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SCIENCE DIRECT
Protein Expression and Purification 46 (2006) 274–284
Protein Expression & Purification
www.elsevier.com/locate/yprep

Extracellular production of *Streptomyces lividans* acetyl xylan esterase A in *Escherichia coli* for rapid detection of activity

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Received 28 June 2005, and in revised form 2 September 2005
Available online 6 October 2005

Abstract

Acetyl xylan esterase A (AxeA) from *Streptomyces lividans* belongs to a large family of industrially relevant polysaccharide esterases. AxeA and its truncated form containing only the catalytically competent domain, $AxeA_{tr}$, catalyze both the deacetylation of xylan and the *N*-deacetylation of chitosan. This broad substrate specificity lends additional interest to their characterization and production. Here, we report three systems for extracellular production of $AxeA_{tr}$: secretion from the native host *S. lividans* with the native signal peptide, extracellular production in *Escherichia coli* with the native signal peptide, and in *E. coli* with the OmpA signal peptide. Over five to seven days of a shake flask culture, the native host *S. lividans* with the native signal peptide secreted $AxeA_{tr}$ into the extracellular medium in high yield (388 mg/L) with specific activity of 19 U/mg corresponding to a total of 7000 U/L. Over one day of shake flask culture, *E. coli* with the native secretion signal peptide produced 84-fold less in the extracellular medium (4.6 mg/L), but the specific activity was higher (100 U/mg) corresponding to a total of 460 U/L. A similar *E. coli* culture using the OmpA signal peptide, produced 10 mg/L with a specific activity of 68 U/mg, corresponding to a total of 680 U/L. In 96-well microtiter plates, extracellular production with *E. coli* gave ~30 and ~86 μg/mL in *S. lividans*. Expression in *S. lividans* with the native signal peptide is best for high level production, while expression in *E. coli* using the OmpA secretion signal peptide is best for high-throughput expression and screening of variants in microtiter plate format.

Keywords: Acetyl xylan esterase; Secretion; *Streptomyces lividans*; *Escherichia coli*; OmpA; Deacetylase activity

Acetyl xylan esterase A (AxeA)[1] [EC 3.1.1.72] from *Streptomyces lividans* hydrolyses the ester linkages of the acetyl groups at positions 2 and 3 of the xylose moieties of natural xylan [1]. The *axe*A gene is located downstream from *xln*B, which encodes xylanase B [2], consistent with its role in the xylanolytic enzyme system leading to the complete hydrolysis of xylan. AxeA consists of a C-terminal substrate-binding domain and an N-terminal catalytic domain, separated by a glycine-rich linker. This C-terminal substrate-binding domain of AxeA shares high sequence similarity with the substrate binding domains of xylanases XlnB and TFXA, which are also located at the C-terminus. The N-terminal catalytic domain of AxeA contains a catalytically competent NodB homology domain, universally conserved among the enzymes belonging to carbohydrate esterase family 4 (CE4) [3]. CE4 includes chitin deacetylases (EC 3.5.1.41), rhizobial NodB chitooligosaccharide deacetylases (EC 3.5.1.-), peptidoglycan *N*-acetylglucos-

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[1] *Abbreviations used:* AxeA, Acetyl xylan esterase A; $AxeA_{tr}$, C-terminally truncated AxeA; CE4, carbohydrate esterase family 4; SP, signal peptide; BSA, bovine serum albumin; TCA, trichloroacetic acid.

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doi:10.1016/j.pep.2005.09.008

amine deacetylases (EC 3.1.1.-), acetyl xylan esterases (EC 3.1.1.72) and xylanases (EC 3.2.1.8) [4].

AxeA catalyzes the deacetylation of the *O*-acetyl group of polysaccharides [5] as well as the *N*-acetyl group in soluble chitinous substrates such as glycol chitin and chitin 50 (a 40% deacetylated chitin with an average M_r of approximately 160,000) [6]. The AxeA substrate-binding domain is not required for substrate hydrolysis since the truncated form of acetyl xylan esterase, $AxeA_{tr}$, which lacks the xylan binding domain, is also active toward xylan and chitinous substrates [6]. Other hydrolases such as chitinases are also catalytically competent in the absence of the chitin binding domain [7]. We have recently shown that the activity of AxeA and $AxeA_{tr}$ varies with the length and degree of acetylation of chitinous substrates, where short oligomers (DP approx 5, 24% acetylation) were the best substrates [34]. Addition of cobalt (II) increases the deacetylase activity of AxeA and $AxeA_{tr}$ fivefold toward chitinous substrates [6].

Considering the broad substrate specificity of *S. lividans* AxeA and its homology to other carbohydrate esterases, *S. lividans* AxeA constitutes a good target for structure–function studies. This will allow protein engineering for improvement of AxeA and other industrially relevant hydrolases. AxeA [5] and its truncated, catalytically competent N-terminal domain, $AxeA_{tr}$ [6], have already been successfully over-expressed in *S. lividans*. Despite the high protein production (>350 mg/L; see Results), the week-long growth period required for protein secretion and the hard texture of colonies of *S. lividans* on solid media, which makes automated colony picking difficult, make *S. lividans* poorly suited to structure–function studies requiring high-throughput screening of thousands of mutants. We therefore set out to establish alternative systems for rapid over-expression and easy recovery of $AxeA_{tr}$.

We have established two systems for extracellular production of the truncated enzyme $AxeA_{tr}$ in *Escherichia coli* using either the natural signal peptide of *S. lividans* AxeA or the signal peptide from the *E. coli* major outer membrane protein OmpA. The OmpA signal peptide efficiently directs proteins to the bacterial periplasm [8,9]. A high level of periplasmically located protein has also been associated with high extracellular production. The mature form of wild-type TEM-1 β-lactamase fused to the OmpA signal peptide was recently recovered extracellularly at a concentration of 140 mg/L of culture medium from *E. coli* BL21(DE3) [10]. Other examples of periplasmic or extracellular production of functional eukaryotic proteins from *E. coli* using the OmpA signal sequence include recombinant human tissue plasminogen activator derivatives [11], salmon calcitonin [12], and chicken avidin [13]. We compared the intracellular and extracellular production of $AxeA_{tr}$ from the two systems in *E. coli* with production in *S. lividans*. The yield remains much greater in the *S. lividans* system (over 350 mg/L of culture medium, relative to 10 mg/L in *E. coli*), but production in *E. coli* offers a 3- to 5-fold improvement of the specific deacetylation activity. Extracellular production in *E. coli* thus provides a rapid and efficient route to obtain more highly active $AxeA_{tr}$ with no refolding steps, allowing for rapid enzyme characterization.

Materials and methods

Materials

All reagents used were of the highest available purity. Restriction enzymes and DNA-modifying enzymes were from New England Biolabs and MBI Fermentas. The Sequenase 2.0 DNA sequencing kit was purchased from GE Healthcare. Synthetic oligonucleotides were synthesized in-house with a Gene Assembler (GE Healthcare). The infrared dye-labeled sequencing primers were from Li-Cor Biotechnology Division. All aqueous solutions were prepared using water purified with a Millipore BioCell system. Reagents (coenzyme A, L-malate dehydrogenase, citrate synthase, acetyl-CoA synthetase) for the acetate assay were purchased from Sigma–Aldrich (Oakville, ON). Chitosan oligomers (degree of polymerization approx 6.4, 20% acetylated) were provided by ISM Biopolymer (Granby, QC).

For expression studies, *E. coli* strain BL21 [F^- *ompThsdS*$_B$($r_B^-m_B^-$)*galdcm*] carrying the plasmid pREP4 (Qiagen, *lacI*^{q}, Kan^r) and *S. lividans* IAF10-164 [*msiK*$^-$] [14,15] were used.

Construction of expression plasmids

The truncated *axe*A gene (*axe*A_{tr}) was obtained from plasmid pIAF44, as described previously [6]. Briefly, the *axe*A_{tr} gene was constructed by overlapping PCR to introduce a unique *Eco*RI site in the structural gene immediately 5′ to the sequence encoding the mature secreted protein. The primers CD200 and CD202 (Table 1; introduced restriction sites are underlined) anneal to the signal peptide-coding region. They were used in a PCR to introduce a 5′ *Sph*I restriction site and an *Eco*RI restriction site downstream of the signal peptide-coding sequence. The primers CD201 and CD204, the latter being complementary to CD202, anneal to the coding region of the structural gene. They were used in a PCR to introduce the *Eco*RI site (described above) and a *Sac*I restriction site at the 3′ end. Finally, a portion of both PCR products were mixed and amplified with the external primers CD200 and CD201. The PCR product was digested with *Sph*I and *Sac*I and purified by agarose gel electrophoresis followed by extraction (QIAEXII, Qiagen). The purified *axe*A_{tr} coding sequence was inserted in the pIJ702-derived [16] plasmid pIAF550 yielding the vector pIAF935 (Table 2). This vector harbours the sequence encoding the native AxeA signal peptide (SP), between the restriction sites *Sph*I (5′) and *Eco*RI (3′).The SP was thus in-frame with the *axe*A_{tr} gene, allowing secretion of $AxeA_{tr}$ in *S. lividans*. The entire *sp-axe*A_{tr} coding sequence was then extracted with the restric-