Published in final edited form as:

J Biotechnol. 2013 March 10; 164(1): 1–8. doi:10.1016/j.jbiotec.2012.11.007.

Engineering *Escherichia coli* for Soluble Expression and Single Step Purification of Active Human Lysozyme

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Abstract

Genetically engineered variants of human lysozyme represent promising leads in the battle against drug-resistant bacterial pathogens, but early stage development and testing of novel lysozyme variants is constrained by the lack of a robust, scalable and facile expression system. While wild type human lysozyme is reportedly produced at 50 – 80 kg per hectare of land in recombinant rice, this plant-based system is not readily scaled down to bench top production, and it is therefore not suitable for development and characterization of novel lysozyme variants. Here, we describe a novel and efficient expression system capable of producing folded, soluble and functional human lysozyme in *E. coli* cells. To achieve this goal, we simultaneously co-express multiple protein folding chaperones as well as harness the lysozyme inhibitory protein, Ivy. Our strategy exploits *E. coli*'s ease of culture, short doubling time, and facile genetics to yield upwards of 30 mg/L of soluble lysozyme in a bioreactor system, a 3000-fold improvement over prior efforts in *E. coli*. Additionally, molecular interactions between lysozyme and a his-tagged Ivy allows for one-step purification by IMAC chromatography, yielding as much as 21 mg/L of purified enzyme. We anticipate that our expression and purification platform will facilitate further development of engineered lysozymes having utility in disease treatment and other practical applications.

1. Introduction

Lysozymes represent a cornerstone of protein biochemistry (Jolles, 1996), and they possess both historical and ongoing technological significance. Alexander Fleming's 1922 description of the protein was a springboard that helped launch the field of antibiotic drug development (Fleming, 1922). Hen egg white lysozyme (HEWL) yielded the very first x-ray crystal structure of an enzyme (Blake et al.), and lysozymes have subsequently been powerful models for the study of protein structure-function relationships (Imoto, 1996; Vocadlo et al., 2001). More recently lysozyme has provided a toehold for mechanistic studies of amyloid fibril diseases (Swaminathan et al., 2011), and HEWL has received international approval for use in food and over the counter applications (Barroso, 2012; Lake, 1998; Speijers and van Apeldoorn). Additionally, lysozymes are now re-emerging as therapeutic candidates for the treatment of drug-resistant pathogens (Bhavsar et al., 2010; Bhavsar et al., 2011; Donovan, 2007), one of the foremost challenges facing modern

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medicine (Taubes, 2008). Thus, it seems likely that the use of lysozymes in practical applications will continue to grow in the coming years.

The utility of lysozymes, or any other protein, is in part a function of material accessibility. HEWL is produced at the metric tonne scale via purification from eggs, and recent advances in transgenic plant technologies have resulted in similarly low production costs for recombinant human lysozyme (hLYZ) (Wilken and Nikolov, 2011). These wild type enzymes thus benefit from economies of scale, but the native proteins suffer from inherent limitations with respect to some emerging applications, e.g. disease therapy (Parisien et al., 2008). To address the inadequacies of natural lysozymes, biomolecular engineering has been leveraged to create designer enzymes tailored to therapeutic niches (Gill et al., 2011; Ibrahim et al., 2002; Lu et al., 2010; Scanlon et al., 2010). Unfortunately, preclinical development of such novel drug candidates can be bottlenecked by material limitations. That is, researchers cannot readily access sufficient protein for all of the required testing and analysis. Validated high level production platforms, such as transgenic chickens or rice, are simply not feasible for early stage experimental enzymes, and therefore researchers are typically relegated to recombinant expression in microbial hosts. For example, hLYZ has been expressed in *Saccharomyces cerevisiae* (Hayano et al., 1995), *Kluyveromyces lactis* (Iwata et al., 2004; Maullu et al., 1999), and *Pichia pastoris* (Wei et al., 2012). Unfortunately, many of these studies neglected to purify the recombinant protein, and the reported yields were often based on activity assays of culture supernatant conducted under varied conditions. This fact complicates comparison of expression yields, but our review of the literature indicates that maximum yields of purified hLYZ from yeast are approximately 20 mg/L of culture (Maullu et al., 1999). To attain such high levels, Maullu *et al.* were obliged to screen libraries of chromosomal integrants to identify the highest level expressers, they developed a high cell-density fermentation strategy using complex media formulation, and they subsequently undertook a multistep purification procedure. Importantly, replicating this approach for each and every lysozyme of potential interest would be a prohibitively time-intensive process. Thus, there remains a need for a scalable and easily implemented expression and purification system for research scale production of interesting lysozyme proteins.

Escherichia coli is one of the most widely used and cost-effective expression hosts for recombinant protein production (Braun and LaBaer, 2003; Busso et al., 2011; Tolia and Joshua-Tor, 2006). The organism's ability to rapidly overexpress desired biologics in a scalable fashion has made it useful in the pharmaceutical and biotechnology industries, and its ease of culture combined with an extensive molecular genetics toolkit have rendered it a preferred expression host for academic laboratories. Unfortunately, expression of soluble lysozymes in *E. coli* results in rapid cellular lysis and poor yields (Fischer et al., 1993). In this host, therefore, the enzymes can only be produced as insoluble and inactive inclusion bodies (Casaite et al., 2009; Koshiba et al., 1998; Schlorb et al., 2005). Isolation of pure, active material from such a system requires a tedious, inefficient, multistep refolding and purification process. To circumvent the limitations of lysozyme production in *E. coli*, we have engineered a multicomponent expression system that leverages (i) a modified strain capable of forming and isomerizing disulfide bonds within the cytoplasmic compartment, (ii) overexpression of complementary protein folding chaperones, and most importantly (iii) co-expression of an antitoxin protein that both sequesters hLYZ bactericidal activity and serves as a trans-acting purification handle for immobilized metal affinity chromatography (IMAC). The antitoxin, inhibitor of vertebrate lysozyme (Ivy), is a 129-residue *E. coli* protein whose native form is secreted to the periplasmic space where it forms homodimers (Abergel et al., 2007). Each Ivy homodimer is capable of binding two C-type lysozyme molecules, and in doing so it acts as a high-affinity inhibitor with a $K_i=1$ nM for HEWL (Monchois et al., 2001). Here, we co-opt this endogenous antitoxin to engineer a lysozyme

expression system that improves upon prior soluble expression efforts in *E. coli* by more than three orders of magnitude, equaling or besting the purified yields from top performing yeast systems. Our one-step IMAC purification of folded and functional lysozyme from overnight *E. coli* cultures should prove useful for facilitating the study and development of various C-type lysozymes and their engineered variants.

2. Materials and Methods

2.1 Materials

The SHuffle T7 Express *E. coli* strain, restriction enzymes, Phusion polymerase and T4 ligase were purchased from New England Biolabs (Ipswich, MA). Oligonucleotides were ordered from IDT (Coralville, IA), and were purified by standard desalting methods. Duet expression vectors were obtained from EMD Millipore (Billerica, MA) and plasmid purification kits were purchased from QIAGEN (Valencia, CA). Gel extraction and DNA clean-up kits were obtained from Zymo Research (Orange, CA). Ni-NTA columns were from GE Healthcare Life Sciences (Piscataway, NJ). Recombinant human lysozyme standards (92% pure) were purchased from Sigma-Aldrich (St. Louis, MO) and all other reagents were from Fischer Scientific (Pittsburgh, PA), unless otherwise noted.

2.2 Cloning of the Human Lysozyme and Ivy Genes

The human lysozyme gene (*hlyz*) was amplified from the p4GM-LYZ vector (Table 1) with oligonucleotides that appended a 5'-NdeI restriction site at the ATG start codon and a 3'-HindIII restriction site immediately following the native stop codon. The *E. coli* Ivy gene was amplified from the *E. coli* strain JM105 with oligonucleotides that appended a 5'-NdeI restriction site and a 3'-XhoI restriction site (appends non-native, C-terminal LeuGlu-hexahistidine sequence in pET26b). Both genes were purified by agarose gel electrophoresis, digested, and ligated separately into similarly digested pET26b expression vectors (EMD Millipore, Billerica, MA). The resulting pET26b-hLYZ and pET26b-Ivy-his constructs were transformed into electrocompetent DH5 α [F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ^-] individual clones were sequence verified, and purified plasmids were transformed into electrocompetent SHuffle T7 Express *E. coli* [fhuA2 lacZ::T7 gene1 [lon] ompT ahpC gal λ att::pNEB3-r1-cDsbC (Spec^R, lacI^q) Δ trxB sulA11 R(mcr-73::miniTn10--Tet^S)2 [dcm] R(zgb-210::Tn10 --Tet^S) endA1 Δ gor Δ (mcrC-mrr)114::IS10].

2.3 Construction of the Human Lysozyme and Ivy Co-expression Platform

To facilitate co-expression of the hLYZ and Ivy-his proteins, both genes were subsequently cloned into the pRSFDuet-1 co-expression vector. The *ivy-his* gene was amplified from pET26b-Ivy-his with oligonucleotides that appended 5'-NcoI and 3'-HindIII restriction sites. The digested gene was then ligated into MCS1 of pRSFDuet-1 to yield pRSF-Ivy-his. The *hlyz* gene was amplified from pET26b-hLYZ with oligonucleotides that appended 5'-NdeI and 3'-XhoI restrictions sites. The digested gene was then ligated into MCS2 of pRSF-Ivy-his generating pRSF-Ivy-his-hLYZ. This co-expression construct was transformed into electrocompetent DH5 α , individual clones were sequence verified, and purified plasmid was retransformed into electrocompetent SHuffle T7 Express for expression studies.

To facilitate cytoplasmic folding of the hLYZ and Ivy-his genes, a panel of chaperones was cloned into the SHuffle T7 Express cells containing pRSF-Ivy-his-hLYZ. Expression vector derivatives of pAR3 contained genes encoding Skp, GroEL/ES, BiP or DnaK/J, while pBAD33 vector derivatives bore the gene encoding Trigger Factor. Chaperones that demonstrated the greatest increase in expression of hLYZ were then amplified with oligonucleotides that appended either 5'-NcoI and 3'-HindIII restriction sites

(complementary to pACYCDuet-1 MCS1) or 5'-NdeI and 3'-XhoI restriction sites (complementary to pACYCDuet-1 MCS2). Pairs of these genes were then digested and ligated into pACYCDuet-1. Additionally, the Skp chaperone gene and a second copy of *hlyz* were ligated into pACYCDuet-1. All dual chaperone pACYCDuet-1 expression constructs were transformed into electrocompetent DH5 α , individual clones were sequence verified, and dual chaperone expression constructs were then transformed into electrocompetent SHuffle T7 Express containing pRSF-Ivy-his-hLYZ.

2.4 Co-expression of Human Lysozyme and Ivy-his

Overnight cultures of SHuffle T7 Express bearing the pRSF-Ivy-his-hLYZ expression construct were grown at 37°C in 3 ml of LB supplemented with 30 μ g/ml kanamycin (LB-Kan). Overnights were sub-cultured 1:100 into 50 ml of fresh media, grown to mid-log ($OD_{600} = 0.6$) at 37°C, and shifted to 30°C with a 30 minute equilibration prior to induction with 0.5 mM IPTG for 20 hours. Following induction, cell cultures were centrifuged at 6000g and 4°C for 10 minutes and cell pellets were resuspended in 5 ml of native lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole pH 8). Cell suspensions were sonicated on ice (Fisher 550 Sonic Dismembrator), transferred to 2ml eppendorf tubes and centrifuged at 17,000g and 4°C for 20 minutes. Where noted, insoluble fractions were taken into 6 M urea and analyzed by HPLC. Soluble fractions were filtered through 0.22 μ m PES membranes and stored at 4°C before analysis by HPLC or further purification. Additionally, expression hosts containing pRSF-Ivy-his-hLYZ combined with a complementary vector encoding various chaperones were grown, induced, and lysed under the same conditions. However, dual-vector hosts required the addition of 34 μ g/ml chloramphenicol to the media (LB-Kan-Cm), and those hosts bearing the pAR3 or pBAD33 vector derivatives were induced with both 0.2% arabinose and 0.5 mM IPTG.

2.5 Scale Up and Optimization of Expression Conditions

Induction temperature for the pRSF-Ivy-his-hLYZ + pACYC-Skp-TF expression host was assessed at 16, 22, 30 and 37°C. Subcultures of overnights were again grown at 37°C to mid-log and then equilibrated at the desired temperature prior to induction. Other details were as described above. Following optimization of the induction temperature, favorable expression hosts were scaled up to 500 ml shake flask expression or 1 L high cell density fermentation.

High cell density fermentation was carried out in a 3 L ez-Control bioreactor (Applikon, Schiedam, Netherlands) with the accompanied BioXpert controller software. Briefly, a 15 ml overnight culture containing the desired expression host was grown at 37°C in LB-Kan-Cm. The overnight was then sub-cultured 1:100 into 1 L of Riesenberg media (Riesenberg et al., 1991) containing 10 g of dextrose and appropriate antibiotics. The initial batch phase growth was continued at pH 6.5 and 37°C until a dissolved oxygen spike was observed (~17.5 hours post sub-culture), at which point the fed-batch phase was initiated and continued for an additional 7.5 hours. A specific growth rate of 0.06 h⁻¹ was used to determine the exponential dextrose feed rate. At 25 hours post sub-culture, the temperature was reduced to 30°C, the cells were induced with 0.5 mM IPTG, and expression was continued for another 20 hours. Following induction, the cell culture was centrifuged at 6000g, 4°C for 15 min and cell pellets were resuspended in 40 ml of native lysis buffer. Filtered soluble cell free extract was then procured as before and stored at 4°C prior to further purification and analysis.

2.6 Purification of Human Lysozyme

An FPLC Ni-NTA column (1 ml or 5 ml column depending on the level of expression) was pre-equilibrated with native lysis buffer and soluble cell free extract was then loaded at 1 ml/

min. After complete loading of the soluble cell free extract, the column was washed with native lysis buffer before eluting hLYZ with 0.1 M Tris-HCl pH 12. Fractions were tested for lytic activity as described below (dilution ratio of 1:50). Following alkaline hLYZ elution, Ivy-his was eluted with 50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole at pH 8. Fractions containing hLYZ were dialyzed into 20 mM NaH₂PO₄ pH 6.5 and stored at 4°C.

2.7 HPLC and Activity Analysis

Soluble and insoluble protein expression levels and purity were assessed by reverse phase chromatography. Samples were loaded at 1 ml/min onto a C4 column (Grace Vydac 214TP, Deerfield, IL) equilibrated with 0.1% trifluoroacetic acid and 22.5% acetonitrile in water. The hLYZ and Ivy-his proteins were eluted with a 30 minute 22.5% to 40.5% acetonitrile gradient. Protein concentrations in experimental samples were determined by comparing integrated peak areas to those of hLYZ and Ivy-his standards run at varying concentrations.

Enzymatic activity of purified hLYZ was assessed by light scattering in 96-well microtiter plates (Lee and Yang, 2002). Briefly, 5 µl containing 25 – 100 ng of hLYZ was added to each well in the first row of a microtiter plate and then brought up to 175 µl with activity buffer (66 mM KHPO₄ pH 7). Next, 75 µl of 1 mg/ml freeze-dried *Micrococcus luteus*, resuspended in activity buffer, was added to each well in the first row with a multichannel pipette and the plate was then placed in a UV/Vis plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA). The decrease in absorbance at 450 nm (correlating to lysed *M. luteus*) was measured every 15 seconds over the course of 10 minutes. Initial slopes were used to determine specific activity. Experimental samples were compared to solutions of commercial hLYZ from Sigma-Aldrich.

3. Results

3.1 Co-expression of Human Lysozyme and Ivy-his with Chaperones

To enhance soluble expression of hLYZ in *E. coli*, we adopted a three-pronged approach. First, we expressed the human protein within the cytoplasmic compartment, where the enzyme would be isolated from its natural peptidoglycan substrate. The hLYZ protein, however, contains eight cysteines that pair into four disulfide bonds. Thus, the decision to sequester the enzyme within the cytoplasm necessitated the use of a *trxB gor aphC* mutant strain capable of forming disulfides in this normally reducing environment (Besette et al., 1999). Given the moderate complexity of the hLYZ disulfide bonding pattern and our ultimate desire to employ a strong T7 promoter, we elected to use the Shuffle T7 Express strain, which bears the requisite *trxB gor aphC* mutations, produces the T7 RNA polymerase, and overexpresses the disulfide bond isomerase DsbC in the cytoplasm. Initial efforts to express hLYZ in this strain caused a decrease in shake flask culture densities yet yielded several milligrams of enzyme per liter culture. However, the resultant hLYZ protein was found almost exclusively as insoluble inclusion bodies (data not shown).

In theory, cytoplasmic expression should separate hLYZ from the labile cell wall peptidoglycan, but our results indicated that the protein remained toxic despite expression in the inner cellular compartment. To mitigate the inherent toxicity associated with hLYZ production, we next co-expressed a cytoplasmic version of *E. coli*'s endogenous lysozyme inhibitor, Ivy (Fig. 1). We anticipated that the high affinity Ivy-hLYZ interaction would effectively sequester lysozyme's innate bactericidal activity and at the same time might also improve hLYZ solubility through a templating effect. Additionally, a hexa-his tag was appended to the Ivy C-terminus so as to facilitate rapid purification of the protein complex. To insure equivalent copy numbers of the *hlyz* and *ivy-his* genes in each individual host cell,

we constructed a pRSF-Ivy-his-hLYZ duet vector that drives hLYZ and Ivy co-expression from two independent T7 promoters on a single vector backbone. Initial results in 50 ml shake flasks showed that expression from the duet vector yielded 2 mg/L of soluble hLYZ and 21 mg/L of soluble Ivy-his. This represented a 200-fold improvement over previous reports of soluble lysozyme production in *E. coli* (Fischer et al., 1993), but western blot analysis indicated that approximately half of the desired hLYZ product remained in the insoluble fraction (data not shown). Thus, there remained a substantial opportunity to improve the soluble expression of hLYZ by enhancing folding efficiency.

In an effort to partition more hLYZ to the soluble fraction, we examined several ternary expression systems that included various molecular chaperones in addition to the pRSF-encoded hLYZ and Ivy-his proteins. The chaperones were each encoded on a second vector having a compatible pACYC origin of replication and an orthogonal chloramphenicol antibiotic marker (Table 1). It bears emphasizing that the Shuffle[®] T7 Express strain constitutively produces cytoplasmic copies of the DsbC protein disulfide isomerase, and the plasmid borne chaperones from Table 1 were therefore expressed in addition to the genomically-encoded DsbC foldase. Introducing any one of the additional chaperones increased mean soluble expression of hLYZ, but there was no significant difference between the various chaperones (Fig. 2A).

Given the improved hLYZ production via plasmid-based overexpression of individual molecular chaperones, we contemplated the potential for further cooperative enhancements via co-expression of chaperone combinations. Based on their distinct activities and modes of action, Skp, Trigger Factor, and GroEL/ES were grouped into all pairwise combinations and cloned into the pACYCDuet-1 vector. The modified pACYCDuet-1 vectors, each bearing two chaperones under control of separate T7 promoters, were then transformed into the pRSF-Ivy-his-hLYZ expression host, and soluble production of hLYZ and Ivy-his were assessed as before (Fig. 2B). Soluble hLYZ expression was generally similar to that of the single chaperone systems, although the Skp & GroEL/ES combination reproducibly yielded decreased hLYZ levels. Perhaps more striking was the trend towards reduced soluble Ivy-his yields exhibited by all chaperone combinations. Ultimately, this latter effect played a fortuitous role in efficient downstream purification of hLYZ, as described below.

In a final effort to further enhance soluble hLYZ expression, we generated a pACYCDuet-1 vector that encoded the Skp chaperone and a second copy of the *hlyz* gene. When transformed into the pRSF-Ivy-his-hLYZ expression host, this dual *hlyz* gene system yielded results similar to those of the combined chaperone systems described above (Fig. 2B). Thus, it appeared that soluble hLYZ expression had been largely optimized within the context of the current approach.

3.2 Purification and Analysis of Human Lysozyme

In addition to its primary function as a protective antitoxin, we designed our cytoplasmic Ivy protein with a C-terminal hexa-histidine tag so as to facilitate separation of the hLYZ-Ivy-his duplex using IMAC. Following clarification of cell lysates by centrifugation and filtration of the supernatant, the his-tagged duplex was readily bound to Ni-NTA metal affinity resin. Standard washing procedures removed non-specifically adsorbed proteins, and hLYZ was dissociated from the immobilized Ivy-his using an alkaline elution buffer. This resulted in the elution of two peaks, both of which contained hLYZ (see supplemental figures). The initial peak migrated with the alkaline buffer front but was not fully eluted prior to the appearance of the second peak. Peak 1 was invariably a mixture of hLYZ and Ivy-his. Peak 2 contained up to 98% pure hLYZ when loading cell lysate with a 2:1 ratio of Ivy-his to hLYZ. In contrast, expression conditions that resulted in 3:1 or greater ratios of Ivy-his to hLYZ yielded Ivy-his contamination of the second peak and lower specific

activity for the eluted enzyme. Fractions from both peaks were found to form some white precipitate shortly after elution from the column, and this insoluble material was removed by centrifugation and filtration prior to analysis of the soluble protein. Despite these losses, the final purified yields of hLYZ remained relatively high (details below).

High levels of hLYZ purity in peak 2 were obtained with cells harboring the pRSF-Ivy-his-hLYZ and pACYC-Skp-TF constructs. While the individual Skp or Trigger Factor chaperones yielded similar soluble hLYZ levels (Fig. 2), simultaneous expression of both chaperones from the pACYC-Skp-TF accessory plasmid reduced soluble Ivy-his production and reproducibly gave an optimal 2:1 Ivy-his to hLYZ expression ratio. As noted above, this ultimately yielded 98% pure hLYZ in the second peak eluted from the IMAC column. Following buffer exchange into 20 mM NaH₂PO₄ pH 6.5, the lytic activity of this material was assessed with Gram-positive *Micrococcus luteus* cells, and it was found to be greater than that of commercially produced hLYZ from recombinant rice (Fig. 3). With the exception of a putative N-terminal methionine resulting from the ATG start codon, our enzyme is the same as the commercial material. Therefore, the enhanced specific activity of our hLYZ preparation likely stems from slightly higher purity as opposed to greater inherent catalytic power.

3.3 Optimizing Induction Temperature and Scale Up

The yields of hLYZ from the pRSF-Ivy-his-hLYZ + pACYC-Skp-TF expression host were examined as a function of temperature (16, 22, 30 and 37°C). Exponential phase shake flask cultures were induced with 0.5 mM IPTG for 20 hours, and the soluble yields of hLYZ and Ivy-his were quantified by HPLC (Fig. 4). Induction at 22°C produced the highest levels of soluble hLYZ, but the molar ratio of Ivy-his to hLYZ, a key determinant of final hLYZ purity, was most favorable at either 30 or 37°C. Thus, an induction temperature of 30°C was employed for all additional studies, as it generated the greatest hLYZ yields while still maintaining the optimal 2:1 ratio of Ivy-his to hLYZ.

To obtain larger quantities of pure enzyme, shake flask culture volumes of the pRSF-Ivy-his-hLYZ + pACYC-Skp-TF expression host were scaled from 50 ml to 2×500 ml. As before, the soluble cell free extract was loaded onto a Ni-NTA column and two lysozyme peaks were eluted with the alkaline buffer and analyzed by HPLC. The first peak contained less than 50% pure hLYZ, and a substantial portion of the eluted material precipitated in the harsh alkaline buffer. The second peak contained 1.6 mg of 98% pure hLYZ, measured after dialysis into storage buffer. The imidazole elution yielded 6.2 mg of pure Ivy-his. Thus, soluble hLYZ expression yields were largely unaffected by the 10-fold increase in culture volume, and moreover, nearly one third of the soluble enzyme could be isolated in a single step at 98% purity.

To further assess the scalability of the co-expression platform, the pRSF-Ivy-his-hLYZ + pACYC-Skp-TF expression host was used to inoculate a one liter bioreactor fermentation. A fed-batch strategy employing an initial 10 g bolus of dextrose followed by an exponential dextrose feed resulted in 8.3 mg/L of soluble hLYZ and 93 mg/L of soluble Ivy-his. Relative to shake flask expression, fermentation increased soluble hLYZ yield by 66%. At the same time, however, soluble Ivy-his yields increased by 10-fold resulting in an 11:1 ratio of Ivy-his to hLYZ. As a result, excess Ivy-his was bound to the IMAC column during purification and the alkaline elution contained predominantly Ivy-his protein.

It was not intuitively obvious what factors were causing differential yield profiles in the shake flask *versus* fermentation cultures of pRSF-Ivy-his-hLYZ + pACYC-Skp-TF, and it seems likely that the physiological origins of this effect were numerous and complex. To readjust the relative yields of hLYZ and Ivy-his in the bioreactor expression runs, we

therefore elected to bypass tedious optimization of the manifold fermentation variables and instead make a simple change to the cellular expression system. Specifically, we combined the base pRSF-Ivy-his-hLYZ expression vector with a supplemental pACYC-Skp-hLYZ vector that encoded the single chaperone Skp and an additional copy of the *hlyz* gene. While use of the supplemental pACYC-Skp-hLYZ vector did not significantly increase soluble hLYZ yields in shake flask cultures (Fig. 2), in the bioreactor it resulted in drastically improved soluble hLYZ yields and a near optimal 2:1 ratio of Ivy-his to hLYZ (75.4 mg/L and 32.8 mg/L, respectively). Purification of this material by IMAC and alkaline elution once again resulted in two peaks, with 21.2 mg of hLYZ in the second peak, as determined by A₂₈₀.

4. Discussion

Applications of wild type HEWL and hLYZ benefit from economies of scale, as both enzymes can be produced by the hundreds or thousands of kilograms via purification from chicken eggs or from recombinant rice, respectively (Wilken and Nikolov, 2011). In contrast, the study of other natural lysozymes or engineered variants can be stalled at preliminary stages due to limited material accessibility. For prospective analysis of early stage candidates, it is simply impractical to create a transgenic rice strain, gain approval for large scale growth, plant and harvest fields of rice, and process the grain to obtain pilot scale quantities of purified enzyme. Similar constraints are encountered with production in transgenic animals, which have been employed to produce wild type lysozymes (Maga et al., 2006; Yang et al., 2011; Yu et al., 2006) but are impractical for systematic production of experimental candidates. Thus, recombinant expression in cellular systems represents the only feasible strategy for obtaining pilot scale quantities of novel and potentially useful lysozyme molecules. Numerous yeast-based systems have been developed, and moderate purified yields have been demonstrated in some of these hosts (Maullu et al., 1999). To date, however, there has been no report of efficient lysozyme production in an *E. coli* platform. Prior expression efforts in *E. coli* have either produced negligibly small quantities of the soluble, active enzyme (Fischer et al., 1993) or have been relegated to isolation of inclusion bodies and refolding (Casaite et al., 2009; Koshiba et al., 1998; Schlorb et al., 2005). Motivated by *E. coli*'s broad accessibility, ease of culture, rapid growth rates, and proven scalability, we sought to develop an efficient expression and purification system for C-type lysozymes in this bacterial host.

The most immediate barrier to soluble expression of lysozymes in *E. coli* is the enzymes' inherent bactericidal activity. To protect our *E. coli* host from the toxic effects of recombinant hLYZ expression, we sequestered the enzyme in the cytoplasm and simultaneously overexpressed cytoplasmic copies of the endogenous lysozyme inhibitor, Ivy. Ivy's potent inhibitory activity towards lysozymes was first reported in 2001 (Monchois et al., 2001), and structural insights into Ivy's mode of action were later elucidated by x-ray crystallography (Abergel et al., 2007). The *E. coli* Ivy protein inhibits both C-type and G-type lysozymes from the animal kingdom (Callewaert et al., 2005), and we therefore anticipate that our expression and purification system may prove useful with diverse enzymes from these two lysozyme families (but likely not the third class of animal lysozymes, I-type). In considering the utility of our system with engineered lysozyme variants, we note the potential for function-enhancing mutations to interfere with Ivy sequestration and thus undermine our antitoxin strategy. We emphasize, however, that Ivy possesses confirmed inhibitory activity towards hLYZ, HEWL and goose egg white G-type lysozyme, which between them exhibit a mere 6% amino acid identity and a low 59% homology. Structurally, the two C-type lysozymes hLYZ and HEWL possess essentially identical α/β folds (rmsd=0.7 Å for backbone atoms of 1JWR (Higo and Nakasako, 2002) and 193L (Vaney et al., 1996), respectively). G-type lysozymes also have a two-domain α/β

fold, but G-type structures are not readily superposed upon C-type structures. Thus, the Ivy antitoxin accommodates highly divergent primary amino acid sequences within a given fold as well as divergent lysozyme structures. To be sure, adapting our system to lysozyme families other than C- and G-type will likely require different antitoxin proteins, but conceptually our strategy has broad applicability to an array of lytic enzymes.

While co-expression of the Ivy antitoxin was critical to production of soluble hLYZ, incorporating various molecular chaperones and foldases into our platform resulted in even greater soluble yields. The DsbC protein disulfide isomerase was expressed from the chromosome of our Shuffle T7 Express strain, and was therefore a constant among our various system configurations. Additional chaperones/foldases were assessed in combination with DsbC in an effort to enhance hLYZ folding. Trigger Factor is a native cytoplasmic chaperone that binds exposed hydrophobic peptides in a co-translational fashion as they exit the ribosome (Kaiser et al., 2006). Trigger Factor also exhibits peptidyl-prolyl cis/trans isomerase activity and requires no nucleotide cofactors. GroEL/ES together form the archetype “Anfinsen cage” chaperonin that acts by an ATP-dependent but passive mechanism (Horwich et al., 2009). In its native form, Skp is a periplasmic chaperone best known for its role in outer membrane protein biogenesis, although its capacity to facilitate folding of soluble proteins has also been leveraged to great effect (Entzminger et al., 2012; Levy et al., 2001). Interestingly, the Skp chaperone has been shown previously to enhance *in vitro* folding efficiency of HEWL (Walton and Sousa, 2004), which has been shown to preferentially fold via pathways involving long-lived intermediates (Kiefhaber, 1995). In our system, it seems likely that Skp reversibly traps off-pathway folding intermediates thereby preventing their aggregation and improving soluble yields of hLYZ *in vivo*. This hypothesis is consistent with recent insights into the mechanism by which Skp facilitates *in vitro* refolding of HEWL and single chain antibody fragments (Entzminger et al., 2012).

In an effort to improve soluble hLYZ yields further, we leveraged compatible Duet expression vectors to generate *E. coli* hosts that simultaneously co-expressed five recombinant proteins: the desired hLYZ enzyme, the critical Ivy antitoxin, the DsbC isomerase, and combinations of two additional chaperones/foldases. In shake flask cultures, these highly engineered expression systems produced soluble hLYZ at levels similar to that obtained with the simpler four component systems comprised of hLYZ, Ivy, DsbC and one other chaperone/foldase. One possible explanation for the lack of improved expression is that simultaneous transcription of mRNA from all four T7 promoters of our dual-vector Duet system eventually overwhelms the translational capacity of our *E. coli* host. Consistent with this hypothesis, the five component systems do not enhance hLYZ expression but they do noticeably decrease soluble yields of the Ivy-his protein in shake flasks. Importantly, efficient downstream purification of hLYZ was dependent upon a 2:1 expression ratio of Ivy-his to hLYZ, and achieving this ratio required different host strain configurations in shake flasks *versus* the bioreactor. Namely, the five component hLYZ, Ivy, DsbC, Skp and Trigger Factor system produced an optimal ratio in shake flasks, but the four component hLYZ (two separate copies), Ivy, DsbC and Skp system was far superior in the bioreactor. The differences likely stem from altered physiological and metabolic states in the two environments, although evidence to support this hypothesis awaits more detailed experimental analysis.

While our multicomponent systems enhanced soluble lysozyme expression as assessed by HPLC, our ultimate goal was a platform for one-step purification of active lysozyme from *E. coli*. To this end, our Ivy antitoxin was designed with a C-terminal hexa-histidine tag so as to facilitate efficient capture of the Ivy-his protein from the complex milieu of whole cell lysates. Because Ivy-his binds hLYZ with high affinity, non-tagged hLYZ hitchhikes upon the antitoxin and is thereby indirectly captured on the IMAC resin. The interaction between

Ivy-his and lysozyme is disrupted in alkaline buffer (Callewaert et al., 2005), while at the same time the hexa-histidine tag retains high affinity for the nickel resin. Under optimal conditions, 98% pure hLYZ can be eluted from the column following binding of soluble cell lysate. The Ivy-his protein thus serves multiple functions in our expression and purification platform: (i) it sequesters lysozyme's inherent bactericidal activity and protects the expression host, (ii) we speculate that it improves lysozyme's solubility by serving as a template for the properly folded structure, and (iii) it constitutes a purification handle that facilitates one-step isolation of unmodified lysozyme in good yield and with high purity. Because our system employs the near ubiquitous *E. coli* expression host and standard IMAC purification, it can be implemented in even the most minimally equipped biotech laboratory. As a result, we anticipate that this expression and purification platform will facilitate further development of novel lysozymes having utility in disease treatment and other practical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the Wallace H. Coulter Foundation, the National Center for Research Resources (5P20RR018787-10) and the National Institute of General Medical Sciences (8 P20 GM103413-10) from the National Institutes of Health. John W. Lamppa was also supported in part by a Renal Function and Disease Training Grant (T32 DK007301) from the NIH. Sam Tanyos was supported in part by a Mazilu Engineering Research Fellowship.

The authors thank Jonathan Guerrette for assistance optimizing HPLC conditions.

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First report of Ivy and lysozyme co-expression in *Escherichia coli*.
Ivy sequesters lysozyme activity and protects host cells from lysis.
His-tagged Ivy can be leveraged for downstream 1-step purification of lysozyme.
Use of molecular chaperones increased soluble lysozyme expression.
Soluble lysozyme expression was improved by 3,000-fold over previous reports.

MGRISSGGMMFKAITTVAALVIATSAMAQDDLTISSLAKGETTKAAFNQMVQGHKLPWVMKG
 GTYTPAQTVTLGDETYQVMSACKPHDCGSQRIAVMWSEKSNQMTGLFSTIDEKTSQEKLTLWL
 NVNDALSIDGKTVLFAALTGSLENHPDGFNFK

Figure 1.

Sequence and Structure of Ivy Antitoxin. (Top) Ivy (ribbon diagram) binds hLYZ (molecular surface) through numerous interactions adjacent to the active site cleft, and hLYZ activity is sequestered via insertion of the Ivy CKPHDC peptide loop into the active site. The Ivy H60 residue, rendered as a stick figure, forms hydrogen bonds with the hLYZ E35 and D52 catalytic residues. Image rendered from PDB file 1GPQ (Abergel 2007) using YASARA Structure. (Bottom) The complete amino acid sequence of Ivy is shown with the leader sequence as white text on black background and the mature protein as black text. The cytoplasmic Ivy construct from this work was generated by replacing the N-terminal leader peptide with a methionine.

Figure 2.

Effects of *E. coli* chaperone overexpression on soluble hLYZ and Ivy yields. Soluble protein yields were determined by HPLC analysis of soluble cell lysates and are shown for hLYZ (white bars) and Ivy (black bars). (A) Effects of individual chaperones. (B) Effects of chaperone combinations. The broken horizontal line indicates baseline hLYZ expression levels in the absence of plasmid-encoded chaperones. Note that in all cases the DsbC chaperone is simultaneously co-expressed from an integrated genomic cassette.

Figure 3. Specific activity of hLYZ from a commercial source and from this study. Enzyme from this study, produced in *E. coli* and purified in a single step IMAC procedure, exhibited an 18% increase in specific activity compared to the commercial protein ($p=0.01$).

Figure 4. Soluble protein yields as a function of induction temperature. Cells bearing the pRSF-Ivy-his-hLYZ + pACYC-Skp-TF constructs were induced across a broad temperature range, and soluble yields of hLYZ (closed circles) and Ivy-his (open squares) were determined by HPLC. A temperature of 30°C produced the highest levels of soluble hLYZ while still maintaining the desired 2:1 ratio of Ivy-his to hLYZ.

Table 1

A comprehensive list of the strains and plasmids used in this study.

Strains and Plasmids	Description	Source
Strains		
JM105	thi, rpsL (StrR), endA, sbcB15, hsdR4, supE, Δ(lac-proAB), F'(traD36, proAB+, lacIq, lacZΔM15)	ATCC# 47016
DH5α	F-(f80dlacZΔM15,) Δ(lacIZYA-argF)U169 deoR recA1 endA1 hsdR17(rk-,mk+) supE44, thi-1 gyrA96, relA1	Invitrogen
SHuffle T7 Express	<i>thiA2 lacZ::T7 gene1 flon1 ompT ahpC gal λatt::pNEB3-r1-cDsbC(Spec^R, lac^R) ΔtrxB sulA11 R(mcr-73::miniTn10--Tet^S)2 ldcM1 R(zgb-210::Tn10--Tet^S) endA1 Δgor Δ(mcrC-mrr)114::IS10</i>	New England Biolabs
Plasmids		
p4GM-LYZ	Yeast expression vector containing the Gal 1 promoter, alpha mating factor leader sequence and human lysozyme	Scanlon et al., 2009
pET26b	<i>E. coli</i> expression vector containing the T7 promoter, pBR322 ori, and kanamycin resistance	EMD Millipore
pET26b-hLYZ	pET26b derivative containing the human lysozyme gene with a methionine start codon and dual stop codon	This study
pET26b-Ivy-his	pET26b derivative containing the <i>E. coli</i> Ivy gene with a non-native C-terminal LeuGlu-hexaHis sequence	This study
pRSFDuet-1	Co-expression vector containing 2 T7 promoters, 2 RBS, 2 MCS, 1 T7 terminator, RSF 1030 ori and kanamycin resistance	EMD Millipore
pRSF-Ivy-his	pRSFDuet-1 derivative containing the Ivy-his gene from pET26b-Ivy-his in MCS1	This study
pRSF-Ivy-his-hLYZ	pRSF-Ivy-his derivative containing the hLYZ gene from pET26b-hLYZ in MCS2	This study
pAR3	<i>E. coli</i> expression vector containing the paraB promoter, pACYC184 ori and chloramphenicol resistance	Perez-Perez et al., 1995
pAR3-Skp	pAR3 derivative containing the Skp chaperone with a Met start codon and no leader sequence	Levy et al., 2001
pAG	pAR3 derivative containing the GroEL chaperone and GroES co-chaperone	Perez-Perez et al., 1995
pAR3-BiP	pAR3 derivative containing the BiP chaperone	Makino et al., 2011
pAKJ	pAR3 derivative containing both DnaK and DnaJ chaperones	Perez-Perez et al., 1995
pBAD33	<i>E. coli</i> expression vector containing the araC promoter, p15A ori and chloramphenicol resistance	Guzman et al., 1995
pBAD33-Tig	pBAD33 derivative containing the Trigger Factor chaperone Co-expression vector containing 2 T7 promoters, 2 RBS, 2 MCS, 1 T7 terminator, p15A ori and chloramphenicol resistance	EMD Millipore
pACYC-Skp-TF	pACYCDuet-1 derivative containing Skp in MCS1 and Trigger Factor in MCS2	This study
pACYC-Skp-GroEL/ES	pACYCDuet-1 derivative containing Skp in MCS1 and GroEL/ES in MCS2	This study
pACYC-GroEL/ES-TF	pACYCDuet-1 derivative containing GroEL/ES in MCS1 and Trigger Factor in	This study
pACYC-Skp-hLYZ	pACYCDuet-1 derivative containing Skp in MCS1 and hLYZ in MCS2	This study