supernatant and pellet. As shown in Figure 3A, strong binding to ball-milled cellulose and insoluble oat-spelt xylan was interestingly observed when 10 mM EDTA was present. To further investigate the effect of EDTA on the binding ability of r*Px*TW1CBM36-CFP, 50 mM EDTA was used in this experiment (Figure 3B). It was clear that r*Px*TW1CBM36-CFP remained bound to both insoluble carbohydrates.

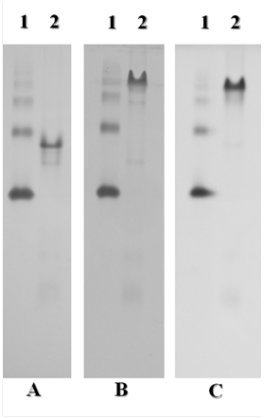

Figure 2. Affinity gel electrophoresis of r*Px*TW1CBM36-CFP from *P. xylaniclasticus* TW1 on non-denaturing polyacrylamide gels prepared with (A) no ligand, (B) birchwood xylan, or (C) oat-spelt xylan. As a non-carbohydrate-binding control, BSA was included in lane 1; r*Px*TW1CBM36-CFP was loaded in lane 2.

Sequence-based relatives of *Px*TW1CBM36 were analyzed using BLAST, as shown in Figure 4. The results revealed that most of the related proteins contained xylanase catalytic modules, indicating that xylan is the primary target for CBM family 36. Among them, proteins from *Clostridium phytofermentans*, *Ruminococcus albus*, and *Streptomyces thermoviolaceus* contained carbohydrate esterase family 4 (CE4), known for xylan-specific esterases (Montanier *et al*., 2010; see URL http://www.cazy.org/CE4_characterized.html). Additionally, CBM36 sequence alignment showed conserved metal-binding sites, with residues coordinating Ca^{2+} (Tyr29, Asp105, Trp109, and Asp110) being invariant in all members. Moreover, Tyr15, an essential component of the second sugar-binding site, was also conserved among members of the family (Montanier *et al*., 2010).

Table 2. Determining the semi-quantitative ligand-binding specificity of r*Px*TW1CBM36-CFP via affinity gel electrophoresis

Soluble carbohydrate	Binding ability*
Birchwood xylan	+++
Oat-spelt xylan	+++
Sugar beet arabinan	++
Laminarin	+
Tamarind seed xyloglucan	+
Methylcellulose	+
Carboxymethylcellulose	-
Lichenan	+
Barley β-glucan	-
Guar gum	-
Pectin	-
Starch	-

* Binding ability was assessed based on the extent of protein migration in the gel: tight (+++), moderate (++), weak (+), or no detectable binding (–).

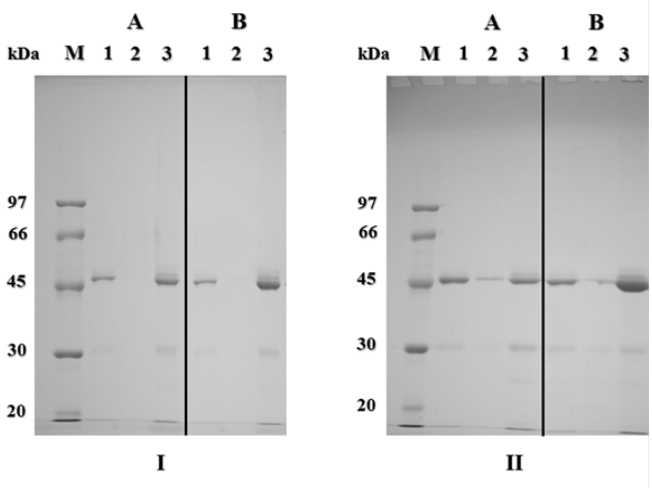

Figure 3. Effect of EDTA on the binding ability of CBM36 from *P. xylaniclasticus* TW1 to insoluble carbohydrates: r*Px*TW1CBM36-CFP was incubated with either ball-milled cellulose (I) or insoluble oat-spelt xylan (II) with 10 mM EDTA (A) or 50 mM EDTA (B). SDS-PAGE was employed to examine the proteins in the supernatant (lane 2) and pellet (lane 3) following centrifugation. Lane 1 shows r*Px*TW1CBM36-CFP as a positive control

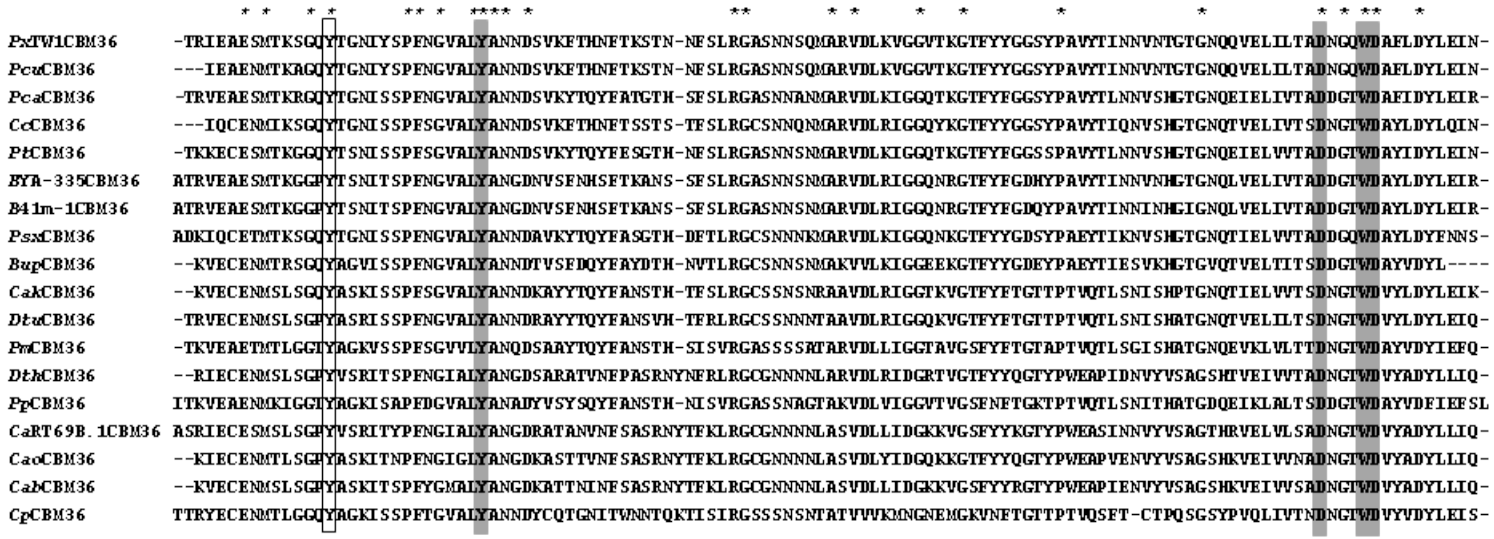

Figure 4. Multiple sequence alignment of CBM36 domains

The alignment was generated using BLASTp with *Px*TW1CBM36 as the query against the NCBI protein database. Conserved residues within the CBM36 family are marked with asterisks (*). Grey highlights indicate amino acid residues implicated in calcium-dependent ligand binding. An open box indicates the aromatic residue that contributes to the second sugar-binding subsite. The sequences are as follows: *Px*TW1CBM36 from *P. xylaniclasticus* TW1 xylanase, *Pcu*CBM36 from *P. curdlanolyticus* B-6 xylanase 11A (GenBank ID: ADB54799.1), *Pca*CBM36 from *P. campinasensis* G1-1 xylanase (GenBank ID: AEI54132.1), *Cc*CBM36 from *Clostridium clariflavum* DSM19732 GH11 (GenBank ID: YP_005047554.1), *Pt*CBM36 from *P. terrae* HPL-003 xylanase/chitin deacetylase (GenBank ID: YP_005077359.1), *B*YA-335CBM36 from *Bacillus* sp. YA-335 endo-1,4-β-xylanase (GenBank ID: CAA41784.1), *B*41m-1CBM36 from *Bacillus* sp. 41m-1 XynJ (GenBank ID:2DCJ¬_A), *Psx*CBM36 from *Pseudobutyrivibrio* xylanivoransxylanase/deacetylase (GenBank ID: P83513.2), *Bup*CBM36 from *Butyrivibrioproteoclasticus* B316 endo-1,4-β-xylanase (GenBank ID: YP_003830062.1), *Cak*CBM36 from *Caldicellulosiruptor kronotskyensis* 2002 endo-1,4-β-xylanase (GenBank ID: YP_004022816.1), *Dtu*CBM36 from *Dictyoglomusturgidum* DSM 6724 endo-1,4-β-xylanase (GenBank ID: YP_002352183.1), *Pm*CBM36 from *P. mucilaginosus* KNP414 XynD (GenBank ID: YP_004642759.1), *Dth*CBM36 from *D. thermophilum* H-6-12 endo-1,4-β-xylanaseA (GenBank ID: YP_002251776.1), *Pp*CBM36 from *P. polymyxa* ATCC 842 Xyn43A (GenBank ID: P45796.1), *Ca*RT69B.1CBM36 from *Caldicellulosiruptor* sp. Rt69B.1 XynD (GenBank ID: AAB95327.1), *Cao*CBM36 from *Ca. owensensis* OL endo-1,4-β-xylanase (GenBank ID: YP_004001463.1), *Cab*CBM36 from *Ca. bescii* DSM 6725 endo-1,4-β-xylanase (GenBank ID: YP_002572022.1) and *Cp*CBM36 from *C. phytofermentans* ISDg polysaccharide deacetylase (GenBank ID: YP_001558798.1).

Binding of CBM36 from P. xylaniclasticus TW1 to sweet potato root cell walls

The ability of CBM36 to bind plant cell walls was evaluated using CFP-tagged recombinant protein (r*Px*TW1CBM36-CFP) applied to root sections of sweet potato. Fluorescence analysis showed that r*Px*TW1CBM36-CFP displayed a clear cell wall recognition pattern, specifically binding strongly to the epidermal tissue of sweet potato roots (Figure 5B).

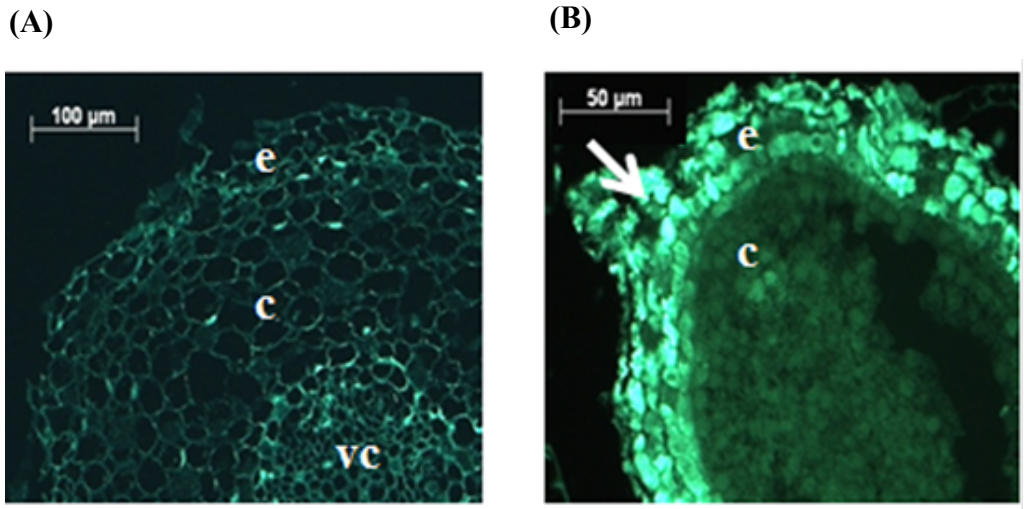

Figure 5. Binding of r*Px*TW1CBM36-CFP to sweet potato root cell walls. (A) the plant cell walls were non-labeled, whereas (B) the cell walls were labeled with r*Px*TW1CBM36-CFP; Arrows indicate recognition of epidermal tissues. The regions corresponding to epidermis (e), cortex (c) and vascular cylinder (vc) were indicated

Discussion

All twelve SDS-PAGE bands of the isolated cellulose-bound proteins were characterized through MALDI-TOF/TOF MS analysis. The peptide mass fingerprints of the unknown components from SDS-PAGE closely matched those of proteins with unknown functions. This suggests that these proteins may represent new families of cellulose-bound proteins. It is anticipated that the findings from this study, along with future research, will contribute to a better understanding of these proteins. Among them, only CBP12 was identified as xylanase, showing similarity to *P. curdlanolyticus* B-6 xylanase, which belongs to glycoside hydrolase family 11 and contains a CBM family 36 (GenBank no. gi|283970946; http://www.ncbi.nlm.nih.gov/BLAST/).

The family 36 CBM of CBP12 shares high homology with CBM36 from *P. curdlanolyticus* B-6 (98% identity, GenBankID: ADB54799.1), *P. campinasensis* G1-1 (79% identity, GenBank ID: AEI54132.1) and *Paenibacillus* sp. Aloe-11 (76% identity, GenBank ID: ZP_09772596.1), respectively. However, no characteristics of CBM36 from these bacteria were reported. Until the present, only CBM36 from *P. polymyxa* ATCC 842 xylanase 43A (GenBank ID: P45796.1) was characterized, showing the calcium-dependent binding of xylans and xylooligosaccharides (Jamal-Talabani *et al*., 2004). In addition, CBM36 from *P. xylaniclasticus* TW1 showed only 50% amino acid sequence similarity with CBM36 of the strain ATCC 842. Consequently, CBM36 of CBP12 from the strain TW1 was interesting to be further studied.

The ligand specificity of CBM36 from *P. xylaniclasticus* TW1 showed tight binding to ball-milled cellulose, as well as to both birchwood and oat-spelt xylans. It bound moderately to sugar beet arabinan (α-linked arabinan) and weakly to laminarin (β-1,3-glucan), tamarind seed xyloglucan, methylcellulose, and lichenan. However, r*Px*TW1CBM36-CFP was unable to bind to barley β-glucan. Despite barley β-glucan being a mixed-linkage β-1,3-1,4-glucan with a structure similar to lichenan, it is generally categorized as having more β-1,3-linked cellotetraosyl units than lichenan (Wood *et al*., 1994). These findings imply that the frequency and composition of substituted groups may influence the binding ability of CBM36. Additionally, the results revealed that r*Px*TW1CBM36-CFP showed relatively tight binding to xylans from different sources, similar to the ligand binding observed in CBM36 of xylanase 43 from *P. polymyxa* ATCC 842 (Jamal-Talabani *et al*., 2004). However, r*Px*TW1CBM36-CFP also demonstrated the ability to bind to cellulose and other carbohydrates, in addition to xylans. Therefore, CBM36 from *P. xylaniclasticus* TW1 exhibits broader binding specificity compared to CBM36 from *P. polymyxa* ATCC 842.

In addition, Jamal-Talabani and coworkers (2004) reported that the CBM36 of *P. polymyxa* ATCC 842 is a calcium-dependent type. Due to the low amino acid sequence similarity (50%), the binding properties of CBM36 from *P. xylaniclasticus* TW1 probably differ from those of the strain ATCC 842. To investigate ligand recognition, the ability of CBM36 from *P. xylaniclasticus* TW1 to bind to insoluble carbohydrates in the presence of EDTA was examined. It was apparent that r*Px*TW1CBM36-CFP still bound to both insoluble cellulose and insoluble xylan. These results indicated that the chelating agent had minimal effects on the binding of r*Px*TW1CBM36-CFP to both insoluble substances. Carbohydrate binding remained strong even when a high concentration of EDTA was added to the protein. Furthermore, these observations also showed that

r*Px*TW1CBM36-CFP had a high binding affinity for both insoluble xylan and cellulose. The ligand specificity of CBMs typically corresponds to the polysaccharide substrate that their linked catalytic domains target (Carvalho *et al*., 2004). CBM36 domains are predominantly associated with xylanases, suggesting that xylan is a biologically relevant ligand for this CBM family (see: http://www.cazy.org/CBM36_all.html). Therefore, the ability of the CBM36 from *P. xylaniclasticus* TW1 to bind both xylan and cellulose is particularly unusual. Moreover, the role of calcium in CBM function has been reported. In fact, calcium-dependent CBMs interact with two or three sugars but cannot bind to four or more sugar ligands. A critical role for calcium appears to be the stabilization of tight binding interactions between CBMs and internal carbohydrate ligand regions, mainly via charged interactions between dipoles (Fersht *et al*., 1985). This mechanism has been demonstrated in several CBMs, including CBM4-2 from *Rhodothermus marinus* xylanase 10A (Abou-Hachem *et al*., 2002), CBM36 from *P. polymyxa* ATCC 842 xylanase 43A (Jamal-Talabani *et al*., 2004), and CBM60 from *Cellvibrio japonicus* (Montanier *et al*., 2010). In sharp contrast, however, r*Px*TW1CBM36 still bound to insoluble carbohydrates in the presence of 50 mM EDTA. Thus, it is possible that the ligand recognition site of CBM36 from *P. xylaniclasticus* TW1 differs from that of strain ATCC 842. Ligand recognition by *Px*TW1CBM36 persisted even after the addition of chelating agents.

In vitro analyses demonstrated that *Px*TW1CBM36 exhibits high affinity for both xylan and cellulose. The findings suggest that *Px*TW1CBM36 could recognize both xylan and cellulose, the main polysaccharides found in plant cell walls. Consistently, r*Px*TW1CBM36-CFP was shown to bind specifically to the epidermal tissue of sweet potato roots, likely reflecting structural variation in cell wall composition and carbohydrate architecture within different plant tissues (O'Neill and York, 2003). The considerable diversity in CBM ligand specificity across different families is well-known. This enables the targeting of their associated catalytic modules to distinct areas within the plant cell wall (McCartney *et al*., 2006). Although it is relatively uncommon for xylanases to possess multiple xylan-binding CBMs from different families, such arrangements have been reported, for example, CBM6 and CBM22 in *Caldicellulosiruptor* sp. Rt69B.1 (Morris *et al*., 1999) and CBM6 and CBM36 in *P. polymyxa* ATCC 842 (Jamal-Talabani *et al*., 2004). In contrast, many xylanases harbor both xylan- and cellulose-binding CBMs, which allow them to interact with heterogeneous plant cell wall environments (Waeonukul *et al*., 2009). These results support the view that individual xylanases are evolutionarily adapted to target specific plant cell wall architectures in nature. The recognition profile of r*Px*TW1CBM36-CFP within plant cell walls likely reflects regions

where target polysaccharides are more accessible to the catalytic domain. Its specific association with the epidermal tissue of sweet potato roots suggests that this region contains a concentrated or structurally available pool of xylan and/or cellulose that can be recognized by the CBM. Crucially, this selective binding may enhance enzymatic hydrolysis by bringing the catalytic module closer to its substrates. Through its dual affinity for xylan and cellulose, *Px*TW1CBM36 may facilitate the stable localization of its cognate xylanase on the plant cell wall surface, enhancing catalytic efficiency by anchoring the enzyme at strategic sites within the complex plant matrix.

In conclusion, this study characterizes a family 36 of CBM from *P. xylaniclasticus* TW1 that predominantly targets xylan, likely facilitating the localization of its appended catalytic module to highly substituted regions of the polysaccharide. Notably, r*Px*TW1CBM36-CFP exhibited a strong and specific binding affinity for the epidermal tissue of sweet potato roots, suggesting a selective capacity to direct catalytic activity to targeted locations on the plant cell wall. Thus, the specificity presented by this CBM could represent a valuable tool for probing the elaborate organization of plant cell walls and for guiding enzymes to precise sites on plant tissue surfaces.

Acknowledgements

This study is an outcome of the program supported by the 2005 World Exposition in Aichi, Japan and implemented by the International Center for Environmental Technology Transfer (ICETT). The program was hosted by the Advanced Fusion Technology Unit, Graduate School of Regional Innovation Studies, Mie University. The program titled as "Project on Invitation to Japan for Environmental Research (PIER)" invites research fellows from developing countries to extend their studies more in cooperation with advice of Japanese Institutes in the area of waste management and recycling issues. The authors would also like to acknowledge the support of the King Mongkut's University of Technology Thonburi, Thailand, through the Higher Education Research Promotion and National Research University Project of Thailand, Office of Higher Education Commission. Additional support was provided by the Thailand Research Fund (TRF Grant) and King Mongkut's Institute of Technology Ladkrabang.

The authors wish to express their gratitude to Prof. Dr. Shuichi Karita and Prof. Dr. Tohru Tsuchiya for their assistance in MALDI-TOF/TOF MS and analytical works and CBM-labeling analysis. We also thank Ms. Julie Koldewyn for carefully proofreading the manuscript of this work.

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(Received: 28 September 2024, Revised: 20 June 2025, Accepted: 1 July 2025)