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An *E. coli* Expression System for the Extracellular Secretion of Barley α-Amylase

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Libraries of modified genes are often screened during the process of genetically engineering enzymes with specifically tailored activities. It is important, therefore, to create expression systems which allow for the rapid screening of many clones. We developed an *Escherichia coli* expression system which will secrete enzymes into the growth medium. We describe the first reported expression of barley α-amylase in *E. coli.* The enzyme is secreted onto solid media containing starch to produce easily visualized halos. In addition, the enzyme is secreted into liquid media in an intact, active form.

KEY WORDS: Secretion; α-amylase; barley; *E. coli.*

1. INTRODUCTION

One of the main strategies for improving the efficiencies of biochemical industrial processes is the development of recombinant enzymes that possess novel or enhanced activities. A common method for engineering better enzymes is by random mutagenesis of the corresponding gene followed by screening of expression libraries to isolate the desired mutants. Many methods exist to generate mutations in the gene of interest. Error-prone PCR[3] is frequently used to create libraries of mutants (Cadwell and Joyce, 1992). In addition, the advent of several "DNA shuffling" strategies has dramatically improved the generation of modified enzymes by recombining multiple useful mutations (Judo *et al.,* 1998; Shao *et al.,* 1998; Stemmer, 1994; Zhao *et al.,* 1998). Once a library of mutant genes has been obtained, it can be screened, and the clones with the desired activities can be isolated and subjected to additional rounds of mutation and selection.

A limiting step is the expression screen. In general, the library is transformed into a microorganism, such as yeast, *Bacillus subtilis,* or *Escherichia coli.* There are several signal sequences that have been characterized in both yeast and *B. subtilis* that can direct the extracellular secretion of a recombinant enzyme (Castelli *et al.,* 1994; Cregg *et al.,* 1993; Hemila *et al.,* 1992; Lam *et al.,* 1998; Scorer *et al.,* 1993). These signal sequences are excised from the recombinant protein during secretion. In contrast, *E. coli* does not have such a well-defined excisable signal sequence which will direct secretion of the protein to the growth medium. However, *E. coli* does have advantages over yeast and *B. subtilis.* It has a much higher transformation efficiency than *B. subtilis* and yeast, and thus a larger population of the library of gene mutants can be screened. In addition, *E. coli* grows at a faster rate than yeast, thus ensuring a high-throughput screen. Finally, plasmid preparations from *E. coli* are simpler and result in much higher DNA yields, allowing for the direct characterization of clones. In contrast, yeast plasmids need to be amplified in bacteria.

We have developed an *E. coli* expression system that secretes recombinant enzyme into the growth medium. We present the first report of the successful *E. coli* expression and secretion of active barley low-pI α-amylase. This enzyme is critical to the brewing

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[3] Abbreviations: BRP, Bacteriocin-release protein; HlyA, hemolysin; IPTG, isopropyl-β-D-thiogalactopyranoside; kil, colicin-release protein; MOPS, 3-(N-morpholio)butanesulfonic acid; PCR, polymerase chain reaction; pelB, pectate lyase B.

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industry and has been the focus of many studies. When colonies were grown on solid media containing starch, the amylase was secreted, and the enzyme digested the starch to form easily visualized halos. In liquid culture, the barley amylase was secreted into the media in a full-length active form.

2. MATERIALS AND METHODS

2.1. Plasmid Construction

The pSE420-gIII expression vector was generated from portions of the pSE420 and pBAD-gIIIB plasmids (Invitrogen). The gene III signal sequence and the polylinker sequence were PCR-amplified from pBAD-gIIIB using PfuTurbo DNA polymerase (Stratagene) and the primers 5′-AAAAAACTGCTGTTCGCGATTCCG-3′ and 5′-GTATGCGGCCGCGGCAAATTCTGTTTTATCAGACCGCTTC-3′. The resulting PCR product was digested with *Not*I to produce a DNA fragment with one blunt and one staggered end. This fragment was cloned into the pSE420 vector backbone which had been treated with *Nco*I, then T4 DNA polymerase (to create blunt ends), and finally *Not*I (to generate a staggered end).

The pSE420-gIII–LpI amylase expression vector was generated by PCR amplification of the barley low-pI α-amylase gene [isolated from a barley cDNA library (Strategene)] with PfuTurbo DNA polymerase and the primers 5′-GGCGGTACCCATATGCACCAAGTCCTCTTTCAGGG-3′ and 5′-GGCTCTAGACTGCAGTCAGCTCCGTTGTAGTGTTGC-3′. The PCR product was then digested with *Kpn*I and *Xba*I and cloned into the pSE420-gIII backbone that was also digested with *Kpn*I and *Xba*I.

All enzymes were purchased from New England Biolabs unless otherwise stated.

2.2. Expression of Barley α-Amylase

To express the barley amylase enzyme, the pSE420-gIII–LpI plasmid was transformed into the *E. coli* strain BL21-Gold (Stratagene). To observe amylase secretion onto solid media, individual clones were spotted onto LB agar plates (Sambrook *et al.,* 1989) that were supplemented with 0.5% starch (Sigma), 50 mM MOPS (pH 7.0), and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Because the barley low-pI amylase requires calcium for stability and activity (Bush *et al.,* 1989), 3.5 mM $CaCl_2$ was also added to the media. The plates were incubated at 30°C overnight and then stained with iodine vapor to visualize the regions of starch digestion.

To analyze amylase secretion into liquid media, individual colonies were inoculated and grown at 25°C in LB broth (Sambrook *et al.,* 1989) that was supplemented with 50 mM MOPS (pH 7.0), 1 mM IPTG, and 10 mM $CaCl_2$. A titration of various concentrations of $CaCl_2$ (3.5–25mM) had demonstrated that a higher amount of $CaCl_2$ was required in the liquid media as compared to the solid for the maximum level of activity.

At various time intervals, aliquots of the bacterial liquid culture were removed and spun down, and the media and whole-cell fractions were collected. The relative levels of amylase enzyme were determined by Western blot analysis. Equivalent culture volumes of media and whole-cell fractions were electrophoresed through a 10% SDS–PAGE gel. The protein was then transferred to nitrocellulose membrane (Schleicher & Schuell), and the blot was sequentially probed with anti-amylase antibody and anti-rabbit HRP antibody (Biorad). The protein bands were visualized using the Lumi-Light Western Blotting Substrate (Boehringer Mannheim) according to manufacturer's instructions. The intensities of the protein bands were quantified on a gel imaging system (Alpha Innotech, San Leandro, CA).

2.3. Measurement of Amylase Activity

Amylase activity in the liquid media was measured using slight modifications of a previously published protocol (Wong *et al.,* 2000). In brief, 160 μl of culture supernatant was added to 56 μl of a solution of Phadebas, a dye-labeled starch substrate (Pharmacia & Upjohn), and incubated at 37°C for 30 min. The reaction was then spun down, and 150 μl of the supernatant was measured at 620 nm on a 96-well microplate scanner (Labsystems, Vista, CA). Enzyme activity units were determined by plotting a standard curve using purified endogenous barley α-amylase of known activity.

3. RESULTS

In order to express and secrete the barley low-pI α-amylase enzyme extracellularly from *E. coli,* an expression vector (pSE420-gIII) was created which consisted of the pSE420 vector backbone with the gene III signal sequence tag and polylinker from the pBAD-gIIIB plasmid (see Section 2). Gene III encodes a capsid protein of the *E. coli* filamentous phage fd, which is assembled at the bacterial membrane (Schaller *et al.,* 1978). When the gene III protein is secreted

from the cytoplasm, the signal peptide comprising the N-terminal 18 amino acids is cleaved from the protein (Goldsmith and Konigsberg, 1977). When the gene III signal sequence is fused to a recombinant enzyme, it can direct secretion of the protein out of the cytoplasm and then be cleaved. Thus, any activity interference caused by extraneous amino acid residues should be minimized.

Transcription of the fusion protein from pSE420-gIII is controlled by the *lac* operator and *trc* promoter, which is inducible by IPTG, a nonhydrolyzable lactose analog. Transcription from the original pBAD-gIIIB plasmid is driven by the *araBAD* promoter, which was activated by arabinose. In order to achieve a steady level of gene expression from pBAD-gIIIB, a constant intracellular level of arabinose must be maintained. Thus, it is important to use a bacterial strain which is unable to metabolize arabinose, such as TOP10. This requirement dramatically limits the number of bacterial strains that can be used with the pBAD-gIIIB vector. With the increased flexibility provided by the IPTG-inducibility of pSE420-gIII, 18 different *E. coli* strains were screened to determine which would give the least amount of background activity (i.e., bacterial carbohydrase activity) on our starch plate halo assay (Table I). The BL21-Gold cell line was chosen because this strain gave little background in the starch activity assay and had very high transformation efficiency.

The barley low-pI α-amylase gene was subcloned into the pSE420-gIII vector (see Section 2). BL21-Gold cells were transformed with either the pSE420-gIII vector or the pSE420-gIII–LpI amylase expression construct. To determine whether the amylase enzyme could be secreted onto solid media, individual colonies were spotted on an LB agar plate containing 0.5% starch and 0.1 mM IPTG. After an overnight incubation at 30°C, the colonies containing the pSE420-gIII–LpI plasmid clearly secreted amylase enzyme as evidenced by the halo of digested starch around the colonies (Fig. 1). Incubation at 37°C resulted in smaller halos (data not shown).

The formation of the halos resulting from starch digestion indicated that the expression system could be used to screen libraries. However, it was also important to demonstrate that the amylase enzyme was secreted into the media in liquid cultures. Amylase that is secreted into the media can be readily purified so that differences in the specific activities among promising mutant enzymes can be determined. BL21-Gold cells transformed with either the pSE420-gIII vector or the pSE420-gIII–LpI expression plasmid were grown in liquid culture containing 1 mM IPTG at 25°C. It was necessary to buffer the growth media with 50 mM MOPS (pH 7.0) to observe amylase activity. Without buffering, the pH of the culture media exceeded 8.5, which was incompatible with the amylase activity (Sogaard and Svensson, 1990).

At different time points, aliquots of the cultures transformed with either the pSE420-gIII or the pSE420-gIII–LpI plasmid were spun down, and the media supernatant was assayed for activity (Fig. 2). Media from the culture containing cells transformed with the amylase expression vector showed increasing levels of enzyme activity up to 48 h. The vector control culture showed no activity.

To determine the relative amount of amylase that was secreted into the liquid media, Western blot analysis was conducted on samples of the culture supernatant and

Table I. Analysis of Background Carbohydrase Activity from Various *E. coli* Cell Lines[a]

Cell line	Activity	Cell line	Activity
AD494(DE3)[4]	++	JM101[1]	+++
BL21-Gold[5]	−	JM109[1]	++
BL21[5]	−	LMG194[3]	+++
BL21(DE3)[5]	−	MC1061[1]	++
BL21(DE3)pLysE[4]	−	RR1[1]	−
BL21(DE3)pLysS[5]	−	Top10[3]	++
DH5α[1]	+	TST3-MC4100[6]	−
FB2[2]	+++	XLmutS[5]	++
HB101[1]	−	XL1 Blue (MRF′)[1]	++

[a] Individual bacterial colonies were spotted and grown overnight on LB agar plates containing 1% starch and 1 mM IPTG. Plates were stained with iodine vapor to detect background starch degradation. "−" indicates very little starch degradation. "+" indicates increasing levels of starch degradation. Strains were obtained from [1]ATCC, [2]*E. coli* Genetic Stock Center (New Haven, CT), [3]Invitrogen, [4]Novagen, [5]Stratagene, and [6]Dr. T. Silhavy (Princeton University).

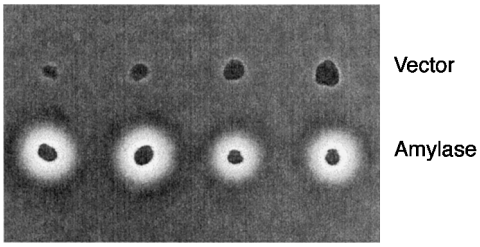

Fig. 1. Starch digestion on solid media. *E. coli* clones transformed with either pSE420-gIII vector (top row) or pSE420-gIII–LpI amylase expression vector (bottom row) were spotted onto LB agar plates containing starch. After overnight incubation at 30°C, starch digestion was visualized by staining plates with iodine vapors.

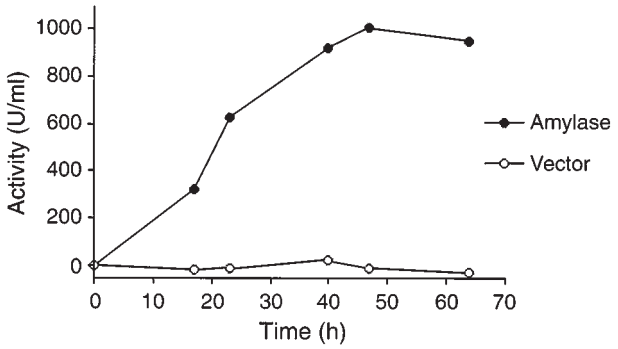

Fig. 2. Amylase activity in liquid media. Cultures of *E. coli* transformed with either pSE420-gIII vector (open circles) or pSE420-gIII–Lpl amylase expression vector (closed circles) were grown at 25°C. At various time intervals, aliquots of the culture media were collected and assayed for activity (international enzyme units).

the whole-cell fraction (Fig. 3). Approximately 65% of the amylase enzyme was secreted into the liquid media. In addition, the enzyme was primarily full-length, and very little proteolysis was evident.

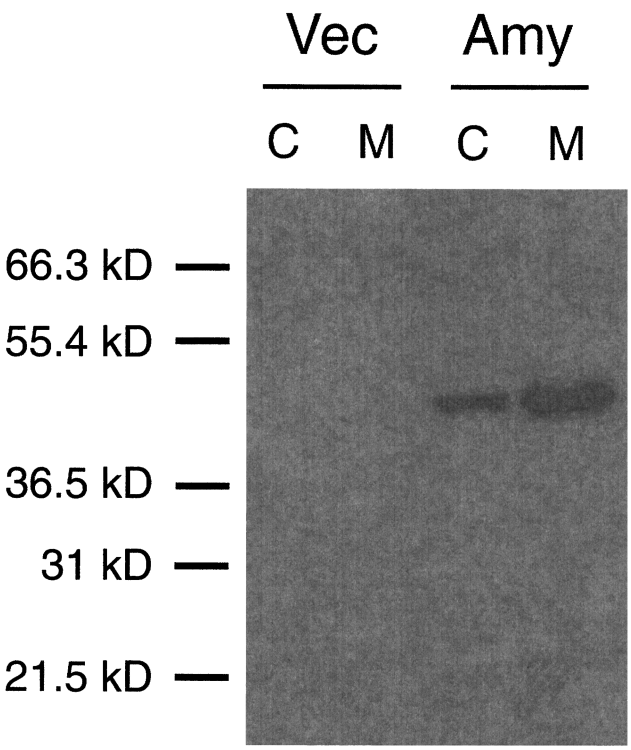

Fig. 3. Western blot of *E. coli* culture fractions. Aliquots (100 μl) of *E. coli* culture transformed with either pSE420-gIII vector (Vec) or pSE420-gIII–LpI amylase expression vector (Amy) were harvested at 24 hr, and the samples were spun down. The medium supernatant was separated from the cell pellet, which was resuspended in 100 μl of SDS sample buffer. Two μl of the medium (M) and the resuspended cell pellet (C) were subjected to SDS–PAGE on a 10% gel, blotted onto nitrocellulose membrane, and probed with anti-amylase antibody.

4. DISCUSSION

In summary, the pSE420-gIII expression vector provides a vehicle to rapidly screen enzyme variants. We demonstrate that active barley low-pI α-amylase enzyme can be secreted extracellularly from *E. coli*. Previous reported attempts to express the barley low-pI α-amylase in *E. coli* were unsuccessful (Sogaard, 1989). Although this enzyme has also been expressed and secreted from yeast (Juge *et al.*, 1993, 1996; Sogaard *et al.*, 1991; Sogaard and Svensson, 1990) and *Aspergillus niger* (Juge *et al.*, 1998), using *E. coli* offers great advantages in terms of higher transformation efficiencies, faster growth rates, and direct clone analysis without the need for an intermediate host to amplify the plasmid.

We used the gene III signal sequence to secrete recombinant protein from the cell. There are many other means to release *E. coli*-produced proteins that are trapped in the cell, but these methods all have drawbacks. One approach is to use external physical force. For example, exposing the cells to a high-voltage electrical (Ohshima *et al.*, 2000) or osmotic (Ausubel *et al.*, 1999) shock has proven successful in releasing recombinant proteins from *E. coli.* Unfortunately, these techniques are unwieldy to use on a large-scale expression screen and will not work on an LB agar assay. The ability of the gene III signal sequence to direct extracellular secretion on solid media dramatically increases the throughput of an expression screen.

Another strategy that has been employed is the use of proteins that permeabilize the *E. coli* cell membrane, such as bacteriocin-release protein (BRP) (van der Wal *et al.*, 1995) and colicin-release protein (kil) (Robbens *et al.*, 1995). We tested whether BRP would release the barley low-pI α-amylase. An expression vector carrying the α-amylase gene was cotransformed with the BRP expression vector pSW1 (Display Systems Biotech, Vista, CA). Although the BRP did release the α-amylase enzyme, there was significant background from the vector controls (data not shown). Presumably, BRP was nonselectively releasing many *E. coli* cellular proteins that had carbohydrase activity. In addition, cell growth was impaired. Attempts at titrating the amount of BRP produced did not resolve these problems.

Another technique for secreting protein into the growth medium involves the use of the *E. coli* hemolysin (HlyA) secretion system (Hahn *et al.*, 1998; Jarchau *et al.*, 1994; Kern and Ceglowski, 1995). The HlyA signal sequence directs a fusion protein through both the inner and outer membranes in a one-step process, thus eliminating the common problem of protein entrapment in the periplasm. One drawback to this method is that the HlyA signal sequence is not excised from the fusion pro-

tein, so there could be interference of the enzyme activity by these residues. In this regard, our use of the gene III signal sequence which is excised by the cellular machinery is a distinct advantage.

Others have used excisable signal sequences in *E. coli* to secrete protein. For instance, proteins fused with the pelB signal sequence were secreted into the growth medium at relatively high levels (Kipriyanov *et al.*, 1997; Lucic *et al.*, 1998). However, when we tested the ability of the pelB signal sequence to secrete recombinant barley low-pI α-amylase, we were unable to detect any activity (data not shown). Thus, the pSE420-gIII vector, which employs the gene III signal sequence, serves as a useful alternative general strategy, especially when other *E. coli* excisable tags fail to direct extracellular recombinant protein secretion.

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REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds.). (1999). *Short Protocols in Molecular Biology,* J Wiley, New York.

Bush, D. S., Sticher, L., van Huystee, R., Wagner, D., and Jones, R. L. (1989). *J. Biol. Chem.* **264**, 19392–19398.

Cadwell, R. C. and Joyce, G. F. (1992). *PCR Meth. Appl.* **2**, 28–33.

Castelli, L. A., Mardon, C. J., Strike, P. M., Azad, A. A., and Macreadie, I. G. (1994). *Gene* **142**, 113–117.

Cregg, J. M., Vedvick, T. S., and Raschke, W. C. (1993). *Biotechnology* (NY) **11**, 905–910.

Goldsmith, M. E. and Konigsberg, W. H. (1977). *Biochemistry* **16**, 2686–2694.

Hahn, H. P., Hess, C., Gabelsberger, J., Domdey, H., and von Specht, B. U. (1998). *FEMS Immunol. Med. Microbiol.* **20**, 111–119.

Hemila, H., Pakkanen, R., Heikinheimo, R., Palva, E. T., and Palva, I. (1992). *Gene* **116**, 27–33.

Jarchau, T., Chakraborty, T., Garcia, F., and Goebel, W. (1994). *Mol. Gen. Genet.* **245**, 53–60.

Judo, M. S., Wedel, A. B., and Wilson, C. (1998). *Nucleic Acids Res.* **26**, 1819–1825.

Juge, N., Sogaard, M., Chaix, J. C., Martin-Eauclaire, M. F., Svensson, B., Marchis-Maouren, G., and Guo, X. J. (1993). *Gene* **130**, 159–166.

Juge, N., Andersen, J. S., Tull, D., Roepstorff, P., and Svensson, B. (1996). *Protein Expr. Purif.* **8**, 204–214.

Juge, N., Svensson, B., and Williamson, G. (1998). *Appl. Microbiol. Biotechnol.* **49**, 385–392.

Kern, I. and Ceglowski, P. (1995). *Gene* **163**, 53–57.

Kipriyanov, S. M., Moldenhauer, G., and Little, M. (1997). *J. Immunol. Meth.* **200**, 69–77.

Lam, K. H., Chow, K. C., and Wong, W. K. (1998). *J. Biotechnol.* **63**, 167–177.

Lucic, M. R., Forbes, B. E., Grosvenor, S. E., Carr, J. M., Wallace, J. C., and Forsberg, G. (1998). *J. Biotechnol.* **61**, 95–108.

Ohshima, T., Hama, Y., and Sato, M. (2000). *Biochem. Eng. J.* **5**, 149–155.

Robbens, J., Raeymaekers, A., Steidler, L., Fiers, W., and Remaut, E. (1995). *Protein Expr. Purif.* **6**, 481–486.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schaller, H., Beck, E., and Takanami, M. (1978). In *The Single-Stranded DNA Phages* (Denhardt, D. T., Dressler, D., and Ray, D. S., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scorer, C. A., Buckholz, R. G., Clare, J. J., and Romanos, M. A. (1993). *Gene* **136**, 111–119.

Shao, Z., Zhao, H., Giver, L., and Arnold, F. H. (1998). *Nucleic Acids Res.* **26**, 681–683.

Sogaard, M. (1989). M. Sc. Thesis, University of Copenhagen.

Sogaard, M. and Svensson, B. (1990). *Gene* **94**, 173–179.

Sogaard, M., Olsen, F. L., and Svensson, B. (1991). *Proc. Natl. Acad. Sci. USA* **88**, 8140–8144.

Stemmer, W. P. (1994). *Nature* **370**, 389–391.

van der Wal, F. J., Luirink, J., and Oudega, B. (1995). *FEMS Microbiol. Rev.* **17**, 381–399.

Wong, D. W. S., Batt, S. B., and Robertson, G. H. (2000). *J. Agric. Food Chem.* **48**, 4540–4543.

Zhao, H., Giver, L., Shao, Z., Affholter, J. A., and Arnold, F. H. (1998). *Nat. Biotechnol.* **16**, 258–261.