0273-2289/1559-0291 (Online)/07/136/0001/$30.00

Biochemical Properties of Genetic Recombinant Xylanase II

MENG-YUN TUNG, CHEN-TIEN CHANG, AND YUN-CHIN CHUNG*

Department of Food and Nutrition, Providence University, 200 Chungchi Road, Shalu, Taichung 43301, Taiwan, E-mail: ycchun@pu.edu.tw

Received November 24, 2005; Accepted February 2, 2006

Abstract

The aim of this study was to overexpress the xylanase II gene of *Trichoderma reesei* in *Escherichia coli* and determine the characteristics of the recombinant enzyme. Recombinant xylanase II gene was constructed by ligating the cDNA of xylanase, obtained from reverse transcriptase-polymerase chain reaction, and fused with NusA protein of pET-431b plasmid. An Ni^{2+}-NTA affinity column was used to further purify the recombinant xylanase II. The molecular mass of the recombinant enzyme measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis was approx 76 kDa (including 55 kDa of NusA and 21 kDa of xylanase II), and the isoelectric point and specific activity were 7.5 and 225 U/mg, respectively. The optimal reaction temperature and pH for the recombinant enzyme were 50°C and 4.0, respectively. The recombinant enzyme was stable at a pH range of 5.0–10.0 and maintained 95% residual activity after incubating at 30–35°C for 30 min. The kinetic parameters K_M and V_{max} of the recombinant xylanase II were 13.8 mg/mL and 336 μmol/(mg·min), respectively, using birchwood xylan as the substrate.

Index Entries: *Trichoderma reesei;* xylanase; overexpression; cloning; *Escherichia coli.*

Introduction

Recently there has been increasing interest in the prebiotic effects of nondigestible oligosaccharides, such as xylooligomers, stachyose, galactooligosaccharide, transgalatosyl-oligosaccharide and fructooligosaccharide.

*Author to whom all correspondence and reprint requests should be addressed.

The benefits of oligosaccharide ingestion include induction of the growth of indigenous bifidobacteria in the colon and suppression of some pathogenic bacteria *(1–7)*.

Xylooligomers are composed of β-1,4-linked D-xylopyranose residues and are nondigestible by humans. It has been demonstrated that xylooligosaccharides can be extensively utilized by several species of bifidobacteria and can stimulate the growth of bifidobacteria *(8–10)*. Our previous studies showed that oral administration of xylooligosaccharides could increase significantly the moisture content of feces, the total cecum weight, and the population of bifidobacteria and decrease the pH level of feces in ICR mice *(11)*. Similar prebiotic effects were also found in Sprague-Dawley rats *(12)*. Hsu et al. *(13)* reported that xylooligosaccharides significantly inhibited precancerous lesions of the colon induced by DMH (a carcinogen) in Sprague-Dawley rats, and that supplementation of xylooligosaccharides was more effective in increasing bifidobacteria population than fructooligosaccharide.

The production of xylooligosaccharides, such as xylobiose and xylotriose, can be accomplished by digesting hemicellulose (xylanes) with high-pressure steam, acid or alkali, or enzymes *(14)*. Chemical digestion of xylan is considered a simple and time-saving process, but it is less applicable because it may produce undesirable byproducts. To produce xylooligosaccharides with high purity, enzymatic digestion of xylan has the advantage of high-specific reaction, which makes it more desirable.

Xylanases, the enzymes that degrade xylan specifically, are found in living organisms, especially in bacteria (such as the genus of *Clostridium*, *Streptomyces*, and *Bacillus*), fungi (such as *Aspergillus*, *Penicillium*, and *Trichoderma*), and yeast *(15)*. The major difficulty in applying xylanases in food processes is the economic consideration. The low production rate of xylanase makes the cost of xylanase extremely high. Microbes with commercial genetically constructed vector having strong promotors capable of producing a high level of encoded protein. To construct a desired gene with strong promotor in a microbial system not only increases the yield and production rate of the target protein, but also simplifies the purification processes with the aid of an Ni^{2+}-NTA affinity column when the target protein is fused with a 6-His tag.

Xylanase II, which degrades xylan in the form of endo cleaving and belongs to endo-β-1,4-xylanases (EC 3.2.1.8), is commonly found in plants, bacteria, and filamentous fungi. Xylanase II attacks xylan on its backbone without substituted group or side chain and, thus, different lengths of xylooligosaccharides can be produced after enzymatic digestion *(16,17)*. High xylanase activity was found in *Trichoderma reesei* when its xylanase II gene was induced by xylan *(18–20)*. In the present study, we overexpressed the *T. reesei* xylanase II gene in *Escherichia coli* and determined the characteristics of the recombinant xylanase II.

Materials and Methods

Fungus and Chemicals

T. reesei (ATCC 32924) was purchased from Bioresource Collection and Research Center, Food Industry Research and Development Institution, Shinchu, Taiwan. *E. coli* AD494(DE3), pET-43.1b vector, and *E. coli* TOP10 were purchased from Novagen (Madison, WI). Total RNA isolation reagent (TriZol reagent), ELONGASE enzyme mix (proofreading DNA polymerase), T4 DNA ligase, reverse transcriptase (SuperScript II RT), Luria Bertani (LB) media, X-Gal, and protein ladder (protein marker, 10 kDa) were from Life Technologies (Gaithersburg, MD). *Taq* DNA polymerase (AmpliTaq Gold) was purchased from Perkin-Elmer (Norwalk, CT). Restriction enzymes, pGEM-T Easy vector, and DNA purification system were from Promega (Madison, WI). Isopropyl-D-thiogalactopyranoside (IPTG), ampicillin, and kanamycin were from Sigma (St. Louis, MO).

Molecular Cloning With pGEM-T Easy Vector

Stock of *T. reesei* was cultured in xylan-containing YPD broth (1% oat, 1% yeast extract, 1% potato, and 2% glucose) for 3 d, and then total RNA was isolated with TriZol reagent following the manufacturer's instructions. Standard molecular cloning techniques were performed according to the guidelines of Sambrook et al. *(21)*. Xylanase II cDNA of *T. reesei* was synthesized by reverse transcription from the total RNA using oligo-dT as primer. Target gene was cloned with 30 cycles of polymerase chain reaction (PCR), which was initiated by 30 s of denaturation at 94°C, 30 s of annealing at 50°C, and 30 s of extention at 70°C, and then by 10 min extention at 70°C in a DNA thermal cycler (GeneAmp PCR system 2400; Perkin-Elmer). Primers used for the PCR procedure were designed on the basis of the *T. reesei* xylanase II nucleotide sequence reported by Törrönen et al. *(18)*: forward primer with *Him*III recognition sequence, 5N-GAGCTCCAG ACGATCAGCCC; and reverse primer with *Sac*I sequence, 5N-AAGCTT GCTGACGGTGATGGA. After PCR reaction, the PCR product was ligated with pGEM-T Easy vector and transformed into cloning host, *E. coli* Top 10, according to the methods of Hanahan and Meselson *(22)*. The transformed host was screened by blue/white selection, *Him*III/*Sac*I digestion of plasmid, and DNA sequencing using T7 and SP6 as sequencing primers.

Construction of NusA-Xylanase-pET-43.1b Expression Vector

The xylanase II nucleotide fragment released from pGEM-T Easy vector was ligated into a pET-43.1b expression vector by using T4 DNA ligase (named xyl-pET-43.1b) and was in frame with NusA gene in the downstream of operon-controlled Lac Z promoter. The constructed plasmid was then transformed into an expression host, *E. coli* AD494(DE3).

Overexpression and Purification of Recombinant Xylanase II

E. coli AD494(DE3) with xyl-pET-43.1b was cultivated in 10 mL of LB broth (10 g of tryptone/L, 5 g of yeast extract/L, and 10 g of NaCl/L) containing 100 g/mL of ampicillin and 15 g/mL of kanamycin in a 50-mL flask at 37°C overnight using a shaking incubator (200 rpm). One milliliter of the activated culture was inoculated into 250 mL of fresh LB broth containing 100 g/mL of ampicillin and 15 g/mL of kanamycin in a 1-L flask. During incubation, the absorbance at 600 nm (OD_{600}) was measured. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 1 m*M* to induce the synthesis of recombinant xylanase II. After 8 h of incubation, the transformed *E. coli* cells were harvested by centrifuging for 10 min at 4000*g* and suspended in 14 mL of binding buffer (pH 8.0; 50 m*M* NaH_2PO_4, 10 m*M* imidazole, and 0.3 *M* NaCl). The harvested cells were sonicated under 240 W for 10 s and then immersed in an ice bath for 20 s. Sonication was performed for 240 cycles using a sonicator VCX400/600 system (Sonics and Materials Inc., CT, USA).

For further purification, recombinant xylanase II was isolated from the soluble fraction of the sonicated sample by centrifuging for 20 min at 5000*g*. After the supernatant was filtered through a 0.45-μm sterilized membrane (Gelman, Ann Arbor, MI), the filtrate was chromatographed on an Ni^{2+}-NTA agarose affinity column (2.6 × 2 cm) preequilibrated with binding buffer. After washing with 6 vol of wash buffer (pH 8.0; 50 m*M* NaH_2PO_4, 20 m*M* imidazole, and 0.3 *M* NaCl), recombinant xylanase II was eluted with 2 to 3 vol of elution buffer (pH 8.0; 50 m*M* NaH_2PO_4, 250 m*M* imidazole, and 0.3 *M* NaCl) and then dialyzed against 50 m*M* citrate buffer (pH 4.0) in order to measure its enzyme activity and characteristics.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Zymogram Analysis

The molecular mass of recombinant xylanase II was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% polyacrylamide according to the method of Laemmli *(23)*. Zymogram analysis was performed by native PAGE according to the method of Wang et al. *(24)* with modification. A 12.5% native PAGE gel containing 0.1% birchwood xylan was used for the zymogram analysis as described by Ali et al. *(25)*.

Determination of Protein Concentration

Protein concentration was determined by bicinchoninic acid method using bovine serum albumin as the standard *(26)*.

Assay of Enzyme Activity

Xylanase activity was assayed using the method of Royer and Nakas *(27)* by mixing 0.5 mL of an appropriately diluted enzyme solution with 0.5 mL of 4.5% birchwood xylan in 50 m*M* citric-citrate buffer (pH 4.0) at

50°C for 30 min. Reducing sugar was determined by the dinitrosalycylic acid (DNS) method with D-xylose as the standard *(28)*. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the liberation of 1 μmol of reducing sugar equivalent/min under the assay conditions.

Assay of Isoelectric Point

The isoelectric point (p*I*) of xylanase II was determined with a PhastGel IEF 3–9 and an isoelectric focusing calibration kit, suitable for a pI range of 3–10 (Pharmacia, Uppsala, Sweden), using Pharmacia Phast system following the manufacturer's instructions. After isoelectric focusing, the gels were fixed in 20% trichloroacetic acid for 5 min before visualizing the protein bands with Coomassie brilliant blue 250. pI marker proteins of values of 3.55–9.30 were purchased from Sigma.

Determination of Kinetic Parameters

Recombinant xylanase II was incubated with various concentrations of birchwood solution (0.25–3.0%) in 50 mM citric-citrate buffer (pH 4.0) at 50°C, and the kinetic parameters, K_M and V_{max}, describing the enzyme activity were calculated by linear regression using Lineweaver-Burk plots *(29)*. Activation energy of xylanase II was also calculated from the plot of the log V_{max} vs 1/T1/OK) (Arrhenius plot, 25–50°C). All assays were performed in triplicate.

Optimum pH for Enzyme Reaction and pH Stability

The effect of pH on the activity of recombinant xylanase II was determined by measuring the enzyme activities in different buffer systems: pH 2.0–6.0 (50 m*M* sodium citrate buffer), pH 7.0 (50 m*M* sodium phosphate buffer), pH 8.0 (50 m*M* Tris buffer), and pH 9.0–10.0 (50 m*M* glycine buffer). For evaluating the pH stability of xylanase II, the enzyme was incubated with the buffer systems at 25°C for 30 min prior to determining its activity.

Optimum Temperature for Enzyme Reaction and Thermal Stability

The effect of temperature on the activity of recombinant xylanase II was determined by measuring the enzyme activities at 30, 35, 40, 50, 60, and 80°C. The thermal stability of xylanase II was evaluated by incubating the enzyme in 50 m*M* citrate buffer (pH 4.0) at 30, 35, 40, 50, or 60°C for 30 min prior to determining its activity.

Substrate Specificity

Substrates (2% of birchwood, polyglucuronic acid, starch, pectin, methyl cellulose, and oat spelt xylan; 0.75% of 4-*O*-methyl-D-glucuro-D-xylan, [w/v]) suspended in 50 m*M* citric-citrate buffer (pH 4.0) were incubated with recombinant xylanase II at 50°C for 30 min, and then the enzyme activity was determined by the DNS method.

Effects of Metal Ions and Chemical Reagents on Enzyme Activity

Various metal ion solutions (7.5 m*M*, 350 μL) mixed with 50 μL of recombinant xylanase II were incubated at 25°C for 30 min and then assayed for enzyme activities. For assaying the effects of chemical reagents on enzyme activity, 50 μL of each chemical reagent (2.5 m*M*) dissolved in its appropriate solvent was mixed with 50 μL of its appropriated buffer (Table 1), and then 60 μL of the enzyme solution was added to the mixture and incubated at 25°C for 30 min. Enzyme activity was determined after dialysis of the chemical-treated enzyme with 50 m*M* citric-citrate buffer (pH 4.0).

Results and Discussion

Molecular Cloning

Our DNA sequencing data showed that our cloned xylanase II contained 570 bp and had a homology of 99% with xylanase II sequence reported by Törrönen et al. *(18)*. Compared to the reported xylanase II, the cloned xylanase II had two different gene bases (Fig. 1). One of the differences in gene base made no change in the gene codo™(of proline (CCA vs CCC) whereas the other changed the gene codon of alanine (GCG) to the gene codon of valine (GTG). Fortunately, the two different gene bases did not locate at the gene sequences for the catalytic residues of the enzyme (Glu 86 and Glu 177) *(30)*. After IPTG induction, the *lac* repressor was released from the *lac* operator; thus, a high level of recombinant xylanase II protein was expressed in the cytoplasm of the transformed *E. coli* AD494(DE3).

Purification

Recombinant xylanase II, tagged with an additional six histidine residues in the C-terminal of the protein, could be purified to electrophoretic homogeneity by using Ni^{2+}-NTA agarose affinity chromatography. After purification, its specific enzyme activity was increased from 14.6 to 225 μmol/(min·mg), achieving 15.4-fold purification (Table 2). The amount of recombinant xylanase II was about 2.0% of the total soluble protein expressed in the cytoplasmic fraction of the *E. coli* transformant after IPTG induction. This high-level expression of the soluble form of NusA-xylanase in the cytoplasm of *E. coli* AD494(DE3) and its rapid purification procedure give the expression system much more potential for use in food processing.

According to SDS-PAGE, recombinant xylanase II had a molecular mass of 76 kDa, which consisted of 55 kDa of NusA and 21 kDa of xylanase II (Fig. 2A). Native gel electrophoresis showed a unique band that corresponded to xylanase activity, as shown by the hydrolysis zone in the zymogram (Fig. 2B). The p*I* of recombinant xylanase II determined by isoelectrofocusing was 7.5 (data not shown), indicating that the enzyme

Table 1
Solvents and Reaction Buffer for Various Chemical Modification Agents

Modification agent	Solvent[a]	Reaction buffer	Reference
p-Hydroxymercuribenzoic acid (PHMB)	Water	0.05 *M* Imidazole buffer, pH 7.0	*31*
N-Acetylimidazole (NAI)	Water	0.025 *M* Phosphate buffer, pH 7.0	*33*
1,2-Cyclohexanedione (CHD)	DMSO	0.2 *M* Phosphate buffer, pH 8.0	*34*
2,4-Dinitro-1-fluorobenzene (DNFB)	DMSO	0.2 *M* Phosphate buffer, pH 8.0	*31*
Ethyl acetimidate (EA)	Water	0.01 *M* Phosphate buffer, pH 7.0	*35*
Diethylpyrocarbonate (DEPC)	Absolute alcohol	0.06 *M* Phosphate buffer, pH 6.0	*36*
Phenylmethylsulfonyl fluoride (PMSF)	DMSO	0.2 *M* Phosphate buffer, pH 8.0	*37*
Woodward's reagent (WRK)	Water	0.5 *M* Phosphate buffer, pH 6.0	*38*

[a]DMSO, dimethyl sulfoxide.

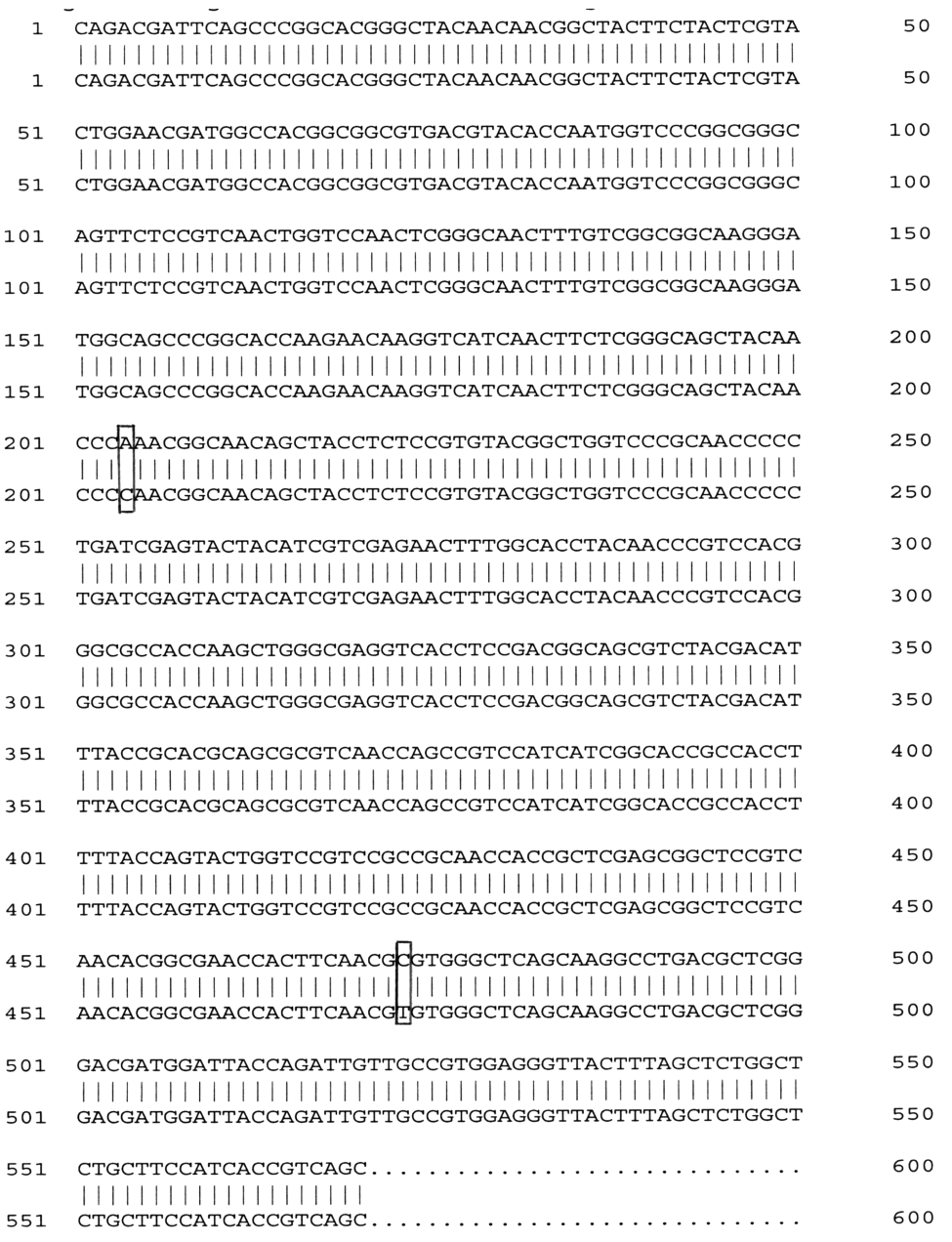

Fig. 1. Comparison of DNA sequence for xylanase II obtained from this study (top sequence) with that published by Törrönen et al. *(18)* (bottom sequence). Two different gene bases were found between these two sequences (shown in boxes).

was a neutral protein. This pI was different from that of wild type (WT) (9.0), possibly owing to the fact that recombinant xylanase II was fused with NusA protein *(18–20)*.

Table 2
Purification of Recombinant Xylanase II From Crude Xylanase Preparation

Procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	471.8	32.4	14.6	1	100
Ni^{2+}-NTA chromatography	147.4	0.66	225	15.4	31.2

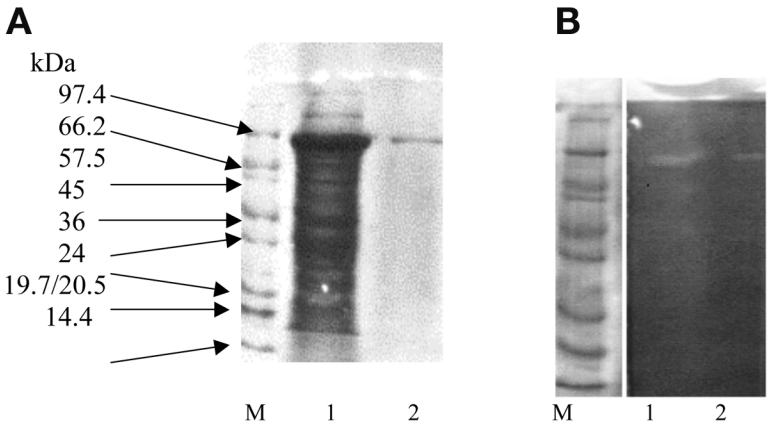

Fig. 2. Polyacrylamide gel electrophoresis of recombinant xylanase II: **(A)** SDS-PAGE and Coomassie blue staining; **(B)** native-PAGE and xylanase activity staining. Lane M, protein molecular mass markers; lane 1, crude xylanase II; lane 2, Ni^{2+}-NTA column–purified xylanase II.

Kinetic Parameters

Apparent K_M and V_{max} values for recombinant xylanase II were 13.8 mg/mL and 336 μmol/(mg·min), respectively, using birchwood xylan as the substrate (data not shown). The activation energy of recombinant xylanase II was 9.0 kcal/mol. The K_M value for recombinant xylanase II using birchwood xylan as the substrate was much higher than that of WT xylanase II (Table 3), possibly owing to the steric effect of the NusA protein.

pH and Thermal Stability

With respect to pH and temperature, in citric-citrate buffer, recombinant xylanase II showed optimum activity at pH 4.0 and 50°C (Figs. 3A and 4A). The pH stability data showed that recombinant xylanase II remained at almost constant high activity (remaining more than 90% activity) in a pH range of 5.0–10.0 (Fig 3B). About 70% of the original activity remained even after 30 min of incubation at pH 2.0–4.0. The results indicated that recombinant xylanase II seemed to be stable at broad pH range.

No significant loss in the activity of recombinant xylanase II was observed after 30 min of incubation at 30–40°C (Fig. 4B). However, enzyme

Table 3
Properties of Xylanase II From *T. reesei*

Xylanase	Moleculer mass (kDa)	p*I*	Activity assay method	Activity assay substrate	K_M (mg/mL)	Xylanase V_{max} (μmol/[mg·min])	Xylanase V_{max}/K_M (U/min·mg/mL)	Reference
Recombinant xylanase II	76	7.5	DNS	Birchwood xylan	13.8	336	24.7	This study
WT xylanase II (*T. reesei* Rut C 30)	20	9.0	DNS	Birchwood xylan	6.8	767	112.8	*19*
WT xylanase II (*T. reesei* Rut C 30)	20	7.5 and 8.1	DNS	Birchwood xylan	3.7	193	52.2	*20*
	20	9.0	DNS	Birchwood xylan	8.0	166	20.8	*20*

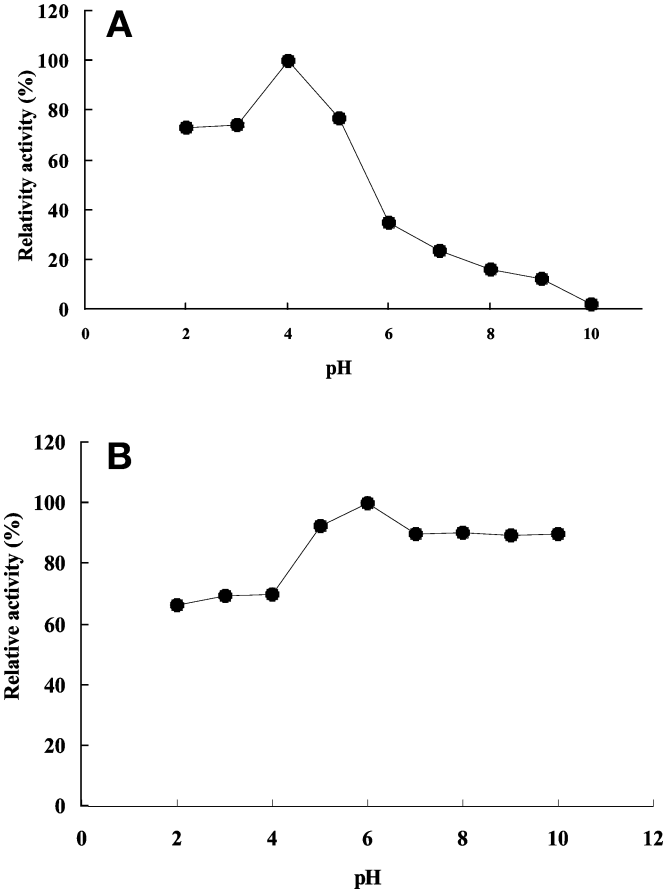

Fig. 3. Effect of pH on **(A)** activity and **(B)** stability of recombinant xylanase II.

activity decreased sharply and nearly denatured when the temperature was greater than 50°C. A conflict between the results shown in Fig. 4A and 4B was noted: recombinant xylanase II was very unstable after incubation for 30 min at 50°C, which was the optimum temperature for the enzyme toward birchwood xylan. To further clarify this conflict, two additional experiments were conducted. The first was conducted to determine the enzyme activity after incubating the enzyme in citric-citrate buffer (pH 4.0) at various temperatures for different time intervals. The purpose of this experiment was to evaluate the denaturation rate of recombinant xylanase II at various temperatures. The second experiment was conducted to determine the enzyme activity at pH 4.0 and 50°C for different time intervals. As shown in Fig. 5A, recombinant xylanase II showed no significant denaturation at low temperatures (30, 35, and 40°C). However, significant denaturation was found at 50°C for time intervals equal or greater than 10 min. After 20 min or longer of incubation at 50°C, enzyme activity became extremely low as a result of heat denaturation. Moreover, Fig. 5B shows

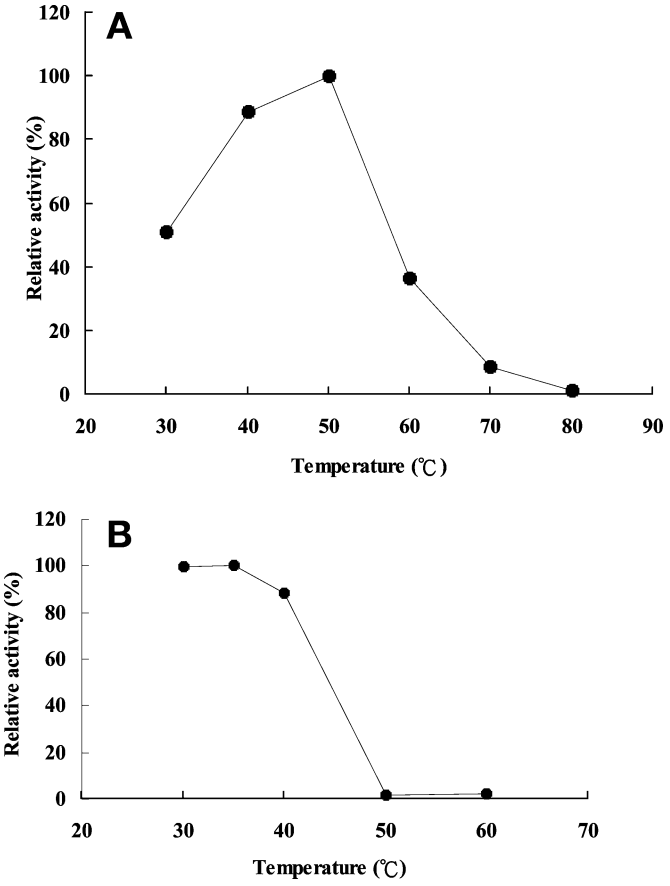

Fig. 4. **(A)** Effect of temperature on activity of recombinant xylanase II; **(B)** effect of temperature on stability of recombinant xylanase II.

that recombinant xylanase II could hydrolyze birchwood xylan at a constant rate at 50°C for even 60 min. These results propose that xylan (the substrate) might enhance the thermal stability of recombinant xylanase II. Most endo-β-1,4-xylanases produced by filamentous fungi show optimum reactions at pH 4.0–6.0 and 45–60°C *(32)*. Therefore, recombinant xylanase II is a somewhat heat-labile protein.

Substrate Specificity

Table 4 shows the substrate specificity of recombinant xylanase II. The activities of the enzyme toward different xylan substrates followed the order birchwood xylan > oat spelts xylan > 4-*O*-methyl-D-glucuro-D-xylan. Less than 10% of starch could be hydrolyzed by the recombinant xylanase II, while methyl cellulose was undegradable by the enzyme. Surprisingly, our data indicated that recombinant xylanase II could hydrolyze 36% of polyglucuronic acid.

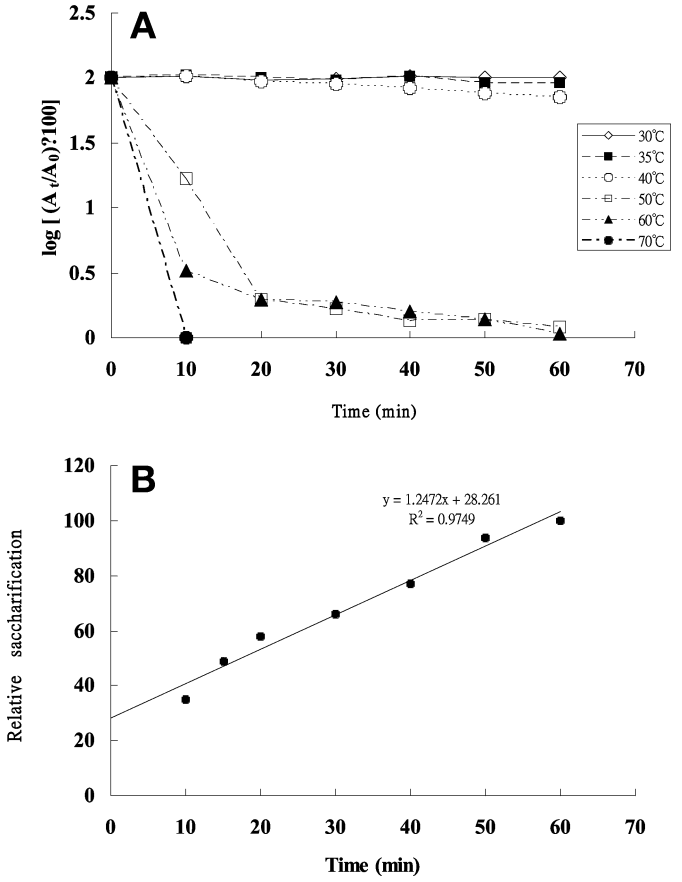

Fig. 5. **(A)** Thermostability and **(B)** kinetics of recombinant xylanase II. The plot of the thermostability is the log of % activity remaining vs incubation tine at various temperatures for the purified recombinant xylanase II. A_0, initial enzyme activity; A_t, enzyme activity after preincubating for time t. The plot of kinetics is the saccharification of birchwood xylan by purified recombinant xylanase II.

Table 4
Substrate Specificity of Recombinant Xylanase II

	Relative activity (%)
Substrate	Recombinant xylanase II
2.0% Birchwood xylan	100
2.0% Oat spelts xylan	74.3
2.0% Soluble starch	9.16
2.0% Pectin	18
2.0% Polyglucuronic acid	36
0.75% 4-*O*-Methyl-D-glucuro-D-xylan	57.6
2.0% Methyl cellulose	0.61

Table 5
Effects of Various Metal Ions on Activity of Recombinant Xylanase II

Chemical	Concentration (m*M*)	Relative activity (%)
		Recombinant xylanase II
None	6.6	100
NaCl	6.6	111.9
$MgCl_2$	6.6	113.3
$ZnCl_2$	6.6	121.3
$MnSO_4$	6.6	163.9
$CuSO_4$	6.6	92.1
$FeSO_4$	6.6	73.0
$FeCl_3$	6.6	36.3
$CaCl_2$	6.6	91.0
EDTA·2Na	6.6	103.3
$HgCl_2$	6.6	3.4

Table 6
Effects of Various Chemical Reagents on Activity of Recombinant Xylanase II

Chemical[a]	Concentration (m*M*)	Relative activity (%)
		Recombinant xylanase II
None	0.75	100
DNFB	0.75	24.1
WRK	0.75	80.6
EA	0.75	75
PMSF	0.75	101.7
CHD	0.75	27.5
DEPC	0.75	74.9
PHMB	0.75	85.6

[a]See Table 1 for definitions of abbreviations.

Effects of Metal Ions and Chemical Reagents on Enzyme Activity

Metal ions of K^+, Na^+, Fe^{3+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} were the activation agents for recombinant xylanase II, and Ca^{2+}, Fe^{2+}, Cu^{2+}, Hg^{2+}, and Hg^{2+} showed inhibitory effects on the enzyme (Table 5). The activity of recombinant xylanase II was inhibited to a great extent by lysine-modifying reagent (2,4-dinitro-1-fluorobenzene) and arginine-modifying reagent (1,2-cyclohexanedione), suggesting that lysine and arginine were important to the active site of this enzyme (Table 6). Phenylmethylsulfonyl fluoride had no effect on enzyme activity, indicating that serine was not located at the active site of recombinant xylanase II. We expected that recombinant xylanase II would be inhibited by WRK because the catalytic residue of this enzyme was glutamic acid. Surprisingly, enzyme activity decreased only 20% by WRK, possibly owing to the steric effect of NusA protein.

Conclusion

Soluble recombinant xylanase II was successfully cloned, overexpressed in *E. coli* AD494(DE3), purified with a simple affinity column chromatography, and characterized. Recombinant xylanase II protein was found to be a soluble and bioactive form in bacteria. Even the cloned xylanase II had two gene bases different from those reported by Törrönen et al. *(18)*, but this did not affect the overexpression of the xylanase II gene because the two different gene bases did not locate at the gene sequences for the active site of the enzyme. The genetic recombinant enzyme could be used for the production of xylooligosaccharides in the future.

Acknowledgment

We greatly appreciate the support of this research by the National Science Council, Taiwan (NSC92-2313-B-126-012).

References

1. Okazaki, M., Fujikawa, S., and Matsumoto, N. (1990), *Bifidobact. Microflora* **9,** 77–86.
2. Hidaka, H., Tashiro, Y., and Eida T. (1991), *Bifidobact. Microflora* **10,** 65–79.
3. Wada, K., Wattabe, J., Mitzutani, J., Suzuki, H., Kiriu, N., Hayakawak, C., and Yamaguchi, C. (1991), Bifidus **4,** 135–140 (in Japanese).
4. Isehibashi, N. and Shimamura, S. (1993), *Food Technol.* **47,** 126, 129, 130, 132–134, 136.
5. Delzenne, N. M., Kok, N., Fiordaliso, M. F., Deboyser, D. M., Goethals, F. M., and Roberfroid, M. B. (1993), *Am. J. Clin. Nutr.* **57,** 820S.
6. Howard, M. D., Gordon, D. T., Garleb, K. A., and Kerley, M. S. (1995), *J. Nutr.* **125,** 2604–2609.
7. Loo, J. V., Coussement, P., Leenheer, L. D., Hoebregs, H., and Smits, G. (1995), *Food Sci. Nutr.* **35,** 525–552.
8. Mutai, M. (1978), *N Food Ind.* **20,** 17–20.
9. Ohku, T. (1992), Süntory Limited report, Japan.
10. Okazaki, M., Koda, H., Izumi, R., Fujikawa, S., and Matsumoto, N. (1991), *J. Jpn. Soc. Nutr. Food Sci.* **44,** 41–44.
11. Chung, Y. C., Hsieh, C. P., and Chan, Y. C. (2002), *Taiwanese Agric. Chem. Food Sci*. **40,** 377–384.
12. Chan, S. H., Chung, Y. C., Chang, C. T., and Chan, K. C. (2004), *Taiwanese Agric. Chem. Food Sci*. **42,** 440–447.
13. Hsu, C. K., Liao, J. W., Chung, Y. C., Hsieh, C. P., and Chan, Y. C. (2004), *J. Nutr*. **134,** 1523–1528.
14. Reilly, P. J. (1981), in *Trends in the Biology of Fermentations for Fuels and Chemicals,* Hollaender, A. E. and Robson, R., eds., Plenum, New York, pp. 111–129.
15. Mcdermid, K. P., Forsberg, C. W., and Mackenzie, C. R. (1990), *Appl. Environ. Microbiol*. **56,** 3805–3810.
16. He, L., Bickerstaff, G. F., Paterson, A., and Buswell, J. A. (1993), *Enzyme Microb. Technol*. **15,** 13–18.
17. Arja, L., Matti, S. A., Nisse, K., Richard, F., and Maija, T. (2000), *Biotechnol. Appl. Biochem.* **31,** 61–68.
18. Törrönen, M. R. L., Messner, M. R., Gonzalez, R., Kalkkinen, N., Harkki, A., and Kubicek, C. P. (1992), *Biotechnology* **10,** 1461–1465.
19. Tenkanen, M., Puls, J., and Poutanen, K. (1992), *Enzyme Microb. Technol*. **14,** 566–574.
20. Lappalainen, A., Siika-Aho, M., Kalkkinen, N., Fagerstrom, R., and Tenkanen, M. (2000), *Biotechnol. Appl. Biochem.* **31,** 61–68.

21. Sambrook, J., Fritch, E. F., and Maniatis, T. (1989), *Molecular Cloning*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. Hanahan, D. and Meselson, M. (1980), *Gene* **10,** 63–67.
23. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
24. Wang, P., Mason, J. C., and Broda, P. J. (1993), *Gen. Microbiol.* **139,** 1987–1993.
25. Ali, B. R. S., Romaniec, M. P. M., Hazlewood, G. P., and Freedman, R. B. (1995), *Enzyme Microb. Technol*. **17,** 705–711.
26. Smith, P., Krohn, R. I., Hermanson, G. T., et al. (1985), *Anal. Biochem*. **15,** 76–85.
27. Royer, J. C. and Nakas, J. P. (1989), in *Forest and Crop Biotechnology, Progress and Prospects,* Valentine, F. A., ed., Springer-Verlag, New York, pp. 363–381.
28. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
29. Lineweaver, H. and Burk, D. J. (1934), *Am. Chem. Soc.* **56,** 658–666.
30. Törrönen, A., Harkki, A., and Rouvinen, J. (1994), *EMBO J.* **13,** 2493–2501.
31. Subramaniyan, S. and Prema, P. (2002), *Crit. Rev. Biotechnol*. **22,** 33–64.