การทำให้บริสุทธิ์และศึกษาสมบัติในการยึดเกาะของ xylan-binding endoxylanase ขนาดโมเลกุลเล็ก (29 กิโลดาลตัน) จาก *Bacillus circulans* B-6

ภัทรา ผาสอน[1] รัตติยา แววนุกูล[1] กนก รัตนะกนกชัย[2*] และ คิน เลย์ คู[3]

มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี บางมด ทุ่งครุ กรุงเทพ 10140

รับเมื่อ 6 มิถุนายน 2548 ตอบรับเมื่อ 17 ตุลาคม 2548

บทคัดย่อ

Bacillus circulans B-6 ผลิต extracellular xylanolytic และ cellulolytic enzymes เมื่อเพาะเลี้ยงในอาหารที่มีไซแลนเป็นแหล่งคาร์บอน ไซลาเนสที่ยึดเกาะกับไซแลนที่ไม่ละลายน้ำสามารถทำให้บริสุทธิ์โดยอาศัยเทคนิคการยึดเกาะและหลุดออกอย่างจำเพาะกับไซแลนที่ไม่ละลายน้ำ ไซลาเนสบริสุทธิ์มีขนาด 29 กิโลดาลตัน ซึ่งแสดงออกเพียงกิจกรรมของไซลาเนส เมื่อศึกษาสมบัติของไซลาเนสบริสุทธิ์ พบว่ามีเสถียรภาพสูงในช่วงพีเอช 6.0 ถึง 7.0 และอุณหภูมิที่ต่ำกว่า 40 องศาเซลเซียส มีพีเอชและอุณหภูมิที่เหมาะสมในการทำงานที่พีเอช 7.0 และ 60 องศาเซลเซียส ตามลำดับ เอนไซม์บริสุทธิ์ย่อยไซแลนได้ไซโลโอลิโกแซ็กคาไรด์ที่มีขนาดเล็กต่างๆ เป็นผลิตภัณฑ์ แสดงว่าจัดอยู่ในกลุ่มเอนโดไซลาเนส xylan-binding endoxylanase ย่อยไซแลนที่ไม่ละลายน้ำซึ่งมีโซ่กิ่งน้อยจาก birch wood ได้ดีกว่า larch wood และ oat spelt xylans ไซลาเนสบริสุทธิ์สามารถยึดเกาะกับไซแลนและอะไวเซลที่ไม่ละลายน้ำ ซึ่ง พีเอช น้ำตาล และไอออนโลหะมีผลต่อการยึดเกาะระหว่างเอนไซม์กับพอลิแซ็กคาไรด์ที่ไม่ละลายน้ำ

คำสำคัญ : การยึดเกาะและหลุดออกกับไซแลนที่ไม่ละลายน้ำ / *Bacillus circulans* B-6 / การทำให้ไซลาเนสบริสุทธิ์ / Xylan-binding endoxylanase

---

**Corresponding author: E-mail: khanok.rat@kmutt.ac.th*

[1] นักศึกษาระดับบัณฑิตศึกษา สายวิชาเทคโนโลยีชีวเคมี คณะทรัพยากรชีวภาพและเทคโนโลยี

[2] รองศาสตราจารย์ สายวิชาเทคโนโลยีชีวเคมี คณะทรัพยากรชีวภาพและเทคโนโลยี

[3] ผู้ช่วยศาสตราจารย์ สายวิชาเทคโนโลยีชีวเคมี คณะทรัพยากรชีวภาพและเทคโนโลยี

Purification and Binding Properties of a Low Molecular Weight Xylan-Binding Endoxylanase (29 kDa) from *Bacillus circulans* B-6

Patthra Pason[1], Rattiya Waeonukul[1], Khanok Ratanakhanokchai[2*], and Khin Lay Kyu[3]

King Mongkut's University of Technology Thonburi, Bangmod, Toongkru, Bangkok 10140

Received 6 June 2005 ; accepted 17 October 2005

Abstract

Bacillus circulans B-6 produced extracellular xylanolytic and cellulolytic enzymes when grown in a medium containing xylan as a carbon source. Purification of xylan-binding xylanase was performed from the culture medium by specific adsorption and desorption on insoluble xylan. The molecular mass of purified xylanase was estimated to be 29 kDa, which showed only xylanase activity. The enzyme was stable at pH 6.0 to 7.0 and temperature up to 40 °C. The pH and temperature optima were 7.0 and 60 °C, respectively. The purified enzyme hydrolyzed xylan to a series of short-chain xylooligosaccharides as the product, indicating that the enzyme was an endoxylanase. The xylan-binding endoxylanase could effectively hydrolyze low substituted insoluble xylan of birch wood more than larch wood and oat spelt xylans. The purified enzyme bound to insoluble xylan and avicel but the binding was affected by pH, sugars and metal ions.

Keywords : Adsorption and Desorption on Insoluble Xylan / *Bacillus circulans* B-6 / Purification of Xylanase / Xylan-binding Endoxylanase

**Corresponding author: E-mail: khanok.rat@kmutt.ac.th*

[1] Graduate Student, Division of Biochemical Technology, School of Bioresources and Technology.

[2] Associate Professor, Division of Biochemical Technology, School of Bioresources and Technology.

[3] Assistant Professor, Division of Biochemical Technology, School of Bioresources and Technology.

1. Introduction

β-Xylanase (EC 3.2.1.8) hydrolyzes β-1,4-glycosidic linkages within the xylan backbone to yield of short chain xylooligosaccharides with different length. Xylan-degrading enzymes have attracted much attention because of their important practical applications in various industrial processes [1], including improving the digestibility of animal feedstocks [2], the modification of cereal-based foodstuffs [3], and the delignification of paper pulp [4]. Recently, 99 glycoside hydrolase families exist (see the carbohydrate-active enzyme CAZY server at http://afmb.cnrs-mrs.fr/~cazy/CAZY/) and showed 6 families of xylanase at 5, 7, 8, 10, 11 and 43. In general, xylanases are composed of a catalytic domain, and are divided into two major families (F/10 and G/11) based on amino acid sequence similarities and they also have several non catalytic functional domains. Most of which are carbohydrate-binding domains (CBMs) [5].

The CBMs of plant cell wall hydrolases play an important role in the efficient hydrolysis of insoluble cellulosic substances [6 - 8]. The function of the CBM is largely to attach the enzyme to its substrate, and therefore it enhances the rate of catalysis by increasing the probability of enzyme/substrate interaction [9]. Enzyme containing CBM was able to purify by using its substrate [10]. Binding is mediated via several co-planar, solvent-exposed aromatic rings and hydrophobic surfaces that from stacking interactions with the sugars in the polysaccharide and also through hydrogen bond [11]. CBMs are utilized in many different applications such as biospecific affinity purification [5, 8, 12] and as an affinity tag which is economical support-matrix (insoluble polysaccharide) for large-scale protein purification or protein immobilization. In this study, we describe the purification and binding properties of the xylan-binding endoxylanase from Bacillus circulans B-6 to apply in efficiently hydrolysis of insoluble xylans.

2. Materials and methods

2.1 Bacterial strain and culture medium

Bacillus circulans B-6 was isolated from an anaerobic digester fed with pineapple waste [13]. The culture medium used was Berg's mineral salt medium [14] supplemented with 0.5% xylan. The culture was incubated in the incubator shaker at 200 rpm and 37 °C for 3 days.

2.2 Enzyme assays

The xylanase activity was measured by determining the amount of reducing sugar released from oat spelt xylan (Sigma-Aldrich Inc.). The reaction mixture consisted of 0.5 ml of 1% oat spelt xylan in 100 mM Tris-HCl buffer (pH 7.0) and 0.1 ml enzyme [15]. After incubation for 10 min at 50 °C, reducing sugar was determined by using the Somogyi-Nelson method with xylose as a standard [16].

One unit of the xylanase activity was defined as that amount of enzyme that liberated 1 μmole of reducing sugars in 1 min under the above conditions.

Cellulase activity was measured under the same condition as described above with carboxymethyl cellulose (Sigma-Aldrich Inc.) as a substrate and glucose as a standard.

2.3 Protein determination

Protein concentration was determined by the method of Lowry *et al.* [17]

2.4 Preparation of insoluble and soluble polysaccharides

The preparation of insoluble polysaccharides was performed by the method of Ghangas *et al.* [18]. 10 g of commercial polysaccharides (birch wood, larch wood or oat spelt xylan (Sigma-Aldrich Inc.) or avicel (Fluka Chemika)) was suspended in 200 ml of distilled water and adjusted to pH 10.0 by 1 M NaOH. Then, the mixture was stirred for 60 min at room temperature, and centrifuged for 10 min at 10,000 rpm. After centrifugation, the supernatant was adjusted to pH 7.0 by 1 M acetic acid, then lyophilized and kept at -20 °C (soluble polysaccharides). The precipitate after centrifugation was suspended in 200 ml of distilled water and adjusted to pH 7.0 by 1 M acetic acid. Then the precipitate was filtered by Whatman No.1 and washed many times with distilled water before lyophilized (insoluble polysaccharides).

2.5 Binding assay

Binding assays was conducted by the method of Irwin *et al.* [19]. The crude or purified enzyme and 2% insoluble oat spelt xylan or avicel in 100 mM phosphate buffer saline (150 mM NaCl) were shaken periodically at 4 °C for 30 min before centrifugation. The amount of the protein remained in the supernatant was determined by the standard protein determination method. The protein lost from the supernatant was assumed to be the protein bound.

2.6 Purification of xylan-binding xylanase

Purification method was modified by the method of Ratanakhanokchai et al. [10]. The culture supernatant was used as a source of xylan-binding xylanase. Two steps of affinity chromatography were used. In the first step, 25 mg of protein from the culture supernatant was incubated with 2% insoluble oat spelt xylan in 10 ml of 250 mM phosphate buffer (pH 7.0) containing 100 mM NaCl (buffer A) and shaken periodically at 4 °C for 30 min. The xylan-bound protein complex was washed ten times with the same volume of buffer A and then eluted with 1% triethylamine (TEA). The eluted enzyme was dialyzed, assayed for xylanase activity and used for another step of affinity chromatography. In the second step, the same method was conducted

using 2% insoluble oat spelt xylan in 10 ml of 100 mM phosphate buffer (pH 7.0) containing 50 mM NaCl (buffer B), instead of buffer A for the protein (4.2 mg) from the first step of purification. The unbound protein and bound protein were collected and dialyzed for analysis.

2.7 Gel electrophoresis and zymogram analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli *et al.* [20]. Xylanase zymogram was prepared as described previously [10] by adding 0.1% soluble xylan in the gel. Carboxymethyl cellulase (CMCase) zymogram was prepared in the same manner using carboxymethyl cellulose (CMC) as a substrate.

2.8 Hydrolysis of insoluble xylan

Insoluble xylans of birch wood, larch wood and oat spelt were hydrolyzed by the purified enzyme. Each substance (2% dry weight) was hydrolyzed with 0.2 U of the enzyme at pH 7.0 and 50 °C. After incubation, samples were removed and the amount of reducing sugars produced was determined by Somogyi-Nelson method [16].

2.9 Analysis of xylan hydrolysis product

Xylan hydrolysis products from 2.8 were determined by thin-layer chromatography on silica gel 60 F254 plates (Merck 1.05554; 20 by 20 cm) with a mixture of *n*-butanol-acetic acid-water (2:1:1) as a solvent system [21]. The sugar spots were detected by heating the plates to 100 °C after spraying them with 4 g of α - diphenylamine, 4 ml aniline, 200 ml of acetone and 30 ml of 80% phosphoric acid.

2.10 Effect of pH on binding of enzyme to insoluble substrate

The binding assay was measured under the same conditions as described previously, using various pHs. The pHs were adjusted with the following buffer systems: acetate buffer (pH 4.0 to 6.0), phosphate buffer (pH 6.0 to 7.0) and Tris-HCl buffer (pH 7.0 to 9.0).

2.11 Effect of sugar on binding of enzyme to insoluble substrate

The binding assay was measured under the same conditions as described above, adding various sugars (25 mM) such as xylose, glucose, cellobiose, galactose, arabinose, mannose and sucrose.

2.12 Effect of metal ion on binding of enzyme to insoluble substrate

The purified enzyme solution was mixed with the final concentration of 10 mM ethylenediamine-tetraacetic acid (EDTA) and dialyzed with 10 mM Tris-HCl buffer pH 7.0. Then the

EDTA treated enzyme solution was preincubated in a mixture containing various metal ions (1 mM) at 30 °C for 1 hr. After that the binding of enzyme to insoluble xylan and avicel was measured under the standard assay condition as described above.

3. Results and discussion

3.1 Enzyme production by *Bacillus circulans* B-6

Bacillus circulans B-6 produced the extracellular xylanolytic and cellulolytic enzymes when cultivated in the xylan medium. The crude enzyme showed xylanase and CMCase activities at 7.19 and 0.12 U/mg protein, respectively. Xylanase from crude enzyme was found to have binding ability to insoluble substances such as insoluble xylan and avicel at 77% and 68%, respectively.

3.2 Purification by affinity chromatography

A summary of the purification results is given in Table 1. The 29 kDa xylan-binding xylanase was purified about 1.8-fold and the specific activity of the purified enzyme was 2.8 U/mg of protein.

Table 1 Purification of xylan-binding endoxylanase (29 kDa) from *B. circulans* B6

Purification step	Total protein (mg)	Total xylanase activity (U)	Specific xylanase activity (U/mg protein)	Purification (fold)	Yield (%)
Culture supernatant	25.0	40.0	1.6	1.0	100.0
Affinity chromatography					
First step	4.2	13.3	3.2	2.0	33.2
Second step	2.7	7.5	2.8	1.8	18.8

Xylan-binding xylanase with the molecular weight at 29 kDa was purified from the culture medium by 2 steps of affinity chromatography. In the first step, buffer A was used for equilibrating and washing. Proteins were eluted with 1% TEA from xylan bound protein complex and it showed 2 protein bands on SDS-PAGE (Fig. 1 A, lane 2) and 2 xylanase bands on xylanase zymogram (Fig. 1 B, lane 2) with the molecular weights of 29 and 48 kDa, but no clear zone on CMCase zymogram, indicating that these two proteins had only xylanase activity. After that 2 proteins were separated by affinity chromatography with buffer B. The xylan-binding xylanase (29 kDa) appeared

in the unbound fraction. It showed single protein band on SDS-PAGE (Fig. 1 A, lane 3) and only one band on zymogram for xylanase activity (Fig. 1 B, lane 3), while another xylan-binding xylanase (48 kDa) appeared in bound fraction. This result indicated that xylan-binding xylanase with molecular weight at 29 and 48 kDa have different xylan-binding capacity. The 29 kDa xylan-binding xylanase requires lower hydrophobic interaction to bind to insoluble xylan than the 48 kDa xylan-binding xylanase. Sun *et al.* [11] found that the binding is mediated via several co-planar, solvent-exposed aromatic rings and hydrophobic surfaces, that from stacking interactions with the sugars in the polysaccharide and also through hydrogen bonding. The binding on xylan was also clearly sensitive to ionic strength. Thus increasing buffer concentration increased the binding of xylanase due to hydrophobic interaction [22]. This report describes the study on the 29 kDa xylan-binding xylanase because of its low molecular weight which could efficiently diffuse into the structure of insoluble substrate to hydrolyze. It is also good for application in protein purification as a tag protein when compared with the 48 kDa xylan-binding xylanase.

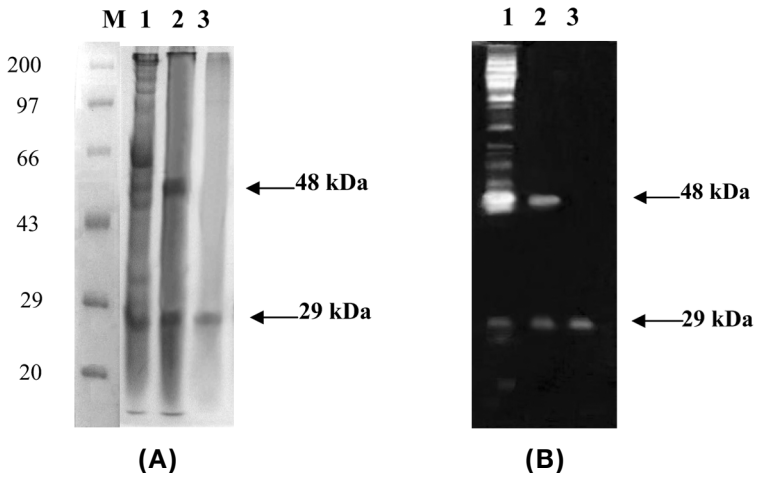

Fig. 1 SDS-PAGE and zymogram of the purified xylan-binding xylanase by adsorption-desorption on insoluble xylan. (A) SDS-PAGE (10% acrylamide gel dyed with Coomassie brilliant blue R-250. (B) Zymogram for xylanase activity (10% acrylamide gel dyed with Congo red); Lane M = standard molecular weight, Lane 1 = culture supernatant (crude enzyme) (50 μg of protein), Lane 2 = bound enzyme in the first step (20 μg of protein), Lane 3 = unbound enzyme in the second step (10 μg of protein).

3.3 Effect of pH on activity and stability

The effect of pH on xylanase activity of the purified xylan-binding xylanase was determined. The optimum pH of the enzyme was 7.0. The stability of enzyme was determined by

incubating the enzyme in the buffer of pH 4-10 at 50 ˚C for 30 min and the residual xylanase activity was determined. It showed that the stability of the enzyme was lost more than 22% at pH lower than 6.0 and 56% at pH higher than 8.0 (Fig. 2).

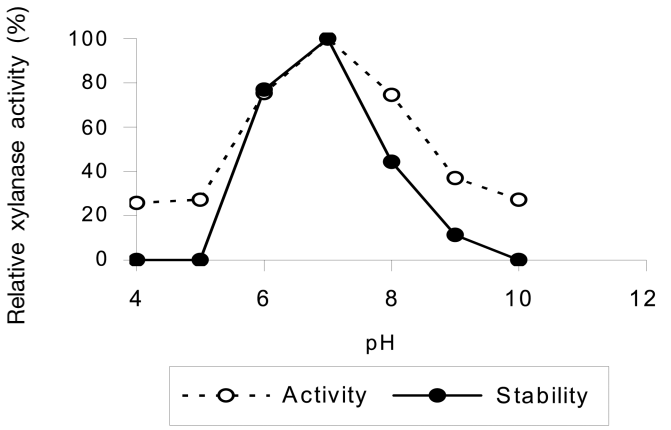

Fig. 2 Effect of pH on activity and stability of the purified xylan-binding xylanase of *Bacillus circulans* B-6. The reaction pH values were adjusted with the following buffer systems: acetate buffer (pH 4.0 to 6.0), phosphate buffer (pH 6.0 to 7.0) and Tris-HCl buffer (pH 7.0 to 9.0).

3.4 Effect of temperature on activity and stability

The optimum temperature of the purified xylan-binding xylanase was determined by varying the reaction temperatures at pH 7.0. The enzyme had an optimum temperature of 60 ˚C (Fig. 3). The thermal stability of the purified enzyme was measured by incubating it at pH 7.0 for 30 min at different temperatures ranging from 30-80 ˚C. The residual activity was determined by the standard assay. The enzyme was stable at temperatures up to 40 ˚C. Below 60 ˚C, the enzyme showed more than 50% of the activity.

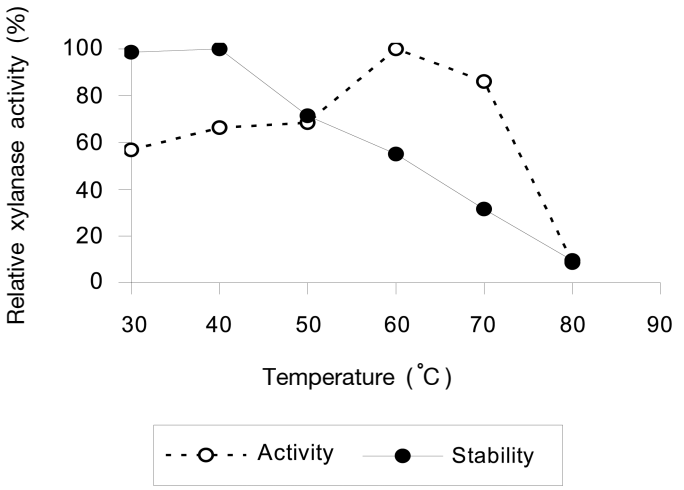

Fig. 3 Effect of temperature on the xylanase activity and stability of the purified xylan-binding xylanase from *Bacillus circulans* B-6.

3.5 Hydrolysis of insoluble xylan

The hydrolysis of insoluble xylans by the purified xylan-binding xylanase (0.2 U) is shown in Fig. 4. The purified enzyme was more effective on hydrolysis of the insoluble xylan from birch wood than those of larch wood and oat spelt xylans. Oat spelt xylan is more substituted, compared to larch wood and birch wood xylans [23]. Therefore, the enzyme prefers to cleave low branch insoluble xylan. The purified enzyme could hydrolyze insoluble xylan due to its xylan-binding ability. Xylan-binding region binds to the insoluble xylan and promotes the hydrolysis of the insoluble substrate [6], suggesting that the substrate-binding domain plays an important role in hydrolysing the insoluble substrate [24, 25].

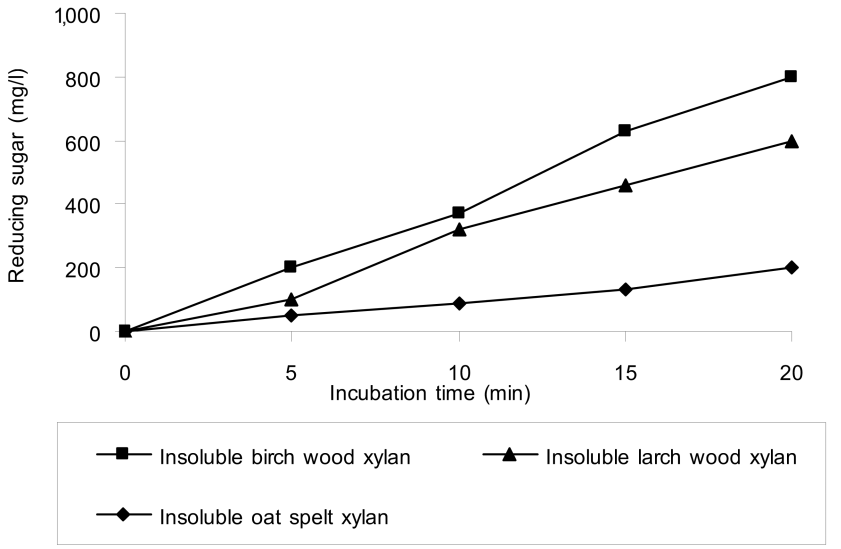

Fig. 4 Hydrolysis of insoluble xylans by the purified xylan-binding xylanase

3.6 Thin-layer chromatography

After insoluble birch wood xylan was incubated with the purified enzyme, the hydrolysis products were analyzed by thin-layer chromatography. The hydrolysis products were a series of short-chain xylooligosaccharides and the smallest product was xylobiose (Fig. 5), indicating that the purified enzyme was an endo type (endoxylanase).

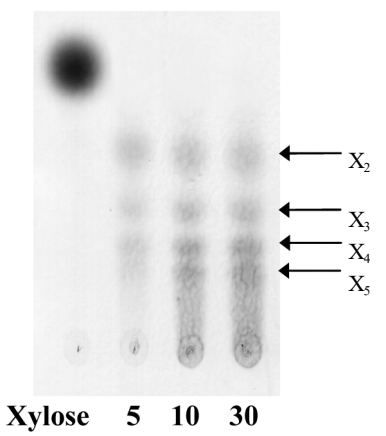

Fig. 5 Thin-layer chromatography of the hydrolysis products of insoluble birch wood xylan with the purified enzyme after incubation for 5, 10 and 30 min.

3.7 Effect of sugars on the binding of the xylan-binding endoxylanase to insoluble polysaccharides

The purified xylan-binding endoxylanase has not only xylan-binding ability but it has also cellulose-binding ability. The effect of sugar on the binding of the purified enzyme to insoluble xylan and avicel (microcrystalline cellulose) were determined using various sugars (Table 2). The binding was affected by all kinds of sugar tested, especially xylose and glucose which are the end hydrolysis products of xylan and avicel, respectively. Decreasing the binding of enzyme to substrates by sugars may be due to the competition of sugars to bind to binding region of the enzyme, reducing binding capacity of the enzyme to insoluble substrates [11, 22].

Table 2 Effect of sugars on the binding of xylan-binding endoxylanase to insoluble polysaccharides

Sugar (25 mM)	% Relative binding	
	Xylan	Avicel
Control	100.00	100.00
Sucrose	96.29	97.45
Mannose	94.97	91.72
Arabinose	94.97	73.25
Galactose	74.84	60.51
Cellobiose	68.55	64.33
Glucose	68.55	40.13
Xylose	65.41	52.87

3.8 Effect of pH on the binding of the xylan-binding endoxylanase to insoluble polysaccharides

Jeffries *et al.* [26] found that the binding of enzyme decreased when pH increased, due to the changes of the conformation of enzyme. The high pH affected to enzyme conformation and its binding ability was lost. In our case, the binding of the purified enzyme to insoluble xylan and avicel was also decreased when pH increased (Fig. 6). It was also found that the binding of enzyme to avicel was higher than insoluble xylan because the enzyme could not hydrolyze avicel, thus no reducing sugars produced. The increase in hydrolyzate sugars was found to decrease the binding of xylanase to insoluble xylan [11].

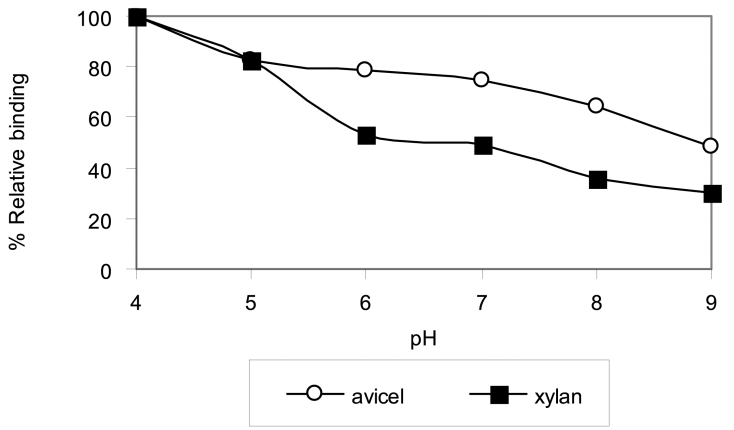

Fig. 6 Effect of pH on the binding of the purified enzyme to insoluble polysaccharides

3.9 Effect of metal ion and EDTA on the binding of the xylan-binding endoxylanase to insoluble polysaccharides

The binding of the enzyme to insoluble xylan and avicel was examined in the presence of various metal ions at the final concentration of 1 mM. As shown in Table 3, the bindings of purified enzyme to both insoluble xylan and avicel were decreased by 10 mM EDTA. Binding ability of the enzyme to insoluble xylan and avicel was reduced at 37.61 and 23.95%, respectively when EDTA was present. It revealed that metal ion was important for binding of the enzyme. However, the binding of purified enzyme to insoluble xylan was decreased by all metal ions tested. It may need other kinds of metal ion. Whereas, the binding of the purified enzyme to avicel was increased by $FeSO_4$ and $CuSO_4$.

Table 3 Effect of metal ions on the binding of xylan-binding endoxylanase to insoluble polysaccharides

Metal ion (1 mM)	% Relative binding	
	Xylan	Avicel
None (-EDTA)	100.00	100.00
None (+EDTA)	62.39	76.05
$FeSO_4$	92.98	133.08
$CaSO_4$	83.19	93.52
$MgSO_4$	80.13	90.43
$CuSO_4$	71.57	119.72
$ZnSO_4$	31.81	86.83

The binding of the purified xylan-binding endoxylanase from *Bacillus circulans* B-6 to insoluble xylan and avicel was decreased by sugars, pH and metal ions. Similarly, the xylanase C from *Bacillus* sp. strain BP-B was eluted from insoluble substrate by 0.2 M glucose, adjusting pH to 9.0 and in the presence of $ZnSO_4$ [27].

4. Conclusion

The low molecular weight xylan-binding endoxylanase (29 kDa) from *Bacillus circulans* B-6 was purified to homogeneity by two steps of affinity chromatography with insoluble xylan. The pH and temperature optima of the purified enzyme were 7.0 and 60 °C, respectively. Due to its xylan-binding ability, the purified enzyme was capable of degrading insoluble xylan especially low substituted insoluble xylan. The binding of the purified enzyme to insoluble xylan and avicel were affected by pH, sugars and metal ions. Therefore, the low molecular weight xylan-binding endoxylanase which has xylan and cellulose-binding ability can be used for applications in hydrolysis of insoluble xylan present in agricultural wastes and in protein purification as a tag protein. The best conditions from each parameter will be selected for using as affinity tag in further study.

5. Acknowledgements

This work was supported by Royal Golden Jubilee Ph.D. program of the Thailand Research Fund and National Center for Genetic Engineering and Biotechnology (BIOTEC) grant.

6. References

1. Wong, K.K.Y., Tan, L.U.L., and Saddler, J.N., 1988, "Multiplicity of β-1,4-Xylanase in Microorganisms: Functions and Applications," *Microbiology Reviews,* Vol. 52, pp. 305-317.

2. Yin, Y.L., Baidoo, S.K., Jin, L.Z., Liu, Y.G., Schulze, H., and Simmins, P.H., 2001, "The Effect of Different Carbohydrase and Protease Supplementation on Apparent (Ideal and Overall) Digestibility of Nutrients of Five Hulless Barley Varieties in Young Pigs," *Livestock Production Science*, Vol. 71, pp. 109-120.

3. Law, B.A., 2002, "The Nature of Enzymes and Their Action in Foods," In: *Enzymes in Food Technology*, Whitehurst, R.J., and Law, B.A. (eds.), CRC Press, Florida, pp. 1-18.

4. Gübitz, G.M., Haltrich, D., Latal, B., and Steiner, W., 1997, "Mode of Depolymerization of Hemicellulose by Various Mannanases and Xylanases in Relation to Their Ability to Bleach Softwood Pulp," *Applied Microbiology and Biotechnology,* Vol. 47, pp. 658-662.

5. Tomme, P., Warren, R.A., Miller, Jr., R.C., Kilburn, D.G., and Gilkes, N.R., 1995, "Cellulose-Binding Domains: Classification and Properties," In: *Enzymatic Degradation of Insoluble Polysaccharides,* Saddler, J.N. and Penner, M. (eds.) American Chemical Society, Washington, pp. 142-163.

6. Black, G.W., Rixon, J.E., Clarke, J.H., Hazlewood, G.P., Ferreira, L.M.A., Bolam, D.N., and Gilbert, H., 1997, "Cellulose Binding Domains and Linker Sequences Potentiate the Activity of Hemicellulases Against Complex Substrates," *Journal of Biotechnology,* Vol. 57, pp. 59-69.

7. Millward-Sadler, S.J., Poole, D.M., Henrissat, B., Hazelwood, G.P., Clarke, J.H., and Gilbert, H.J., 1994, "Evidence for a General Role for High-Affinity Non-Catalytic Cellulose Binding Domains in Microbial Plant Cell Wall Hydrolases," *Molecular Microbiology,* Vol. 11, pp. 375-382.

8. Levy, I. and Shoseyov, O., 2002. "Cellulose-Binding Domains Biotechnological Applications," *Biotechnology Advances,* Vol. 20, pp. 191-213.

9. Gill, J., Rixon, J.E., Bolam, D.N., McQueen-Mason, S., Simpson, P.J., Williamson, M.P., Hazlewood, G.P., and Gilbert, H.J., 1999, "The Type II and X Cellulose-Binding Domains of *Pseudomonas* Xylanase a Potentiate Catalytic Activity against Complex Substrates by a Common Mechanism," *Biochemical Journal*, Vol. 342, pp. 473-480.

10. Ratanakhanokchai, K., Kyu, K.L., and Tanticharoen, K, 1999, "Purification and Properties of a Xylan-Binding Endoxylanase from Alkaliphilic *Bacillus* sp. Strain K-1," *Applied and Environmental Microbiology,* Vol. 65, pp. 694-697.

11. Sun, J.L., Sakka, K., Karita, S., Kimura, T., and Ohmiya, K., 1998, "Adsorption of *Clostridium stercorarium* Xylanase A to Insoluble Xylan and the Importance of the CBDs to Xylan Hydrolysis," *Journal of Fermentation and Bioengineering,* Vol. 85, pp. 63-68.

12. Bayer, E.A., Morag, E., and Lamed, R., 1994, "The Cellulosome a Treasure-Trove for Biotechnology," *Trends in Biotechnology,* Vol. 12, pp. 379-386.

13. Tanticharoen, M. and Cheevadhanarak, S., 1984, "The Production of Cellulase and Xylanase from Cellulolytic Microorganisms Isolated from Pineapple Anaerobic Digester. II. Activities Studies," *Annual Report of ASEAN Working Group on the Management and Utilization of Food Waste Materials*, pp. 493-505.

14. Berg, B., Hofstan, B.V., and Petterson, G., 1972, "Growth and Cellulase Formation by *Cellvibrio fulvus," Journal of Applied Bacteriology,* Vol. 35, pp. 201-214.

15. Kyu, K.L., Ratanakhanokchai, K., Uttapap, D., and Tanticharoen, M., 1994, "Induction of Xylanase in *Bacillus circulans* B-6," *Bioresource Technology,* Vol. 48, pp. 163-167.

16. Nelson, N., 1944, "A Photometric Adaptation of the Somogyi Method for the Determination of Glucose," *Journal of Biological Chemistry*, Vol. 153, pp. 375-380.

17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., 1951, "Protein Measurement with the Folin Phenol Reagent," *Journal of Biological Chemistry,* Vol. 193, pp. 265-275.

18. Ghangas, G.S., Hu, Y.J., and Wilson, D.B., 1989, "Cloning of a *Thermomonospora fusca* Xylanase Gene and Its Expression in *Escherichia coli* and *Streptomyces lividans," Journal of Bacteriology,* Vol. 171, pp. 2963-2969.

19. Irwin, D., Jung, E.D., and Wilson, D.B., 1994, "Characterization and Sequence of a *Thermomonospora fusca* Xylanase," *Applied and Environmental Microbiology,* Vol. 60, pp. 763-770.

20. Laemmli, U.K., 1970, "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4," *Nature*, Vol. 227, pp. 680-685.

21. Kubata, B.K., Suzuki, J., Horitsu, H., Kawai, K., and Takamizawa, K., 1994, "Purification and Characterization of *Aeromonas caviae* ME-1 Xylanase V, which Produces Exclusively Xylobiose from Xylan," Applied and Environmental Microbiology, Vol. 60, pp. 531-535.

22. Tenkanen, M., Buchert, J., and Viikari, L., 1995, "Binding of Hemicellulases on Isolated Polysaccharide Substrates," *Enzyme and Microbial Technology,* Vol. 17, pp. 499-505.

23. Coughlan, M.P. and Hazlewood, G.P., 1993, "β-1,4-D-Xylan Degrading Enzyme Systems: Biochemistry, Molecular Biology and Applications," *Biotechnology and Applied Biochemistry,* Vol. 17, pp. 259-289.

24. Linder, M. and Teeri, T.T., 1997, "The Roles and Function of Cellulose-Binding Domains," *Journal of Biotechnology,* Vol. 57, pp. 15-28.

25. Subramaniyan, S. and Prema, P., 2002, "Biotechnology of Microbial Xylanases: Enzymology, Molecular Biology, and Application," *Critical Reviews in Biotechnology,* Vol. 22, pp. 33-64.

26. Jeffries, T.W., 1996, "Biochemistry and Genetics of Microbial Xylanases," *Current Opinion in Biotechnology,* Vol. 7, pp. 337-342.

27. Blanco, A., Diaz, P., Zueco, J., Parascandola, P., and Pastor, F.I.J., 1999, "A Multidomain Xylanase from a *Bacillus* sp. with a Region Homologous to Thermostabilizing Domains of Thermophilic Enzymes," *Microbiology,* Vol. 145, pp. 2163-2170.