J. Microbiol. Biotechnol. (2006), **16**(7), 1132–1138

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Purification and Characterization of Novel Bifunctional Xylanase, XynIII, Isolated from *Aspergillus niger* A-25

HONG-GE CHEN[1]*, XIN YAN[1], XIN-YU LIU[1], MING-DAO WANG[1], HUI-MIN HUANG[1], XIN-CHENG JIA[1], AND JIN-AN WANG[2]

[1]*College of Life Sciences, Henan Agricultural University, Zhengzhou 450002, P. R. China*
[2]*Laboratory of Catalysis and Materials, Superior School of Chemical Engineering and Industrial Extractives, National Polytechnic Institute, Mexico City 07738, Mexico*

Received: January 9, 2006
Accepted: March 28, 2006

Abstract Three types of xylanases (EC 3.2.1.8) were detected in the strain *Aspergillus niger* A-25, one of which, designated as XynIII, also displayed β-(1,3-1,4)-glucanase (EC 3.2.1.73) activity, as determined by a zymogram analysis. XynIII was purified by ultrafiltration and ion-exchange chromatography methods. Its apparent molecular weight was about 27.9 kDa, as estimated by SDS-PAGE. The purified XynIII could hydrolyze birchwood xylan, oat spelt xylan, lichenin, and barley β-glucan, but not CMC, avicel cellulose, or soluble starch under the assay conditions in this study. The xylanase and β-(1,3-1,4)-glucanase activities of XynIII both had a similar optimal pH and pH stability, as well as a similar optimal temperature and temperature stability. Moreover, the effects of metal ions on the two enzymatic activities were also similar. The overall hydrolytic rates of XynIII in different mixtures of xylan and lichenin coincided with those calculated using the Michaelis-Menten model when assuming the two substrates were competing for the same active site in the enzyme. Accordingly, the results indicated that XynIII is a novel bifunctional enzyme and its xylanase and β-(1,3-1,4)-glucanase activities are catalyzed by the same active center.

Key words: Bifunctional enzyme, xylanase, β-(1,3-1,4)-glucanase, *Aspergillus niger*, purification

Cellulose, hemicellulose, and lignin are the major components of plant cell walls [24]. When combined, they form the support framework for plants and defend against the invasion of aggressors. To successfully invade or live on plant tissues, microorganisms have to synthesize a number of different enzymes in order to hydrolyze cellulose or hemicellulose. Thus, to effectively degrade the plant cell wall complex, many microorganisms develop a cell-associated multiprotein complex, called cellulosome [14] or xylosome [15], which contains cellulases, xylanases, and cellulose-binding factors. Another strategy is to induce the multifunctionalization of certain enzymes to hydrolyze different kinds of substrates. Such multifunctional enzymes have already been found in many microorganisms. For example, *Trichoderma miride* can produce an exo-CMCase that hydrolyzes xylan, CMC, and cellooligosaccharides [23], *Aspergillus niger* produces a xylanase that can hydrolyze xylan and crystalline cellulose [11], *Pencillium corylopltilum* produces a xylanase that can hydrolyze xylan, CMC, and pNPX [25], and *Caldocellum saccharolyticum* [5] and *Ruminococcus flavefaciens* [4] both produce a bifunctional enzyme with two separate catalytic domains, where the first exhibits activity towards guar bean gum, konjac glucomannan, CMC, oat spelt xylan, and lichenin, and the second is capable of hydrolyzing xylan and lichenin.

In addition to its importance in allowing microorganisms to invade plants and degrade plant residues, xylanase also has prospective applications for the food, animal feed, and paper and pulp industries. Furthermore, we can frequently find such processes as the enhancement of feed nutrient digestibility [19, 20, 22, 26] and improvement of wheat flour quality [8], where xylanase and β-(1,3-1,4)-glucanase are used at the same time. For these applications, a bifunctional enzyme is undoubtedly more valuable than an enzyme complex.

Accordingly, to search for enzymes that can effectively hydrolyze xylan and glucan, the present study investigated the xylanase and β-(1,3-1,4)-glucanase activities of the strain *Aspergillus niger* A-25, and a novel bifunctional enzyme, XynIII, was identified. The purification and characterizations of XynIII are reported in detail, and its kinetics behavior studied using the Michaelis-Menten model.

*Corresponding author
Phone: 86-371-63555153; Fax: 86-371-63555790;
E-mail: honggeyz@163.com

MATERIALS AND METHODS

Strain and Culture Conditions

The *Aspergillus niger* A-25 was obtained from the Department of Microbiology, College of Life Sciences, Henan Agricultural University, Henan Province, China. The cultural medium contained barley flour, 4%; corn steep liquor, 2%; $NaNO_3$, 0.4%; Na_2HPO_4, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.03%; $FeSO_4 \cdot 7H_2O$, 0.01%; $CaCO_3$, 0.5%; and Tween-80, 0.2%; pH 6.0. For enzyme production, the strain was grown at 30°C for 84 h in 300-ml shake flasks, containing 50 ml of the medium.

Assay of Enzymatic Activity

The enzymatic assays were carried out in a 0.1 mol/l sodium acetate buffer (pH 4.6) at 50°C, unless otherwise stated. The xylanase standard assay was performed by mixing 0.1 ml of the enzyme extract, 0.1 ml of 1% (w/v) birchwood xylan (Fluka), and 0.8 ml of the buffer. The reaction mixture was then incubated for 15 min. The reaction was terminated by adding 0.6 ml of a dinitrosalicylic acid reagent [21], and then the mixture was heated in boiled water for 15 min and the final volume adjusted to 5 ml by adding distilled water. The reducing sugars were measured based on the absorbance at 550 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of xylose equivalent per minute. For the β-(1,3-1,4)-glucanase standard assay, lichenin was used as the substrate, and one unit of glucanase was defined as the amount of enzyme releasing 1 μmol of glucose equivalent per minute from the substrate. The specific activity was expressed as units per mg of protein. The protein concentration in the extract was determined using the method of Lowry [18].

Gel Electrophoresis

The PAGE was performed using the method of Davis [2], and the stacking and separating gel concentrations were 5% and 8% of polyacrylamide, respectively. The SDS-PAGE was performed using the method of Laemmli [13] with 5% of polyacrylamide in the stacking gel and 10% in the separating gel. After the electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (Sigma).

Zymogram Analysis

For the xylanase zymogram analysis, after the PAGE was completed, the gel was cut vertically into two halves. In one half, the proteins were stained with Coomassie brilliant blue R-250, whereas the other half was washed with a 0.1 mol/l sodium acetate buffer (pH 4.6), partially dried, and laid on top of a xylan-agarose sheet containing 0.2% birchwood xylan (w/v) and 1.5% agarose (w/v) in the same buffer. After incubating the bilayered sheet for 1 h at 50°C, the xylan-agarose sheet was soaked in a 0.1% Congo red solution for 30 min at room temperature and washed with 1 mol/l NaCl to remove any excess dye from the active bands. Finally, the sheet was submerged in 0.5% acetic acid, at which point the background turned dark purple, and the active bands were observed as clear colorless areas.

The β-(1,3-1,4)-glucanase zymogram was assayed using the method of Beguin [1].

On the zymogram gels, the three bands were numbered XynI, XynII, and XynIII for the xylanases, and GluI, GluII, and GluIII for the β-(1,3-1,4)-glucanases, according to their migration rates (from the lowest to the highest).

Enzyme Purification

All the procedures were performed at 4°C, unless otherwise stated. The crude enzyme obtained from the culture by centrifugation (10,000 ×*g* for 10 min) was ultrafiltrated using a Vivaflow 200 (Sartorius) equipped with a membrane (30,000, MWCO, PES). The filtrated enzyme solution was loaded onto a DEAE-Sepharose CL-6B (Sigma) column (2.6×20 cm), previously equilibrated with a 20 mmol/l Tris-HCl buffer (pH 7.1) and the bound proteins were then eluted with a linear gradient of 0 to 0.5 mol/l NaCl in the same buffer at a flow rate of 0.4 ml/min. Each 5 ml of eluted solution was collected as one fraction and each fraction was detected by electrophoresis and the enzyme activity assayed as mentioned above. The fractions containing XynIII were pooled and further purified with the same column preequilibrated with a 20 mmol/l sodium acetate buffer (pH 5.5). The elution conditions were the same as the first time, except a sodium acetate buffer (pH 5.5) was used instead of the Tris-HCl buffer (pH 7.1).

Enzyme Characterizations

For the substrate specificity analysis of the purified enzymes, the relative hydrolysis rate was determined for each potential substrate (oat spelt xylan, lichenin, barley β-glucan, CMC, avicel, and soluble starch, all from Sigma) at a concentration of 1% (w/v) with the hydrolysis rate for birchwood xylan as 100%.

The optimum pHs for the xylanase activity and β-(1,3-1,4)-glucanase activity of XynIII were determined at 50°C in buffers ranging from pH 1.6 to 10.6, with a pH interval of 1. The enzyme stabilities at different pHs were determined by measuring the residual enzymatic activities after incubation in the same buffers for 24 h at 30°C.

The optimum temperatures for the enzymatic reaction of xylanase and β-(1,3-1,4)-glucanase were determined by assaying the enzymes at temperatures from 20°C to 90°C. The thermostabilities were measured by incubating the enzyme samples in a 0.1 mol/l sodium acetate buffer (pH 4.6) for 1 h at temperatures from 20°C to 80°C. The enzyme solutions were chilled on ice for 5 min, and then examined using a standard assay at 50°C.

The effects of various metal ions, including Ca^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+}, Pb^{2+}, Mg^{2+}, K^{+}, Zn^{2+}, Hg^{2+}, and the reagent EDTA, on the purified enzyme were tested at a

concentration of 1 mmol/l. The residual activities were measured in a 0.1 mol/l sodium acetate buffer (pH 4.6) at 50°C. The enzyme activity measured without any additives was noted as 100%.

Kinetic Studies of Xylanase and β-(1,3-1,4)-Glucanase Activities of XynIII

To determine whether the xylanase and β-(1,3-1,4)-glucanase activities of XynIII were due to the same enzyme, kinetic studies were carried out.

Determination of Kinetic Constants. The Michaelis constants (K_m) and maximum velocities (V_{max}) for the xylanase and β-(1,3-1,4)-glucanase activities of XynIII were estimated from the Lineweaver-Burk plots of their initial velocities of saccharification of the substrates. The initial velocities of birchwood xylan and lichenin hydrolysis were determined at various substrate concentrations (1 to 28 mg/ml) containing 0.86 μg of the purified enzyme. The reaction was performed by incubating the mixture at 50°C for 10 min.

Competition Between Xylan and Lichenin [3]. If XynIII hydrolyzed both substrates at the same active center, the two substrates would competitively inhibit each other during the process of enzyme-substrate binding. In this case, the overall rate of hydrolysis (V_1) of the two substrates would be given as Eq. (1). Conversely, if the substrates were independently hydrolyzed at different active centers or by two different enzymes, no competitive inhibition would be shown. In this case, the overall rate of hydrolysis (V_2) of the two substrates would be given by the simple sum of the velocities of each substrate separately, as in Eq. (2).

$$V_1=V_x+V_g=\frac{\frac{V_{mx}X}{K_{mx}}+\frac{V_{mg}G}{K_{mg}}}{1+\frac{X}{K_{mx}}+\frac{G}{K_{mg}}} \tag{1}$$

$$V_2=V_x+V_g=\frac{\frac{V_{mx}X}{K_{mx}}}{1+\frac{X}{K_{mx}}}+\frac{\frac{V_{mg}G}{K_{mg}}}{1+\frac{G}{K_{mg}}} \tag{2}$$

where V_x and V_g are the velocities of the hydrolysis of xylan and lichenin, respectively, X and G are the concentrations of xylan and lichenin, respectively, V_{mx} and V_{mg} are the maximum velocities for xylan and lichenin, respectively, and K_{mx} and K_{mg} are the Michaelis constants for xylan and lichenin, respectively.

RESULTS

Discovery of Bifunctional Enzyme Through Zymogram Analysis

The PAGE revealed that the culture supernatant of *A. niger* A-25 contained several major protein bands. The xylanase

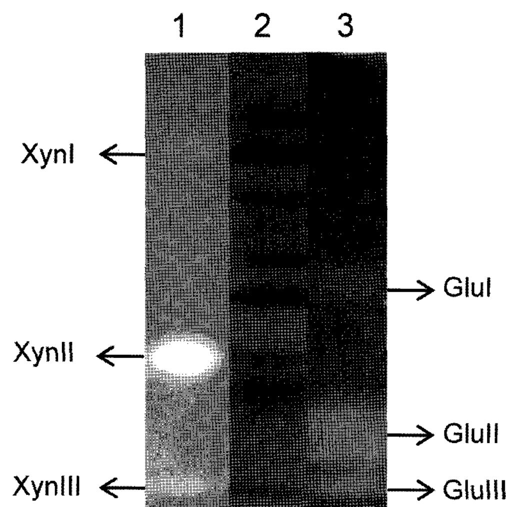

Fig. 1. PAGE and zymograms of xylanase and β-(1,3-1,4)-glucanase.
Lane 1, Xylanase zymogram gel; Lane 2, PAGE of crude enzyme stained with Conmassie brilliant blue; Lane 3, β-(1,3-1,4)-glucanase zymogram gel.

zymogram analysis (Fig. 1, lane 1) revealed three active xylanases (XynI, XynII, and XynIII), and the β-(1,3-1,4)-glucanase zymogram analysis (Fig. 1, lane 3) also revealed three active β-(1,3-1,4)-glucanases (GluI, GluII, and GluIII). When combining the two zymograms, it was found that XynIII and GluIII were coincidently at the same position in the PAGE. Thus, the further purification of XynIII was necessary for additional study.

Enzyme Purification

After purifying the XynIII using ultrafiltration and two consecutive steps of column chromatographic operations,

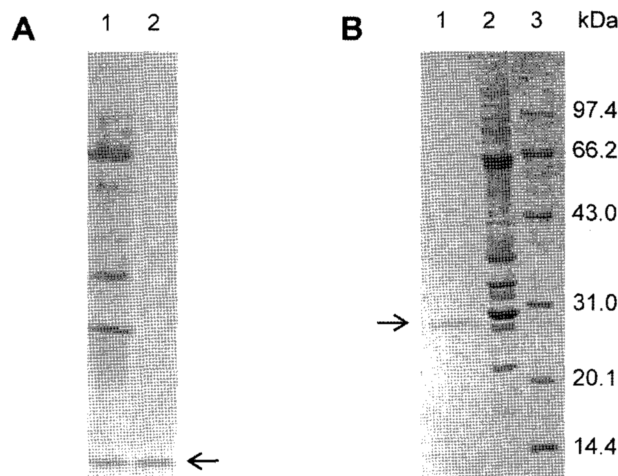

Fig. 2. Results of purification of XynIII.
A. PAGE of purified enzyme XynIII. Lane 1, Crude enzyme; Lane 2, Purified XynIII. **B.** SDS-PAGE of purified XynIII. Lane 1, Purified XynIII; Lane 2, Crude enzyme; Lane 3, Molecular mass standards.

Table 1. Summary of purification procedures for XynIII from culture supernatant of *A. niger* A-25.

Steps	Total activity (U)[a]	Total protein (mg)	Specific activity (U/g)	Purification fold	Yield (%)
Crude extract	9,340.10	429.10	21.77	1.00	100
Ultrafiltration	8,790.60	130.02	67.61	3.10	94.12
DEAE-Sepharose-CL6B (pH 7.1)	593.10	6.87	86.34	3.97	6.35
DEAE-Sepharose-CL6B (pH 5.5)	102.44	1.12	91.63	4.21	1.10

[a]Activities were measured with birchwood xylan as the substrate.

PAGE (Fig. 2A) and SDS-PAGE (Fig. 2B) revealed a single protein band with an apparent molecular weight of 27.9 kDa. A summary of the purification procedures of XynIII from the supernatant of the *A. niger* A-25 culture is given in Table 1. The enzyme was purified 4.21-fold to a specific activity of 91.63 U/mg with a yield of about 1.1%.

Enzyme Characterizations

The purified enzyme XynIII was examined for its ability to hydrolyze various substrates (Table 2). Its relative activities with oat spelt xylan, lichenin, and barley β-glucan were 125.54%, 39.60%, and 32.74%, respectively. It exhibited no activity against CMC, avicel, and soluble starch.

The optimum pHs for the xylanase and β-(1,3-1,4)-glucanase activities of XynIII were 4.6 and 3.6–5.6, respectively. Both activities decreased quickly beyond pH 3.6–5.6 (Fig. 3A). The xylanase activity remained stable at pH 2.6–10.6, whereas the β-(1,3-1,4)-glucanase activity remained stable at pH 3.6–10.6 (Fig. 3B).

The optimum temperatures for both activities were about 50°C (Fig. 4A). The thermostabilities were investigated by measuring the residual enzyme activities after 1 h of incubation at temperatures ranging from 20°C to 80°C. Both enzymes sharply decreased in activity up to 60°C, yet became very stable below 50°C (Fig. 4B).

The effects of various metal ions on the xylanase and β-(1,3-1,4)-glucanase activities of XynIII were investigated (Table 3). Both activities were completely inhibited by the addition of Hg^{2+}. The other ions Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Pb^{2+}, K^{+}, Zn^{2+}, and EDTA had different inhibitive effects on the two activities, whereas Fe^{2+} and Mg^{2+} slightly promoted both activities.

Table 2. Substrate specificity of XynIII.

Substrate	Relative activity (%)
Birchwood xylan	100±0.92
Oat spelt xylan	125.54±1.35
Lichenin	39.60±1.78
Barley β-glucan	32.74±1.13
Avicel	0±0
CMC	0±0
Soluble starch	0±0

Kinetics of Xylanase and β-(1,3-1,4)-Glucanase Activities of XynIII

The V_{max} and K_m values for the xylanase and β-(1,3-1,4)-glucanase activities of XynIII are presented in Table 4. The V_{max} values for xylanase and β-(1,3-1,4)-glucanase were 0.258 and 0.149 ΔA/min/ml, respectively, and the K_m values were 4.54 and 10.02 mg/ml, respectively. The competition results between xylan and lichenin are shown in Table 5. The observed values for the overall rate of hydrolysis agreed well with the theoretical values calculated from Eq. (1), but not with those calculated from Eq. (2). Therefore, the results demonstrated that the hydrolysis of xylan and lichenin took place at the same active center, indicating that the xylanase and β-(1,3-1,4)-glucanase activities of XynIII are controlled by the same active domain.

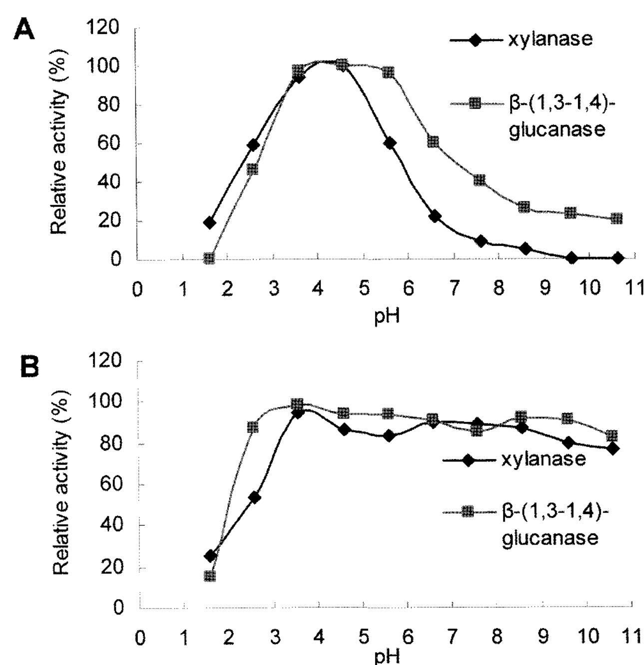

Fig. 3. Effect of pH on the xylanase and β-(1,3-1,4)-glucanase activities of XynIII.
A. Optimum pH for XynIII. **B.** pH stability of XynIII.

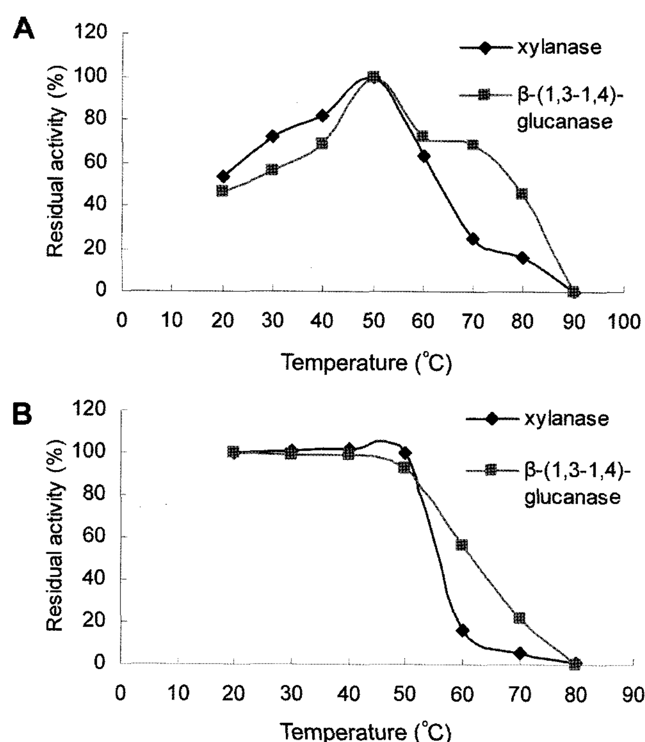

Fig. 4. Effect of temperature on the xylanase and β-(1,3-1,4)-glucanase activities of XynIII.
A. Optimum temperature for XynIII. **B.** Thermostability of XynIII.

DISCUSSION

Based on the xylanase and β-(1,3-1,4)-glucanase zymograms and the substrate specificity of the purified enzyme, a bifunctional enzyme, XynIII, was identified that could hydrolyze two different kinds of substrate: xylan, mainly consisting of xylose units joined by β-1,4 linkages; and β-glucan, such as lichenin and barley β-glucan, consisting of glucose units joined by β-1,4 and β-1,3 linkages. The β-(1,3-1,4)-glucanase activity was 30%–40% of the xylanase activity. The respective V_{max} and K_m values for the xylanase and β-(1,3-1,4)-glucanase activities of XynIII were both very different. The hydrolytic ability and affinity of XynIII for xylan were higher than those for lichenin, and the value of V_{max}/K_m for xylanase was also higher than that for β-(1,3-1,4)-glucanase, implying that xylan was the more optimal substrate for XynIII.

Table 3. Effect of metal ions on the xylanase and β-(1,3-1,4)-glucanase activities of XynIII.

Ions or EDTA	Relative activity (%)[a]	
	Xylan	Lichenin
None	100±0.55	100±0.80
$CaCl_2$	98.24±0.35	90.98±0
$CoCl_2$	81.97±0.49	82.19±1.87
$FeSO_4$	103.30±1.41	120.80±0
$CuSO_4$	74.61±0	44.00±0.39
$MnSO_4$	72.27±1.99	37.29±1.96
$Pb(Ac)_2$	75.36±1.23	79.29±0.78
$MgSO_4$	107.10±1.04	105.30±1.96
KAc	97.12±0.47	95.61±1.43
$ZnSO_4$	97.56±0.71	88.66±0.91
$Hg(Ac)_2$	0±0	0±0
EDTA	88.05±0	79.64±0.24

[a]The activities of XynIII were assayed in the presence of 1 mmol/l metal ions. The activities assayed in the absence of metal ions (None) were considered to be 100%.

Table 4. Kinetic parameters of xylanase and β-(1,3-1,4)-glucanase of XynIII.

Substrate	V_{max} (ΔA/min/ml)[a]	K_m (mg/ml)	V_{max}/K_m
Birchwood xylan	0.258±0.015	4.54±0.23	5.683×10^{-2}
Lichenin	0.149±0.011	10.02±0.37	1.487×10^{-2}

[a]ΔA is the absorbance at 550 nm.

Early in 1993, Flint *et al.* [4] found a similar bifunctional enzyme that could hydrolyze xylan and lichenin, yet this enzyme had two separate catalytic domains responsible for hydrolyzing each substrate. Its amino-terminal domain was related to family G xylanases, whereas the carboxyl-terminal domain was related to β-(1,3-1,4)-glucanases. The two domains were connected by a region with an unknown function that consisted of 309 amino acids and included a 30-amino-acid threonine-rich sequence. As regards the bifunctional enzyme XynIII reported herein, its two enzymatic activities were derived from the same active center, as confirmed by competitive kinetic experiments, and by the similar pH and temperature ranges, as well as the similar reactivity to various metal ions. In addition, since XynIII had an apparent molecular mass of only 27.9 kDa, which is much lower than that of other multifunctional enzymes [4, 6, 7, 11, 12, 23, 25, 27] (most exhibit a xylanase-cellulase bifunction), it could hardly form two active centers. Therefore, it is believed that XynIII is a bifunctional enzyme with only one active center, which is a crucial feature, as a multifunctional enzyme with only a single active center is a true multifunctional enzyme [24].

Jenkins *et al.* [10] previously provided a structure-based explanation for this type of case. He found that the catalytic domains of xylanases and β-(1,3-1,4)-glucanases mostly had an 8-fold β/α-barrel structure, and even included the same catalytic amino acid residues, implying that the enzymes belong to a superfamily of 8-fold β/α-barrels with a common ancestor. From the viewpoint of the substrate structure, the conformation of the polysaccharide chain also plays an important role in enzyme-substrate recognition and hydrolysis [9, 16]. As such, xylan has a three-dimensional helical conformation, cellulose a flat ribbon-like conformation,

Table 5. Overall reaction velocities of XynIII with mixtures of various concentrations of xylan and lichenin.

No.	Concentration of substrates in reaction mixture (mg/ml)		Total velocity of hydrolysis (ΔA/min/ml)		
	Birchwood xylan	Lichenin	Observed values[a]	Theoretical values calculated for[b]	
				same active center	different active center
1	16	4	0.182±0.026	0.197	0.248
2	12	8	0.171±0.007	0.180	0.253
3	8	12	0.154±0.004	0.160	0.245
4	4	16	0.118±0.021	0.134	0.213

[a]The activity was measured, as described in Materials and Methods, using 0.86 μg XynIII.
[b]The theoretical values were calculated from Eqs. (1) and (2) in the text.

and barley β-glucan and lichenin, a more twisted conformation than cellulose [17], owing to the addition of β-1,3 linkages. Therefore, the similarity in the xylan, barley β-glucan, and lichenin structures would seem to suggest that they were all able to fit the same active center of the enzyme.

In the present study, XynIII, an important bifunctional enzyme, was successfully purified and identified, and the data revealed an ability to hydrolyze both xylan and β-glucan at the same active site with a single catalytic domain.

Acknowledgment

The authors are thankful to Dr. E. T. Wang at the National Polytechnical Institute of Mexico for his valuable comments.

REFERENCES

1. Beguin, P. 1983. Detection of cellulase activity in polyacrylamide gels using Congo red-stained agar replicas. *Anal. Biochem.* **131:** 333–336.
2. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum. *Ann. NY Acad. Sci.* **121:** 404–427.
3. Dixon, M. and E. C. Webb. 1964. *Enzyme*, 2nd Ed., pp. 84–87. Longmans, Green, and Co., Ltd, London.
4. Flint, H. J., J. Martin, C. A. McPherson, A. S. Daniel, and J. X. Zhang. 1993. A bifunctional enzyme with separate xylanase and β-(1,3-1,4)-glucanase domains, encoded by the *Xyn*D gene of *Ruminococcus flavefaciens*. *J. Bacteriol.* **175:** 2943–2951.
5. Gibbs, M. D., D. J. Saul, E. Luthi, and P. L. Bergquist. 1992. The β-mannanase from "*Caldocellum saccharolyticum*" is part of a multidomain enzyme. *Appl. Environ. Microbiol.* **58:** 3864–3867.
6. Gomez de Segura, B. and M. Fevre. 1993. Purification and characterization of two 1,4-β-xylan endohydrolases from the rumen fungus *Neocallimastix frontalis*. *Appl. Environ. Microbiol.* **59:** 3654–3660.
7. Haga, K., M. Kitaoka, Y. Kashiwagi, T. Sasaki, and H. Taniguchi. 1991. Purification and properties of a xylanase from *Cellvibrio gilvus* that hydrolyzes *p*-nitrophenyl cellooligosaccharides. *Agric. Biol. Chem.* **55:** 1959–1967.
8. Haros, M., C. M. Rosell, and C. Benedito. 2002. Improvement of flour quality through carbohydrases treatment during wheat tempering. *J. Agric. Food Chem.* **50:** 4126–4130.
9. Hrmova, M. and G. Fincher. 2001. Plant enzyme structure. Explaining substrate specificity and the evolution of function. *Plant Physiol.* **125:** 54–57.
10. Jenkins, J., L. Lo Leggio, G. Harris, and R. Pickersgill. 1995. β-Glucosidase, β-galactosidase, family A cellulases, family F xylanases and two barley glycanases form a superfamily of enzymes with 8-fold β/α architecture and with two conserved glutamates near the carboxy-terminal ends of β-strands four and seven. *FEBS Lett.* **362:** 281–285.
11. John, M., B. Schmidt, and J. Schmidt. 1979. Purification and some properties of five endo-1,4-beta-D-xylanases and a beta-D-xylosidase produced by a strain of *Aspergillus niger*. *Can. J. Biochem.* **57:** 125–134.
12. Kim, C. H. 1995. Characterization and substrate specificity of an endo-β-1,4-D-glucanase I (Avicelase I) from an extracellular multienzyme complex of *Bacillus circulans*. *Appl. Environ. Microbiol.* **61:** 959–965.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
14. Lamed, R. and E. A. Bayer. 1988. The cellulosome concept: Exocellular enzyme reactor centers for efficient binding and cellulolysis, pp. 101–116. *In* J. P. Aubert, P. Beguin, and J. Millet (eds.), *Biochemistry and Genetics of Cellulose Degradation*. Academic Press, London.
15. Lin, L. L. and J. A. Thomson. 1991. An analysis of the extracellular xylanases and cellulases of *Butyrivibrio fibrisolvens* H17c. *FEMS Microbiol. Lett.* **84:** 197–204.
16. Lo Leggio, L., J. Jenkins, G. W. Harris, and R. W. Pickersgill. 2000. X-ray crystallographic study of xylopentaose binding to *Pseudomonas fluorescens* xylanase 10A. *Proteins* **41:** 362–373.
17. Lo Leggio, L., S. Kalogiannis, K. Eckert, S. C. Teixeira, M. K. Bhat, C. Andrei, R. W. Pickersgill, and S. Larsen. 2001. Substrate specificity and subsite mobility in *T. aurantiacus* xylanase 10A. *FEBS Lett.* **509:** 303–308.

18. Lowry, O. H., N. J. Risebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
19. Mathlouthi, N., H. Juin, and M. Larbier. 2003. Effect of xylanase and beta-glucanase supplementation of wheat- or wheat- and barley-based diets on the performance of male turkeys. *Br. Poult. Sci.* **44:** 291–298.
20. Meng, X., B. A. Slominski, C. M. Nyachoti, L. D. Campbell, and W. Guenter. 2005. Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. *Poult. Sci.* **84:** 37–47.
21. Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **31:** 426–428.
22. Omogbenigun, F. O., C. M. Nyachoti, and B. A. Slominski. 2004. Dietary supplementation with multienzyme preparations improves nutrient utilization and growth performance in weaned pigs. *J. Anim. Sci.* **82:** 1053–1061.
23. Shikata, S. and K. Nsizawa. 1975. Purification and properties of an exo-cellulase component of novel type from *Trichoderma miride. J. Biochem. (Tokyo)* **78:** 499–512.
24. Wong, K. K., L. U. Tan, and J. N. Saddler. 1988. Multiplicity of β-1,4-xylanase in microorganisms: Functions and applications. *Microbiol. Rev.* **52:** 305–317.
25. Yang, R. J., S. Y. Xu, and Z. Wang. 2001. Purification and properties of xylanases from *Pencillium corylopltilum. J. Wuxi Univ. Light Industry* **20:** 35–39 (in Chinese with English summary).
26. Yu, P., J. J. McKinnon, and D. A. Christensen. 2005. Improving the nutritional value of oat hulls for ruminant animals with pretreatment of a multienzyme cocktail: *In vitro* studies. *J. Anim. Sci.* **83:** 1133–1141.
27. Zhang, J. X. and H. J. Flint. 1992. A bifunctional xylanase encoded by the XynA gene of the rumen cellulolytic bacterium *Ruminococcus flavefaciens* 17 comprises two dissimilar domains linked by an asparagine/glutamine-rich sequence. *Mol. Microbiol.* **6:** 1013–1023.