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Streamlining the preparation of "endotoxin-free" ClearColi cell extract with autoinduction media for cell-free protein synthesis of the therapeutic protein crisantaspase

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ABSTRACT

An "endotoxin-free" *E. coli*-based cell-free protein synthesis system has been reported to produce therapeutic proteins rapidly and on-demand. However, preparation of the most complex CFPS reagent – the cell extract – remains time-consuming and labor-intensive because of the relatively slow growth kinetics of the endotoxin-free ClearColi™BL21(DE3) strain. Here we report a streamlined procedure for preparing *E. coli* cell extract from ClearColi™ using auto-induction media. In this work, the term auto-induction describes cell culture media which eliminates the need for manual induction of protein expression. Culturing Clearcoli™ cells in autoinduction media significantly reduces the hands-on time required during extract preparation, and the resulting "endotoxin-free" cell extract maintained the same cell-free protein synthesis capability as extract produced with traditional induction as demonstrated by the high-yield expression of crisantaspase, an FDA approved leukemia therapeutic. It is anticipated that this work will lower the barrier for researchers to enter the field and use this technology as the method to produce endotoxin-free *E. coli*-based extract for CFPS.

1. Introductionintroduction

The invention of low-cost, lyophilized, and shelf-stable reagents for cell-free protein synthesis (CFPS) has the potential to transform how therapeutic proteins are produced [1,2]. This technology could enable distributed on-site production of specialized and even personalized protein therapeutics anywhere [3,4]. Recently, researchers have also developed an on-demand cell-free expression platform using an engineered *E. coli* strain reported elsewhere to be "endotoxin-free" [5,6], and this platform was used to produce a complex cytotoxic FDA-approved therapeutic protein [7,8]. This advancement combines the low-cost, high-yield performance of *E. coli* cell lysates with endotoxin-free production traditionally only achieved with eukaryotic cell lysates [9–11], and demonstrates that transformative, on-site protein therapeutic production at low cost is closer to becoming a reality [12,13].

One challenge to employing this endotoxin-free CFPS platform arises from the slow growth kinetics of the ClearColi™BL21(DE3) strain, which require extended monitoring of cell growth during production of the most complex CFPS reagent – the cell extract. Here, we introduce a new fermentation procedure for preparing *E. coli*-based CFPS extract that contains T7 RNA Polymerase (T7 RNAP) using auto-induction media. We successfully apply this procedure to create highly active BL21 Star™(DE3) and endotoxin-free ClearColi™BL21(DE3) cell extract, then use auto-induced endotoxin-free cell extract to produce the therapeutic protein crisantaspase. Erwinaze, the commercial form of crisantaspase has lamentably been out of stock multiple times in recent years, and was out of stock while this manuscript was reviewed. This fact highlights the need for on-demand/magistral strategies to produce this critical therapeutic and the value of this streamlined workflow for cell extract production. We anticipate that it will enable new researchers to enter the field of endotoxin-free CFPS. Our hope is that streamlined protocols for endotoxin-free CFPS systems will ultimately help address the unmet need for global accessibility to protein therapeutics at reasonable costs.

While potential applications of endotoxin-free CFPS have primarily been focused on producing therapeutic proteins using CFPS, the

Abbreviations: **sfGFP**, super-folder green fluorescent protein; **CFPS**, cell-free protein synthesis; **T7 RNAP**, bacteriophage T7 RNA polymerase; **cAMP**, cyclic adenosine monophosphate

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presented streamlined technology also has application to other CFPS products including biosensors [14–16], gene circuits [17–19], biocatalysts [20–22], vaccines [23,24], protein-polymer conjugates [25], and uniquely functionalized proteins with non-canonical amino acids [26–28].

2. Materials and methods

2.1. Investigating induction kinetics in auto-induction media

Auto-induction media was adapted from the protocol reported by Studier [29]. Specifically, solutions of 4% lactose, 10% glucose, 80% glycerol, 1 M $MgSO_4$, 1000x trace metals (50 mM $FeCl_3$, 20 mM $CaCl_2$, 10 mM each of $MnCl_2$ and $ZnSO_4$, and 2 mM each of $CoCl_2$, $CuCl_2$, $NiCl_2$, Na_2MoO_4, Na_2SeO_3, and H_3BO_3 in 60 mM HCl) and 50x Buffer M (1.25 M Na_2HPO_4, 1.25 M KH_2PO_4, 2.5 M NH_4Cl, and 250 mM Na_2SO_4) were filter sterilized. Solutions of 1% tryptone, and 0.5% yeast extract were autoclaved. These solutions were combined with ultra-pure water to achieve final concentrations of 0.2% w/v lactose, 0.05% w/v or 1% w/v glucose, 0.5% v/v glycerol, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 50 mM NH_4Cl, 5 mM Na_2SO_4, 1% w/v tryptone, 0.5% w/v yeast extract, and trace metals at the above concentrations diluted 1000x (0.05 μM $FeCl_3$, etc.). (See Studier auto-induction media "ZYM-5052" [29]). For routine extract preparation with autoinduction media, lactose, glycerol, and glucose may be combined and autoclaved). Calcium competent BL21 Star™(DE3) and ClearColi™ BL21(DE3) cells were transformed with pY71-sfGFP [30]. Overnight (~16 h) starter cultures of E. *coli* BL21 Star™(DE3) (Invitrogen, Carlsbad, CA), and BL21 Star™(DE3)-pY71-sfGFP were grown in LB-Miller media (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v sodium chloride) at 37 °C and 280 rpm in 16 × 100 mm test tubes (Corning Inc., Corning, NY). 1 mL overnight culture was added to 100 mL duplicate auto-induction media [29] with either 0.05 or 1% w/v final glucose concentrations. Kanamycin was added to pY71 cell strains to a final concentration 40 μg/mL to ensure plasmid maintenance. Cultures fermented at 37 °C and 280 rpm in 500 mL baffled shake flasks. Absorbance (OD_{600}) of cultures were read at 1 in 12 dilution 2 or 3 times per hour for 12 h. Simultaneously, fluorescence of 100 μl of cell culture was measured in a Costar® 96-well black polystyrene plate (Corning Inc., Corning, NY) with Synergy-MX Multi-Plate Reader (Biotek, Winooski, VT). ClearColi™ growth and induction kinetics were assessed similarly with the following differences: overnight cultures of ClearColi™ BL21(DE3) (Lucigen, Middleton, WI) and ClearColi™ BL21(DE3)-pY71-sfGFP were grown for ~18 h. OD_{600} and culture fluorescence were measured once per hour for 15 h.

2.2. Extract preparation with autoinduction media

Overnight cultures of E. *coli* BL21 Star™(DE3) (~16 h) and ClearColi™ BL21(DE3) (~18 h) were diluted 1/100 in duplicate 1.5 L auto-induction media ZYM-5052 prepared to 0.05% final glucose concentration as described above in 2.5 L Tunair shake flasks (IBI Scientific, Peosta, IA). Cultures were fermented at 37 °C and 280 rpm in between rapid cell harvests. Cell harvests were performed at 6000 g for 10 min, supernatant was discarded, and cells were immediately flash frozen for storage at −80 °C. 700 mL, 450 mL, and 350 mL of BL21 Star™(DE3) cell culture were harvested at 4 h (OD_{600} = 1.3), 5 h (OD_{600} = 2.93), and 5.9 h (OD_{600} = 5.0) of fermentation, respectively. 700 mL, 300 mL, and 300 mL of ClearColi™ cell culture were harvested at 5.4 h (OD_{600} = 1.0), 7.7 h (OD_{600} = 3), and 9.8 h (OD_{600} = 7.0) of fermentation, respectively. About 0.3 gm cells were harvested per 100 mL culture per OD_{600}. Frozen cell paste was resuspended and washed (10 mL per gram cell) in sterile-filtered Buffer A (10 mM Tris, 14 mM Magnesium Acetate, 60 mM, Potassium Glutamate, 1 mM DTT) and centrifuged at 6000 g for 10 min at 4 °C. Cells were once more resuspended (1 mL per gram cell) in Buffer A before lysis with an Avestin EmulsiFlex-B15 homogenizer (Avestin, Ottawa, Canada), 3 passes at 20,000 psi. Cell lysate was centrifuged at 12,000 g for 10 min, and supernatant was incubated at 37 °C for 30 min immediately prior to flash freezing and storage at −80 °C.

2.3. CFPS expression

CFPS reactions were formulated at 50 or 70 μL in 2 mL microcentrifuge tubes (BioExpress, GeneMate). 25 vol percent cell extract was added to 12 nM plasmid DNA and the following components: 10–20 mM magnesium glutamate (concentration optimized for protein yield), 1 mM 1,4-diaminobutane, 1.5 mM spermidine, 40 mM phosphoenolpyruvate (PEP), 10 mM ammonium glutamate, 175 mM potassium glutamate, 2.7 mM potassium oxalate, 0.33 mM nicotinamide adenine dinucleotide (NAD), 0.27 mM coenzyme A (CoA), 1.2 mM ATP, 0.86 mM CTP, 0.86 mM GTP, 0.86 mM UTP, 0.17 mM folinic acid, and 2 mM of all the canonical amino acids except glutamic acid, and incubated 3 h at 37 °C and 120 rpm. Plasmid DNA (pY71) containing the sfGFP gene under control of the T7 promoter was reported previously [30]. sfGFP expression yields were determined by fluorescence at 485/510 excitation/emission wavelengths according to a standard curve created by C^{14}-leucine labeled proteins, as reported previously [7]. T7 RNAP with an N-terminus hexa-histidine tag was expressed in BL21 Star™(DE3) cells and purified by HPLC with a His-Trap™ HP (GE Healthcare, Pittsburgh, PA). Crisantaspase was expressed in 2 mL microcentrifuge tubes for 6.5 h at 30 °C and 120 RPM with amino acid supplementation [8], and yield was determined using C^{14} labeled leucine as previously described [7].

3. Results and discussion

3.1. Auto-induction media for cell extract preparation

This work combines the rapidly growing techniques of cell-free protein synthesis and auto-induction media to create a streamlined method for preparing the highly active cell extract necessary for CFPS. The purpose of fermenting *E. coli* cells in auto-induction media is to eliminate the need for monitoring and manually inducing T7 RNAP expression before harvest [31], which requires 12–18 h of hands-on time when preparing cell lysate with the ClearColi™ strain [7]. Inducing expression of T7 RNAP during fermentation is preferred because it achieves higher yields in CPFS than adding purified T7 RNAP [31]. In this work, "yield" refers to the mass of protein produced per volume CFPS reaction. While several methods for automatically inducing protein expression have been developed for a variety of applications [32–35], this work utilizes auto-induction media with engineered concentrations of glucose and lactose, as reported by Studier [29]. Briefly, autoinduction media operates on the principle of catabolite repression of the lacUV5 promoter of the DE3 lysogen. When the preferred carbon source glucose is present in culture media, cellular cyclic adenosine monophosphate (cAMP) levels remain low and expression of the lacUV5-promoted protein (T7 RNAP in this work) is kept at a basal level. As glucose is depleted, cellular cAMP levels rise, increasing transcription of the lacUV5 promoter on DE3 lysogen if lactose is also present in the medium [29,36]. While the lacUV5 promoter is not repressed to the same degree as the wildtype lac promoter, experiments have shown that CAP-activated transcription from the lacUV5 promoter increases by more than two-fold in the presence of cAMP [37]).

3.2. Autoinduction of BL21 Star™(DE3) and ClearColi™

To verify catabolite repression of the lacUV5 promoter, as well as to help determine the optimal *E. coli* cell harvest time for CFPS extract production, we first assessed the kinetics of T7 RNAP autoinduction (specifically in the ZYM-5052 formulation [29]). This was performed by fluorescence where a plasmid containing sfGFP under T7 RNAP

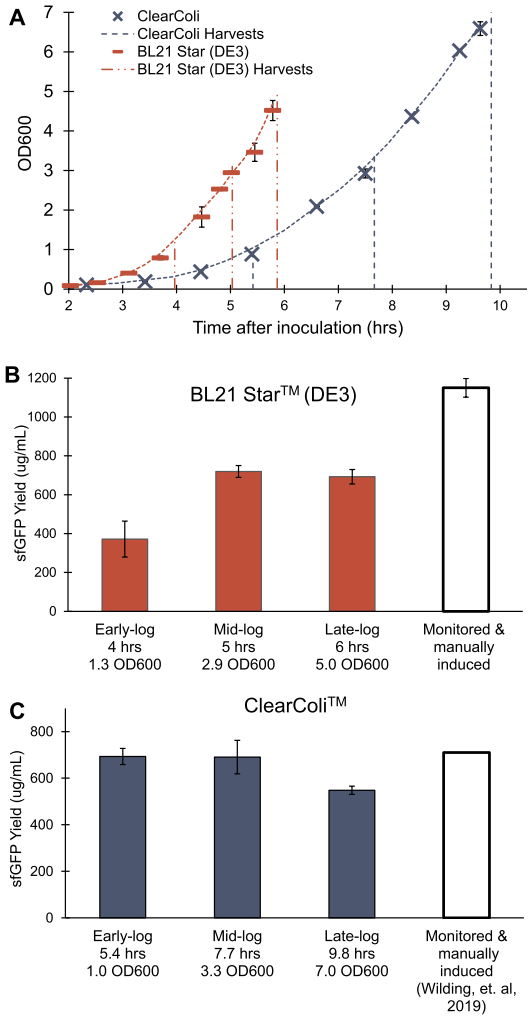

Fig. 1. A. Growth curves (OD600) and harvest times of BL21 Star™(DE3) and ClearColi™ *E. coli* cells fermented in ZYM-5052 auto-induction media for CFPS cell extract preparation. Cells were harvested at early, mid, and late logarithmic growth phase. Error bars on growth curves represent one standard deviation for n = 2 independent fermentations. **B**. Activity of BL21 Star™(DE3) cell extracts produced from early, mid, and late log-phase harvests. Cell extract activity is indicated by expression yield of sfGFP in CFPS reactions. Error bars represent one standard deviation for n = 4 CFPS reactions. Included for comparison is sfGFP expression yield from BL21 Star™(DE3) cell extract prepared from conventional monitoring and manual induction of T7 RNAP expression as previously reported [40]. **C**. Activity of ClearColi™ cell extracts produced from early, mid, and late log-phase harvests. Error bars represent one standard deviation for n = 4 CFPS reactions. Included for comparison is sfGFP expression yield from ClearColi™ cell extract prepared using the traditional method as reported by Wilding et al. [8].

promotion was transformed into the cells. Both a BL21 Star™(DE3) *E. coli* strain and an "endotoxin-free" BL21(DE3) *E. coli* strain (ClearColi™ which is mutated to prevent the production of endotoxin [5]) were investigated. Previously, both *E. coli* strains were reported to produce highly active extract for CFPS after shake-flask fermentation [7,38]. The results of these experiments suggest that induction of the DE3 lysogen likely begins shortly after the onset of log growth phase for both BL21 Star™(DE3) and ClearColi™ (Supplementary Fig. 1). Importantly, these experiments indicate that cells fermented in otherwise identical auto-induction media exhibit prolonged repression of the lacUV5 promoter in 1% glucose when compared with 0.05% glucose.

Using this information, 0.05% glucose auto-induction media (ZYM-5025) was used to ferment both BL21 Star™(DE3) and ClearColi™. Cells were harvested at early, mid, and later in the log growth phase, then homogenized. Growth kinetics and harvest timepoints are shown in Fig. 1A. It is important to note that both *E. coli* strains did not contain the plasmid for sfGFP production used in the above experiment. Extract for CFPS was prepared from the cell product by homogenizing, centrifuging, and incubating a "run-off" reaction to degrade preexisting mRNA as detailed in the methods. The streamlined procedure of preparing extract using auto-induction media relative to similar previously reported procedures is shown in Fig. 2. The auto-induction-produced extract was then assessed for its ability to produce model protein sfGFP with CFPS relative to extract prepared using the traditional method of OD_{600} monitoring, adding IPTG at an OD_{600} of ~0.5 and then harvesting during log-phase. The results are shown in Fig. 1B.

3.3. BL21 Star™(DE3) cell extract with auto-induction

Cells harvested at mid and later into the log phase of BL21 Star™(DE3) strain's growth (at 5 and ~6 h after inoculation) performed equivalently at producing protein in CFPS (Fig. 1B). Alternatively, cells harvested early in the log-phase (4 h after induction) were ~50% less productive (Fig. 1B). Insufficient induction of T7 RNAP was hypothesized as the cause. This is supported by additional experiments where adding purified T7 RNAP doubled the protein producing capability of CFPS reactions using extract harvested at early-log phase (Supplementary Fig. 2). Alternatively, adding T7 RNAP did not increase the yields when added to CFPS reactions from mid to late-log phase (Supplementary Fig. 2). While the auto-induction method could alleviate the need to continuously monitor OD_{600} to time manual IPTG induction, extract harvested from cells using the traditional monitoring and manual induction method had the highest production capabilities (Fig. 1B).

3.4. Endotoxin-free ClearColi™ cell extract with autoinduction

In contrast to BL21 Star™(DE3), CFPS extracts from auto-induced ClearColi™ achieved similar cell-free yields from ClearColi™ extracts obtained using the traditional monitoring and manual induction method (Fig. 1C). This is particularly important as ClearColi™ has slower growth kinetics and thus requires 12–18 h of monitoring when preparing extract using the traditional method. In addition, the window for harvesting is larger (between ~5.5 and 8 h after induction for the early-log and mid-log harvest) (Fig. 1, Supplementary Fig. 3) for obtaining high yielding extract which further simplifies using auto-induction media. Even waiting until 10 h after induction produced decent protein expression yields. Although, at this time point the system may be leaving log phase, which could be the reason for the ~20% drop in performance compared to earlier harvested extract (Fig. 1C). Together, these results demonstrate that preparing ClearColi™ cell extract with auto-induction media produces equivalent-yielding CFPS but is faster and simpler than traditional monitoring and induction. We therefore recommend auto-induction fermentation whenever cell extract is prepared with ClearColi™.

3.5. Endotoxin-free production of crisantaspase

In a previous study, we discovered that residual endotoxin in our BL21*(DE3) cell extract registered limulus amebocyte lysate (LAL) reactivity of ~2 × 10^7 EU/mL [7]. This is not altogether surprising because lipopolysaccharide (LPS) is found in the outer membrane of gram-negative bacteria, and previous reports affirm that *E. coli* cell membrane is not fully removed during lysate clarification [39]. In contrast, ClearColi™ cells incorporate a chemically distinct LPS into their outer membrane which is not capable of eliciting a human immune response [5,6]. To demonstrate protein therapeutic production with inherently endotoxin-free auto-induced ClearColi™ cell lysate, extract from the

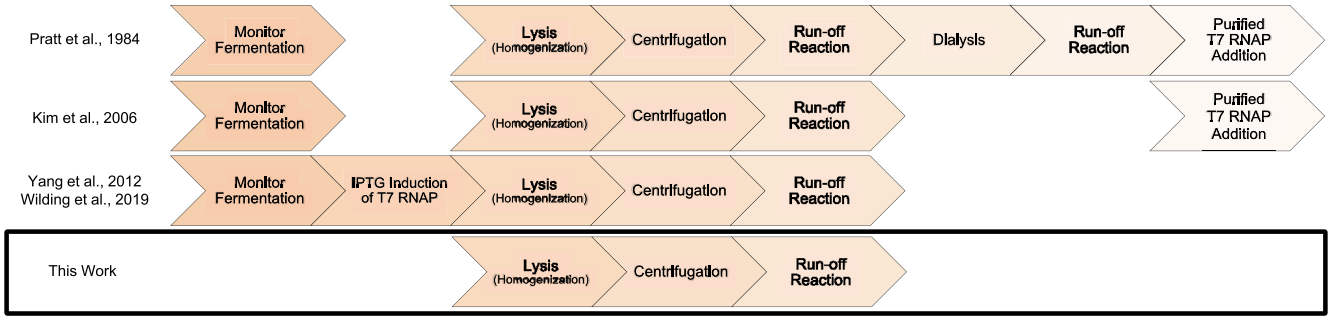

Fig. 2. Pictorial representation of streamlining advancements in extract preparation procedures upon which this work builds. The illustration starts with the seminal method of Pratt [41] that was further developed in the J.R. Swartz lab at Stanford up to this current work which builds upon these methods. Advances include the ability to omit the dialysis step post lysis [42], the production of T7 RNAP during fermentation [43], and the introduction of auto-induction media in this work.

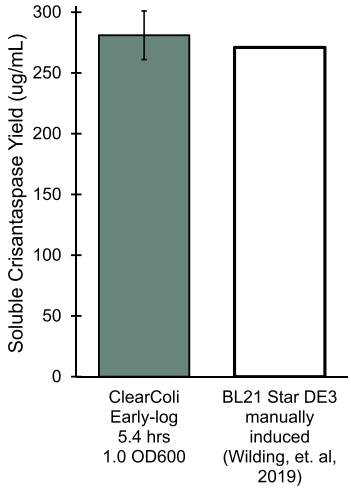

Fig. 3. Soluble protein expression yield of biosimilar to FDA-approved cancer therapeutic crisantaspase using ClearColi™ cell extract prepared with auto-induction media and harvested at early-log phase. For comparison, soluble expression of crisantaspase obtained with BL21 Star™(DE3) cell extract prepared using the traditional method is provided. This reference is the highest reported CFPS yield of tag-less crisantaspase previously reported in literature [8].

early harvest (~5.5 h after induction) was used to synthesize Acute Lymphoblastic Leukemia therapeutic crisantaspase to a final concentration of 280 ng/uL (Fig. 3). The crisantaspase is a tag-less biosimilar to the FDA-approved drug currently on market. Importantly, this yield is similar to yields of crisantaspase from traditionally prepared BL21 Star™(DE3) extract (Fig. 3).

4. Conclusion

Auto-induction media is a productive strategy for preparing cell extract for *E. coli*-based CFPS. The use of auto-induction as presented can significantly reduce the amount of hands-on time required for preparation of cell extract and eliminate the need for manual induction. Previously, optical density had to be continuously measured and the culture manually induced at precisely the correct time. Auto-induction, however, reduces the monitoring needed and offers significant potential for standardizing cell extract preparation. This technique is particularly relevant to extract prepared from the slow-growing "endotoxin-free" ClearColi™ cells, where the larger range of possible harvest times enables overnight culturing. Additionally, the auto-induced endotoxin-free extracts produce protein at similar levels to traditionally prepared endotoxin-free extracts and were used to produce the therapeutic protein crisantaspase at high titers. Streamlined preparation of "endotoxin-free" cell extract improves accessibility to on-demand expression technologies toward alleviating drug shortages and expanding global access to life-saving therapeutics.

CRediT Author Statement

J. Porter Hunt: Project Administration, Conceptualization, Methodology, Formal Analysis, Investigation, Visualization, Supervision, Writing – Original Draft, Writing – Review and Editing, Resources. **Emily Long Zhao**: Methodology, Validation, Investigation, Visualization, Writing – Original Draft, Writing – Review and Editing, Resources. **Mehran Soltani**: Investigation, Writing – Review and Editing. **Madison Frei**: Investigation. **J. Andrew D. Nelson**: Investigation, Methodology. **Bradley C. Bundy**: Funding Acquisition, Conceptualization, Methodology, Supervision, Project Administration, Writing – Original Draft, Writing – Review and Editing.

Declaration of competing interest

The authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2019.11.003.

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