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Recent Advancements in Cell-free Protein Expression: Review Paper

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Abstract

Holding several advantages, cell-free translation is getting applications in a variety of research areas. Advances in traditional expression systems have made it important for several biotechnological studies. Cell free translation systems are being used to improve the protein quality for pharmaceutical, clinical and analytical purposes. The cell-free translation systems not only eradicated troubles like aggregates formation, post-production purification but also provide opportunity to obtain more than 6 mg mL-1 of functional and pure product in small volume of reaction. This article reviews the introduction to cell free systems, their types, applications and advancements for improvement of cell-free translation systems.

Key words: Proteins translation, Cell-free Expression, *In vitro*, Expression Systems

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1. Introduction

Cell-free translation systems were used in 1961 in pioneering studies of Nirenberg and Matthaei, and played a central role in translating the generic code. Recently, CFPT systems have shown a remarkable utility as a protein translation technology. Cell-free proteins synthesis (CFPS) systems can perform two fundamental processes transcription and translation, and produces proteins of interest outside the cell. In this mechanism a template is introduced in the reaction mixture containing all the required apparatus for protein expression. Proteins being translated with high yield by in vivo systems can be suitably subjected to cell-free systems for extraordinary results. Such systems aim to provide (i) a chemical reaction environment mimicking the natural cell settings; (ii) prevent the multi-substrate protein synthesis; (iii) pure and high quality protein product.

Advantages of cell-free translation systems over cell based systems

- The CFPS system provides an opportunity to directly monitor add/remove components with an ease, not possible in cell based system (Jewett *et al.,* 2009).
- One of the fundamental advantages is the production of one product at a time through metabolic resources of the cell-free extract, thus only the protein of interest is synthesized (Miyamoto-sato *et al.,* 2006, Ozawa *et al.,* 2006).
- Difficult to express proteins, harmful to host cell are easily produced in open environment, free of cell boundaries. Overexpressed proteins are not an issue in cell-free systems, while in cellular environment is sometimes lethal to host cell (Henrich *et al.,* 1982).
- Recombinant proteins in vivo are time consuming and along with appropriate expression vectors, they require selection media and incubation, while CFPT systems save time and labor and do not require several hours of incubation and expertise on microbial handling [Stiege and Erdmann, 1995, Wang and Zhang, 2009).
- Single protein is produced in single reaction, and all reaction aspects were monitored. A highly purified protein is obtained after a few hours. While such a practice is not possible in cell-based protein synthesis (Szamecz *et al.,* 2008).
- Some protein products in microbial systems are prone to aggregation and result in formation of inclusion bodies. By cell-free translation systems, the issue has been resolved by synthesis of native protein in complete active and soluble form (Kang *et al.,* 2005, Takashi *et al.,* 2009).

It has been challenging to produce the proteins in a heterologous system for biological research and pharmaceutical improvement. Chemical synthesis, in vivo protein translation has several limitations. Chemical synthesis method is not practiced for long polypeptides and in vivo system produces proteins friendly to host environment. In addition to the production cost, the solubility and yield are compromised due to aggregation and indigenous inhibitors, besides cell-free protein synthesis systems has emerged as efficient means for the translation of genetic information into functional proteins (Spirin, 2004). The future of structural genomics and functional proteomics depends on functional and quality proteins. Traditional methods for the generation of crude proteins through cell based systems have several disadvantages, as being time consuming, laborious, product toxicity and instability to the host cell. In vitro translation system on the other hand has emerged as a striking and appropriate substitute for the protein expression. The system is free to be added with required reagents, and is flexible to generate swift and folded proteins. Utilization of PCR fragments as template made CFPS a robust technique, as in vitro cloning is no longer required by the use of PCR fragments in cell-free system for the expression of large number of proteins (Watzele *et al.,* 2001, Wu *et al.,* 2007, He, 2008). Additionally, in combination with PCR directed systems for template DNA fragments, approximately 50 genes can be translated at a time, yielding 0.1-2.3 mg of protein within two days (Sawasaki *et al.,* 2002). The system also serves as a platform for the preparation of difficult to express proteins, functional membrane proteins and proteins with disulfide bonds (Jackson, 2004). A typical cell-free protein synthesis assembly is shown in Figure 1.

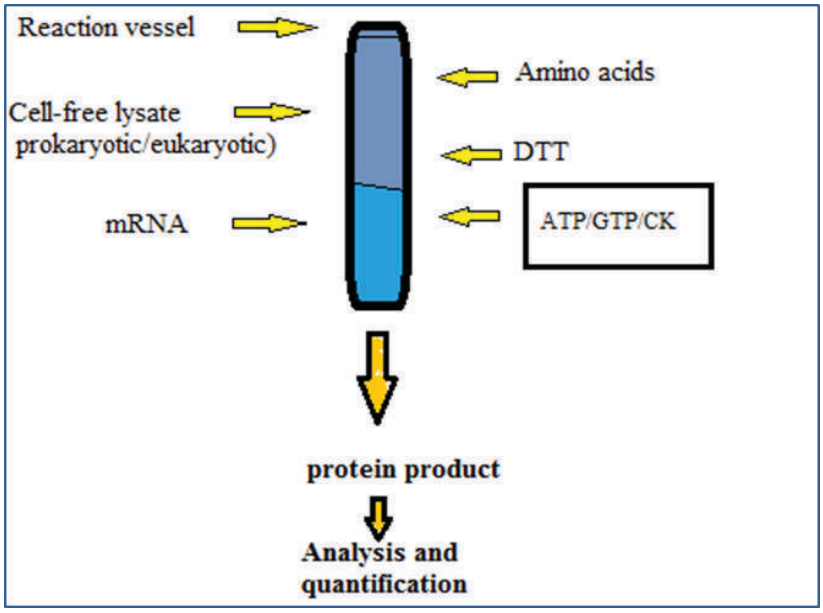

Fig.. 1. Cell-free protein synthesis assembly; blue colored eppendorf vessel contains cell free lysate, mRNA as template for target protein, amino acids, DTT acts as reducing agent (for optimal protein synthesis), and energy source in form of ATP/GTP/CK.

Swasaki *et al.* (2002), Nozawa *et al.* (2007) and several other researchers have reported modified systems for cell-free translation of proteins. With many advantages the modified cell-free systems based on E. coli, rabbit reticulocytes and wheat germ/insects extracts are available commercially with ability to transcribe a DNA/RNA or plasmid. The cell-free translation system "PURE" has been designed by Cosmo Bio. Co., LTD. The system has been reported to have all the necessary constituents for transcription, translation with continuous energy recycling process. PURE system S-S has been found to create a redox environment for the reaction mixture. The absence of contaminants like glutathione reductase and thioredoxin reductase prevents the redox reactions (PURE system®). Dihydrofolate reductase (DHFR), Green fluorescent protein (GFP) and glutathion-S-transferase (GST) were synthesized by using PURE system having marked efficiency (Shimizu *et al.,* 2001). Other popular suppliers of cell-free protein synthesis are Promega and Roche molecular biochemical (Sitaraman *et al.,* 2004).

The Cell- free translation systems are employed for the analysis of proteins having essential biochemical functions and medical applications (He, 2008; Leippe *et al.,* 2010). For example, a little is known about the biochemical function of more than 1300 proteins involved in the ubiquitation in Arabidopsis. Using wheat germ cellular lysates, Takashi *et al.* (2009) reported a cell-

free protein expression method for determination of function of these proteins in ubiquitation. Ma *et al.* (2010) reported a cell-free system for several proteins, while specifically studied enhanced green fluorescent protein. Cell-free translation systems are employed to study structural proteomics by NMR, protein microarrays, preparation of proteins difficult to express, protein-protein interactions, protein-DNA interaction, RNA-protein interaction. This review article describes cell-free expression systems, their types, uses, process optimization and some recent approaches which translate into new lines for cell-free expression.

Cell free lysates

An inclusive list of chemicals is mandatory for the generation of in vitro transcription and translation. The cell-free lysates hold top of this list, for providing a medium that accommodates all necessary components of reaction. He (2008) reported that, after the exogenous supply of genetic materials, the cell-free extracts from any origin can be used for the biosynthesis of functional proteins. Current reported lysates include E. coli, wheat germ, rabbit reticulocytes, xenopus eggs and mammalian cell based extracts [Glenn and Colma, 1991; Shimizu *et al.,*2001; Sitaraman *et al.,* 2004; Mikami, 2006).

Since the nature of translation system in each extract is different, the protein translation might depend on the system employed and cell extracts from variable sources differ in their protein folding ability. Sources of cell extracts for the expression of protein are variable depending on amount and type of protein to be transcribed. Frequently reported cell extracts from E. coli, rabbit reticulocytes, and wheat germ cell lysates, provide 6 mg, 6 µg and 4 mg protein per mL, respectively (Kigawa *et al.,* 1999, Madin *et al.,* 2000), while microgram per milliliter protein was obtained by using lysates from Xenopus eggs (Glenn and Colma, 1991). *E. coli* cell extract is obtained after centrifugation at 30,000×g the extract contains all enzymes and machinery for the translation of target mRNA (Kigawa *et al*, 2004). *E. coli* S30 extracts provide high efficiency rate for translation and overall yield as compared to eukaryotic cell extracts (Zarate *et al.,* 2010).

Comparison of E. coli and Wheat germ lysates

Wheat germ extracts are highly stable, providing reaction time upto weeks for transcribing 10 mg protein per mL (Sawasaki *et al.,* 2002). Nakano *et al.* (1994) reported a high yield of dihydrofolate reductase by condensed wheat germ extract. Rather stable but wheat germ extracts are difficult to obtain because of hard cell wall, indigenous nucleases and translation inhibitors. In contrast to its superiority over E. coli for protein folding, it has slower translation rates and poor yield. Limitations of wheat germ lysates have been overcome which presented its feasibility for pharmaceutical proteins, patient specific vaccines and translation of some viral RNAs (Bhanu *et al.,* 2010; Tsuboi *et al*., 2010). This eukaryotic extract is suitable for translation of proteins harmful to bacteria; moreover, its un-coupled assembly due to compartmentalization, translation occurs independent to transcription, which supports its use for ribonuclease production (Ishikawa *et al.,* 2005). As an effective tool, wheat germ lysates were used for in vitro protein research to create human protein factory, 13364 proteins of human genome translated through this system and most were found with effective biological activities. The system was used for generating cytokines with disulfide bonds (Goshima *et al.,* 2008). Madin *et al.* (2000) reported the production of dihydrofolate reductase, green fluorescent protein and luciferase enzyme causing wheat germ cell-free lysates. Shin and Noireaux (2010) used *E. coli* cell extract for translation of proteins; which supplied endogenous protein synthesizing machinery. *E. coli* S12 extract was found rather beneficial than S30 extract, providing more stable means by using economical sources. A variety of proteins with distant origins could be produced by the system reported by Kim *et al.* (2008).

Post translational modifications cannot take place in *E. coli* extracts as larger eukaryotic proteins are folded incorrectly ending into inclusion bodies. While eukaryotic extracts like wheat germ and rabbit reticulocytes perform peptide cleavage, methylation, phosphorylation, acetylation and protein folding after translation (Denman, 2008). To prevent the expression of background proteins translation in case of rabbit reticulocytes and insects, endogenous mRNA is degraded by RNase supplementation, once endogenous mRNAs are removed, inhibitors for RNase are introduced into the system, thus preventing the degradation of exogenous mRNA, so that only the exogenous mRNA is specifically transcribed by the system (Schwarz, 2008). Lysates from such sources call for more research to make these economical. Hillebrecht and Chong (2008) investigated the difference in protein synthesis in prokaryotic and eukaryotic cell lysates, and produced fusion protein that folded well in eukaryotic system while failed to fold correctly in *E. coli* lysates.

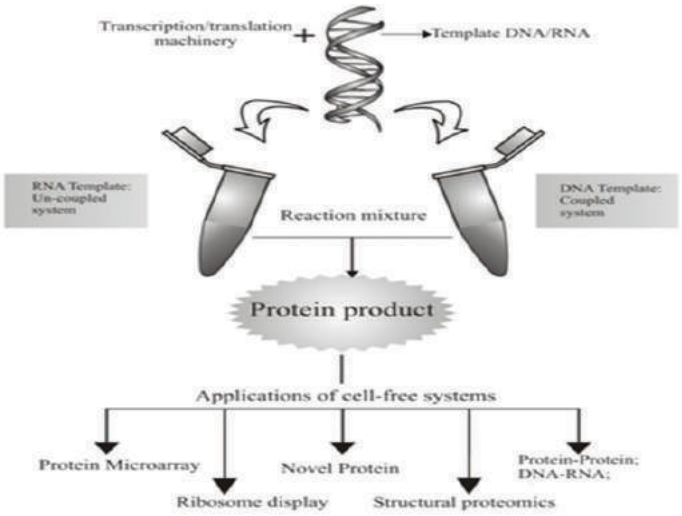

Fig.2. Cell-free protein coupled and un-coupled translation systems; use of cell free protein translation systems for various purposes.

Types of cell-free systems

In addition to other reaction components, cell-free systems hold either a DNA or RNA as a target for translation. Before translation of RNA, when DNA is to be transcribed into RNA, and transcription is immediately followed by translation, the system is called coupled transcription-translation system. Un-coupled system on the other hand is supplied with exogenous mRNA, to be translated into specific protein (Figure 2). The un-coupled translation system allows the optimization of transcription and translation separately. He and Khan (2005) optimized the cell free translation of antibodies by using the un-coupled system, to avoid disruptive effects of DTT on protein folding. The systems may have DNA fragments or PCR constructs having promoter and translation-initiation signal. Yang *et al.* (1980) reported that efficiency of transcription and translation is influenced by the structure of DNA in which the gene of interest is present. Quick coupled transcription-translation systems are supplied by Promega, the production and purification of RNA is not necessary. Un-coupled systems are also suitable for studying the optimization of transcription and translation individually.

Two modes of cell-free protein translation are semi-continuous flow cell-free translation (SFCF) and continuous flow cell-free translation (CFCF) system. The continuous mode of translation bears an ultrafiltration membrane separating the pure product from reaction mixture, so that recovery of the protein is relatively easy. Semi-continuous system contains two separated cambers for reaction and feeding separated by semipermeable membrane. mRNA machinery for protein production is present in reaction chamber, while a relatively larger feeding chamber contains dNTPs, and amino acids. Final protein products accumulate in reaction chamber (Madina *et al.,* 2006) Continuous exchange cell-free system (CECF) separates proteins from low molecular weight products, and is involved in cost reduction of the system along with CFCF, and SFCF. Proteins produced in preparative amounts can be scaled up to industrial level using continuous flow and continuous exchange cell-free systems (Revathi *et al.,* 2010).

Advances in cell-free protein systems

On the record of the cell-free translation systems, several adaptations of the systems have been reported based on suitable cell-free extracts. The most robust and perhaps the efficient one has been derived from E. coli cell lysates. For the last few decades researchers have focused on the advancement of traditional strategies for cell-free expression. With the advent of time, high-throughput proteins have been achieved with low volume of extracts for enhanced periods.

Through modifications and innovations, traditional cell-free translation systems have invigorated to meet the challenging demands for high quality proteins. For such approaches, cell-free systems have been directed towards the maximum synthesis of proteins and thus the stability of mRNA has been considered as well. Describing the history of cell-free protein synthesis Katzen *et al.* (2005) reported that advancements in cell-free protein synthesis (CFPS) systems are based on the foundations of Nirenberg and Matthaei. By the time of their invention CFPS systems has been a significant means of in vitro translation of toxic molecules and antibiotic drug discovery (Orhan and Heinecke, 2006). The expansion of numerous applications especially in vital areas of research has revolutionized the old techniques into superior ones. More robust methods for the continuous energy supply, stronger and

modified reaction designs have made it possible to surpass the milligram quantity of protein to per milliliter. Moreover, the opened system, allows the scientists to have a tight control over the synthesized peptide to direct the system towards preferred conditions.

Continuous cell-free translation system

Due to unsuitably low yields of conventional CFPS systems, a variety of reaction systems are generated. Continuous exchange cell-free translation systems are engaged on preparative scale. In addition to traditional protein translational techniques, these systems are helping for better protein yields. The reaction mixture is incubated in a simple dialyzing bag which reloads the substrates and removes the by-products through dialysis. The system provides a freedom to supplement the reaction, and allows the dilution of the by-products as well (Bhanu *et al.,* 2010). The reaction time is extended by supplementation of the reaction mixture. Several mg mL-1 product recoveries after short duration of time are possible in continuous exchange system. The complexity of system however, poses handling problems, which limit its exercise in high-throughput applications.

Enhanced production by larger reaction components

Batch systems work with a constant small volume of reaction mixture, without addition of feeding mixture (as in continuous system). Research has been focused to introduce variations in conventional systems to enhance the protein yields; in this regard batch systems have been improved by introducing the larger amounts of reaction components (Kim *et al.,* 2006). Introduction of polyethylene glycol (PEG) and phosphoenol pyruvate (PEP) also enhanced reaction product; while mRNA lifetime has been extended by the addition of polyguanylic acid 5' (Shen *et al.,* 1999).

Continuous energy supply

Energy source depletion, nucleotides and amino acids degradation has been reported as major factors resulting in halt of protein expression (Kim and Swartz 2000; Jewett and Swartz 2004; Reydellet *et al.,* 2004). A dual energy system has been reported by Kim and Kim (2009), using glucose as energy source; which is converted to pyruvate giving away two ATP molecules. The requirement of ATP in glycolytic cycle is satisfied by creatine phosphate/glucose system; which regenerates ATP in the reaction. Within 3 hours, proteins are produced at 2-3 times higher rate, through this dual energy approach.

Maintenance of translational speed

Protein translation period can be extended by maintaining the concentration of magnesium ions; which decline due to inorganic phosphates accumulation even at higher ATP concentrations. One milligram per milliliter of recombinant protein was obtained after two hours reaction. The systems for cell-free translation have been improved by increased expression level of translation product. Creatine phosphate has been reported as potent source for energy regeneration; but the source also supplies unwanted phosphate ions. The problem has been solved by a concomitant addition of magnesium ions (Stoevesandt *et al.,* 2009). Cell-free expression system using cAMP/CP/CK system proposed by Ma *et al.* (2010) showed higher expression levels. Moreover, the system presented feasibility to express several kinds of proteins, but simultaneous supplementation of system with substrates is mandatory.

Applications of cell-free protein synthesis systems

Cell-free translation systems remain a pool of divergence for several techniques. Its advantages endow with new perspectives of applications. Some of these are discussed as under

Protein Microarrays

Cell-free protein expression for protein microarrays has been reported by many researchers (Angenendt *et al.,* 2006; Chandra and Srivastava 2009; Doolan *et al.,* 2010). The proteomics is represented by an entire complement of proteins expressed by an organism's genome under the defined set of conditions. As high throughput technologies, protein based microarrays proposal present a significant promise. Protein microarrays and its applications in diagnostics and analysis of proteins within short time and economical approach has been intensively studied (Stoevesandt *et al.,* 2009). Challenges and promises for the protein microarrays have been reviewed by Talapatra *et al.* (2002).

As the protein microarrays demand the optimal transcription, translation and protein folding, these have been less preferred as compared to the DNA microarrays, so, cell-free protein systems became a paramount for the production of self-assembling protein microarrays (Zarate *et al.,* 2010). It has been suggested that a protein translation system with higher efficiency for the protein folding and effective yield would optimize protein translation for protein microarray. Microarray technology allows the analysis of protein

and nucleic acids for interaction at larger scale. Protein in situ immobilization arrays termed as PISA was generated by He and Taussig (2001). Initiating with a PCR generated DNA construct, the protein expression and in situ immobilization was performed in single vessel. Protein expression for Protein microarrays is important for the production of functional-recombinant proteins. Aggregation and degradation of variety of proteins occur in biological system, while PISA system avoids these effects offering proteins with greater activity and solubility.

Production of novel proteins and antibodies

Proteins carrying unnatural amino acids can be utilized for protein folding, structure, stability, protein-protein interaction and protein localization studies (Kohrer and RajBhandary, 2000). As Jackson *et al.* (2004) reported the production of novel proteins for proteome applications, incorporation of un-natural or chemically modified amino acids at desired positions is allowed by cell-free translation systems. In *E. coli* system enzymatically active novel proteins were generated by Goerke and Swartz (2009) after site specific modification. Shimizu *et al.,* (2001) introduced unnatural amino acids in mutant DHFR mRNA in *E. coli* S30 extract. In recent studies, it is demonstrated that cell-free translation systems are sufficiently progressed to facilitate the generation of a wide-range of renewable resources of antibodies encoded by approximately 22500 human protein coding genes. The adjustment of environment during selection of antibodies, the confirmation, affinity and unwanted cross reactivity can be controlled over (Dubel *et al.,* 2010). Oh *et al.* (2010) reported the production of active antibodies to neutralize Botulinum neurotoxin serotype B from *E. coli* cell-free lysate.

A tool for production of protein conjugates

Protein conjugates, with functional groups at specific sites of protein conjugates play a powerful role for the characterization of protein function and development of protein tools for therapeutics. Protein conjugates, a tool for biophysical and biomedical research, diagnostics and therapeutically active substances are being produced by cell-free translational systems.

Structural proteomics

It is believed that three dimensional structures of proteins regulate the molecular and cellular functions of living cells. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy have ruled for the structural analysis of biological molecules. NMR spectroscopy is a potent technique for three dimensional structural analyses of proteins (Wuthrich, 2002). Obviating cell lysis, and purification which are time consuming, cell-free systems successfully allow the protein expression for NMR spectra without separation from reaction mixture (Guignard *et al.,* 2002). Structural biology demands the quantity and quality of the target sample because undesired proteins hinder structural evaluation, which is provided by in-vitro translation systems.

To achieve effective labeling of the target protein, deficiency of natural amino acids is necessary (Guignard *et al.,* 2002). The cell-free expression allows efficient incorporation of selectively labeled amino acids with satisfactory yield for NMR analysis (Klammt *et al.,* 2004); while in vivo isotope labeling of proteins results in dilution and scrambling of isotope (Kigawa *et al.,* 1995). Moreover, highlighted advantages of proteins through cell-free systems are; purified product with no need to proceed for chromatographic operations, low volumes of isotope labeled amino acids are sufficient because of small volumes of cell-free expression systems and higher protein yield (Kigawa *et al.,* 1999; Ozawa *et al.,* 2004).

After a direct use of PCR templates, relatively high protein yields by continuous cell-free transcription/translation systems (*E. coli* extract) were obtained by Wu *et al.* (2007). Dengue virus protease construct was subjected to NMR analysis after site directed mutagenesis through PCR. Cell-free synthesis of PpiB declined after a depletion of natural amino acids. The un-natural amino acids labeled with 15N were employed for the NMR spectral analysis (Ozawa *et al.,* 2004); they also provide data for rebalancing the amino acids concentrations. As the structural proteomics demands for more efficient protein expression methods, a system proposed by CESG (Centre for Eukaryotic Structural Genomics) based on wheat germ lysate was utilized for the protein expression and NMR analysis (Vinarov *et al.,* 2004).

In recent studies Acton *et al.* (2011) proposed advanced, automated and parallel cloning, expression and purification system which is suitable for the structural and functional biology, proteomics, ligand screening and structural genomics investigations. Cloning, expression and purification of the 6×His-tagged protein were performed using T7 based E. coli expression system. Homogeneity level of 97%, which is sufficient for the NMR analysis, was achieved. Toxic proteins by in vivo systems are difficult to obtain in a quantity requested for structural determination through X-rays crystallography. Cell-free translation system resolves this toxicity setback by using wheat germ lysates (Watanabe *et al.,* 2010).

Protein interaction and ribosome display

Another phase of purified recombinant protein evaluation is the analysis of protein-protein interactions. Protein interactions are studied by incubation of one protein synthesized in CFPS system, with labeled protein. The complexes are subjected to immune-precipitation or studied by electrophoretic mobility, where complexed compounds are retarded (He, 2008). For protein-protein interactions cross linking, co-immunoprecipitation, co-fractionation has also been practiced (Fields and Song, 1989). For high throughput analysis and protein-protein interaction, cell-free translation of the tagged bait and prey protein is beneficial (Miyamoto-sato *et al.,* 2005). Hurst *et al.* (2011) studied protein-protein interactions by combining cell-free protein expression system with HaloLinkTM protein arrays. The method was helpful to evaluate binary and complex protein interactions in a single day.

For selection and directed evolution of proteins, ribosome display is a powerful tool. Cell-free translation has been utilized for ribosome display in a way to provide link between genotype and phenotype, thus allowing the genes encoding proteins with desired properties to be identified by selection. The coupling of genotype and phenotype through cell-free translation produced stabilized ribosome complexes. The translated protein and their encoding mRNA remain attached to ribosome (Villemagne *et al.,* 2006). *E. coli* cell extracts have been extensively used for ribosomal display, while eukaryotic extracts are also reported with good results (Douthwaite, 2012). Since it overcomes the limitations of cell based display method by using cell-free system, ribosome display is representative of next generation of display technology. It offers advantages of screening large libraries and continuously expanding new diversity during selection (He and Khan, 2005).

He and Taussig (2007) reported that cell-free systems are utilized for ribosome display and protein in situ arrays; which are utilized for screening of large libraries to separate protein binding molecules. Such techniques can be combined to a swift detection of protein interactions. Other methods for screening of large protein libraries are based on living cells like cell surface display, yeast two hybrid system and plasmid display. Such systems invite several difficulties; like limited complexities by transformation efficiency, non-diversification. In most recent studies, cell-free protein expression system has been utilized for the expression of MORC2 proteins, which are involved with decrease in acetylation of histone H3 at CAIX promoter. The confirmation of histone is affected by post translational modifications like acetylation, methylation, ubiquitylation and phosphorylation. In addition, gene silencing mechanism of the enzyme peptidye argentine deaminase type IV has been elucidated by its expression in cell-free expression system (Denis *et al.,* 2009; Shao *et al.,* 2010).

Factors affecting protein yield by in vitro translation

The efficiency and quality of the cell-free protein is determined by its folded form, which in turn resembles to naturally present form. Cell-free protein synthesis is retarded due to accumulation of inhibitors in the system. Wheat germ endosperm for example contains inhibitors for translation like proteases, deoxyribonucleases, thionin, and RNA-N-glycosidase. High quantity of protein with stability and activity was obtained after extensive washing of wheat germ (Sawasaki *et al.,* 2002).

Energy supply for the translation system has been most expensive but vital component of the translation process. Continuity of the reaction necessitates efficient ATP regeneration systems. For years, efforts have been made to reduce the cost and timing of the reactions for cell-free system (Calhoun and Swartz 2008; Liu *et al.,* 2008; Sitaraman and Chatterjee 2009). Lowering the energy contents ceases the reaction, which ultimately affects the yield. Kawarasaki *et al.,* (1994) reported that exhaustion of source of ATP regeneration system and observed pause in reaction after decreased ATP contents. Better recycling of free phosphate levels leads to a highly efficient cell-free translation system. Previously reported ATP regeneration sources are glucose, glucose—phosphate, creatine phosphate, 3-phosphoglycerate, fructose-1, 6-bis-phosphate (Madina *et al.,* 2006). Kim *et al.* (2007) used creatine phosphate and glucose used simultaneously in a dual energy supply to significant regeneration of ATP and lowers the accumulation of phosphate ions. Phosphate released from CP and used in glycolytic pathway for glucose utilization, thus increased ATP supply and decreased accumulation of inorganic phosphate. Magnesium ions which are critically essential for NTPs synthesis, translation, and reaction termination are precipitated by elevated phosphate levels, which severely affect the translation mechanism (Levit *et al.,* 2002). Increment of magnesium ions inside the reaction mixture could extend the reaction. ATP de-phosphorylation reaction depends on pH of the reaction mixture. Kawarasaki *et al.* (1994) reported a two fold increase in protein synthesis at pH 7.6. Accumulation of free phosphate drastically slows down the cell-free translation phenomenon, as in case of pyruvate energy regeneration source. Kim and Swartz (2000) not only replaced costly energy sources with phosphate free secondary energy providers like glucose and pyruvate, but also introduced pyruvate oxidase to prevent buildup of free-phosphates.

Protein yields are affected by building units (NTPs), stability of mRNA, and amino acids concentration (Madina, 2006). Rapid consumption of NTPs is observed in cell-free expression system, while a turn down in NTPs concentration ceases the reaction. NMPs could be utilized as cost effective source of nucleotides. Such an economical system was developed by Calhoun and Swartz (Calhoun and Swartz, 2008) where glucose and nucleotides contents were kept consistent for a 3 h reaction, resulting into high yields of chloramphenicol acetyl transferase (CAT) and β-glucosidase.

Dithiothreitol (DTT), a reducing reagent maintains reducing environment, and T7 RNA polymerase in active forms. Reducing conditions prevent the formation of disulfide bond; on the other hand, oxidative conditions can be maintained for the disulfide bonds formation (Allen *et al.,* 1995). DTT introduction in the reaction functions for the maintenance of reduced cytoplasmic environment and optimal protein synthesis (Jackson, 2004). Addition of a freshly prepared DTT solution to the reaction mixture caused maintenance of ATP level (Kawarasaki *et al.,* 1994). PEG introduces crowding effect on molecules for better reactions and high level of protein per volume of extract could be obtained after its addition. In an optimization study, Kim *et al.* (1996) reported that polyethylene glycol (PEG) and phosphoenol pyruvate (P-pyruvate) as independent at co-optimized essential components for optimal protein expression.

Suitable systems for translation of several proteins are generated at optimal activity of T7 promoter which is affected more by the lower temperature as compared to the ribosomes as reported by Madina *et al.* (2006). Ozawa *et al.* (2004) reported a decline in yields insignificantly by lowering the temperature from 37 to 30 ºC.

Conclusion

The discussion concludes that, cell-free system provides opportunity to generate high quality proteins in small volumes and short duration. Cell-free extracts from *E. coli* are for small peptides, while eukaryotic systems are a good opportunity for larger peptides. The optimization of CFPSs by continuous energy supply and enhanced production using extended lifetime of mRNA in batch mode has encouraged the scientists for more advantageous utilizations of these systems. Furthermore, the discouraging factors, affecting the yield have been solved by scientific efforts. Applications area has broadened due to standardization of factors for CFP synthesis. A variety of cell-free translational systems have been reported by several with significant yields of functional proteins, So, it is strongly recommended to use these optimized systems for prokaryotic and eukaryotic proteins. Further research for CFPSs optimization could be useful for optimum expression of challenging protein.

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References

Acton, T.B., R. Xiao, S. Anderson, J. Aramini, W. A. Buchwald, C. Ciccosanti, K. Conover, J. Everett, K. Hamilton, Y. J. Huang, H. Janjua, G. Kornhaber, J. Lau, D. Y. Lee, M. Maglagui, L. Ma, L. Mao, D. Patel, P. Rossi, S. Shadev, R. Sahdev, R. Shastry, G.V. Swapna, Y. Tang, S. Tong, D. Wang, H. Wang, L. Zhao, G. T. Montelione. 2011. Preparation of protein samples for NMR structure, function, and small-molecule screening studies. Meth. Enzymol. 493: 21-60.

Allen, S., H. Y. Naim and N. J. Bulleid. 1995. Intracellular folding of tissue-type plasminogen activator; Effect of disulfide bond formation on N-linked glycosylation and secretion. J. Biol. Chem. 270: 4797-4804.

Angenendt, P., J. Kreutzberger, J. Glokler and J. D. Hoheisel. 2006. Generation of high density protein microarrays by cell-free in situ expression of unpurified PCR product. Mol. Cell. Proteom. 5: 1658-1666.

Bhanu, R. K., K. V. A. Ramaiah, A. G. Bhuyan, S. KSR, A. Sudhakar and L. Burela. 2010. Trends in wheat germ cell-free protein expression system with an emphasis on up-scaling and industrial application. Ind. J. Sci. Tech. 3: 349-354.

Calhoun, K. and J. R. Swartz. 2008. An economical method for cell-free protein synthesis using glucose and nucleotide monophosphates. Biotechnol. Prog. 21: 1146-1153.

Chandra, H. and S. Srivastava. 2009. Cell-free protein synthesis based microarrays and their applications. Proteom. 10(4): 717-730.

Denis, H., R. Deplus, P. Putmans, M. Yamada, R. Metivier and F. Fuks. 2009. Functional connection between deimination and deacetylation of histones. Mol. Cell Biol. 29: 4982-4993.

Denman, R. 2008. Protein methylatransferase activities in commercial in vitro translation systems. J. Biochem. 144: 223-233.

Doolan, P., P. Meleady, N. Barron, M. Henry, R. Gallagher, P. Gammell, M. Melville, M. Sinacore, K. McCarthy, M. Leonard, T. Charlebois and M. Clynes. 2010. Microarray and Proteomics expression profiling identifies several candidates, including the

valosin-containing protein (VCP), involved in regulating high cellular growth rate in production CHO cell lines. Biotechnol. Bioeng. 106: 42-56.

Douthwaite, J. A. 2012. Eukaryotic ribosome display selection using rabbit reticulocyte lysate. Meth. Mol. Biol. 805: 45-57.

Dubel, S., O. Stoevesandt, M. J. Taussig and M. Hust. 2010. Generating recombinant antibodies to the complete human proteome. Trends Biotechnol. 28: 333-339.

Fields, S. and O. K. Song. 1989. A novel genetic system to detect protein-protein interactions. Nature. 340: 245-246.

Glenn, M. and A. Colma. 1991. A highly efficient, cell-free translation/translocation system prepared from Xenopus eggs. Nucleic Acids Res. 19: 6405-6412.

Goerke, A. R. and J. R. Swartz. 2009. High-level cell-free synthesis yield of proteins containing site-specific non-natural amino acids. Biotechnol. Bioeng. 102: 400-416.

Goshima, N., Y. Kawamura, A. Fukumoto, A. Miura, R. Honma and R. Satoh. 2008. Human protein factory for converting the transcriptme into an in vitro-expressed proteome. Nat. Methods. 5: 1011-1017.

Guignard, L., K. Ozawa, S. E. Pursglove, G. Otting and N. E. Dixon. 2002. NMR analysis of in vitro synthesized proteins without purification: a high-throughput approach. FEBS Lett. 524: 159-162.

He, M. 2008. Cell-free protein synthesis: applications in proteomics and biotechnology. New Biotechnol. 25: 126-132.

He, M. and F. Khan. 2005. Ribosome display: next generation display technologies for production of antibodies in vitro. Expert. Rev. Proteomics. 2: 421-430.

He, M. and M. J. Taussig. 2001. Single step generation of protein arrays from DNA by cell-free expression and in situ immobilization (PISA method). Nucleic acid Res. 29: e73-3.

He, M. and M. J. Taussig. 2007. Rapid discovery of protein interactions by cell-free protein technologies. Biochem. Soc. Trans. 35. 962-965.

Henrich, B., W. Lubitz and R. Plapp. 1982. Lysis of Eschrichia coli by induction of cloned Ø X174 genes. Mol. Gen. Genet. 185: 493-497.

Hillebrecht, J. R. and S. Chong. 2008. A comparative study of protein synthesis in in-vitro systems: from the prokaryotic reconstitured to the eukaryotic extract-based, BMC. Biotechnol. 8: 58.

Hurst, R., R. Ohana and N. Nath. 2011. Protein Interactions Analysis Using Cell-Free Protein Expression Systems and HaloLinkTM. Protein arrays Internet. [http://www.promega.com/resources/articles/pubhub/protein-interactions-analysis-with-cell-free-expression-systems-and-halolink-arrays/].

Ishikawa, K., M. Watanabe, T. Kuroita, I. Uchiyama, J. M. Bujnicki, B. Kawakami, M. Tanokura and I. Kobayashi. 2005. Discovery of a novel restriction endonuclease by genome comparison and application of a wheat germ-based cell-free translation assay: Pabl (5o-GTA/C) from the hyperthermophilic archaeon Pyrococcusabyssi. Nucleic acid Res. 33: e112.

Jackson, A. M. 2004. Review: cell-free protein synthesis for proteomics. Brief. Funct. Genomic. Proteomic. 2: 308–319.

Jewett, M. C. and J. R. Swartz. 2004. Rapid expression and purification of 100 nmol quantities of active protein using cell-free protein synthesis. Biotechnol. Prog. 20: 102-109.

Jewett, M. C., M. L. Miller, Y. Chen and J. R. Swartz. 2009. Continued Protein Synthesis at Low [ATP] and [GTP] Enables Cell Adaptation during Energy Limitation. J. Bacteriol. 1083-1091.

Kang, S. H., D. M. Kim, H. J. Kim, S. Y. Jun, K. Y. Lee and H. J. Kim (2005). Cell-free production of aggregation-prone proteins in soluble and active forms. Biotechnol. Prog. 21(5): 1412-9.

Katzen, F., G. Chang and W. Kudlicki (2005). The past, present and future of cell-free protein synthesis. Trends Biotechnol. 23: 150-156.

Kawarasaki, Y., H. Nakano and T. Yamane. 1994. Prolonged cell-free protein synthesis in a batch system using wheat germ extract. Biosci. Biotech. Biochem. 58: 1911-1913.

Kigawa, T., T. Yabuki, N. Matsuda and T. Matsuda. 2004. Preparation of Escherichia coli cell extract for highly productive cell-free protein expression, J. Strucu. Funct. Genomics. 5: 63-68.

Kigawa, T., T. Yobuki, Y. Yoshida, M. Tsutsui, T. Ito, T. Shibata and S. Yokoyama. 1999. Cell-free production and stable-isotope labeling of milli-gram quantities of proteins. FEBS Letters. 422: 15-19.

Kigawa, T., Y. Muto and S. Yokoyama. 1995. Cell-free synthesis and amino acid selective stable isotope labeling of proteins for NMR analysis. J. Biomol. NMR. 6: 129-134.

Kim, D. M. and J. R. Swartz. 2000. Prolonging cell-free protein synthesis by selective reagent additions. Biotechnol. Prog. 16: 385-390.

Kim, D. M., T. Kigawa, C. Y. Choi and S. Yokoyama. 1996. A highly efficient cell-free protein synthesis system from E. coli. Eur. J. Biochem. 239: 881-886.

Kim, H. C. and D. M. Kim. 2009. Methods for energizing cell-free protein synthesis. J. Biosci. Bioeng. 108: 1-4.

Kim, T. W., D. M. Kim and C. Y. Choi. 2006. Rapid production of milligram quantities of proteins in batch cell-free protein synthesis system. J. Biotechnol. 124: 373-380.

Kim, T. W., I. S. Oh, J. W. Keum, Y. C. Kwon, J. Y. Byun, K. H. Lee, C. Y. Choi, D. M. Kim. 2007. Prolonged cell-free protein synthesis using dual energy sources: combined use of creatine phosphate and glucose for efficient supply of ATP retarded accumulation of phosphate. Biotechnol. Bioeng. 97: 1510-1515.

Kim, T.W., H. C. Kim, I. S. Oh and D. M. Kim. 2008. A highly efficient and economical cell-free protein synthesis system using the S12 extract of Escherichia coli. Biotechnol. Bioproc. Eng.13: 464-469.

Klammt, C., F. Löhr, B. Schafer, W. Haase, V. Ruterjans and C. Glaubitz. 2004. Bernhard F., High level cell-free expression and specific labeling of integral membrane proteins. Eur. J. Biochem. 271: 568-580.

Kohrer, C. and U. L. RajBhandary. 2000. Proteins carrying one or more unnatural amino acids. Madame Curie Biosci. Database.

Leippe, D. M., K. Q. Zhao, K. Hsiao and M. R. Slater. 2010. Cell-free expression of protein kinase A for rapid activity assay. Anal. Chem. Insights. 5: 25-26.

Levit, M. N., B. M. Abramczyk, J. B. Stock and E. H. Postel. 2002. Interactions between Escherichia coli nucleaoside-diphosphate kinase and DNA. J. Biol. Chem. 277: 5163-5167.

Liu, D. V., J. F. Zawada and J. R. Swartz. 2008. Streamlining Escherichia coli S30 extract preparation for economical cell-free protein synthesis. Biotechnol. Prog. 2: 460-465.

Ma, R., Z. Yang, L. Huang, X. Zhu, L. Kai, J. Cai, X. Wang and Z. Xu. 2010. Construction of an efficient Escherichia coli cell-free system for in vitro expression of several kinds of proteins, Engineering. Life. Sci. 10: 333–338.

Madin, K., T. Swasaki, T. Ogasawara and Y. Endo. 2000. A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. Pro. Natl. Acad. Schi. USA. 97: 559-564.

Madina, B.I., W. Szaflarski, M. Dreyfus, J. Remme, K. H. Nierhaus. 2006. Trouble shooting coupled in vitro translation system derived from Escherichia coli: synthesis of high-yield fully active proteins. Nucleic acids Res. 34(19): e135.

Mikami, S., M. Maustani, N. Sonenberg, S. Yokoyama and H. Imataka. 2006. An efficient mammalian cell-free translation system supplemented with translation factors. Protein Expr. Purif. 46: 348–357.

Miyamoto-sato, E., M. Ishizaka, K. Horisawa, S. Tateyama, H. Takashima and S. Fuse. 2005. Cell-free cotranslation and selection using in vitro virus for high-throughput analysis of protein-protein interactions and complexes. Genome. Res. 15710-717.

Nakano, T., H. T. Spencer, J. R. Appleman and R. L. Balkely. 1994. Critical role of phenylalanine 34 of human dihydrofolate reductase in substrate and inhibitor binding and in catalysis. Biochem. 23: 9945-9952.

Nozawa, A., H. Nanamiya, T. Miyata, N. Linka, Y. Endo, A. P. M. Weber and Y. Tozawa. 2007. A cell-free translation and proteoliposome reconstitution system for functional analysis of plant solute transporters. Plant Cell Physiol. 48: 1815-1820.

Oh, I. S., J. C. Lee, M. S. Lee, J. H. Chung and D. M. Kim. 2010. Cell-free production of functional antibody fragments. Bioproc. Biosys. Eng. 33: 127-132.

Orhan, P. H. and J. W. Heinecke. 2006. Misincorporation of m-tyrosine into cellular proteins: a potential cytotoxic mechanism for oxidized amino acids. Biochem J. 15: 277-284.

Ozawa, K., M. J. Headlam, P. M. Schaeffer, B. R. Henderson, N. E. Dixon and G. Otting. 2004. Optimization of an Escherichia coli system for cell-free synthesis of selectively 15N-labelled proteins for rapid analysis by NMR spectroscopy. Eur. J. Biochem. 271: 4084-4093.

Ozawa, K., P. S. C. Wu, N. E. Dixon and G. Otting. 2006. 15N-labelled proteins by cell-free protein synthesis strategies for high-throughput NMR studies of proteins and protein-ligand complexes. FEBS. J. 273: 4154-4159.

Revathi, B., K. V. A Ramaiah, A. G. Hinnebusch, A. K. Bhuyan, S. K. S. R. A. Sudhakar and L. Burela. 2010. Trends in wheat germ cell-free expression system with an emphasis on up-scaling and industrial application. Ind. J. Sci. Technol. 3: 349-354.

Reydellet, M. N., K. Calhoun and J. Swartz. 2004. Amino acid stabilization for cell-free protein synthesis by modification of the Escherichia coli genome. Metab. Eng. 6: 197-203.

Sawasaki, T., T. Ogasawara, R. Morishita and Y. Endo. 2002. A cell-free protein synthesis system for high-throughput proteomics. PNAS. 99: 14652-14657.

Schwarz, D., V. Dotsch and F. Bernhard. 2008. Production of membrane proteins using cell-free expression system .Proteomics. 8: 3933-3946.

Shao, Y., Y. Li, J. Zhang, D. Liu, F. Liu, Y. Zhao, T. Shen and F. Li. 2010. Involvement of histidinedeacetylation in M/ORC2-mediated down-regulation of carbonic anhydrase IX. Nucleic acids Res. 38: 2813-2824.

Shen, X. C., S. L. Yao, H. Fukano, S. Terada, A. Kitayama, T. Nagamune and E. Suzuki. 1999. Poly[G] improved protein productivity of cell-free translation by inhibiting mRNased in wheat germ extract. J. Biotechnol. 75: 221-228.

Shimizu, Y., A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa and T. Ueda. 2001. Cell-free translation reconstituted with purified components. Nat. Biotechnol. 19: 751–755.

Shin, J. and V. Noireaux. 2010. Efficient cell-free expression with the endogenous E. coli RNA polymerase and sigma factor 70. J. Biol. Eng. 4: 2-9.

Sitaraman, K. and D. K. Chatterjee. 2009. High-throughput protein expression using cell-free system. Meth. Mol. Biol. 498: 229-244.

Sitaraman, K., D. Esposito, G. Klarmann, S. F. Grice, J. L, Hartley and D. K. Chatterjee. 2004. A novel cell-free p rotein synthesis system. J. Biotechnol.110: 257-263.

Spirin, A. 2004. High-throughput cell-free systems for synthesis of functionally active proteins. Trends Biotechnol. 22: 538–545.

Stiege, W. and V.A. Erdmann. 1995. The potential of the in vitro protein biosynthesis system. J. Biotechnol. 41 (2-3): 81-90.

Stoevesandt, O., M. J. Taussig, M. He. 2009. Protein microarrays: high-throughput tools for proteomics. Expert Rev. Proteomics. 6: 145-157.

Szamecz, B., E. Rutkai, L. Cuchalova, V. Munzarova, A. Herrmannova, K. H. Nielsen, L. Burela, A. G. innebusch and L. Valasek. 2008. ElF3a cooprates with sequence 5' of Uorf1 to promote resumption of scanning by post-termination ribosomes for reinitiationon GCN4 Mrna. Genes Dev. 22: 2414-2425.

Takashi, H., A. Nozawa, M. Seki, K. Shinozaki, Y. Endo and T. Sawasaki. 2009. A simple and highly-sensitivity method for analysis of ubiquination and polyubiquination based on wheat-cell free protein synthesis. BMC. Plant Biol. 9: 39.

Talapatra, A., R. Rouse and G. Hardiman. 2002. Protein microarray: challenges and promises. Pharmacogen. 3: 527-536.

Tsuboi, T., S. Takeo, T. U. Arumugam, H. Otsuki and M. Torii. 2010. The wheat germ cell-free protein synthesis system: a key tool for novel malaria vaccine candidate discovery. Acta. Trop. 114: 171-176.

Villemagne, D., R. Jackson, J. A. Douthwaite. 2006. Highly efficient ribosome display selection by use of purified components for in vitro translation. J. Immunol. Meth. 30: 140-108.

Vinarov, D. A., B. L. Lytle, F. C. Peterson, E. M. Volkman and J. L. Markley. 2004. Cell-free protein production and labeling protocol for NMR-based structural proteomics. Nat. Meth. 1: 149-153.

Wang, Y. and Y. H. Zhang. 2009. Cell-free protein synthesis energized by slowly-metabolized maltodextrin. BMC. Biotechnol. 9: 58.

Watanabe, M., K. Miyazono, M. Tanokura, T. Sawasaki, Y. Endo, I. Kobayashi. 2010. Cell-free protein synthesis for structural determination by X-ray crystallography. Meth. Mol. Biol. 607: 149-160.

Watzele, M., C. Nemetz, T. Metzler, E. Fernholz, T. Hoffmann and W. Mutter. 2001. Rapid translation system RTS 100-a high throughput cell-free protein expression. Biochemica. 3: 27-28.

Wu, P. S. C., K. Ozawa, S. P. Lim, S. G. Vasudevan, N. E. Dixon and G. Otting. 2007. Cell-free transcription/translation from PCR-Amplified DNA high-throughput NMR studies. Chem. Int. Ed. 46: 3356-3358.

Wuthrich, K. 2002. NMR studies of structure and function of biological macromolecules. Noble Lecture. Eidgenossishe Technische Hochschule Zurich, CH-8093 Zurich. Switzerland. The Scripps Research Institute. 10550 N. Torrey Pines Rd. La Jolla CA 92037. USA.

Yang, H. L., L. Ivashkiv, H. Z. Chen, G. Zubay and M. Cashel. 1980. Cell-free coupled transcription-translation system for investigation of linear DNA segments, Proc. Nati Acad Sci., 1980, 77, 7029-7033.

Zarate, X., D. C. Henderson, K. C. Phillips, A. D. Lake and D. W. Galbraith. 2010. Development of high-yield auto fluorescent protein microarrays using hybrid cell-free expression with combined *Escherichia coli* S30 and wheat germ extracts. Proteome Sci. 8: 32.