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Overexpression and single-step purification of a thermostable xylanase from *Bacillus stearothermophilus* T-6

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Abstract

Xylanase T-6 is a thermostable alkaline-tolerant enzyme that is produced by *Bacillus stearothermophilus* T-6. Xylanase T-6 was found to bleach pulp effectively at pH 9 and 65°C and was used successfully on an industrial-scale mill trial. To facilitate the future characterization of the protein via X-ray analysis and protein engineering, it was necessary to overexpress the enzyme in *Escherichia coli*. The xylanase gene was cloned into T-7 polymerase expression vectors and its expression was optimized. The enzyme was found to constitute over 70% of the cell protein and it was efficiently purified from the host proteins by a single heating step. Over 2 g of soluble and active enzyme per l culture were achieved. Copyright © 1996 Elsevier Science B.V.

Keywords: Bacillus stearothermophilus; *Escherichia coli*; Overexpression; Xylanase

1. Introduction

During the process of producing white paper, the unbleached pulp is usually treated with chlorine-based compounds. Bleaching with chlorine is a highly effective and economical process due to the selectivity of chlorine. However, this process generates organic chlorine compounds that are formed from the reaction between the chlorine compounds and residual lignin, present in the wood fibers. Due to the large volumes of bleached pulp produced by the pulp and paper industry, the release of these chlorinated organic compounds into the environment is of major concern (Amato, 1993; Samadni, 1991). The paper and pulp industries are actively seeking new bleaching technologies that will reduce or eliminate chlorine in the bleaching process. One of the emerging technologies of chlorine-free bleaching involves the use of enzymes (Grant, 1991; Koponen, 1991). Viikari et al. (1986, 1987) were the first to demonstrate that treating unbleached pulp with hemicel-

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lulases (in particular xylanases) facilitates the bleaching process and reduces the amount of chlorine needed for bleaching. This enzymatic approach is considered to be applicable for large-scale facilities, and indeed the utilization of hemicellulases in the pulp and paper industry turns out to be a new field for industrial enzymes (Grant, 1991; Koponen, 1991; Lundgren et al., 1994; Viikari et al., 1994). The economics of enzymatic bleaching is made up of several factors, including the cost of the enzyme, the need for new facilities and control elements in the bleaching lines, and the yield loss due to hemicellulose solubilization. Xylanases that are active at high temperatures and pH are of great interest, since they can be introduced freely into many bleaching lines in facilities which utilize the Kraft process (alkaline pulping; Viikari et al., 1994). Recently, we have characterized an alkaline-tolerant, thermostable xylanase produced by *Bacillus stearothermophilus* T-6 (Fishman et al., 1995; Khasin et al., 1993). Xylanase T-6 was found to bleach pulp effectively at pH 9 and 65°C and was used successfully on an industrial-scale mill trial (Hogman et al., 1992; Lundgren et al., 1994; Shoham et al., 1993a). In order to further study the enzymatic properties of the enzyme and to improve its catalytic and stability properties via X-ray analysis and protein engineering, we were interested in overexpressing the xylanase gene in *Escherichia coli*. This study describes the overproduction of xylanase T-6 in *E. coli*.

2. Materials and methods

2.1. Bacterial strains and plasmids

B. stearothermophilus T-6 (NCIMB 40222) was isolated by using an enrichment procedure which selects for bacteria capable of producing extracellular thermostable xylanases (Shoham et al., 1993a,b). *E. coli* strains HMS174 and BL21(DE3) (pLysS) and the T-7 RNA polymerase expression vectors pET3d and pET9d were described elsewhere (Studier et al., 1990) and were obtained from Novagen (Madison, WI).

2.2. Media and growth conditions

Cells were grown in either Luria broth (LB), containing per l: 10 g tryptone, 5 g yeast extract x and 5 g NaCl, or Terrific broth (TB), containing per l: 12 g tryptone, 24 g yeast extract, 4 ml glycerol, 2.31 g KH_2PO_4 and 12.54 K_2HPO_4 (Sambrook et al., 1989). Antibiotics were added at (μg ml^{-1}): ampicillin, 50; kanamycin, 25; and chloramphenicol, 34. Growth was carried out at 37°C in 2-l shake flasks (150 rpm) containing 400 ml of media.

2.3. DNA manipulation

DNA was manipulated by standard procedures (Ausubel et al., 1992; Sambrook et al., 1989). DNA transformation was performed using either the calcium chloride method or by electroporation using Gene Zapper (IBI, New Haven, CT). *B. stearothermophilus* T-6 genomic DNA was isolated by the Marmur method (Marmur, 1961) as outlined by Johnson (Johnson, 1981). Plasmid DNA was purified with the Qiagen kit (Qiagen, Chatsworth, CA). DNA sequencing was performed by the dideoxy chain termination method (Sanger et al., 1977) with the ^{T7}Sequencing Kit (Pharmacia, Uppsala, Sweden).

2.4. Miscellaneous methods

Protein determinations were carried out according to Bradford (1976), using ovalbumin as a standard. Enzyme purity was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) and by gel filtration on a Superose 12 HR 10/30 column (Pharmacia, Uppsala, Sweden). Xylanase activity was assayed as described elsewhere (Khasin et al., 1993) using 1% oat spelt xylan (Sigma, St. Louis, MO) in 50 mM phosphate buffer (pH 7.0) at 65°C. 1 U of activity was defined as that amount of enzyme which produces 1 μmol of reducing xylose-equivalent per min.

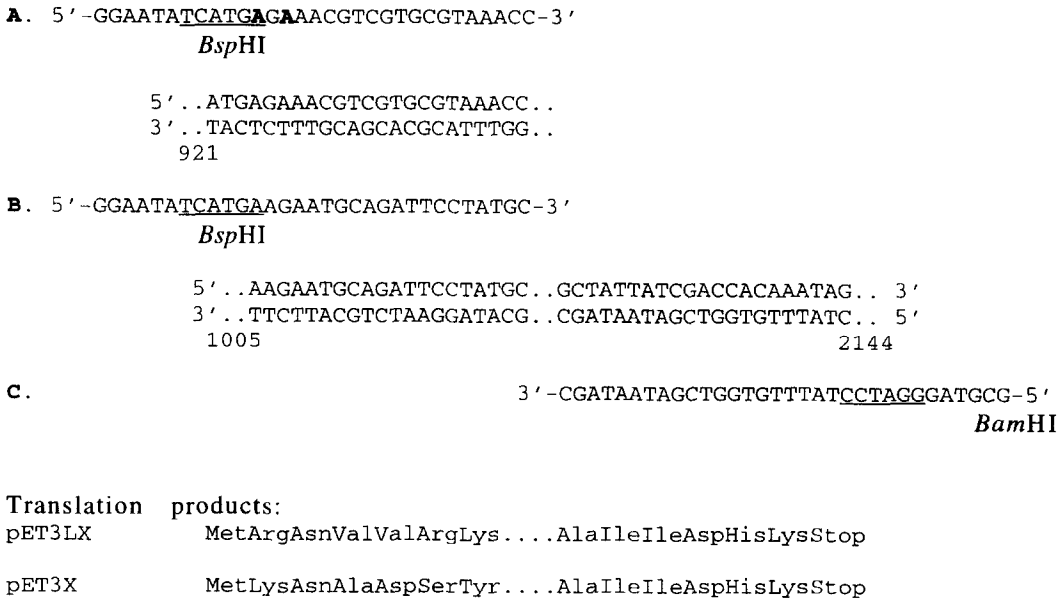

Fig. 1. PCR primers for the cloning and expression of the xylanase gene. (A) N-terminal primer for the pre-xylanase gene (containing the leader sequence). The Arg codon CGG (following the ATG codon) was changed to the Arg codon AGA, to improve expression (Looman et al., 1987); (B) N-terminal primer sequence for the mature xylanase; (C) C-terminal primer sequence. The numbers correspond to the nucleotide position in the sequenced gene (Gat et al., 1994) (accession number Z29080).

3. Results and discussion

3.1. Cloning the xylanase gene into T-7 RNA polymerase expression vectors

We have previously cloned and sequenced the xylanase gene (Gat et al., 1994). The gene codes for a 412 amino-acid protein, in which the first 28 amino acids comprise the signal peptide. Based on the DNA sequence of the gene, we designed polymerase chain reaction (PCR) primers that allow the in-frame cloning of the gene, with or without the signal peptide, into the pET vectors. The N-terminal primers were designed to contain an ATG translational start codon inside a *Bsp*HI restriction site, and the C-terminal primer was designed to contain a stop codon (TAG) and a *Bam*HI restriction site at the end of the gene (Fig. 1). Following PCR amplification, the two constructs were cloned into vector pET3d (linearized with *Nco*I and *Bam*HI), resulting in plasmids pET3LX (containing the xylanase gene with its leader sequence) and pET3X (without the leader sequence), which were maintained in a T-7 RNA polymerase-free host (*E. coli* HMS174). DNA sequencing indicated that the gene was cloned intact in both plasmids. The DNA of the two constructs was used to transform *E. coli* strain BL21(DE3) (pLysS), harboring the T-7 RNA polymerase gene. Only plasmid pET3X produced viable transformants, indicating that the leader sequence of the xylanase gene is highly toxic to *E. coli*. This result is consistent with our observations that (a) the intact xylanase gene cannot be maintained in *E. coli* on a high copy number plasmid, and (b) λ-EMBL3 phages, containing the intact gene, form very small plaques and are highly unstable (Gat et al., 1994). The cloned gene without the leader peptide was expressed well in the pET system and was used for further analysis and optimization.

3.2. Expression of the xylanase gene in E. coli

The induction and expression of the xylanase gene was studied first with *E. coli* cells growing in

LB medium. Following 4 h of induction with 4 mM IPTG (isopropyl-β-D-thiogalactopyranoside), xylanase activity reached 140 U ml^{-1} compared with less then 20 U ml^{-1} in uninduced cultures (Fig. 2). The activity was detected in the soluble fraction of the cell extracts (following sonication) and very small activity could be detected in the insoluble fraction, even after complete solubilization with 6 M guanidine–HCl and subsequent dilution in buffer. SDS-PAGE of samples from whole cells and from the soluble and insoluble fractions of the cell extract indicated that indeed the overexpressed enzyme remained soluble. Microscopic observation of induced intact cells did not reveal inclusion bodies (results not shown). Based on enzymatic activity, SDS-PAGE and protein determinations, the amount of enzyme obtained was about 0.35 g l^{-1} culture.

3.3. Optimization of expression and purification

To improve further the expression of the xylanase gene in *E. coli*, the effect of several parameters was examined. The concentration of the inducer (IPTG) can affect the expression in several ways. Low concentrations may not be sufficient for full induction, whereas high concentrations of the inducer may affect the growth of the cells and indirectly the overall yield. Following a 4-h induction period with 4.0, 0.5, 0.2, 0.05 and 0.02 mM IPTG, the relative xylanase activities were 100, 103, 99, 86 and 87 U ml^{-1}, respectively. Thus, it appears that 0.2 mM IPTG is sufficient for complete induction.

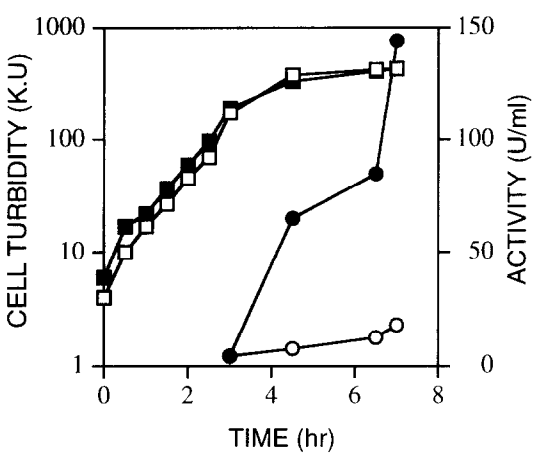

Fig. 2. Growth and xylanase expression in *E. coli* cells. *E. coli* BL21(DE3) (pLysS, pET3X) cultures were grown in LB media in shake flasks and induced (4 mM IPTG) after 3 h growth. Symbols: turbidity of induced (■) and uninduced (□) cultures; xylanase activity of induced (●) and uninduced (○) cultures.

Initially, LB was used as the growth medium for xylanase expression. This medium can support only modest growth in shake flasks, since it is not well buffered. To improve the growth characteristics of the cells, we tested the richer and better-buffered medium, TB. With TB as the growth medium, both cell and enzyme yields improved several fold. When the inducer was added at cell turbidity of about 500 Klett units (KU), enzyme activity increased continually for a period of over 8 h, reaching a level of about 400 U ml^{-1} culture (approximately 1 g l^{-1} enzyme).

The antibiotic rifampicin can be used in the pET expression systems in order to reduce the background synthesis of host RNA and proteins (Studier et al., 1990). We have tested the effect of the antibiotic (added at 200 μg ml^{-1} together with the inducer) on the expression and the relative amount of the xylanase gene (judged by SDS-PAGE) and found no detectable difference between rifampicin-free and rifampicin-containing cultures (results not shown).

Plasmid pET3X encodes ampicillin resistance via its β-lactamase gene. The use of ampicillin as a selective antibiotic is problematic with dense cultures, due to the fact that the β-lactamase is a periplasmic enzyme that can hydrolyze efficiently the ampicillin in the growth medium (Studier et al., 1990). We have found that at the end of the fermentations, a considerable percentage of the cells were ampicillin-sensitive, thus lacking pET3X. To avoid the loss of the expression vector during growth, the xylanase gene was recloned into vector pET9d which encodes for kanamycin resistance. The resulting plasmid, pET9X, was then introduced into *E. coli* BL21(DE3). Cells harboring pET9X did not lose their resistance phenotype, even after prolonged incubation, thereby allowing efficient expression of the xylanase gene.

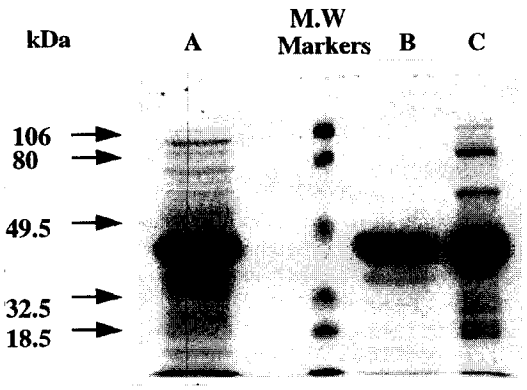

Fig. 3. Overexpression in *E. coli* and purification via heat treatment of xylanase T-6. *E. coli* BL21(DE3) (pET9X) cultures were grown on TB media and induced for 8 h with 1 mM IPTG. The cells were broken by passage through a French Press, and the soluble fraction was subjected to SDS-PAGE before (lane A) and after (lane B) heat treatment. Lane C contained xylanase T-6 purified from *B. stearothermophilus* T-6. All lanes contained equal amounts of enzyme (11 μg).

Following the optimization of the various factors, we routinely used the following protocol for the expression of the xylanase gene. Cultures of *E. coli* BL21(DE3) (pET9X) were grown in TB medium and kanamycin (400 ml in a 2-l shake flask) at 37°C. Induction (1 mM IPTG) was carried out at a cell turbidity of 7.0 OD_{600} (125 KU $\approx$ 1 OD_{600}). After 8–12 h, during which culture turbidity reached 10–12 OD_{600}, the cells were centrifuged, resuspended in phosphate buffer (15–20 ml of 50 mM buffer, pH 7.0, per 1 culture) and disrupted by two passages in a French Press. Following centrifugation of the cell extract (24 000 $\times$ *g* for 30 min), the soluble fraction contained about 2 g enzyme per 1 culture. Based on culture turbidity and the total amount of soluble protein in the cell extracts, we estimate that xylanase T-6 constituted over 70% of the total host proteins.

Since the xylanase gene is thermostable and remains soluble in the host cell, it was possible to remove many of the *E. coli* proteins by heat treatment. The efficiency of this treatment depends on the protein concentration in the cell extract. The soluble fraction of the cell extract was heated at 60°C for 30 min, centrifuged (24 000 $\times$ *g* for 30 min), and analyzed via SDS-PAGE analysis (Fig. 3) and specific activity determination. The heat treatment was highly efficient and removed most of the host proteins without detectable losses of activity. Following the heat treatment, xylanase T-6 was over 98% pure and was used directly for obtaining crystals suitable for X-ray analysis.

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