African Journal of Biotechnology Vol. 7 (14), pp. 2454-2461, 18 July, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Over-expression of xylanolytic α-glucuronidase from *Thermotoga maritima* in the pHsh system of *Escherichia coli* for the production of xylobiose from xylan

Yemin Xue*, Jinjin Yu, Xiangfei Song, Jingjing Peng, Weilan Shao

Jiangsu Key Laboratory for Biodiversity and Bio-resources, the Key Laboratory of Microbial Engineering, Nanjing Normal University, 1 Wenyuan Rd, Nanjing 210046, P.R. China.

Accepted 9 June, 2008

The GH67α-glucuronidase encoded by *aguA* of *Thermotoga maritima* is one of the most thermostable α-glucuronidases described to date and thus has considerable potential in industrial application. The enzyme was higher expressed by using the novel pHsh expression vector than in pET vector in *Echerichia coli*. The site directed mutations changed codons for the first two arginines, a leucine and a proline in the 5' flanking region of *aguA* to CGU, CUG and CCG respectively, resulted in a maximum activity of 7.1 U mg^{-1} in pHsh system. The results of calculation using MFOLD showed the introduction of replacement of the nucleotides encoding the N-terminal region of the protein by optimizing rare codons based on reducing the mRNA secondary structures in TIR is a useful approach to increase the expression level of heterologous proteins in *E. coli* cells. The α-glucuronidase of *T. maritima* was clearly able to remove 4-*O*-methylglucuronic acid groups from polymeric xylan and its fragment oligosaccharides. The enzyme acts synergistically with xylanase and beta-xylosidase in the hydrolysis of birchwood xylan and 4-*O*-methyl-D-glucuronoxylan. Enzymatic hydrolysis of corncob xylan examined by HPLC showed that more xylobiose was produced by xylanase hydrolysis in the presence of α-glucuronidase.

Key words: α-Glucuronidase, free energy, overexpression, pHsh, rare codon, secondary structure xylobiose.

INTRODUCTION

Hemicellulases in general, and xylan-degrading enzymes in particular, have attracted much attention because of their potential industrial use in the biobleaching of paper pulp, the bioconversion of lignocellulose material to fermentative products as well as the improvement of animal feedstock digestibility. A recent production of xylooligosaccharides (XOSs), especially xylobiose, can be extensively applied in industry because they have a stimulatory effect on the selective growth of bifidobacterium (Hsu et al., 2004; Palframan et al., 2003). XOSs are released from xylan, the predominant form of hemicellulosic polysaccharides in plant cell walls hydrolysed by xylanase. Because xylan backbone is substituted with α-1,3-linked L-arabinofuranosyl and α-1,2 linked 4-*O*-methylglucuronic acid residues as well as with acetic, *p*-coumaric and ferulic acids, xylanases cannot bind and cleave the xylan backbone close to these modifying groups in xylan degradation. Removal of the side groups is catalyzed by α-D-glucuronidases (EC 3.2.1.139), α-L-arabinofuranosidases (EC 3.2.1.55) and acetylesterases (EC 3.1.1.6). Therefore, α-D-glucuronidases is one of the rate-limiting enzymes for the

*Corresponding author. E-mail: xueyemin@njnu.edu.cn. Tel: +86-025-85891275.

Abbreviations: AguA, α-glucuronidase; Ara, α-L-arabinofuranosidase; Xyl, β-xylosidase; XynB, endoxylanase B; *aguA*, the α-glucuronidase gene; *aguA*1, the N-terminal mutated forms of the *aguA*; *aguA*2, the interior mutated forms of the *aguA*; *aguA*3, the N-terminal plus interior mutated forms of the *aguA*; *aguA*4, the deleted forms of four ammonia acids at N-terminals of AguA; GluA, glucuronic acid; TIR, translational initiation region; RBS, ribosome binding site; XOSs, xylooligosaccharides; PPB, potassium phosphate buffer.

Table 1. Nucleotide sequences of used primer.

Primer	Nucleotide sequence
P1	ccgttccatggactacaggatgtgc
P2	ccgctcgagcggatatatctttcttccctt
P3	ccgttccatggactac**cgt**atgtgctggctggagtac**cgt**ggt**ctgccg**gctgatgtcgccggaa
P4	ccgctcgagcggatatatctttcttccctt
P5	gaa**cg**ttcgattggcatcactcc**gc**g**t**tttattcc**cg**tcc**g**ttgaagaaagaaaaatacatcatgg
P6	agaaaa**a**c**ga**c**g**gatctcgtctttca**g**aactgaacc**c**ggttccagaatggaaacacttgaaaac
P7	atgtgctggctggagtacagaggtttacca

Sever primers for PCR amplification designed and used for the construction of the aguA, aguA1, aguA2, aguA3, aguA4 genes, the replaced nucleotides marked by bold.

degradation of xylan (Ruile et al. 1997; Shao et al., 1995). Recently, it has been revealed that *Bifidobacterium adolescentis* has no α-glucuronidase activity, and more effective proliferation of *Bifidobacterium* was obtained using XOSs pretreated with α-glucuronidase in addition to endoxylanase (Zeng et al., 2007). Therefore, XOSs production by the addition of α-glucuronidase to endoxylanase is necessary.

Thermotoga maritima is an extremely thermophilic eubacterium with an optimum growth temperature of 80°C (Huber et al., 1986). The xylanolytic α-glucuronidase (AguA) found in this organism is one of the most thermostable α-glucuronidases described to date, and thus has considerable potential in industrial applications (Ruile et al., 1997; Xue et al., 2004). However, the expression of the gene from this organism in *E. coli* was very low, even in an expression plasmid construct with optimized transcription and translation start sequences (Ruile et al., 1997). Previous researches have shown that rare codons for arginine (AGA and AGG) and isoleucine (AUA) affected the AguA from *T. maritima* in *E. coli* (Ruile et al., 1997; Xue et al., 2004).. In order to raise gene expression to meet industrial requirements, we report here replacement of the rare codons in the N-terminal region of the AguA from *T. maritima*, and the over-expression of the *aguA* gene using the heat-inducible vector pHsh (Shao and Wu, 2006). The application of the recombinant enzyme in the production of xylobioses from xylan is also described.

MATERIALS AND METHODS

Bacterial strain, plasmids and enzymes

T. maritima (ATCC43589) was grown anaerobically at 80°C in modified Luria-Bertani (LB) medium (pH 7.0), which contained (per litre): 10 g Tryptone, 5 g Yeast extract, 27 g NaCl, 1 mg resazurin, 15 ml trace minerals (as in Difco Maritima Broth medium), 0.5 g Na_2S. The anaerobic condition was generated by degassing, boiling and adding Na_2S (Xue et al., 2006). *E. coli* JM109 (DE3) and JM109 (Promega, Wisconsin, WI, USA) were used as hosts for the expression of *aguA,* via the plasmids of the T7 system pET-28a (Novagen, Madison, WI, USA) and pHsh (Shao and Wu, 2006), respectively. Plasmid pHsh is a new vector of a gene expression system regulated by alternative σ factor σ^{32} (Shao and Wu, 2006), and employed for α-glucuronidase expression. Recombinant enzyme was induced by a temperature shift from 30 to 42°C. *E.coli* was grown in LB supplemented with 100 μg ampicillin ml^{-1} or 30 μg kanamycin ml^{-1}.

DNA manipulation

Plasmid constructions were carried out according to standard procedures (Sambrook et al., 1989). DNA transformation was performed by using electroporation. Genomic DNA was isolated as described by Sambrook et al. (1989), and DNA restriction and modification enzymes were purchased from TaKaRa. Plasmid DNA was purified using the Qiagen Plasmid kit, or QIAquick Gel Extraction Kit, respectively.

Computer analysis

Nucleotide and amino-acid sequences were analyzed with Dnaman version 6.0 of the sequence analysis software package (Lynnon Biosoft, USA). DNA sequencing was performed by Biological Services Unit of Shanghai. The secondary structure of the mRNA in the translational initiation region of the 77 nt from promoter to the first 42 codons in the 5′ flanking region of *aguA* was predicted with MFOLD (www.bioinfo.rpi.edu/applications/mfold).

Plasmid construction

A 2.0 kb chromosomal fragment encoding the putative α-glucuronidase (TM0055) from *T. maritima* (SWISS-PROT accession no. P96105) was amplified by polymerase chain reaction (PCR) using two oligonucleotide primers P1 and P2. Amplicons were ligated into pET-28a or pHsh at restriction sites *Nco*I and *Xho*I, resulting in plasmids pET-28a-*aguA* and pHsh-*aguA,* each with 8 nt spacing between ATG and the Shine-Dalgarno sequence. To raise the expression level of AguA, inverse PCR was carried out by using Pyrobest with pET-28a-*aguA* or pHsh-*aguA* as templates. The primers designed for this purpose were in Table 1.

The first mutated form of *aguA* contained codons for the first two arginines Arg-4,11 (AGG, AGA), leucine Leu-13 (UUA) and proline Pro-14 (CCA) in the N-terminal AguA changed into CGU, CUG and CCG, respectively. The second mutated forms of *aguA* were replacement of the nucleotides encoding Pro-33,54,60, Leu-38 and Arg-43,44,48,55,59 in AguA with CCG, CTG and CGT, respectively. The PCR products were purified using QIAquick PCR purification kit, and phosphated with T_4 polynucleotide kinase, and ligated with T_4 DNA ligase, resulting in the expression plasmids pHsh-*aguA*1

(the N-terminal mutated form) pHsh-*aguA*2 (the interior mutated form) and pHsh-*aguA*3 (the double mutated form). In the process of mutagenesis of *aguA*, the four amino acids at N-terminal were cut off in order to maintain one ATG of target protein, producing the plasmids pHsh-*aguA*4. These recombinant plasmids were verified by DNA sequencing, and used for the over-expression of recombinant α-glucuronidase.

Gene expression

E. coli JM109 (DE3) and JM109 cells were freshly transformed with the expression vectors pET-28a-*aguA*, pET-28a-*aguA*1, respectively, and grown at 37°C in 100 ml LB media until the early exponential phase (OD_{600} = 0.6 - 0.8), and induced with 0.8 mM IPTG at 37°C for 5 h. *E.coli* JM109 cells harboring pHsh-*aguA,* pHsh-*aguA*1, pHsh-*aguA*2, pHsh-*aguA*3 and pHsh-*aguA*4 were grown at 30°C to an OD_{600} of 0.6 to 0.8, and then transferred into a 42°C water-bath shaker, and grows for a further 8 h at 42°C. *E. coli* cells in 1 ml of culture were harvested by centrifugation at 9,600 g for 10 min, and resuspended in 300 µL of 50 mM potassium phosphate buffer (PPB, pH 6.0, 75°C). The mixture was immediately sonicated by using four 15 s pulses while kept on ice. The cell debris was separated from the protein supernatant (crude cell extract) by centrifugation at 12,000 *g* and 4°C for 10 min. The resulting supernatant was used for the α-glucuronidase activity assay and monitor by SDS-PAGE (10%). The gel was stained with coomassie blue R-250, and the protein bands were analyzed with an image analysis system (Bio-rad).

Enzyme assays

The substrate for α-glucuronidase was 1% (w/v) 4-*O*-methyl-D-glucurono-D-xylan (Sigma, X0502) pretreated with recombinant XynB of *T. maritima* (10 units per gram xylan) for 12 h at 75°C. α-Glucuronidase activity was determined by measuring the release of 4-*O*-methyl-α-D-glucuronic acid from the substrate. The amount of enzyme to produce 1 □mol of glucuronic acid per min was defined as 1 unit. The reaction mixture contained 10 µl substrate, 85 □l of 100 mM PPB (pH 6.0, 75°C), and 5 µl of α-glucuronidase. After 10 min of incubation at 75°C, the reaction was stopped through the addation 0.3 ml of copper reagent (Milner and Avigad, 1967). The reaction mixture was then boiled for 10 min and cooled in ice bath. The supernatant was mixed with 0.2 ml of arsenomolybdate reagent (Nelson, 1944), and the absorbance at 620 nm was measured. Glucuronic acid (Sigma, G5269) was used as the standard for quantification of 4-*O*-methylglucuronic acids. The amount on release of 4-*O*-methylglucuronic acid from different xylan was determined by this colorimetric method. Protein concentrations were determined by the bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Xylanase, α-L-arabinofuranosidase and β-xylosidase activities were measured using 10 g l^{-1} birchwood glucuronoxylan (Sigma, X0502), *p*-nitrophenyl α-L-arabinofranoside (Sigma, N3641) and *p*-nitrophenyl β-D-xylopyranoside (Sigma, N2132), respectively, as substrates (Xue et al et al., 2003, 2004, 2006). All enzyme activities were expressed in SI units.

Action of α-glucuronidase with other enzymes

The substrates used for the hydrolysis experiments were 4-*O*-methyl-D-glucuronoxylan (Sigma, M5144), birchwood xylan (Sigma, X0502), and oat spelt xylan (Sigma, X0627). The α-glucuronidase (35 Ug^{-1}) was incubated with substrates (5 g l^{-1}) in 50 mM PPB, pH 6.0 at 75℃ for 5 h. The other enzymes used in the hydrolysis experiments were xylanase, β-xylosidase, and α-arabinofuranosidase of *T. maritima*.

Application tests of enzymatic hydrolysis

Xylans from 100 g of corncob meal were extracted for 1 h at 120°C in 1 l of 2% (w/v) KOH. The corncob residue was separated from the xylan supernatant by nylon fabric. The resulting supernatant was mixed with 3 volumes of cold ethanol to precipitate and harvest the corncob xylans. Water content of the corncob xylans was determined after drying for 16 h at 105°C. For quantitative assay of reaction rates, a milliliter of 2% (w/v) corncob xylan in 50 mM PPB was hydrolyzed at 75°C for 1 to 26 h with purified 0.03 U xylanase B with or without 0.02 U α-glucuronidase. For HPLC analysis, 1 g corncob xylan was dissolved in 20 ml of 100 mM PPB (pH 6.0, 75°C) in a 100-ml bioreactor, 24 U xylanase B was added with or without 6 U α-glucuronidase. The enzymatic reaction was carried out for 12 h at 75°C and stopped by cooling at -20°C before analysis.

Analysis of the degradation products

For quantitative assay of reaction rates, reducing ends in the reaction mixture was measured using a modified 3, 5-dinitrosalicyclic acid assay (Miller, 1959). The amount of 4-*O*-methylglucuronic acid from different xylan by acting of α-glucuronidase with other enzymes was determined by the colorimetric method (Milner and Avigad, 1967; Nelson, 1944). For HPLC analysis of xylose and xylo-oligosaccharides, two milliliters of the reaction mixture were mixed with 6 ml of ethanol to precipitate and remove the xylooligosaccharides of higher DP. The samples were then lyophilized in a Speed-Vac lyophilizer (1Z58Q FTS system, USA), and the pellets re-suspended in 500 µl water. A 10 µl sample of the re-suspended hydrolysate was injected into the carbohydrate analysis column (Waters Sugarpak1, USA) with water as a mobile phase, and the xylo-oligosaccharides were detected by a refractive-index detector (Waters Co., USA). Xylose, xylobiose and xylotriose (Suntory Ltd., Japan) were used as standards.

RESULTS

Over-expression of α-glucuronidase gene by the Hsh promoter

The *aguA* gene of *T. maritima* from pET-28a-*aguA* was subcloned into a 2442-bp plasmid called pHsh, which is a novel over-expression vector for *E.coli* and controls the expression of foreign gene by the unique Hsh promoter, induces the expression of foreign gene by transferring recombinant cells from 30 to 42°C. After inducted at the exponential phase (OD_{600} of 0.6 - 0.8), the activity of thermostable AguA reached the maximum in an 8-h induction period of JM109 / pHsh-*aguA* (data not shown). Based on the optimal induction, the expression level of gene *aguA* from plasmid pHsh-*aguA*, appeared higher than that from the plasmid pET-28a-*aguA* (Figure 1, compare lanes 2 and 7). The activity of α-glucuronidase detected in recombinant *E.coli* cells harboring pET-28a-*aguA* and pHsh-*aguA* was 4.5 Umg^{-1}, 5.1 Umg^{-1} of total soluble proteins.

Site directed mutagenesis of *aguA*

Silent mutations were introduced into the 5' flanking regi-

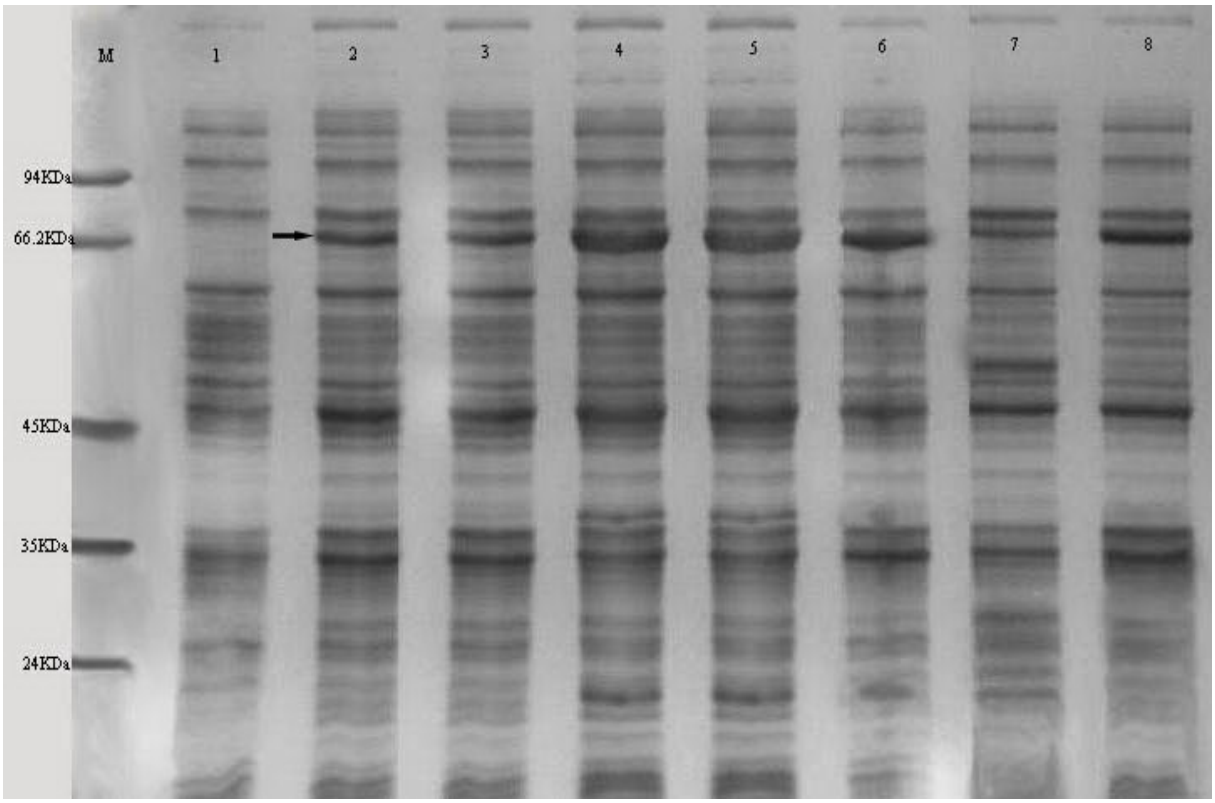

Figure 1. SDS-PAGE analysis of total proteins prepared form the recombinant *E. coli* strains expressing the α-glucuronidase of *T. maritima*. 8 µg protein samples were applied. Lane M, low molecular weight marker; Lane 1, *E. coli* JM109 containing the plasmid pHsh; Lane 2, *E. coli* JM109 containing the plasmid pHsh-*aguA*; Lane 3, *E. coli* JM109 containing the plasmid pHsh-*aguA*2; Lane 4, *E. coli* JM109 containing the plasmid pHsh-*aguA*1; Lane 5, *E. coli* JM109 containing the plasmid pHsh-*aguA*3; Lane 6, *E. coli* JM109 containing the plasmid pHsh-*aguA*4; Lane 7, *E. coli* JM109 (DE3) containing the plasmid pET-28a-*aguA*; Lane 8, *E. coli* JM109 (DE3) containing the plasmid pET-28a-*aguA*1.

on of *aguA*, since codons for the Arg-4,11 (CGG, AGA), Leu-13 (AUA) and Pro-14 (CCA) in AguA were changed to CGU, CUG and CCG, which are frequently used in *E. coli*. The expression of AguA was monitored by activity measurements and SDS-PAGE, and the results are given in Figure 1. The SDS-PAGE analysis of the total soluble proteins from recombinant *E.coli* cells harboring pET-28a-*aguA*1, pET-28a-*aguA*, pHsh-*aguA*1 and pHsh-*aguA* indicated that the expression level of the *aguA* gene from plasmid pET-28a-*aguA*1 and pHsh-*aguA*1 was much higher than that from the plasmid pET-28a-*aguA* and pHsh-*aguA* (Figure 1, compare lanes 8 and 7, 4 and 2), respectively. And a band of about 74 kDa, which agrees with the expected theoretical molecular weight of the α-glucuronidase could be observed on the SDS gel (Figure 1. arrowhead). The activity of α-glucuronidase detected in recombinant *E.coli* cells harboring pET-28a-*aguA*1, pHsh-*aguA*1 was 5.3 Umg^{-1} and 7.1 U mg^{-1} of total soluble proteins, respectively. The expression level of the α-glucuronidase in the double mutated form was not further improved (Figure 1, compare lanes 3 and 2, 5 and 4). The *aguA* expression level from pHsh-*aguA*4 slightly higher than that from pHsh-*aguA* (Figure 1, compare lanes 6 and 2).

The presence of endoxylanase and β-xylosidase enhances the activity of α-glucuronidase on xylan

The influence of endoxylanase, α-arabinosidase and β-xylosidase from *T. maritima* on α-glucuronidase activity was studied by incubating combinations of these enzymes with birchwood xylan, 4-*O*-methyl-D-glucuronoxylan and oat spelt xylans, respectively, and the results are given in Table 2. The purified recombinant α-glucuronidase of *T. maritima* was clearly able to remove 4-*O*-methylglucuronic acid groups from birchwood xylan, 4-*O*-methyl-D-glucuronoxylan and oat spelt xylans, was clearly enhanced in the presence of xylanase B from *T. maritima*. The enzyme also showed slight synergism with xylanase and β-xylosidase while acting on birchwood xylan and 4-*O*-methyl-D-glucuronoxylan but not oat spelt xylans. It did not show any synergy with the α-L-arabinofuranosidase of *T. maritima* (Table 2).

Table 2. Synergistic effects of α -glucuronidase (AguA, 35 U g^{-1}), endoxylanase (XynB, 35 U g^{-1}), β-xylosidase (Xyl, 35 U g^{-1}), and α-L-arabinofuranosidase (Ara, 35 U g^{-1}) activity on release of 4-*O*-methylglucuronic acid from different xylan (5 g l^{-1})[a].

Xylan	Enzyme(s) used in reaction mixtures	MeGlcA liberated (mg l^{-1})[b]
4-*O*-methyl-D-glucuronoxylan	None	120
	AguA	375
	XynB	130
	Xyl	145
	AguA+XynB	525
	AguA+Xyl	380
	AguA+XynB+Xyl	535
Birchwood xylan	None	130
	AguA	405
	XynB	140
	Xyl	180
	AguA+XynB	585
	AguA+Xyl	430
	AguA+XynB+Xyl	700
Oat spelt xylan	None	240
	AguA	475
	XynB	190
	Xyl	245
	Ara	215
	AguA+XynB	555
	AguA+ Xyl	435
	AguA+Ara	465
	AguA+ XynB+Xyl	555
	AguA+XynB+Ara	565
	AguA+XynB+Ara+Xyl	555

[a]Five hour hydrolysis at pH 6.0 and 75˚C. The XynB, Xyl and Ara used in the hydrolysis experiments were recombinant endoxylanase, β-xylosidase and α -L-arabinofuranosidase from *T. maritima,* respectively.
[b]The amount of 4-*O*-methylglucuronic acid was determined by the colorimetric method.
We measured the amount of 4-*O*-methylglucuronic acid for usually three or more independent experiments.

Application in enzymatic production of xylooligosaccharides from xylan

Production of xylooligosaccharides from corncob xylan by xylanase and α-glucuronidase was examined. The purified xylanase (0.033 U) was incubated with 0.5 mL of 2% (w/v) corncob xylan dissolved in 100 mM PPB (pH 6.0) at 75^{o}C; reducing sugars were increased with the reaction time. With the addition of α-glucuronidase (0.048 U) to xylanase, the hydrolysis rate was increased rapidly in 3 h, and slowed down after 3 h of reaction (Figure 2). HPLC measurement revealed that the hydrolysis of corncob xylan by xylanase was enhanced by the addition of α-glucuronidase, and 1.6 times more xylobiose were 4.7 times xylose produced by the xylanase in the presence of the α-glucuronidase (Figures 3A, B).

DISCUSSION

Over the past few decades, it has been most thoroughly documented that the gene expression level can be caused by the foreign genes through forming secondary structures of mRNA, inducible promoters, spacing between the Shine-Dalgarno sequence and start codon, or using rare codons of *E. coli* (Nivinskas et al., 1999; Spanjiaard et al., 1990; Kimura and Iyanagi, 2003). Many strong inducible promoters have been developed in different expression vectors, such as the *lac*, *tac*, *trc*, p_L/p_R and T7 promoter. In this work, we used a new vector, pHsh carring the unique Hsh promoter, resulting in higher expression of α-glucuronidase in pHsh vector than in pET vector (Figure 1, compare lanes 2 and 7, 4 and 8). With MFOLD algorithm online

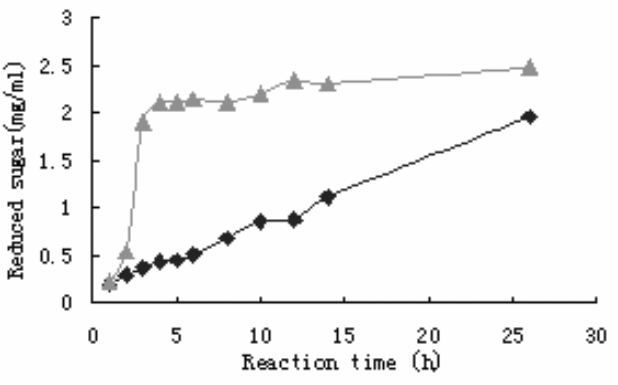

Figure 2. Effect of α -glucuronidase on the hydrolysis of corncob xylan (a). The purified xylanase in the presence or absence of the α -glucuronidase at 75ºC, in different time, was incubated with 0.5 mL 2 % (w/v) corncob xylan dissolved in 100 mM potassium phosphate buffer (pH 6.0) at 75ºC. The reducing sugars in the reaction mixtures were analyzed by 3,5-dinitrosalicyclic acid (DNS). ■ xylanase (0.033 U), ▲ xylanase + α-glucuronidase (0.048 U).

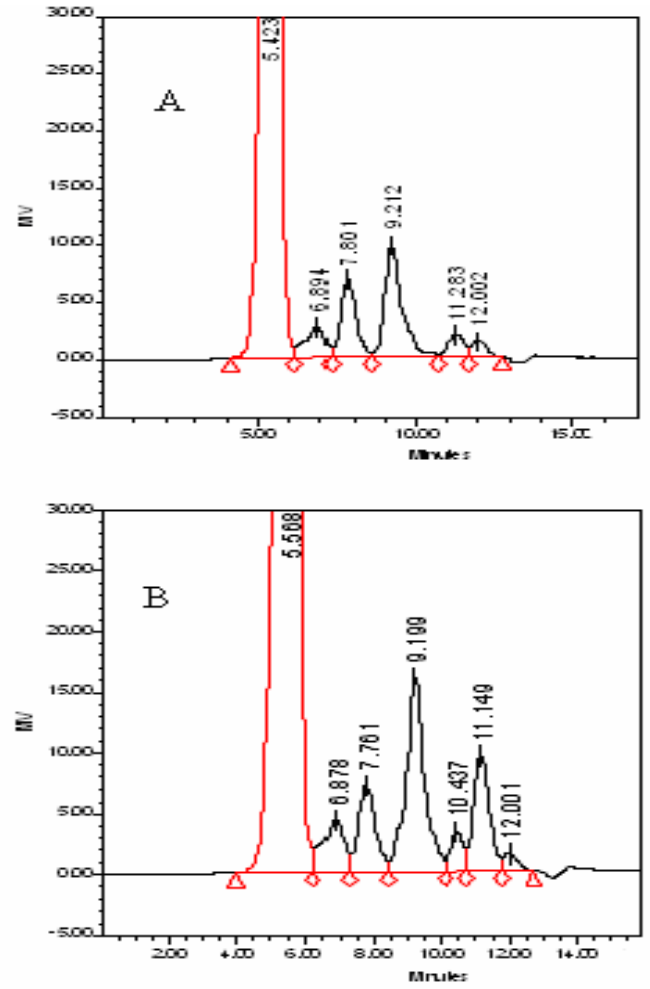

Figure 3. HPLC of products of corncob xylan hydrolysed by the purified xylanase (A) or by xylanase plus α-glucuronidase (B). Details of reaction conditions are described in Materials and Methods. A 10 µl sample of the resuspended hydrolysate was injected into the carbohydrate analysis column with water as a mobile phase, and the xylooligosaccharides were detected by a refractive-index detector. The migration times of xylose, xylobiose and xylotrise, were 11.283, 9.212, and 7.801 min in Figure 3A, or 11.149, 9.199, and 7.761 min in Figure 3B, respectively.

(www.bioinfo.rpi.edu/applications/mfold/rna/forml-2.3.cgi), the Gibbs free energy (ΔG) of secondary structure of mRNAs in TIR was predicted to be almost the equal in the pHsh-*aguA* (ΔG = -7.17 kcal/mol) and pET-28a-*aguA* (ΔG = -7.31 kcal/mol), while their Shine-Dalgarno site (AAGGAG) was also the same, and the SD sequence and AUG are located at the optimum spacing of 8 bp. The pHsh vector was constructed by combining the synthesized Hsh promoter with a synthesized terminator and the replicon of pUC19, the critical characteristic element of pHsh vector was the Hsh promoter which was designed based on the conserved sequences of promoters of heat shock genes of *E. coli* (Shao and Wu, 2006). Thus, the result showed that high-level expression of *aguA* genes was related with the pHsh vector carrying Hsh promoter and the replicon of pUC19, which was also confirmed by overexpressing of the gene encoding thermostable xylanase in our laboratory (Wu et al., 2008).

Previous results have shown that rare codons for arginine (AGA/AGG), isoleucine (AUA), leucine (CUA), and proline (CCC) affected the amount of AguA from *T. maritima* produced in *E. coli*. Therefore, it is worthwhile to raise gene expression level to meet industrial requirements by sequence modification on the gene, and silent mutations were introduced into the 5´ flanking region of *aguA*, since codons for the first arginines (CGG, AGA), leucine (AUA) and proline (CCA) in AguA were changed to CGU, CUG and CCG, which are frequently used in *E. coli*. The resulting *aguA* expression level from pHsh-*aguA*1, pET-28a-*aguA*1 was much higher than that from pHsh-*aguA*, pET-28a-*aguA,* respectively. And the *aguA* expression level appeared a maximum activity of 7.1 U mg^{-1} in pHsh-*aguA*1, which almost corresponds to the activity of 8.4 U mg^{-1} from pET-28a-*aguA* in *E. coli* BL21-CodonPlus (DE3)-RIL. Furthermore, the results of calculation using MFOLD showed that the free energy of least the 77 nt was changed from -7.17 (pHsh-*aguA*), -7.31 (pET28a-*aguA*) kcal mol^{-1} to -3.26 (pHsh-*aguA*1), -4.16 (pET28a-*aguA*1) kcal mol^{-1}, respectively, resulting in the negative value of ΔG in the pHsh-*aguA*1 (ΔG = -3.26 kcal/mol). Because less negative the value of the free energy of an mRNA secondary structure is, more unstable the mRNA secondary structure forms (Wu et al., in press; Zuker, 2003), which can be used for efficient translational initiation. Subsequently, increasing the expression level of the protein by replacement of the nucleotides encoding Pro-33,54,60, Leu-38 and Arg-43,44,48,55,59 in AguA with CCG, CTG and CGT, respectively, was expected, but which was not confirmed. Therefore, these data indicate that the rare arginine codons (AGA/AGG), leucine codon (TTA) and proline

(CCA) in the 5´ flanking region of *aguA* strikingly restricted its expression, and the introduction of site directed mutations of the gene encoding the N-terminal region of the protein by optimizing rare codons based on reducing the mRNA secondary structures in TIR, is a useful approach to increase the expression level of heterologous proteins in *E. coli* cells.

The specific activity of the α-glucuronidases from *T. maritima* on aldotriuronic acid $MeGlcAX_2$ has been determined, but its activity against polymeric substrate has not been studied (Ruile et al., 1997). The recombinant α-glucuronidase of *T. maritima* purified in this work was clearly able to remove 4-*O*-methylglucuronic acid groups from polymeric xylan, but its action was clearly enhanced in the presence of xylanase of *T. maritima*, which belongs to glycoside hydrolase family 10. Thus, the α-glucuronidase from *T. maritima* favored water-soluble oligomeric substrates over polymeric xylan. The enzyme acts synergistically with xylanase and β-xylosidase in the hydrolysis of birchwood xylan and 4-*O*-methyl-D-glucuronoxylan. Arabinoxylans from oat spelt xylan were also tested as substrates for the α-glucuronidase of *T. maritima*. The enzyme did not show any synergy with the α-L-arabinofuranosidases of *T. maritima.* Similarly, Tenkanen and Siika-aho (2000) reported that the α-glucuronidase of *Schizophyllum commune* acting on polymeric xylan did not show any synergy with the α-arabinosidase of *A. terreus* even though the α-arabinosidase efficiently removed arabinose from the softwood pulp (Tenkanen and Siika-aho, 2000).

Production of xylobiose from xylan by the purified endoxylanase and α-glucuronidase was examined. With corncob xylan hydrolysis, the addition of α-glucuronidase to endoxylanase gave xylobiose as the major component in the hydrolysates, and its proportion increased with the reaction time. Without addition of α-glucuronidase, xylan hydrolysis was weakened (Figure 2: compare A and B). The main product of enzyme hydrolysis, xylobiose, is a selective growth stimulant to intestinal *Bifidobacterium*, which is beneficial for the maintenance of healthy intestinal microflora (Hsu et al., 2004). The selective stimulative effect of xylobiose on *Bifidobacterium* was greater than that of other oligosaccharides (Palframan et al., 2003). Previously, we found that three bifidobacterias cannot utilize parts of XOS decorated with 4-*O*-methyl-D-glucuronic acid, and more effective XOS from xylan will be obtained by the addition of α-glucuronidase to endoxylanase (Zeng et al., 2007). These results show that α-glucuronidase can be used effectively to produce xylobiose from xylan by endoxylanase. Therefore, the high level of the thermostable α-glucuronidase from *T. maritima,* combined purification by a simple heat treament, has a considerable potential in the production of xylooligosaccharide, especially xylobiose.

On the other hand, the addition of α-glucuronidase to endoxylanase shows an increase of the xylose in this study. It is possible because the recombinant XynB of *T. maritima* was found to cleave *p*-nitrophenyl-β-xyloside (Jiang et al. 2004) and much more xylose is produced by this β-xylosidase activity and its synergism with the α-glucuronidase (Shao et al., 1995). Therefore, the endoxylanases without β-xylosidase activity can produce more xylobiose and less xylose in the production of xylooligosaccharide.

In this application test, we have studied the hydrolysis of only corncob xylan. The corncob xylans generally contain more L-arabinose than 4-*O*-methyl-D-glucuronic acid (or D-glucuronic acid) residues. In terms of the efficiency of hydrolysis, α-L-arabinofuranosidases might be more important than α-glucuronidase, which was also confirmed by the enzymatic production of xylobiose from corncob xylan by the endoxylanase and α-L-arabinofuranosidase (Xue et al., 2006). These indicate that the synergism of α-L-arabinofuranosidase and α-glucuronidase with endoxylanase are needed in order to effectively produce xylobiose from corncob xylan. The cooperation between these enzymes may be improved by hybrid enzyme to simplify the reactions of multi-step and multi-enzyme. It cannot only reduce the synthesis cost, but also accelerate the reaction rate and have great significance in industry (An et al., 2005). As further investigation to produce a more efficient enzyme capable of degrading heteroxylan to xylobiose, we are carrying out bifunctional fusions of α-L-arabinofuranosidase (ǫrα-glucuronidase) and xylanase and applications thereof.

ACKNOWLEDGEMENTS

This project work, BK2006220 was supported by NSF of Jiangsu Province of China, and also by a grant from The Nanjing Normal University (2006104XYY0163).

REFERENCES

An JM, Kim YK, Lim WJ, Hong SY, An CL, Shin EC, Cho KM, Choi BR, Kang JM, Lee SM, Kim H, Yun HD (2005). Evaluation of a novel bifunctional xylanase-cellulase constructed by gene fusion. Enzyme Microb. Technol. 36:989-995.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgrom quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Hsu CK, Liao JW, Chung YC, Hsieh CP, Chan YC (2004). Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats. J. Nutr. 134: 1523-1528.

Huber R, Langworthy TA, Helmut K, Thomm M, Woese CR, Sleytr UB, Stetter KO (1986). *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up 90□. Arch. Microbiol. 144: 324-333.

Jiang ZQ, Zhua YP, Li L, Yu XH, Kusakabe I, Kitaoka M, Hayashi K (2004). Transglycosylation reaction of xylanase B from the hyperthermophilic *Thermotoga maritima* with the ability of synthesis of tertiary alkyl-□-xylobiosides and xylosides. J. Biotechnol. 114: 125-134.

Kimura S, Iyanagi T (2003). High-level expression of porcine liver cytochrome P-450 reductase catalytic domain in *Escherichia coli* by modulating the predicted local secondary structure of mRNA. J. Biochem. 134: 403-413.

Milner Y, Avigad GA (1967). Copper reagent for the determination of

hexuronic acids and certain ketohexoses. Carbohydr. Res. 4: 359-361
Miller GL (1959). Use of dinitrosalicyclic acid reagent for determination of reducing surgars. Anal. Chem. 31: 426-428.
Nelson NA (1944). Photometric adaptation of the Somogyi method for determination of glucose. Biol. Chem. 159: 375-380.
Nivinskas R, Malys N, Klausa V, VaisÏkunaite R, Gineikiene E (1999). Post-transcriptional control of bacteriophage T4 gene 25 expression: mRNA secondary structure that enhances translational initiation. J. Mol. Biol. 288: 291-304.
Spanjiaard RA, Chen K, Walker JR, Duin (1990). J Frame shift suppression at tandem AGA and AGG dodons by cloned tRNA genes: assigning a codon to argU tRNA and T4 tRNA ^{Arg}. Nucleic Acids Res. 18: 5031-5036.
Palframan RJ, Gibson GR, Rastall RA (2003). Carbohydrate preferences of Bifidobacterium species isolated from the human gut. Curr. Issues Intest. Microbiol. 4: 71-75.
Ruile P, Winterhalter C, Liebl W (1997). Isolation and analysis of a gene encoding α-glucuronidase an enzyme with a novel primary structure involved in the breakdown of xylan. Mol. Microbiol. 23: 267-279.
Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual, 2th ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Shao WL, Obi SKC, Puls J, Wiegel J (1995). Purification and characterization of the α-glucuronidase from *Thermoanaerobacterium* sp. Strain JW/SL-YS485, an important enzyme for the utilization of substituted xylan. Appl. Environ. Microbiol. 61: 1077-1081.
Shao WL, Wu HW (2006). Plasmid Vectors Regulated by sigma 32 in *E.coli*. PCT patent, 002574A1.
Tenkanen M, Siika-aho (2000) M An alpha-glucuronidase of *Schizophyllum commune* acting on polymeric xylan. J. Biotechnol. 78: 149-161.
Wu HW, Pei JJ, Wu GG, Shao WL (2008). Overexpression of GH10 endoxylanase XynB from T. *maritima* in *E. coli* by a novel vector with potential for industrial application. Enzyme Microb. Technol. 42: 230-234.
Xue YM, Mao ZG, Shao WL (2004). Expression and purification of thermostable α-glucuronidase from *Thermotoga maritima*. Chin. J. Biotechnol. 20: 554-560.
Xue YM, Wu AL, Zeng HY, Shao WL (2006). High-level expression of an β-L-arabinofuranosidase from *Thermotoga maritima* in *Escherichia coli* for the production of xylobiose from xylan. Biotechnol. Lett. 28: 351-356.
Xue YM, Mao ZG, Shao WL (2003). Expression of xylanase B gene of *Thermotoga maritima* in *Escherichia coli*. Food Ferment Ind. 29: 20-25.
Xue YM, Shao WL (2004). Expression and characterization of a thermostable β-xylosidase from hyperthermophile *Thermotoga maritima*. Biotechnol. Lett. 26: 1511-1515.
Zeng HY, Xue YM, Peng TT, Shao (2007). WL Properties of xylanolytic enzyme system in bifidobacteria and their effects on the utilization of xylooligosaccharides. Food Chem. 101: 1172-1177.
Zuker M (2003). Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31: 3406-3415.