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Cloning and Expression Analysis of Two Endo-1,4-β-Xylanase Genes from *Phanerochaete chrysosporium*

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Abstract

The complete sequence analysis of two β-1,4-xylanase genes, *xyn*A and *xyn*B, from the model lignocellulose degrading white rot fungus *Phanerochaete chrysosporium* to the GenBank database (accession number AF301902 to AF301905 has been reported. Here we report the cloning and the successful expression of both genes to significant levels in a pET-based *E. coli* expression system. The size of the expressed proteins, XYNA and XYNB were approximately 48 and 37 kDa, respectively. In both cases, the vast majority of the expressed protein was located in the insoluble fraction presumably within the inclusion bodies. Finally, the presence of the recombinant proteins was confirmed by Western Blot analysis.

Introduction

The naturally occurring lignocellulosic plant biomass consists of 20 - 30% hemicellulosic materials that are heterogeneous polysaccharides found in association with cellulose and lignin. Xylan is a major constituent of hemicellulose and is the second most abundant renewable resources with a potential for degradation to useful end products. They consist of a polymeric backbone of β-1,4-linked β-D-xylose residues which is decorated with substituents to generate a branched structure. Common substituents are acetyl residues at positions 2 or 3, α-1,3-linked L-arabinofuranosyl residues and α-1,2-linked 4-*O*-methyl D-glucuronyl residues. Thus complete degradation of xylan involves removal of several different substituents as well as backbone cleavage and involves a number of different, synergistic, enzyme activities. Typically,

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these include α-glucuronidase, α-L-arabinofuranosidase, acetyl esterase together with ferulic and coumaric acid esterases for side chain removal, as well as the main chain cleaving endo-1,4-β-xylanases.

Xylan has the potential to make a significant contribution to meeting the global demands for raw materials in the near future. However, the cost of enzymatic hydrolysis is one of the factors currently limiting the economic feasibility of xylan utilization. The production of xylanases, the preferred catalyst for xylan hydrolysis, must therefore be improved by finding organisms that can hyperproduce the enzymes.

Despite the relatively high cost of current xylanase preparations, industry is already using commercial enzyme preparations. The use of hemicellulolytic enzymes as a substitute for chlorine in pulp and paper industries has recently attracted considerable interest because of environmental concerns. Xylanases also play key roles in the maceration of vegetable matter (Beck and Scoot 1974), isolation of protoplasts from plant cells, clarification of juices and wine (Biely 1985), liquefaction of coffee mucilage for making liquid coffee, recovery of oil from subterranian mines, extraction of flavors and pigments, plant oils and starch (McCleary 1986) and improving the efficiency of agricultural silage production (Wong and Saddler 1992).

Xylanases are also of interest in their own right. Thus, purified xylanase from *Trichoderma viride* was found to induce the biosynthesis of ethylene and two other pathogen-related proteins in tobacco, suggesting that xylanases may play a role in induction of plant defence mechanisms (Bailey et al. 1995)

The white rot fungus *Phanerochaete chrysosporium* is one of the limited classes of organisms able to degrade lignin, cellulose and hemicellulose. The ability of this organism to degrade all component of lignocellulose is achieved through the concerted action of a large number of extracellular enzymes including two distinct classes of peroxidases believed to degrade lignin, a number of several cellulases and hemicellulases like xylanases (Broda et al. 1995). Extensive molecular biological studies have shown that the synthesis of the extracellular enzymes for the degradation of lignin and cellulose is tightly controlled (Broda et al. 1996). However, our understanding of the degradation of hemicellulose by this organism is currently much less complete. This is unfortunate since the natural substrate of the organism is the tripartite complex, lignocellulose, and if its degradation is to be properly understood, the molecular details of the degradative systems of all three components will be required.

The xylanolytic system of *P. chrysosporium* has been studied at the protein level and shown to be multi-component, including endo-1,4-b-xylanase, a-L-arabinofuranosidase, 4-*O*-methyl α-glucuronidase and β-xylosidase/ glucosidase (Copa Patino et al. 1993; Dobozi et al. 1992; Castanares et al. 1995). However, there is very little information concerning the genes encoding hemicellulase activities and their expression in this important model organism, with only the α-galactosidase (one of the debranching hemicellulase) described at this level (Hart et al. 2000).

The first description of endo-1,4-β-xylanase genes from this model organism has been completed at the sequence level (the GenBank database accession numbers AF301902 to AF301905; Khan et al. 2001). Here we describe the expression of these genes, *xynA* and *xynB*, classified as endo-1,4-β-xylanases and belonging to glycosyl hydrolase families 10 and 11 respectively, at high levels in *E. coli*.

Materials and Methods

Organisms: E. coli (a xylanase negative species) strain MSD2658 (W3110 carrying the DE3 prophage) was used as host for protein expression. For all other genetic manipulations, strain XL1-Blue was used.

RNA extraction and cDNA preparation: The culture medium used for RNA extraction was the liquid medium used for growth of fungal cultures as previously described (Broda et al. 1995), except that it was buffered with 10 mM Na acetate buffer (pH 4.5) and did not contain veratryl alcohol. Cultures for RNA extraction were inoculated with 2.5 x 10^5 spores per ml and grown without shaking in 250 ml Erlenmeyer flasks at 37_C. The carbon source added (at a final conc. 0.2%) was a mixture of wood pulp, wheat bran and sugar beet. Total RNA from *P. chrysosporium* was isolated using the RNeasy plant mini kit (Qiagen, Crawley, UK). The fungal mycelium was filtered through a coarse nylon cloth and washed with ice-cold, autoclaved, distilled water. The mycelium was immediately frozen in liquid nitrogen and then stored at – 80°C. Samples (maximum 100 mg) were ground in liquid nitrogen using a mortar and pestle and then processed according to the manufacturer's recommendations. The RNA thus obtained (30 - 50 µl containing 6 mg) was stored at – 80°C. For RT-PCR analysis, 1 mg of total RNA was converted to ssDNA using a first strand cDNA synthesis kit (Roche Diagnostics, Lewes, U. K.). Aliquots (approximately 50 ng) of this material were then used in standard PCR using

primer pairs that amplified full length cDNA of *xynA* and *xynB* gene. These fragments were cloned into pGEM-T vector and transformed into XL1-Blue strain of *E. coli.*

Oligonucleotide primers: Oligonucleotide primer pairs 7x8HetExp1 plus 7x8HetExp2 and 1x3HetExp1 plus 1x3HetExp3 were used to amplify cDNA templates of the *xynA* and *xynB* genes, respectively. Primer sequences are detailed below:

Name	Sequence [a, b]
7∇8HetExp1	GTGCAT**ATG**CAGTCGCCTGTTTGGGGCCAATGC
7∇8HetExp2	GGAACGCGAATTCGAATTGCCGAAGCCAATAGC
1∇3HetExp1	GCCCAT**ATG**TTYCCCTTCGAGTTCCACAACGG
1∇3HetExp3	ACCCTCCATCCTGCACGCCGAATTCAGGCAC

[a]Underlining indicates restriction sites used for cloning into pET22b. [b]Bold type indicates the start codon for methionine introduced to initiate translation in pET22b constructs.

The primers were used for heterologous expression of either Family 10 (symbolized as 7 x 8 according to the initial experiments in gene sequencing) or Family 11 (symbolized as 1x3 accordingly) and thus named either 7x8HetExp or 1x3 HetExp. Both the primers were designed from the full-length cDNA sequence of *xynA* and *xynB* gene. In both cases, the primers contained additional sequence to include restriction endonuclease cleavage sites and thus yield amplification products flanked by 5′*Nde*I and 3′ *EcoR*I sites, to facilitate directional cloning into plasmid pET-22b. In addition to the restriction sites, the primers introduced a methionine residue immediately upstream of the predicted signal peptide cleavage sites and removed the normal stop codon. Translation of either construct is then expected to start at the introduced methonine and continue through the amplified fragment exiting via the *EcoR*I site, in such a way that a hexahistidine tag, encoded by the vector sequence is added to the C-terminus of each of the mature xylanases.

The 1193 bp (*xynA*) and 845 bp (*xynB*) PCR products were cloned into the pGEM-T cloning vector (Promega) and transformed into *E. coli* XL1-Blue. They were then subjected to digestion with *EcoR*I and *Nde*I, followed by gel purification of the insert. The pET vector was similarly digested with *EcoR*I and *Nde*I and was also gel purified prior to insert ligation. The final constructs were first transformed into *E. coli* XL1-Blue strain for storing and propagation and also to minimise plasmid instability problems.

Expression of recombinant proteins in E. coli: E. coli strain MSD2658 was transformed with pET-22b(+) (Novagen, Madison, U.S.A.) his-tagged recombinant xylanase gene containing plasmids. A key advantage of the pET (plasmid for expression by T7 RNA polymerase) system, driven by the strong T7 bacteriophage promoter is that host cell RNA polymerase and T7 RNA polymerase recognise completely different promoters. Therefore, any cDNA sequence or gene cloned in-frame to the T7 promoter, when transformed into an appropriate host will be transcribed actively and selectively under the control of the T7 RNA polymerase. This system is able to maintain target genes transcriptionally silent in the uninduced state. However, upon induction almost all of the cell resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein after a few hours. The cost of such expression is a drastic decrease in cell growth. Cultures are thus grown to late log phase to acquire sufficient biomass before induction.

Target genes are initially cloned using hosts that do not contain the T7 RNA polymerase gene, so that they are virtually unexpressed and cannot cause plasmid instability due to the production of proteins potentially toxic to the host cell. Once established, plasmids are transferred into expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under the *lac*UV5 control, and expression is induced by the addition of IPTG.

The pET vectors contain 'His-tags' facilitating the detection and purification of the target protein. This six histidine domain enables convenient, economical single step purification using positively charged metal chelating chromatography. For the vector to be used, the host cell must contain an inducible form of the gene that codes for T7 RNA polymerase. This is usually provided in the form of the lambda prophage DE3 within the *E. coli* host chromosome.

2 ml of LB medium containing carbenicillin (50 μg/ml) (Sigma) was inoculated with a single colony from a freshly transformed recombinant clone of the expression host, *E. coli* strain MSD 2658. The culture was grown at 37_C with shaking until the $O.D_{600}$= 0.2 - 0.6. The cells were collected by centrifugation (30 seconds in a microcentrifuge) and the pellet was resuspended in 1 ml fresh medium. 50 μl of this culture was added to 8 ml LB medium containing carbenicillin (200 μg/ml) and the culture was grown at 37_C until $O.D_{600}$ reached at 0.2 - 0.6. 1 ml of medium was taken as the uninduced culture. Then the remaining culture was made 1 mM with respect to IPTG and it was then immediately transferred to 30_C. The IPTG-induced 1 ml of sample was

collected after 3 hours. The remaining cell suspension was lysed by heating at 95_C for 10 to 15 min. The lysate was spun down for 20 min at top speed in a microfuge and the supernatant was transferred to a clean Eppendorf tube. A 10 µl aliquot of each whole cell protein sample was electrophoresed through a SDS polyacrylamide gel (Sambrook et al. 1989). Separated proteins in gels were stained in Coomassie Brilliant Blue R250 or transferred to a nitrocellulose membrane for Western Blotting.

To investigate the intracellular localization of the expressed protein *E. coli* cells were lysed and fractionated into soluble and insoluble material using BugBuster (Novagen) and the manufacturer's recommended protocol. BugBuster protein extraction reagent is formulated for the gentle disruption of the cell wall of *E. coli*, resulting in the liberation of soluble protein. It provides a simple, rapid and low cost alternative to mechanical methods such as French press or sonication for releasing proteins in preparation for functional studies or purification. The proprietary formulation utilises a mixture of non-ionic detergents that is capable of cell wall perforation without denaturing soluble proteins.

Western blotting: Western blotting (Towbin et al. 1979) was performed using a Bio-Rad Trans-Blot SD semi-dry transfer cell. The primary antibody was a polyclonal rabbit anti-his IgG (Santa Cruz Biotechnology, Santa Cruz, U.S.A.) used at a dilution of 1 : 1,000 which was detected with a goat anti-rabbit IgG alkaline phosphatase-conjugate (Promega, Southampton, U.K.) at a dilution of 1 : 10,000 according to the manufacturer's instructions.

Results and Discussion

Duplicate cultures of the expression clones (*E. coli* strain MSD2658 transformed with pET-22b (+) his-tagged recombinant *xynA* and *xynB* gene containing plasmids) were set up for this experiment to rule out the possibility that expression of recombinant protein might be impaired by the host transcription. To this end rifampicin (Sigma) was added to a final concentration of 200 µg /ml in one of the duplicates, 45 minutes after IPTG induction, to stop host transcription.

The sizes of the expressed XYNA (Fig. 1, lanes 4 and 8) and XYNB (Fig. 2, lanes 4 and 8) protein were approximately 48 kDa and 37 kDa, respectively. There was no expression 15 minutes after IPTG induction (Fig. 1A, lanes 3 and 7; Fig. 1B, lanes 3 and 7). Both transformants from each type showed significant

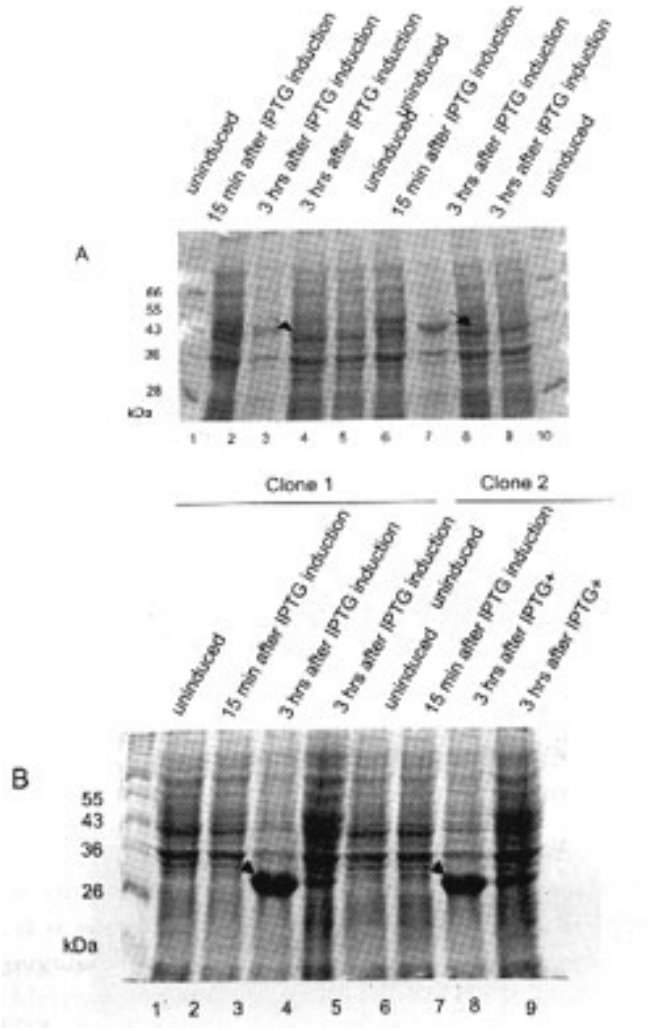

Fig. 1. Preliminary expression analysis of two transformants from each recombinant plasmid of *P. chrysosporium xynA* and *xynB* in *E. coli* MSD 2658. Above are two Coomasie Blue-stained SDS-12.5% polyacrylamide gels. *E. coli* cells containing either *xynA* (A) or *xynB* (B) gene expression constructs were grown in 8ml LB medium containing 200 μg/ml carbenicillin at 37_C to OD_{600} = 0.5. An aliquot (1 ml) was taken and the cells pelleted (uninduced total protein, lane 2 and lane 6). The remaining cultures were transferred to 30_C immediately after induction by addition of 1 mM IPTG. After 15 minutes and 3 hours aliquots were removed and cell pelleted (15 minutes induced total protein, lane 3 and lane 7) and (3 hours induced total protein, lane 4 and lane 8). Lane 5 and lane 9 show the induced cell lysates at the end of 3 hours of IPTG+rifampicin induction. The sizes of the molecular weight markers (lane 1) are indicated to the left of the figures.

expression of a single novel protein 3 hours after the IPTG induction. The gels also reveal that expression of recombinant protein was, in both cases, far higher in the absence of rifampicin (Fig. 1A, lanes 5 and 9; Fig. 1B, lanes 5 and 9).

The presence of His-tagged protein in these samples was confirmed by Western blot analysis using anti-His antibody as a probe (Figs. 2A and 2B). Signals were absent in any of the lanes loaded with uninduced sample (lanes 2, 4, 6) and the sample of both the uninserted, uninduced and induced pET-22b (lanes 8 and 9, respectively). Significant signals were seen from the induced

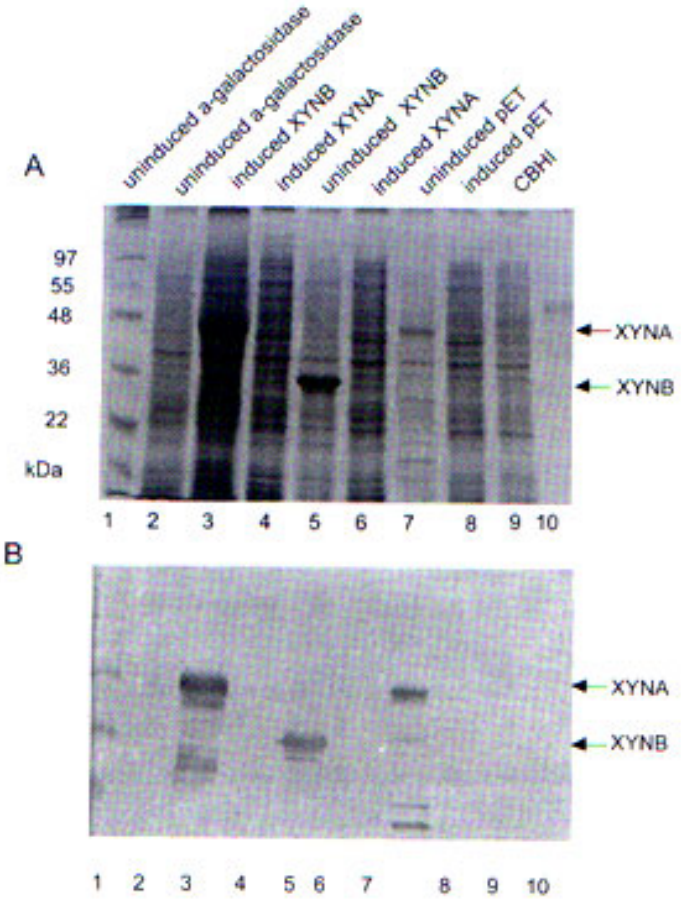

Fig. 2. Expression of 6xHis-tagged *P. chrysosporium* XYNA and XYNB in *E. coli* MSD 2658. Panel A is a Coomassie Blue-stained SDS-12.5% polyacrylamide gel; panel B, Western analysis of the same gel using anti-hexahistidine primary antibody. *E. coli* cells containing an α-galactosidase gene, xynA, xynB and uninserted pET expression constructs were grown in 8 ml LB medium containing 200 μg/ml carbenicillin at 37_C to OD_{600} = 0.5. An aliquot (1 ml) from each of the -galactosidase, xynB, xynA and uninserted pET was taken and the cells pelleted (uninduced total protein, lane 2, 4, 6 and 8, respectively). The remaining cultures were transferred to 30_C immediately after induction by addition of 1 mM IPTG. After 3 hr, a second aliquot was removed from each and the cells pelleted (induced total protein, lane 3, 5, 7, 9). A purified *P. chrysosporium* CBHI (lane 10) was loaded as a negative control. Molecular weight markers (lane 1) are indicated herewith.

samples of xynA and xynB and alpha-galactosidase (one of the debranching hemicellulase used as positive control of his tagged recombinant protein) (lanes 7, 5 and 3, respectively). A purified *P. chrysosporium* protein CBH1 (lane 10), was used as a negative control and as expected, gave no signal. This result demonstrates that the expressed proteins were definitely derived from the inserted vector. Moreover, since the his-tags recognized by the antibody are located at the extreme 3' ends of the genes, the proteins induced by IPTG are most likely to be full-length XYNA and XYNB.

The intracellular localisation of the expressed protein was then investigated. After induction, cell lysates were treated with BugBuster reagent (Novagen) according to the manufacturer's recommendation. Cells are harvested by centrifugation as usual, followed by suspension in BugBuster reagent at room temperature. During the brief incubation, soluble proteins are released. The soluble fraction and any inclusion bodies that may be present can be separated by centrifugation. Several washing steps with diluted BugBuster reagent were then carried out to get the final recovery of the pellet. The insoluble fraction associated with the pellet was solubilised in 6 M Guanidine HCl and was then ready for IMAC (immobilized metal affinity chromatography) purification. Fig. 3 is a SDS-12.5% polyacrylamide gel analysis that demonstrates that the vast majority of both the XYNA and XYNB proteins produced is located in the insoluble fraction presumably within inclusion bodies (lanes 9 and 5, respectively).

For the commercial realization and economic viability of xylanase production, it is necessary to identify organisms that can hyperproduce the enzymes. Recombinant DNA technology offers the means to enhance protein production. Moreover, in order to obtain unambiguous data about the structure and function of such enzymes, highly pure proteins are required which is possible using this technology. Recombinant DNA technology therefore presents a powerful alternative approach for the production of highly homogeneous protein. The objective of these experiments was to develop an *E. coli*-based expression system which, it was hoped, would, allow for successful production of heterologous *P. chrysosporium* xylanases. This would enable rigorous study of the enzymology of individual pure xylanases. To reduce the effects of protein toxicity on cell growth prior to induction, the level of basal transcription that occurs in the absence of induction (leakiness) were repressed as much as possible by keeping the number of generations to a minimum. This was accomplished by setting up cultures from fresh transformation plates by inoculating a small starter culture (2 ml) which was grown for 2 - 3 hours, until mid-log phase. The starter culture was then diluted in prewarmed medium and

grown at 37_C to an O.D.$_{600}$ of approximately 0.5 - 0.6 before induction. Furthermore, this procedure helped rule out instability of the expression construct.

Poor plasmid maintenance in the cells can lead to low expression levels. Ampicillin is an unstable antibiotic and is rapidly depleted in growing cultures due in part to the - lactamase secreted by resistant bacterial cells. To maintain drug selection throughout the culture period, the culture medium was replenished with fresh antibiotic containing a high concentration (200 μg/ml) of drug 2 - 3 hours after the starter cultures were inoculated. In addition, the cells were grown in the presence of carbenicillin, a more stable β-lactam compared to ampicillin.

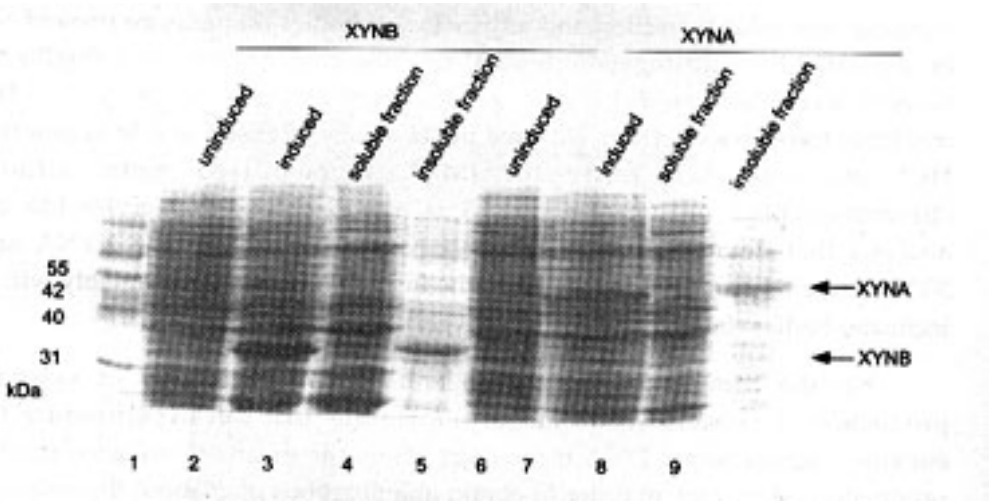

Fig. 3. Demonstration of the location of *P. chrysopsorium* XYNA and XYNB expressed in *E. coli* MSD 2658. A Coomassie Blue stained SDS-12.5% polyacrylamide gel showing the uninduced *xynA* (lane 6) and *xynB* (lane 2), plus induced cell lysates of *xynA* (lane 7) and *xynB* (lane 3) after 3 hr of IPTG induction at 30_C. Harvested cell pellets were treated with BugBuster reagent. The yielded supernatant (induced soluble fraction, lanes 4 and 8) and pellet (induced insoluble fraction, lanes 5 and 9) solubilised in 6M Guanidine HCl were loaded. Molecular weight markers are indicated beside the gel.

Eukaryotic proteins expressed intracellularly in *E. coli* are frequently sequestered into insoluble inclusion bodies. The intermolecular association of hydrophobic domains during folding is believed to play a role in the formation

of inclusion bodies. For proteins with cysteine residues, improper formation of disulfide bonds in the reducing environment of the *E. coli* cytoplasm may also contribute to incorrect folding and formation of inclusion bodies. The presence of a protein secretory sequence may obstruct folding, resulting in misfolded molecules and the inability of *E. coli* to glycosylate the protein may also reduce the solubility of the folding intermediates resulting in insoluble aggregates.

In a study on expression of proteins from bacteria, yeast, virus and animal cells in *E. coli,* no relationship was observed between the formation of intracellular aggregates and the origin of the protein, the promoter used and the hydrophobicity of the polypeptide (Kane and Hartley 1988). Inclusion bodies invariably limit the utility of standard purification procedures, which rely on the protein's native form. However, purification of 6 ∇ His-tagged proteins by metal affinity chromatography can be performed under native or denaturing conditions, and is not affected by problems arising from protein insolubility. Ongoing studies of this type will complement the present work.

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