Overcoming difficulties on synthesis of cardiac troponin-I

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ABSTRACT
Human cardiac troponin-I (cTnI) is one of the most sensitive and specific indicators, used in the diagnosis of myocardial infarction. To produce the protein efficiently, *Escherichia coli* and *Pichia pastoris* systems were used. Initial trials for the expression in *E. coli* were not successful, although different expression vectors with different promoters were tested. This led us to use *P. pastoris* for the expression. After several trials with two different expression strains of *P. pastoris*, it was concluded that *P. pastoris* was also not an optimal expression host for cTnI. Comprehensive analysis of the expression systems indicated that an efficient expression is only possible when the gene is optimized for expression in *E. coli*. For this purpose, the gene was optimized in-silico, but edited manually afterwards. It was synthesized and cloned into pQE-2 vector. Expression was performed using routine experimental conditions. Thus, cTnI could be efficiently expressed from the optimized gene in *E. coli*. The expression and purification were practical and may be used for commercial purposes since a total yield of 25 µg highly pure protein per milliliter of culture could be obtained. The protein was in its ready-to-use form for many biological applications, including as a standard in diagnostic tests and an antigen for antibody production.

KEYWORDS
Cardiac troponin-I; *Escherichia coli*; gene optimization; *Pichia pastoris*; protein expression; protein purification

Introduction

Myocardial infarction (MI) is the main cause of mortality in the western world and costs the EU economy €192 million per year. In the continuum of acute coronary syndrome, cardiac biomarkers play a key role in the diagnosis, prognosis, and risk stratification of patients. Currently, several biomarkers are in use, those that are tissue-specific [cardiac troponin-I (cTnI)] and nontissue-specific [creatine kinase (CK), myoglobin (MB) isoenzyme of CK (CK–MB), lactate dehydrogenase (LDH), and MB].

Among the nontissue-specific biomarkers, CK–MB is one of the most commonly used one. The disadvantages of this biomarker are that it remains elevated only for 2–3 days in serum after the onset of chest pain and its serum levels may also elevate after skeletal muscle injuries, which limits its use as a specific diagnostic tool. Other serum enzymes such as LDH and glutamic oxaloacetic transaminase are used in the diagnosis of myocardial infarction, but frequent serial measurements are required in the early hours after the onset of chest pain, that represents difficulties for definitive diagnosis. Compared to the nontissue-specific ones, cTnI has a higher specificity for cardiac tissue as a biomarker. cTnI is released before any other cardiac biomarkers are released into the blood stream, making it the most sensitive and specific indicator of cardiac injury. In addition, cTnI levels in healthy individuals are very low or undetectable, which adds its usefulness as a biomarker for myocardial infarction.

In previous studies, regarding in vitro usage of pure cTnI protein, mostly its extraction from animal tissues, e.g., bovine and beef heart tissues, was preferred. However, the methods for isolation of cTnI from cardiac tissues were laborious and yielding only small quantities of pure protein. Later, cTnI expression using recombinant DNA technology has replaced these methods. In the literature, there are several studies and patents regarding the expression and purification of cTnI. In those studies, *Escherichia coli* and *Pichia pastoris* systems were used as the preferred expression hosts. Between these two, *E. coli* allows a rapid production process with a low cost, but there is a contamination risk of the target protein with intracellular *E. coli* proteins, and the risk increases dramatically if the expression yield is not sufficient. On the other hand, *P. pastoris* system offers secretory production with a eukaryotic glycosylation pattern for the target protein. However, the production cost is relatively high.

Although there are some studies regarding the production of cTnI in the literature, most of them do not reveal crucial aspects of the expression and purification steps because of its commercial value or other patent-relevant issues. The highly hydrophobic nature of this protein makes it a troublesome candidate for routine laboratory production. In this study, we developed a practical method for expression and purification of cTnI in *P. pastoris* that could be used for commercial purposes.
approach to produce cTnI with high efficiency in *E. coli*. The highly purified protein may be used for the production of anti-cTnI antibodies or as a standard in ELISA tests or for many other biological or biotechnological applications. We presented all the necessary details concerning the problems of various production and purification stages of cTnI.

**Experimental**

**Isolation of cTnI gene (TNNI3)**

Human cDNA pool was purchased from Life Technologies, USA. IcDNA of the target gene was amplified from the cDNA pool using sense and antisense primers with Pfu enzyme through PCR (polymerase chain reaction (Table 1)). PCR conditions were as follows: 5 min at 94°C, for initial denaturation. Then, 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C applied for 35 cycles. Finally, 72°C for 10 min was applied. The amplified gene product was purified from an agarose gel by using a commercial kit (Roche, Germany) and sequenced for confirmation (Iontek, Turkey).

**Cloning of cTnI**

The PCR product encoding cTnI was used in the cloning experiments. The cloning was performed using routine molecular biology approaches.[13,14] Two different vectors were used: A strong T7 promoter-containing pACYC-Duet1 (Novagen, USA) and a strong T5 promoter-containing pQE-2 (Qiagen, Germany). Both vectors provided a 6×His-Tag to the N-terminal region of the protein. For creation of recombinant plasmids, EcoRI & HindIII and SacI & HindIII restriction enzymes were used for insertion of the gene into pACYC-Duet1 and pQE-2, respectively. The plasmids were then transferred into *E. coli* DH10B competent cells through electroporation. The clones were selected with appropriate antibiotics. Plasmid isolations were performed from the clones and the inserts of the plasmids were sequenced to confirm in-frame cTnI (Iontek, Turkey).

**Codon optimization of the cTnI gene for optimal expression**

Geneious software was used for codon optimization (Eurofins Genomics, Ebersberg, Germany). Codon randomization using the Kazusa database and equal distribution of G–C content were also performed using the Geneious software. Sequences leading to creation of possible secondary mRNA structure and codon changes leading to creation of restriction sites were avoided. In addition to the parameters taken into account by the software, Shine–Dalgarno (SD) and SD-like sequences were eliminated from the gene by manual editing.[15] Rare codons found at the 5′-end of the designed gene were preferentially replaced with frequently found codons in *E. coli*. The optimized cTnI gene was then synthesized by Eurofins (Ebersberg, Germany).

**Cloning of codon-optimized cTnI**

The codon-optimized cTnI gene (from now on, it will be referred to as OcTnI) was synthesized by Eurofins (Germany) and shipped by the company as a clone in a standard vector (pEX-A2, Eurofins). OcTnI was then subcloned into pQE-2 using SacI & HindIII and into pETM14 (EMBL, Germany) using NcoI & XhoI restriction enzymes. A strong T7 promoter-containing pETM14 introduces 6×His-tags to N- and C-termini with an N-terminal 3C proteolytic site. pETM14–OcTnI and pQE2–OcTnI were transformed into *E. coli* DH10B and clones were selected by ampicillin.

**Protein expression in *E. coli***

For protein expressions, *E. coli* BL21 (DE3) Star and *E. coli* M15 (pREP4) strains were used with pACYC-Duet1, pETM14, and pQE-2, respectively. Cells were cultured in LB-antibiotic medium in baffled flasks (Carl Roth, Germany) until OD₆₀₀ reached to 0.5 ± 0.1 at 37°C. After induction with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside), cultures were maintained at 30°C for 3 hr in an orbital shaker. The cells were harvested by centrifugation at 4,000g for 20 min at 4°C and stored at −80°C.

**Cloning of cTnI into pPIC9**

Cardiac troponin-I was also cloned into pPIC9 to achieve high-level expression and extracellular secretion in *P. pastoris*. For creation of recombinant pPIC9–cTnI, cTnI was digested with EcoRI & NotI and ligated into EcoRI & NotI-digested pPIC9 and transformed into *E. coli* DH10B after linearization with a single digest using SacI. One of the clones harboring the insert was selected for plasmid isolation.

**Protein expression in *P. pastoris***

The recombinant plasmid, carrying cTnI gene, was initially transformed into electrocompetent KM71 Mut⁻ strain to achieve a slow expression rate. Colony PCR was used for selection of the positive colonies of KM71 Mut⁻ grown on minimal dextrose (MD) agar plates. For expression, the selected clones were initially cultured in minimal glycerol medium and then transferred into minimal methanol (MM) medium.

To achieve high expression level, GS115 Mut⁻ strain was used. Recombinant colonies were initially grown on MD plates and then transferred onto MM plates for selection of fast growing (Mut⁺) colonies. Fast growing colonies were subjected to colony PCR to confirm the presence of the insert. To find the

| Vector name | Forward primer sequence | Reverse primer sequence |
|-------------|-------------------------|------------------------|
| pACYC-Duet1 | GAATTCAATTCATGGCGATGGGAAGCAGCC | AAGCTTAAGCTTCGCTCTCATCAAAGCTTCTTCGGGCG |
| pQE-2 | GAGCTCGAGCTCGCATGGCGGATGGGAGCAGCG | AAGCTTAAGCTTCGCTCTCATCAAAGCTTCTTCGGGCG |
| pPIC9 | GAATTCAATTCATGGCGATGGGAAGCAGCC | GCGGCCGCGGGCCGCTACGTCTCTCTACAAAGCTTCTTCGGGCG |
most productive cTnI expressing colonies, expression screening experiments were performed in 5 ml of media in 50-ml conical tubes. A total of five colonies were selected based on their 24-hr expression profiles. The most productive colonies were used for the large-scale expression. The large-scale cultures were maintained at 37°C for 4 days. At the end of the 4th day, cells were harvested by centrifugation at 3,000g for 5 min, then the supernatant was snap-frozen and stored at -80°C.

**Immobilized metal affinity column purification**

Purifications of cTnI and OcTnI were performed under native and denatured conditions as described in QIAexpressionist handbook. Prepacked HisTrap columns (GE Healthcare, USA) coupled to an automated chromatography system (NGC, Bio-Rad, USA) were used. When batch purifications were performed, 50% (v/v) Ni²⁺-NTA resin (Qiagen, Hilden, Germany) was used. Fractions containing cTnI and OcTnI were located by monitoring the absorbance increase at 280 nm. The effect of various urea concentrations on binding efficiency. The highest yield of purification was obtained with 0.1M NaH₂PO₄ pH 7.4 buffer containing 8M urea, 1 mM DTT (dithiothreitol) and 2 mM EDTA (ethylenediaminetetraacetic acid). The elution was performed with a linear NaCl gradient (0–1M).

**Ion-exchange column purification**

HiTrap SP column (GE Healthcare, USA) containing cation exchange resin for the purification of cTnI from P. pastoris was used. For optimization of ion exchange (IEX), several buffer systems were tested to increase binding efficiency. The highest yield of purification was obtained with 0.1M NaH₂PO₄ pH 7.4 buffer containing 8M urea, 1 mM DTT (dithiothreitol) and 2 mM EDTA (ethylenediaminetetraacetic acid). The elution was performed with a linear NaCl gradient (0–1M).

**Verification of cTnI/OcTnI by MALDI-TOF/TOF**

In some cases where cTnI expression was very low and purification was limited and nonhomogenous, the cTnI-presence and identities of the impurities were revealed by MALDI-TOF/TOF analysis. Fifteen microliters of each collected fractions were subjected to 12% SDS-PAGE after purification. An unstained protein marker was used to estimate the molecular weight (Fermantas, Waltham, Massachusetts, USA). The resulting gels were stained with colloidal coomassie. Stained bands were cut from the gels using EXQuest spot cutter (Bio-Rad, USA), and samples were subjected to MALDI-TOF/TOF analysis using AB SCIEX MALDI-TOF/TOF 5800. Mascot search was performed to identify the proteins as described by Akpinar et al.

**Western blot analysis**

Western blotting (WB) was performed as described in Clarity Western ECL Kit Handbook (Bio-Rad, Hercules, California, USA). Proteins were transferred to nitrocellulose membranes using a semidyne electroblotting system (Bio-Rad, USA). Monoclonal antibodies against cTnI (Novus Biologicals, NB100-73085 and NB110-2546) and 6×His-Tag (Invitrogen, Carlsbad, California, USA) were used, respectively. The anti-mouse HRP (horseradish peroxidase)-conjugated antibody was from Bio-Rad and used as recommended. To detect the chemiluminescence signal, a hypersensitive film was directly placed onto the membrane in a cassette holder. The film was developed, dried, and scanned with a simple scanner.

**Results**

**Expression of cTnI in E. coli**

Two different inducible expression vectors, pACYCDuet-1 and pQE-2, were used for the expression of cTnI in E. coli. There was no notable difference between the two vector systems in terms of their expression yield. Therefore, the pQE-2-based E. coli system was used for cTnI expression. Initial screening of the eluted fractions during purification indicated the presence of low levels of cTnI both in the soluble and insoluble fractions as determined by the analysis of coomassie-stained SDS-PAGE gels. The absence of an overexpressed cTnI band in the 25–30 kDa region was evident in cell-free extracts (Figure 1a). We were able to confirm cTnI expression only by WB analysis after prolonged exposure of the films to the HRP signal (Figure 1b). Comparison of the band intensities in the WB indicated that cTnI was predominantly present in its insoluble form. In spite of several trials, the efficiency of protein synthesis could not be increased. Despite the low level of cTnI expression, purification experiments were performed. Under native purification conditions, purified cTnI was eluted at a very low concentration. A hardly observable cTnI band was detected in colloidal coomassie blue-stained SDS-PAGE gels when the collected fractions were examined (Figure 2a). However, the cTnI band could be detected by WB (Figure 2b).

A mass spectrometry analysis was performed to the putative cTnI band in addition to the major bands observed on the SDS-PAGE of the selected fractions. Although we were able to detect peptides that might originate from cTnI, most of the peptides originated from the common histidine-rich E. coli expression profile. The most productive colonies were used for the large-scale expression. The large-scale cultures were maintained at 37°C for 4 days. At the end of the 4th day, cells were harvested by centrifugation at 3,000g for 5 min, then the supernatant was snap-frozen and stored at -80°C.

![Figure 1](image-url)
proteins.\textsuperscript{19–21} Apparently, there was not sufficient amount of target protein to bind the resin to prevent binding of the contaminating \textit{E. coli} proteins.

There was an interesting observation when WB was performed with the fractions of Figure 2. The anti-TnI antibody revealed the presence of several other protein bands with molecular weights ranging from 15 to 25 kDa and 70 to 75 kDa (Figure 2b). We suggest that the high molecular weight bands might originate from the multimeric forms of the cTnI and the low molecular weight bands might originate from the prematurely terminated translation products. To eliminate the high molecular weight bands, DTT was successfully used in place of $\beta$-mercaptoethanol in the sample-loading buffer. However, there was no way to eliminate the low molecular weight bands by changing the experimental conditions.

Purification under denaturing conditions was also performed since a more prominent cTnI band was observed in the insoluble fraction. Under denaturing conditions, however, cTnI failed to bind the resin. Thus, the optimization studies performed to test various binding conditions has failed.

**Expression of cTnI in \textit{P. pastoris}**

As an alternative to \textit{E. coli}, which failed to express the cTnI at a respectable level, the use of a low-cost eukaryotic expression system, \textit{P. pastoris}, was tested. Two different strains, KM71 Mut\textsuperscript{+} and GS115 Mut\textsuperscript{+}, were utilized for the expression.

**Expression in KM71 Mut\textsuperscript{+} strain**

The insert for cTnI was cloned into \textit{pPIC9} expression vector and the recombinant clone was transformed into KM71 Mut\textsuperscript{+} strain. Five colonies were selected and screened for their efficiency of expressing cTnI and four of them were positive (Figure 3a). Colony 9 was selected for the production of cTnI. After 4 days of incubation, the cells were collected and a cell-free extract was prepared to evaluate the level of cTnI production. The expression yield was lower than what was expected. The high level of premature peptide expression pattern was also observed in \textit{P. pastoris} as observed in \textit{E. coli} (Figure 3b).
Expression in GS115 Mut\(^+\) strain

The recombinant pPIC9 plasmid harboring cTnI was transformed into GS115 Mut\(^+\) strain. Five colonies were selected and screened for their efficiency of expression. One of the colonies expressed the cTnI at a respectable level and was used for production (Figure 4). Analysis of the expression pattern in scaled up cultures showed a linear increase in the cTnI expression pattern in time. Therefore, the culture supernatant was used for purification of cTnI after completion of the 4-day incubation period. Although the purification was successful, sufficient quantity of purified cTnI was not obtained.

Codon optimization of cTnI and expression of codon-optimized cTnI in E. Coli

Selected codes in cTnI sequence were changed without disturbing the primary amino acid sequence of the polypeptide.

The changes were made based on the codon preferences calculated in E. coli. The codon-optimized cTnI was synthesized and cloned into pQE-2 expression vector.

As opposed to wild-type cTnI, an obvious overexpression pattern of OcTnI was detected in SDS-PAGE gels (Figure 5a). Therefore, immobilized metal affinity column (IMAC) purification of the expressed protein was performed. Initial purification was performed under native conditions. Despite the use of a high imidazole concentration (500 mM), OcTnI could not be eluted from the column, indicating that the protein might have precipitated inside the column. Our optimization efforts such as loading a diluted protein extract to the column, increasing imidazole concentration, changing binding conditions, eluting with pH gradient did not improve the purification of the OcTnI. Therefore, purification under denaturing conditions was performed. Under the denaturing condition, the elution was highly efficient and the protein was purified to homogeneity (Figure 5b). Total yield of the process was approximately 25 µg purified protein per milliliter of culture.

Discussion

Although the E. coli protein expression system is a suitable system to express some human genes, the human cTnI gene was not one of them, displaying a very low-level expression pattern. In this study, we explored the possibility of increasing cTnI expression in E. coli to achieve a low-cost cTnI production. There is a great demand for highly purified cTnI, since the purified protein can be used as a calibrator for immunoassays, as an immunogen for antisera production and as a mass spectrometry standard. Our initial trails in E. coli failed mainly because of the absence of commonly found human codons in E. coli. To overcome this difficulty, there were two possibilities: One was to use a eukaryotic expression system, which is usually less efficient and more expensive, while the other way was to optimize the cTnI gene for expression in E. coli and use the E. coli expression system, which is usually more efficient and less expensive.

To explore the first possibility, we used the eukaryotic P. pastoris expression system. Two different strains of P. pastoris (KM71 and GS115) were tested and cTnI was successfully expressed in both strains. However, the level of cTnI expression was less than satisfactory, indicating that this system might not be economically feasible to obtain sufficient amount of purified cTnI for commercial purposes.

In expression studies, although some heterologous genes do not cause any compatibility problems in their expression hosts, some may lead to ribosomal stalling, frameshifts, and relevant translational problems. In this study, we observed unusual band patterns on WBs in both E. coli and P. pastoris, indicating that the ribosomal machinery and codon preferences of these organisms do not properly support the heterologous expression of cTnI. Bioinformatics analysis of cTnI gene revealed the presence of codons that are rarely found in both E. coli and P. pastoris. The absence of these codons may cause stalling and pausing of ribosomes during translation process. In addition, the rare arginine codons might have contributed to the formation of SD-like sequences creating additional problems.\[^{13}\] There are also some common slippery fragments
in cTnI gene, which may cause $+1$ or $-1$ frameshifts during translation. Whether the cTnI was terminated early with a newly created frameshift-caused stop codon or it was elongated with a different amino acid sequence, it was evident from the western blots that the translation of cTnI formed premature peptides (with or without an additional out-frame elongation). We anticipated that ribosomal translation initiation of cTnI may also be leaky, since there are additional out-of-frame start codons (ATGs) found very close to the initial in-frame start codes. Such a phenomenon causes ribosomes to miss the first start codon and start the translation from the first succeeding AUG (Adenine-Uracil-Guanine) (in-frame or out-frame). Therefore, an alternative open reading frame was translated and it mostly ends up with the minor expression of small peptide chains. Consequently, all these facts in turn caused an inefficient translation process by decreasing the translation of cTnI formed premature peptides. Consequently, all these facts in turn caused a different amino acid sequence, it was evident from the western blots that the translation of cTnI formed premature peptides (with or without an additional out-frame elongation).

References

This study was supported by The East Marmara Development Agency under the grant number of TR42/13/GPD/1.

Acknowledgments

pETM14 vector was kindly donated by EMLB, Heidelberg, Germany. Authors also would like to thank Hüseyin Besir, from Protein Expression and Purification Core Facility (EMBL), for his valuable suggestions.

Funding

This study was supported by The East Marmara Development Agency under the grant number of TR42/13/GPD/1.

Conclusions

In this study, with current optimization strategies, a high-level cTnI production with high purity was achieved in E. coli. Purification of the cTnI from the optimized gene was practical and may be used for commercial purposes. The protein was in its ready to use form for any biological applications, including as a standard in diagnostic tests (ELISA) and as an antigen for antibody production. cTnI is a model gene for codon optimization studies in which the end product is important for commercial purposes.

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