J Ind Microbiol Biotechnol (2011) 38:967–973
DOI 10.1007/s10295-010-0868-5

ORIGINAL PAPER

Signal peptide of *Aureobasidium pullulans* xylanase: use for extracellular production of a fungal xylanase by *Escherichia coli*

Kazuyoshi Ohta · Hidenori Tanaka · Daisuke Yamakawa · Hironori Hamasuna · Hirohisa Fujimoto

Received: 30 June 2010 / Accepted: 28 August 2010 / Published online: 25 September 2010

Abstract An extracellular xylanase XynI of glycoside hydrolase family 11 from the dimorphic fungus *Aureobasidium pullulans* ATCC 20524 possesses an N-terminal extension of 34 amino acids (Ohta et al., J. Biosci. Bioeng. 92:262–270, 2001). The N-terminal extension includes three sites (Ala-X-Ala-X-Ala-X-Ala) that are potentially cleavable by signal peptidase I of *Escherichia coli*. The *A. pullulans xynI* signal sequence was fused in frame to the mature protein region of the equivalent xylanase gene *xynA* from the filamentous fungus *Penicillium citrinum*. The gene fusion *xynI*::*A* was inserted into the plasmid pET-26b(+) to yield pEXP401. An *E. coli* BL21(DE3) transformant harboring the pEXP401 exhibited xylanase activity (per ml of the culture) of 16.8 U in the fraction of culture supernatant as well as 4.29 U in the fraction of cell-free extract after 12 h of growth with isopropyl-*β*-D-thiogalactopyranoside at 30°C. N-terminal amino acid sequence analysis of the secreted recombinant proteins revealed cleavage at four distinct sites within the N-terminal extension of XynI, two of which conformed to the Ala-X-Ala motif prior to the cleavage site. The XynA proteins secreted into the culture medium showed high specific activities from 506 to 651 U/mg, which were twofold higher than that of the native enzyme.

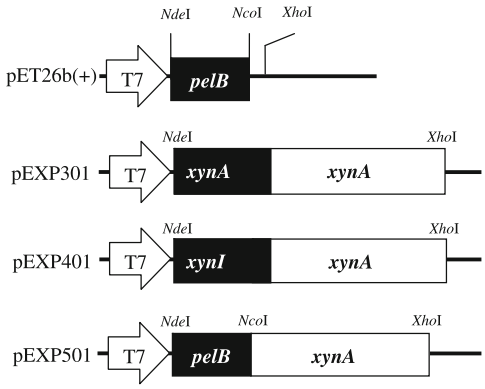
Keywords *Aureobasidium pullulans* · *Escherichia coli* · *Penicillium citrinum* · Signal peptide · Xylanase

K. Ohta (✉) · H. Tanaka · D. Yamakawa · H. Hamasuna · H. Fujimoto
Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai Nishi, Miyazaki 889-2192, Japan
e-mail: k.ohta@cc.miyazaki-u.ac.jp

Introduction

β-1,4-D-Xylans constitute the main polymeric component of the hemicellulose fraction of plant cell wall and serve as a potential feedstock for production of fuels and chemicals [1]. Endo-*β*-1,4-xylanase (*β*-1,4-D-xylan xylanohydrolase; EC 3.2.1.8), designated xylanases, hydrolyze internal *β*-(1→4)-xylosidic linkages in the insoluble xylan backbone to yield soluble oligosaccharides. The majority of xylanases fall into two glycoside hydrolase (GH) families: family-10 xylanases with a high M_r of greater than 30 kDa and family-11 xylanases with a relatively low M_r ranging from 19 to 25 kDa [2]. We have previously purified a GH family-11 extracellular xylanase from the dimorphic fungus *Aureobasidium pullulans* ATCC 20524 [3]. The xylanase gene (*xynI*) encoded a precursor protein (XynI) composed of an N-terminal extension of 34 residues and a mature protein of 187 residues. *A. pullulans* XynI was expressed heterologously and efficiently secreted into the culture medium by its own signal peptide in yeasts *Saccharomyces cerevisiae* [3] and *Pichia pastoris* [4].

The advantages of using *E. coli* for production of recombinant proteins are the ease of growth and genetic manipulation. Burchhardt and Ingram [5] expressed the xylanase gene (*xynZ*) from the thermophilic bacterium *Clostridium thermocellum* in ethanol-producing *Escherichia coli* KO11 that ferments all lignocellulose-derived sugars. However, the recombinant xylanase produced was retained in the cytoplasm. To convert xylan to fuel ethanol in a single-stage process, the xylanase needs to be secreted from the cytoplasm to the outside of the *E. coli* cell by crossing two biological membranes, the inner and outer membranes. Extracellular production of recombinant xylanase by *E. coli* will facilitate the



B

	Xylanase activity (U/ml of the culture)	
	Culture supernatant	Cell-free extract
pET26b(+)	ND	ND
pEXP301	0.623 ± 0.429	1.48 ± 0.28
pEXP401	16.8 ± 2.0	4.29 ± 2.09
pEXP501	10.1 ± 1.1	0.627 ± 0.338

Fig. 1 **a** Schematic representation of expression plasmids. *Arrows* indicate T7 promoter. *Solid and open bars* indicate signal sequences and mature protein regions, respectively. **b** Extracellular and intracellular xylanase activities of *E. coli* BL21(DE3) carrying different plasmids. Culture supernatants and cell-free extracts were prepared from 1-ml portion of each culture after growth with IPTG for 12 h as described in the text. The values of activity are the mean of three independent cultures ± standard deviation. *ND* not detected

metabolic engineering of strains for converting plant biomass directly into a variety of fermentation products. To date, several glycoside hydrolases such as xylanase [6–8], cellulase [7], and pectinase [9] have been shown to be exported from the cytoplasm to the culture medium with various percentages when expressed in *E. coli* using native or heterologous signal peptides. In such cases, translocation of recombinant proteins into periplasmic space through cleavage of signal peptide by signal peptidase is followed by their release through the outer membrane into the external environment by an as yet unknown mechanism. Successful extracellular protein secretion is likely to depend on the *E. coli* host strain and the nature of signal sequence and protein to be secreted [10].

Ala is preferred at positions −3 and −1 with respect to the cleavage site for signal peptidase I [10]. Inspection of the N-terminal extension of *A. pullulans* XynI revealed the presence of three repeats of potential cleavage site, which conforms to the so-called Ala-X-Ala box where X could be almost any residue: Ala-X-Ala-X-Ala-X-Ala. To our knowledge, the use of fungal signal peptide for extracellular production of recombinant proteins by *E. coli* has not been investigated. Recently, we cloned a *xynA* gene encoding a GH family-11 extracellular xylanase from a filamentous fungus *Penicillium citrinum* [11]. In this study, a combination of the signal peptide of *A. pullulans* XynI with the GH family-11 xylanase XynA of relatively small molecular size from *P. citrinum* led to expression of the highly active enzyme in *E. coli* BL21(DE3) and efficient secretion into the culture medium. Here we show for the first time that a signal peptide of fungal origin is functional in *E. coli*.

Materials and methods

E. coli strain, plasmids, and genes

E. coli BL21(DE3) (Stratagene, La Jolla, CA, USA), which is deficient in the outer membrane protease OmpT, was used as the host strain for expression and secretion of xylanase. Plasmid pET-26b(+) (Novagen, Madison WI, USA), which carries the T7 promoter and the signal sequence of a pectate lyase gene, *pelB*, from the gram-negative bacterium *Erwinia carotovora*, was used for construction of the series of expression plasmids (see Fig. 1a). Plasmid pET-26b(+) also includes a gene encoding kanamycin resistance. The *pelB* signal sequence was excised from the pET-26b(+) by digestion with *Nde*I and *Xho*I when other signal sequences were substituted. Full-length cDNAs of the xylanase genes, *xynI* from *A. pullulans* and *xynA* from *P. citrinum*, were previously cloned into pUC18 to give pXYN119 [3] and pXYN303 [11], respectively.

DNA manipulations and analysis

Standard molecular cloning techniques were performed as described by Sambrook and Russell [12]. PCRs were done in a thermal cycler (Takara Bio, Otsu, Japan). DNA was sequenced on both strands with an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Plasmid construction for secretory expression of XynA

On the basis of the nucleotide sequence of the *P. citrinum* *xynA* gene [11], the following PCR primers were designed to amplify the indicated regions of the *xynA* cDNA (letters

Table 1 Amino acid sequences of signal peptides used for the secretion of *xynA* product from *E. coli*

Protein	Sequence[a]	Organism	Source or reference
XynI	MKFFATIAALVVAAVAAPV*AEADAEA*SSPLMIER	*A. pullulans*	[3]
XynA	MPSLTSLFSFFALASGAFS*ATA*DLSKR	*P. citrinum*	[11]
PelB	MKYLLPTAAAGLLLLAAQP*AMA*	*E. carotovora*	pET-26b(+)

[a] The hydrophobic region is underlined. The sequence signature AXA in the C-domain is in italics

in bold type indicate the coding sequence): P1 (forward; 5′-GGGAATTCCAT**ATGAAGTTCTTCGCCACT**-3′) and P2 (reverse; 5′-CCGCTCGAG**CTAGCTAACAGTAAT ATCAGC**-3′) containing *Nde*I and *Xho*I sites (underlined), respectively, for expression of the precursor protein with its own signal sequence; P3 (forward; 5′-CATGCCATG**G AATCTTATACTTCCAGCTCG**-3′) containing *Nco*I site (underlined) and P2 (reverse) for fusion of the mature protein to the PelB signal peptide encoded in the pET-26b(+) (Table 1). PCRs were done with the following program: 25 cycles of denaturation (15 s at 94°C), annealing (30 s at 50°C), and extension (1 min at 68°C). An amplified fragment of the *xynA* ORF was digested with *Nde*I and *Xho*I, and ligated into the corresponding sites of pET-26b(+) to generate pEXP301. Another amplified fragment of the *xynA* mature protein region was digested with *Nco*I and *Xho*I, and ligated into the corresponding sites downstream of the *pelB* signal sequence of pET-26b(+) to construct pEXP501.

Construction of *xynI*::*A* fusion

The *xynI* signal sequence was fused in frame to the *xynA* mature protein region by using overlap extension PCR [13]. The *xynI* signal sequence and the *xynA* mature protein region were separately amplified from *xynI* and *xynA* cDNAs from the pXYN119 [3] and pXYN303 [11], respectively, by the first-round PCR with the following primer pairs (letters in italics and boldface indicate the *xynI* signal sequence and the *xynA* mature protein region, respectively): P1 (forward) and P4 (reverse; 5′-**TATAAGATT***CACG CTCGATA*-3′); P5 (forward; 5′-*TATCGAGCGT***GAA TCTTATA**-3′) and P2 (reverse). Two amplified fragments having overlapping ends were precipitated by ethanol, redissolved, and combined. Subsequent 3′ extension of the complementary strand consisted of a denaturation step at 94°C for 2 min and 5 cycles of the following steps: denaturation (15 s at 94°C), annealing of the denatured fragments at the overlap (30 s at 50°C), and extension (1 min at 68°C). The fusion product was gel-purified by using the QIAquick gel extraction kit (Qiagen, Valencia, CA, USA) and amplified further by the second-round PCR using a pair of primers P1 (forward) and P2 (reverse). The PCR product was digested with *Nde*I and *Xho*I and ligated into the corresponding sites of pET-26b(+) to generate pEXP401. The *xynI*::*A* fusion was verified by the DNA sequence analysis of the relevant region of pEXP401.

E. coli transformation and culture conditions

The plasmids constructed were introduced into *E. coli* BL21(DE3) by electroporation using a Bio-Rad Gene Pulser II (Hercules, CA, USA) according to the manufacturer's instructions. Transformants were recovered on Luria–Bertani plates [12] supplemented with kanamycin at 30 μg/ml to maintain the plasmids.

E. coli transformants were inoculated into 400 ml of a modified M9 medium (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 5 g of NaCl, 1 g of NH_4Cl, 8 g of glycerol, per liter, and 1 mM of $MgSO_4$) supplemented with 30 μg/ml of kanamycin in 2-l Erlenmeyer flasks. The cultures were grown on an orbital shaker (150 rpm) at 30°C. When the OD_{660} of the cultures reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to give a concentration of 1 mM for expression of T7 RNA polymerase in the host, which then transcribes the xylanase gene placed in the plasmid under control of the T7 promoter. The cultures were allowed to grow for another 12 h.

Sampling procedures and preparation of cell-free extracts

Supernatant fractions were recovered after centrifugation of 1.0-ml aliquots of recombinant cultures at 5,000×*g* and 4°C for 5 min and assayed for extracellular enzyme activity. The cell pellets were washed twice with 1.0 ml of ice-cold 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 150 mM NaCl, and resuspended in 1.0 ml of the same buffer. Cells were disrupted by 30-s beating with 50% (v/v) glass beads (350–500 μm) using Vortex for twelve times with 30-s intervals of cooling on ice. The lysates were centrifuged at 20,000×*g* and 4°C for 20 min. The supernatants containing cytoplasmic proteins were recovered as cell-free extracts and assayed for intracellular enzyme activity.

Enzyme and protein assays

The reaction mixture consisted of 0.2 ml of a 1.0% (w/v) suspension of birch-wood xylan (Sigma Chemical, St. Louis, MO, USA) in distilled water, 0.1 ml of a suitably diluted

enzyme solution, and 0.1 ml of either 0.1 M sodium acetate–HCl buffer (pH 2.0) (for *A. pullulans* xylanase XynI) or 0.1 M acetate buffer (pH 5.0) (for *P. citrinum* xylanase XynA). After incubation at 45°C for 10 min, reducing sugars were determined by the Somogyi–Nelson method [14]. One unit (U) of xylanase activity was defined as the amount of enzyme that liberated 1 μmol of xylose equivalents from xylan per minute. Protein concentrations were measured by the method of Lowry et al. [15] using bovine serum albumin (Sigma) as a standard.

Enzyme purification

All purification procedures were carried out at 4°C. The contents of six flasks of *E. coli* (pEXP401) cultures grown for 12 h with IPTG were centrifuged at 5,000×*g* for 5 min. To the 2,327-ml supernatant, ammonium sulfate was added to give 90% saturation with stirring, and the mixture was left overnight. The precipitate was collected by centrifugation at 20,000×*g* for 15 min and dissolved in 80 ml of 20 mM acetate buffer (pH 4.0). Subsequent chromatographic purification of the enzyme was performed on a Bio-Rad BioLogic DuoFlow system. The enzyme solution was subjected to cation-exchange chromatography on a SP Sepharose Fast Flow (GE Healthcare, Buckinghamshire, UK) column (2.6 × 40 cm) that had been equilibrated with 20 mM acetate buffer (pH 4.0). The column was washed with 320 ml of the same buffer. The adsorbed proteins were eluted in a step gradient with 300 ml of 0.05 M NaCl and 200 ml of 0.1 M NaCl in the same buffer at a flow rate of 1.0 ml/min. Fractions of 10 ml were collected and assayed for xylanase activity and A_{280}.

SDS–PAGE and amino acid sequencing

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out with a 4.5% stacking gel and a 12.5% separating gel as described by Laemmli [16]. Gels were stained for protein with Coomassie brilliant blue R-250. The protein bands on SDS–polyacrylamide gels were electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) using Mini Trans-Blot cell (Bio-Rad). The N-terminal amino acid sequences of blotted proteins were identified by using a G1005A Hewlett-Packard protein sequencer (Palo Alto, CA, USA).

Results and discussion

XynI signal peptide has potential recognition sites for signal peptidase I

A typical signal peptide recognizable by the general secretion pathway possesses three distinct domains [17] as observed for that of *E. carotovora* PelB (Table 1). The N-domain harbors a positively charged residue, Arg or Lys, after the initiating Met residue. The hydrophobic H-domain comprises the center of the signal peptide. A polar C-terminal domain bears the cleavage site for signal peptidase. In the PelB signal peptide, the Ala-Met-Ala sequence immediately precedes the cleavage site. However, N-terminal leader sequences of the GH family-11 xylanases of fungal origin contain the Arg residue at position −1 [3, 11] (Table 1) and are not in accordance with the (−3, −1) rule for signal peptidase cleavage sites. Instead, the N-terminal extensions of *P. citrinum* XynA and *A. pullulans* XynI contained a single Ala-X-Ala sequence and three successive Ala-X-Ala sequences, respectively, before the Arg residue at the cleavage site. It is conceivable that in filamentous fungi, following the signal peptidase cleavage of the presequence (signal peptide) in the endoplasmic reticulum (ER), processing of the remaining N-terminal extension, the pro-sequence, after the Arg residue occurs by different proteases in the Golgi apparatus. In this regard, *E. coli* signal peptidase is known to have the same cleavage specificity as ER signal peptidase [17].

Extracellular xylanase production by *E. coli* using XynI signal peptide

We initially attempted the expression and secretion of *A. pullulans* XynI in *E. coli* BL21(DE3), but no xylanase activities were detected in either culture supernatant or cell-free extract of the *E. coli* transformant. Therefore, the equivalent enzyme XynA from *P. citrinum* was used in subsequent experiments.

The expression plasmids constructed and used in this study are listed in Fig. 1a. Growth was similar for all transformants as determined by the OD_{660} of the cultures, irrespective of the quantity of xylanase secreted into the culture medium (data not shown). The enzyme activity per milliliter of culture after 12 h of IPTG induction is shown in Fig. 1b. The *E. coli* (pEXP301) carrying the entire *xynA* ORF, including the sequence for the 27 amino acid secretion signal, showed a low xylanase activity of 0.623 U in the fraction of culture supernatant and an appreciable activity of 1.48 U in the fraction of cell-free extract. The control transformant with the vector plasmid pET-26b(+) had no detectable xylanase activity in either fractions of culture supernatant or cell-free extract. Thus, the majority of enzyme activity (70%) in the fraction of cell-free extract of the *E. coli* (pEXP301) indicated inefficient secretion of the fungal xylanase in this construct.

These observations prompted us to fuse the *xynI* signal sequence to the *xynA* mature protein region. The *E. coli* (pEXP401) carrying the gene fusion *xynI*::*A* showed xylanase activities of 16.8 U in the fraction of culture supernatant

and 4.29 U in the fraction of cell-free extract. Thus, replacement of the native signal peptide of XynA with the XynI signal peptide not only led to the efficient secretion (79%) of the XynA from *E. coli* cells but also resulted in a tenfold increase in the overall enzyme activity in the culture. These results suggest that the signal peptide of xylanase XynA failed to work effectively in *E. coli*. Extracellular xylanase activity of the culture of *E. coli* (pEXP401) was similar to that (17 U/ml) in the culture supernatant of the *P. pastoris* transformant carrying the *xynA* cDNA and fivefold higher than that (3.5 U/ml) of the culture filtrate of the *P. citrinum* in our previous study [11].

The PelB signal peptide from *E. carotovora* is commonly used as periplasmic secretion signal in *E. coli* [10]. As reported by Matsumoto et al. [9], the pectate lyase from *Bacillus subtilis* is expressed and efficiently released into the culture supernatant by the *E. coli* BL21(DE3) using the PelB signal peptide encoded in pET-22b(+), which carries a gene encoding ampicillin resistance. To evaluate the efficiency of XynI signal peptide, the expression and secretion level were compared with those of the PelB signal peptide. For this purpose, the *xynA* mature protein region was fused to the *pelB* signal sequence in pET-26b(+) to yield pEXP501. The *E. coli* (pEXP501) showed xylanase activities of 10.1 U in the fraction of culture supernatant and 0.627 U in the fraction of cell-free extract. While the overall enzyme activity by the use of XynI signal peptide was 1.5 times higher than that by the use of PelB signal peptide, the percentage of extracellular enzyme activity as high as 94% suggests that the PelB signal peptide directed more efficient secretion of the xylanase XynA from *E. coli* cells.

Thus, changes in the signal peptide affected the level of xylanase XynA production in accordance with the previous observation with *Streptomyces lividans* [18]. The transcription levels of *xynA* gene in plasmids pEXP301, pEXP401, and pEXP501 were assumed to be invariant because each construct contains the same promoter and transcriptional terminator. Variations of xylanase XynA production after replacement of its signal peptide could be due to other factors such as translation rates of the gene fusions and secretion efficiency of the fusion proteins.

Purification of secreted XynA proteins

Recombinant XynA proteins secreted from *E. coli* (pEXP401) were purified by a two-step process involving ammonium sulfate precipitation followed by SP Sepharose Fast Flow column chromatography. Elution profile of XynA proteins from the column showed two major protein peaks I and II and a minor protein peak III with xylanase activity as designated by the order of elution at a concentration of 0.1 M NaCl (data not shown). Specific activities of purified recombinant XynA proteins from peak I (fractions 32–35) and peak II (fractions 38–42) were 506 and 651 U/mg (Table 2), respectively, both of which were twofold higher than that of the native enzyme (303 U/mg) from *P. citrinum* [10]. After SDS–PAGE, two discrete protein bands, IA and IB, with apparent M_r values of 24.4 and 23.6 kDa, respectively, were found in the pool of fractions 32–35 of peak I, and a single protein band, II, with an apparent M_r of 23.2 kDa, was found in the pool of fractions 38–42 from peak II (Fig. 2).

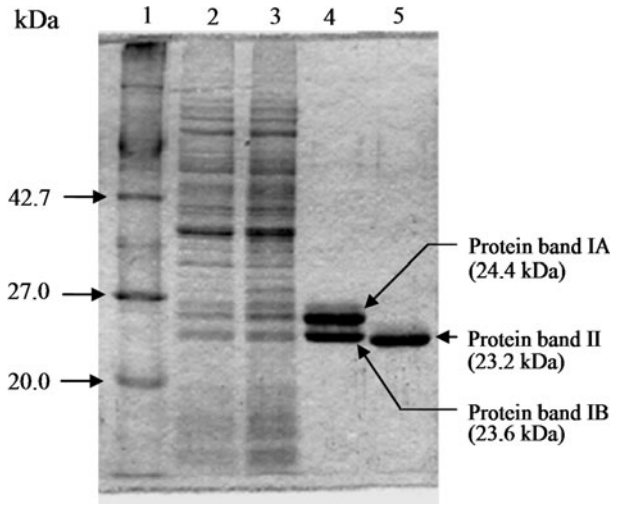


Fig. 2 SDS–PAGE analysis of extracellular xylanase produced by *E. coli* BL21(DE3) carrying the plasmid pEXP401. Protein was visualized by Coomassie brilliant blue R-250 staining. *Lanes*: *1*, marker proteins; *2*, culture supernatant; *3*, sample precipitated with ammonium sulfate; *4* and *5*, the pools of the active fractions 32–35 and 38–42, respectively, after SP Sepharose Fast Flow column chromatography

Table 2 Purification of recombinant xylanases secreted by *E. coli* (pEXP401)

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture supernatant	35,300	449	142	100	1.00
Sample precipitated with ammonium sulfate	24,200	55.1	440	68.6	3.10
SP Sepharose Fast Flow					
Peak I (fractions 32–35)	5,420	10.7	506	15.3	3.57
Peak II (fractions 38–42)	1,860	2.87	651	5.28	4.59

Table 3 Alignment of N-terminal amino acid sequences of extracellular recombinant xylanases

Xylanase	N-terminal amino acid sequence																				M_r (Da)
	10										20									30	
XynI signal sequence[a]	L	V	V	A	A	V	A	A	P	V	A	E	A	D	A	E	A	S	S	P	M	
Protein band IA						V	A	A	P	V	·	·	·	·	·	·	·	·	·	·	·	22,684
Protein band IA							A	A	P	V	A	·	·	·	·	·	·	·	·	·	·	22,583
Protein band IB														D	A	E	A	S	·	·	·	21,974
Protein band II																E	A	S	S	P	·	21,787

[a] Numbers indicate amino acid positions relative to the N-terminus of the XynI precursor protein (see Table 1)

Identification of N-termini of secreted XynA proteins

The N-terminal amino acid sequence was determined for three protein bands on the SDS–polyacrylamide gel: VAAPV and AAPVA for the band IA, DAEAS for the band IB, and EASSP for the band II. As shown in Table 3, sequence alignments indicated that the XynI signal peptide of the hybrid protein was cleaved at four distinct sites. Two sites between Ala-22 and Asp-23 and Ala-24 and Glu-25 are among the three probable recognition sites of signal peptidase I within the C-domain. The site between Ala-22 and Asp-23 was predicted as the most likely signal peptidase cleavage site by the SignalP program [19] using hidden Markov models [20] trained on gram-negative bacteria. These results suggest the flexibility in recognition of the cleavage sites by signal peptidase I. Consistent with our findings, Pratap and Dikshit [21] reported that signal peptidase I recognizes all three potential cleavage sites in an analogous sequence Ala-X-Ala-X-Ala-X-Ala created in the signal peptide of the *E. coli* outer-membrane protein OmpA fused to the *Streptococcus equisimilis* streptokinase protein. On the other hand, two adjacent sites between Ala-14 and Val-15 and Val-15 and Ala-16 within the H-domain seem to have been recognized by some other proteases. It remains to be determined if the cleavage at these two sites was associated with the translocation across the inner membrane. Although processing of the prepro-sequence of XynI in *E. coli* left 10–20 residues of pro-sequence preceding the N-terminus of the mature protein, the specific activities of the purified recombinant XynA proteins suggested that the remaining N-terminal extension did not reduce the enzyme activities.

In contrast, cleavage of the pelB signal peptide in *E. coli* (pEXP501) is expected to occur at a single potential recognition site by signal peptidase that was located at the end of the signal peptide as shown in Table 1. Although four variants of xylanase XynA with respect to the N-terminal extension were secreted from *E. coli* (pEXP401), secretion of the multiple proteins will not hamper the industrial application. In addition, the *A. pullulans* XynI signal peptide has the advantage over the *pelB* signal peptide of bacterial origin owing to the former's versatility to secrete recombinant xylanases from *E. coli* as well as yeasts.

Acknowledgments This study was done as a part of the project entitled "Research and Development of Catalytic Process for Efficient Conversion of Cellulosic Biomass into Biofuels and Chemicals", through Special Funds for Education and Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1. Coughlan MP, Hazlewood GP (1993) β-1,4-D-Xylan-degrading enzyme systems: biochemistry, molecular biology and applications. Biotechnol Appl Biochem 17:259–289
2. Henrissat B, Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316:695–696
3. Ohta K, Moriyama S, Tanaka H, Shige T, Akimoto H (2001) Purification and characterization of an acidophilic xylanase from *Aureobasidium pullulans* var. *melanigenum* and sequence analysis of the encoding gene. J Biosci Bioeng 92:262–270
4. Tanaka H, Okuno T, Moriyama S, Muguruma M, Ohta K (2004) Acidophilic xylanase from *Aureobasidium pullulans*: efficient expression and secretion in *Pichia pastoris* and mutational analysis. J Biosci Bioeng 98:338–343
5. Burchhardt G, Ingram LO (1992) Conversion of xylan to ethanol by ethanologenic strains of *Escherichia coli* and *Klebsiella oxytoca*. Appl Environ Microbiol 58:1128–1133
6. Xue GP, Johnson JS, Bransgrove KL, Gregg K, Beard CE, Dalrymple BP, Gobius KS, Aylward JH (1997) Improvement of expression and secretion of a fungal xylanase in the rumen bacterium *Butyrivibrio fibrisolvens* OB156 by manipulation of promoter and signal sequences. J Biotechnol 54:139–148
7. Shin HD, Chen RR (2008) Extracellular recombinant protein production from an *Escherichia coli lpp* deletion mutant. Biotechnol Bioeng 101:1288–1296
8. Fukuda M, Watanabe S, Kaneko J, Itoh Y, Kamio Y (2009) The membrane lipoprotein LppX of *Paenibacillus* sp. strain W-61 serves as a molecular chaperone for xylanase of glycoside hydrolase family 11 during secretion across the cytoplasmic membrane. J Bacteriol 191:1641–1649
9. Matsumoto T, Katsura D, Kondo A, Fukuda H (2002) Efficient secretory overexpression of *Bacillus subtilis* pectate lyase in *Escherichia coli* and single-step purification. Biochem Eng J 12:175–179
10. Choi JH, Lee SY (2004) Secretory and extracellular production of recombinant proteins using *Escherichia coli*. Appl Microbiol Biotechnol 64:625–635
11. Tanaka H, Nakamura T, Hayashi S, Ohta K (2005) Purification and properties of an extracellular endo-1,4-β-xylanase from

Penicillium citrinum and characterization of the encoding gene. J Biosci Bioeng 100:623–630
12. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor
13. Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61–68
14. Somogyi M (1952) Notes on sugar determination. J Biol Chem 195:19–23
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
16. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
17. Pugsley AP (1993) The complete general secretory pathway in gram-negative bacteria. Microbiol Rev 57:50–108
18. Pagé N, Kluepfel D, Shareck F, Morosoli R (1996) Effect of signal peptide alterations and replacement on export of xylanase A in *Streptomyces lividans*. Appl Environ Microbiol 62:109–114
19. Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. J Mol Biol 340:783–795
20. Nielsen H, Krogh A (1998) Prediction of signal peptides and signal anchors by a hidden Markov model. Proc Int Conf Intell Syst Mol Biol 6:122–130
21. Pratap J, Dikshit KL (1998) Effect of signal peptide changes on the extracellular processing of streptokinase from *Escherichia coli*: requirement for secondary structure at the cleavage junction. Mol Gen Genet 258:326–333