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Comparative expression of wild-type and highly soluble mutant His103Leu of hydroxynitrile lyase from *Manihot esculenta* in prokaryotic and eukaryotic expression systems

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ABSTRACT

Low protein solubility and inclusion body formation represent big challenges in production of recombinant proteins in *Escherichia coli*. We have recently reported functional expression of hydroxynitrile lyase from *Manihot esculenta*, *Me*HNL, in *E. coli* with high *in vivo* solubility and activity using directed evolution. As a part of attempts to clarify the mechanism of this phenomenon, we have described the possibility of expression of the highly active and soluble mutant *Me*HNL-His103Leu as well as wild-type enzyme in several expression systems. Methylotrophic yeast *Pichia pastoris*, protozoan host *Leishmania tarentolae* and two cell-free translations, including an *E. coli* lysate (WakoPURE system) and wheat germ translation system were used to compare expression profiles of the genes. Two distinguishable protein expression patterns were observed in prokaryotic and eukaryotic-based systems. The wild-type and mutant enzyme showed high activity for both genes (up to 10 U/ml) in eukaryotic hosts *P. pastoris* and *L. tarentolae*, while those of *E. coli* exhibited about 1 and 15 U/ml, respectively. The different activity level in prokaryotic systems but the same level among the eukaryotic hosts indicate the phenomenon is specific to the *E. coli* system. Both the wild-type and mutant enzymes were functionally expressed in eukaryotic systems, probably using the folding assistants such as chaperones. Properties of expression systems used in this study were precisely compared, too.

Introduction

There is a long list of advantages which render *Escherichia coli* as the first and most common choice for overexpression of heterologous genes, although many genes cannot be expressed functionally in this work horse of biotechnology [1–2]. Therefore, researchers may change the host cells, use fusion proteins or try to develop new expression systems for realizing expression of more genes in this versatile host, because there are numerous proteins waiting for structural studies as well as biotechnological applications [3].

Asano et al., have created highly active and *in vivo* soluble mutants of *Me*HNL[1] (hydroxynitrile lyase from *Manihot esculenta*, cassava plant) in *E. coli* by directed evolution, where several mutants of this enzyme were generated including His103 and also Lys-Pro mutants [4]. Functionally expressed enzymes show similarities and differences in comparison to the wild-type in the terms of stability to temperature and pH, biochemical kinetics, and in their structures (unpublished data). It was found that protein expression profile reversed from a low soluble wild-type enzyme to highly *in vivo* soluble form by introduction of mutations such as His103Leu, i.e., the main expression form of the wild-type enzyme was as inclusion body while it was present in the soluble fraction following the mutation. The highly active mutants of the enzyme *Me*HNL were found to have both surface and buried substitutions including triple mutant Lys176Pro, Lys199Pro, Lys224Pro as well as single mutants His103Met, His103Leu, His103Ile, His103Val, and His103Cys. There are only rare examples of application of directed evolution in improvement of *in vivo* solubility of overexpressed proteins in *E. coli* in the literature [5–7], although a comparable improvement of *in vivo* solubility level in *E. coli* was only reported by Jenkins et al., with a Phe185Lys substitution in a truncated catalytic domain (50–212) of human immunodeficiency virus type 1 integrase [8]. The mutant was the only truncated soluble enzyme among 29 mutants made by replacing hydrophobic residues within the core domain with either alanine or lysine.

We have been studying the wild-type and mutant *Me*HNL created by directed evolution to clarify the mechanism involved in this phenomenon. One of interesting features would be answering the questions of whether wild-type and mutant genes are expressible in other host cells and do they show the same tendencies in expression profiles; or is this remarkable change in the

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[1] *Abbreviations used:* *Me*HNL, hydroxynitrile lyase from *Manihot esculenta*; CECF, Continuous Exchange Cell-Free.

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expression of the enzyme following the mutations is uniquely specific to the host *E. coli*. In other words, is there any similar behavior among the eukaryotic and prokaryotic expression systems employed in this study for expression of the low soluble wild-type *Me*HNL and its highly *in vivo* soluble mutant His103Leu?

To address this issue, we arranged a comparative expression experiment where two above-mentioned genes were expressed in five different hosts including eukaryotic expression systems methylotrophic yeast *Pichia pastoris*, wheat germ cell-free translation system and trypanosomatid protozoan host *Leishmania tarentolae* (a parasite of lizard, classified as non-pathogenic to human) as well as prokaryotic host *E. coli* and its commercially available lysate named as WakoPURE system. To the best of our knowledge, there is no literature on a comparative heterologous overexpression of genes using broad range of expression systems for wild-type and the mutant genes created by directed evolution.

Materials and methods

Chemicals and commercial kits

All chemicals were purchased from commercial sources and used without further purification, unless specifically stated otherwise. The QIAquick® Gel Extraction Kit (QIAGEN, Valencia, CA, USA) was used for DNA extraction from agarose gel. RTS-100 wheat germ linear template generation set, His_6-tag kit was purchased from Roche Applied Science (Mannheim, Germany). EasySelect™ *Pichia* Expression Kit (Invitrogen, Carlsbad, CA, USA) used for expression of the genes in *P. pastoris*. LEXSYcon2 Expression Kit (Jena Bioscience, Jena, Germany) was used for gene expression in *L. tarentolae*. WakoPURE system and RTS 100 wheat germ CECF kit were purchased from Wako (Osaka, Japan) and Roche Applied Science (Mannheim, Germany), respectively.

Vectors, microorganisms, and culture media

pUC19 vector (Takara Bio Inc., Ohtsu, Japan), pT7 Blue T-vector (Novagen, WI, USA), pPICZA (Invitrogen, CA, USA), and pLEXSY-sat2 (Jena Bioscience, Jena, Germany) were used for cloning of the *hnl* genes. The *E. coli* host strain JM109, *P. pastoris* strains and *L. tarentolae* were purchased from Takara Bio Inc. (Ohtsu, Japan), Invitrogen and Jena Bioscience (respectively) and used for expression of the constructs encoding *S-Me*HNL and its mutant His103Leu. Luria–Bertani (LB) broth amended by 50 μg/ml ampicillin was used for cultivation of *E. coli* cells. In order to induce protein expression in *E. coli*, 0.1–1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were added to the media. Yeast Extract Peptone Dextrose Sorbitol (YPDS) agar and broth contained 1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar, and 100 μg/ml Zeocin™ (Invitrogen). For cultivation of *L. tarentolae*, brain heart infusion (BHI) broth was supplemented by 5 μg/ml hemin, 50 U/ml penicillin and 50 μg/ml streptomycin (Pen–Strep) to prevent bacterial contaminations and incubated at 26 °C under dark conditions, according to the producer's manual.

Restriction enzymes, primers and PCR programs

Restriction enzymes were purchased from Takara Bio Inc. (Ohtsu, Japan), Toyobo Co., Ltd. (Osaka, Japan), New England Biolabs (Ipswich, MA, USA), Roche (Mannheim, Germany), and MBI Fermentas (Vilnius, Lithuania) and used in accordance with producers' instructions. The DNA manipulations were carried out using *Molecular Cloning* as a reference [9], unless otherwise noted. Oligo-nucleotide primers were prepared from Hokkaido System Science Co. Ltd. (Tsukuba, Japan).

Pairs of restriction enzymes *Pst*I & *BamH*I; *Eco*RI & *Kpn*I and *Not*I & *Bgl*II were used for cloning of the *hnl* in pUC19, pPICZA, pLEXSY-sat2 vectors, respectively. The primers used in this study are presented in Supplementary Table 1. ExTaq PCR program was used for amplification of the genes: 98 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 1 min. The only exception is the second PCR for WakoPURE system cell-free translation experiment, in which the KOD plus program was used, consisting 94 °C for 2 min and 30 cycles of 94 °C for 30 s, 52 °C for 30 s, 68 °C for 1 min, and finally 5 min at 72 °C, prior to cooling down to 4 °C.

Transformation and expression of the genes

After preparation of competent cells of *P. pastoris* (according to the producer's instruction), the following protocol was used for transformation of wild-type and mutant *Me*HNL genes in the strains GS115, X-33 and KM71: *Sac*I linearized pPICZA harboring *hnl* gene was transformed into the competent cells by electroporation, as described in Easyselect™ *Pichia* Expression Kit (Invitrogen). About 20 colonies of *P. pastoris* were picked up for each gene from YPDS plates and inoculated in YPD broth containing Zeocin™ at 30 °C for 36 h. Genomic DNA of colonies were extracted by Dr. GenTLE kit for Yeast (Takara, Shiga, Japan), amplified by AOX3′ and AOX5′ primers and the clones having amplification were cultivated in 500-ml baffled Erlenmeyer flasks containing 100 ml MGYH (100 mM potassium phosphate buffer pH 7.0, 1.34% yeast nitrogen base without amino acid, 4×10^{-5}% biotin, 0.004% histidine, and 1% glycerol) with shaking rate 200 rpm, for 1–2 days at 28 °C, then were centrifuged (3500*g*, 5 min, 4 °C) and suspended in 100 ml expression medium BMMH (MGYH containing 0.5% methanol instead of glycerol). Methanol was added to the flasks as inducer every 24 h, following sampling. The activity and protein expression profile were monitored for 7 days. The procedure described in LEXSYcon2 Expression Kit was followed for preparation of competent cells and transformation of *Swa*I linearized pLEXSY-2 vector harboring these two genes. Frozen cells of *L. tarentolae* were added into BHI broth supplemented by hemin and Pen-Strep and incubated at 26 °C for 1–2 days. The grown cells were transformed by the *hnl* genes using electroporation, according to the producer's instruction. Subsequently, the cells were added to 10 ml BHI broth containing hemin and Pen-Strep, incubated for 36 h and checked microscopically for viability of the cells and lack of contamination. The cells were subsequently transferred to a fresh medium of BHI broth containing nourseothricin (NTC) and finally inoculated in 100 ml BHI containing hemin and Pen-Strep (no NTC) and incubated under dark conditions (140 rpm) at 26 °C for up to 4 days. The cells were lysed by a Multi-Beads Shocker MB 601U (S) (Yasui Kikai, Osaka, Japan) to release the enzyme for activity measurement and SDS–PAGE analysis.

In order to generate linear expression constructs, two PCRs were performed. In the first reaction, gene specific primers were used to add overlap regions to the sequence of the target gene. The product was mixed with two flanking primers and DNA fragments coding for the T7 promoter, eukaryotic 5′- and 3′-regulatory elements and a 6×His-tag sequence. The product of the first step annealed with the added DNA fragments and 5′- and 3′-ends were extended in the second PCR named overlap extension PCR. This linear expression construct was finally amplified by the flanking primers. RTS wheat germ linear template generation set, His_6-tag from Roche was used for this purpose (Supplementary Table 1). Product of the 2nd PCR was used for an *in vitro* protein synthesis reaction in a RTS ProteoMaster instrument already incubated at 24 °C and shaking speed of 900 rpm. Feeding solution (1 ml) and 50 μl reaction mixture (including linear DNA template) were added to the system and to initiate the reaction it was incubated for 20 h at 24 °C, stopped by keeping on ice, 5 and 10 μl samples were withdrawn for SDS–PAGE and activity measurement, respectively [10].

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Two PCR steps were also used to prepare the template DNA for expression in WakoPURE system, which is a commercially available cell-free translation system derived from *E. coli*. An adapter containing Shine-Dalgarno sequence at the 5′ end was added in the first PCR using forward and reverse primers designed based on the gene sequence (Supplementary Table 1). Protein synthesis reaction was done using the reaction cocktail mentioned in the kit, incubated at 37 °C for 60 min without shaking and was stopped by incubation on ice [11].

Enzyme assay and SDS–PAGE analysis

We used an HPLC-based hydroxynitrile lyase activity measurement method [12], with slight modifications. A solvent system comprising *n*-hexane: 2-propanol = 85:15 (Wako, Tokyo, Japan) was used for extraction of produced mandelonitrile and also as a mobile phase for HPLC analysis. The retention times for benzaldehyde, *R*- and *S*-mandelonitrile were about 4.4, 9.4, and 11.6 min, respectively. In a cyanohydrin synthesis reaction, 1 U of enzyme activity is defined as amount of the enzyme that produces 1 μmol of optically active cyanohydrin from the corresponding aldehyde or ketone per minute under the standard assay conditions [12]. Fig. 1 demonstrates cyanogenesis in plants, where HNL catalyzes a reversible reaction in the last step. The produced HCN is used mainly for defending the plants against fungi and pests.

SDS–Polyacrylamide gel electrophoresis was used for analysis of protein expression profiles both in the soluble and insoluble fractions. Slab gels of 15% acrylamide were used according to Laemmli method [13]. Protein concentrations were measured by Bio-Rad kit (Hercules, CA, USA) according to the Bradford method using bovine serum albumin (Wako, Osaka, Japan) as protein standard [14].

Results and discussion

Low protein solubility is a bottleneck for overexpression of genes in *E. coli*, an issue that researchers frequently encounter, although they mainly try other options such as changing the host [2,15] or using fusion proteins, codon optimization, and molecular chaperones [1]. Asano et al., created very high soluble mutants of the *Me*HNL by directed evolution [4] where protein engineering methods were employed to solve the problem of low *in vivo* solubility of *Me*HNL in *E. coli*. The gene of hydroxynitrile lyase from *Manihot esculenta* was expressed in *E. coli* JM109 mainly in the form of inclusion bodies. Using directed evolution techniques, highly active and *in vivo* soluble mutants were generated including triple mutant Lys176Pro, Lys199Pro, Lys224Pro and single mutants His103Leu, His103Met, His103Ile, His103Val and His103Cys. The protein expression profile was changed so that the main expression form was observed in the soluble fraction and the activity level for the enzyme was improved up to 17 times (Fig. 2A) [4].

Expressibility and main form of expression

*Me*HNL-His103Leu, one of high *in vivo* soluble and active mutants, was cloned and expressed in various expression vectors and host systems as was the wild-type *Me*HNL. A clear understanding of expression profiles of the genes in various expression systems will be helpful in attempts to find the mechanism of solubility of the mutants. Therefore, a series of expression experiments were arranged for these two genes, which exhibited absolutely different expression and activity levels in the original study in *E. coli*. It was found that the genes were expressed in all employed expression systems but to different extents among prokaryotic and eukaryotic systems (Table 1). Levels of soluble expression were displayed by "+" in the table.

Expression of the genes in the prokaryotic systems – E. coli and WakoPURE system

The wild-type and mutant His103Leu were expressed in all employed systems but the level of solubility was significantly different. As demonstrated in Fig. 2A, the wild-type and the mutant exhibited a reversed expression profile in the SDS–PAGE, i.e. one critical point mutation on the residue His103 affects profoundly the *in vivo* solubility of expressed enzyme in *E. coli*. On the other hand, cell-free protein synthesis systems of extracts of *E. coli*, wheat germ and rabbit reticulocyte have been considered as standards for *in vitro* translation systems of the prokaryotic and eukaryotic genes. The use of *E. coli* extracts (ECE) for cell-free translation and transcription–translation seems to be the most practical and efficient for *in vitro* synthesis of functional protein of both prokaryotic and eukaryotic origin. Vast information on *E. coli* protein synthesis machinery made considerable improvements of bacterial cell-free systems possible [16]. WakoPURE system (Fig. 2B), which is a cell-free translation technique, originally contains the basic protein synthesis machinery of *E. coli*. The system is a reconstruction of *E. coli* protein synthesis machinery containing over 100 purified components including 30 purified enzymes necessary for energy recycling, transcription, and translation, which, in turn, contains translation initiation, elongation and release factors (IF1, IF2 and IF3; EF-G, EF-Tu and EF-Ts; RF1, RF2 and RF3, respectively). Other elements include RRF for termination factors, 20 aminoacyl-tRNA synthetases (ARSs), methionyl-tRNA transformylase (MTF), T7 RNA polymerase, 70S ribosomes, tRNAs, NTPs, amino acids and energy recycling factors. All the components involved in transcription and translation are tagged with 6×His at the N- or C-termini except for ribosomes. The system lacks RNA helicases, nucleases, proteases, and enzymes that hydrolyze nucleoside triphosphates [17]. The wild-type enzyme exhibited no detectable activity and soluble expression in this system, whereas the highly soluble mutant exhibited some functional expression in the soluble fraction (Fig. 2B).

Expression of the genes in eukaryotic systems – P. pastoris

Expression of the wild-type *Me*HNL and highly active mutant His103Leu in three eukaryotic systems including *P. pastoris*, *L. tarentolae*, and wheat germ cell-free translation, showed similar protein expression profiles among them (Fig. 2C–E), indicating a distinguishable expression profile from the prokaryotic systems used in this study. As shown in Fig. 2C, in the SDS–PAGE level, it seems there is no significant difference in expression of soluble and insoluble fractions in comparison of the phenomenon observed for them in *E. coli*. Methylotrophic yeast *P. pastoris* is a single-cell organism and has a fast growth rate such as bacteria.

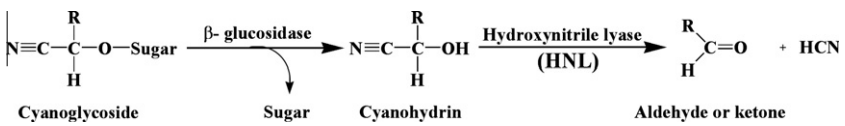

Fig. 1. Cyanogenesis in higher plants: HNL catalyzes cyanohydrin decomposition to aldehyde or ketone and HCN. The enzyme condenses aldehydes or ketones with a cyanide source to produce chiral cyanohydrins in the reverse reaction. The cyanohydrins are being used as intermediates in the production of ranges of valuable chemicals, agrochemicals and pharmaceuticals.

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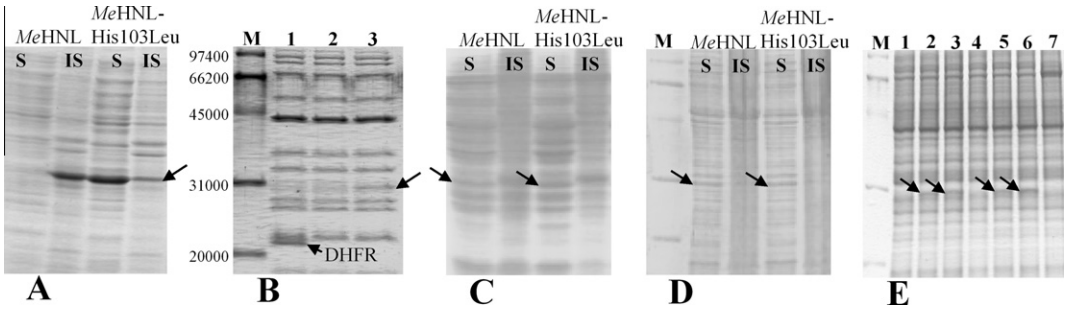

Fig. 2. (A–E) SDS–PAGE demonstrating the expression profiles of the wild-type *Me*HNL and its mutant His103Leu in five prokaryotic (A and B) and eukaryotic (C–E) hosts. The arrows show the location of the expressed enzymes. (A)*Escherichia coli* JM109; (B) WakoPURE system (1: positive control; dihydrofolate reductase; 2: *Me*HNL; 3: *Me*HNL-His103Leu); (C) *Pichia pastoris*; (D) *Leishmania tarentolae*; (E) wheat-germ cell-free translation system (1–3: wild-type; 1: N-term 6×His-tag; 2: C-term 6×His-tag; 3: no 6×His-tag; 4–6: His103Leu; 4: N-term 6×His-tag; 5: C-term 6×His-tag; 6: no 6×His-tag; 7: positive control: glucuronidase); (M, Bio-Rad protein markers; S, soluble fraction; IS, insoluble fraction. The lysate was centrifuged after disrupting the cells and the supernatant was considered the soluble fraction, while the pellet was resuspended in the equal volume of potassium phosphate buffer and named the insoluble fraction).

Table 1
Expressibility and main form of expression for wild-type and mutant *Me*HNL in prokaryotic and eukaryotic systems.

HOST	Soluble expression		Main expression form	
	*Me*HNL	*Me*HNL-His103Leu	*Me*HNL	*Me*HNL-His103Leu
Escherichia coli	+	++++	Insoluble	Soluble
WakoPURE system	–	+	Insoluble	Soluble
Pichia pastoris	++	++	Soluble	Soluble
Leishmania tarentolae	++	++	Soluble	Soluble
Wheat germ cell-free translation	++	++	Soluble	Soluble

+:expression;–:expression.

However, unlike bacteria it is an eukaryote with the similar intracellular environment and many of the same post-translational protein processing capabilities as higher eukaryotes. After selection of a suitable vector and cloning the gene, various strains of *P. pastoris* should be tested for the best expression, which was done in the current work for three strains and it was found that the strain GS115 is much suitable for its production (data not shown). *P. pastoris* differs from bacterial systems because the vector containing the desirable gene will be ultimately integrated in the genome of the host during transformation (homologous recombination) [15].

Expression of the genes in eukaryotic systems – L. tarentolae (LEXSY system)

LEXSY system has the advantages of eukaryotic protein expression/folding with relatively easy usage of prokaryotic expression systems. In trypanosomatid protozoan species, regulation of protein expression occurs mainly at RNA level and might be influenced by the structure of the intergenic regions [18,19]. Intergenic regions used in pLEXSY vectors were optimized for expression of heterologous genes in the eukaryotic host *L. tarentolae* [20]. For high-level expression, the desired genes were supplied with specific splicing signals provided on the expression vector and stably integrated into a chromosomal locus. A recent report describes active expression of an acetyl serotonin methyl transferase using this system. The researchers used this eukaryotic system to overcome inactive expression of the enzyme in *E. coli* [21]. The expression vectors were constructed in *E. coli* and introduced into the *L. tarentolae* by electroporation. After selection steps, it was found that the plant gene and its highly soluble mutant were expressed in the form of soluble proteins, which is different from the results obtained from prokaryotic systems employed in this research (Fig. 2D).

Table 2
Comparison of activity (A) and specific activity (B) of the wild-type and mutant His103Leu in the prokaryotic and eukaryotic expression systems. The assay was done using benzaldehyde as substrate in synthesis of the product mandelonitrile which was detected by HPLC at 254 nm. In Table 2A, another highly active mutant and its activity level is also presented. 0, no expression.

Expression system/gene	Prokaryotic systems		Eukaryotic systems				
	Escherichia coli	WakoPURE system	*Pichia pastoris*	*Leishmania tarentolae*	RTS 100 wheat germ cell-free translation system		
A *U/ml of culture medium or reaction mixture*					N-terminal 6 × His-tag	C-terminal 6 × His-tag	No 6 × His-tag
Wild-type *Me*HNL	0.95	0	9.6	10.2	0	0.31	0.40
*Me*HNL-His103Leu	15.2	0.14	5.6	10.8	0	0.21	0.20
*Me*HNL-His103Met	17.2	0.11	–	–	0	0.21	0.39
B *U/mg protein*							
Wild-type *Me*HNL	1.73	0	2.1	2.03	0	1.55	1.97
*Me*HNL-His103Leu	29.2	0.7	1.1	2.18	0	1.02	1.98

The data presented for cell-free translation systems are average of 3 independent experiments. The data for live host cells are the result of many reproducible experiments with standard errors less than 5%.

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Table 3
Precise comparison of properties of prokaryotic and eukaryotic expression systems employed in this study.

System	Cloning	Promoter (vector)	Competent cell preparation	Transformation	Selection	Genetics	Possibility of functional expression	Expression type and production time	Possibility of scaling up	Main culture conditions			
										Media costs	Optimum temperature (°C)	Agitation (rpm)	Inducer
Escherichia coli	Usual	lacZ (pUC19)	Simple and easy	Easy (e.g., heat shock)	No need	Host chromosome-independent	Not high	Mainly intracellular, 12–24 h	Possible	Cheap	Variable (15–37)	Necessary (mild, 150–200)	IPTG
Pichia pastoris	Usual	P_{AOX1} (pPICZA)	Multi-step	Relatively complicated (e.g., electroporation)	Time consuming	Recombination into host chromosome	Usually high	Intracellular and secretory, 3–7 days or more	Possible	Relatively expensive	28–30	Necessary (mild, 150–200)	Methanol
Leishmania tarentolae	Usual	T7 (pLEXSY-sat2)	Multi-step	Relatively complicated (e.g., electroporation)	Time consuming, contamination-sensitive	Recombination into host chromosome	Usually high	Intracellular, 2–3 days, darkness	?	Expensive	26	Necessary (mild, 120)	No
Wheat germ cell-free translation	2-step PCR, no cloning	T7 (−)	No need	None (direct)	No need	Using protein machinery of lysate	Usually high	20–24 h	Not Possible	Very expensive	24	Necessary (Vigorous, 900)	No
WakoPURE system	2-step PCR, no cloning	T7 (−)	No need	None (direct)	No need	Using protein machinery of lysate	Usually low	1 h	Not Possible	Very expensive	37	No need	No

Expression of the genes in eukaryotic systems: wheat germ cell-free translation

The result of this experiment supports our original assumption that the expression of the wild-type and highly soluble His103Leu mutant might not result in a drastic difference in protein solubility in eukaryotic expression systems. Expression of these two genes in wheat germ cell-free translation system has been shown in Fig. 2E. Continuous Exchange Cell-Free (CECF) system with dialysis format was a diffusion exchange of substrates and low-molecular-weight products through a porous membrane. At the same time, potential inhibitory by products of the reaction were diluted into the 1-ml feeding compartment. In contrast to traditional batch systems, CECF conditions allow protein synthesis to continue for 24 h. Transcription and translation processes take place simultaneously in the 50 μl reaction volume. The important advantage of classical eukaryotic extracts including wheat germ extract is that they have much lower endogenous nuclease activities, and this is the reason that both circular and linearized plasmid DNAs as well as PCR fragments can be used successfully in this system [16].

As it was mentioned in Materials and methods, the *hnl* and its mutant were provided in three forms for expression in this system including no-6×His-tag, N-terminal 6×His-tag and C-terminal 6×His-tag. No and C-terminal added His-tagged genes were expressed functionally whereas there was no expression for N-terminal His tagged gene, as shown in Fig. 2E and Table 2A–B.

Activity and specific activity of the wild-type and mutant enzyme in the five expression systems

The level of activity and specific activity of the genes expressed in the five expression systems were also compared and the results represented in Table 2A and B. The cells of *E. coli* JM109 exhibited 15–17-fold higher enzyme production for the mutant His103Leu and His103Met than the wild-type, while the level of activity for WakoPURE system cannot be compared to the wild-type because we could not detect activity for the wild-type. The mutant exhibited a 17-fold improvement in specific activity in the cell lysate of *E. coli* cells, but we had the same observation for the WakoPURE earlier, i.e., no activity for the wild-type enzyme in this system, perhaps because of lacking of chaperones or folding assistants in the WakoPURE system. It is not surprising that there was no detectable activity and soluble expression visible in SDS–PAGE (Fig. 2B) since we measured only about 1 U/ml activity for the wild-type enzyme in the original observation in *E. coli* JM109, where the soluble fraction of the wild-type was very low (Fig. 2A). On the other hand, there is at least 10-fold higher rate of protein synthesis in live *E. coli* cells in comparison of the cell-free synthesis. The data shown in SDS–PAGE (Fig. 2A and B) are in coordination with the information presented in Table 2A and B for these enzymes in prokaryotic hosts. Activity level of His103Leu mutant is a little lower than the wild-type in *P. pastoris* GS115, as seen with the wheat germ translation system, despite the fact that the proteins expressed in *P. pastoris* exhibited higher activity level. *L. tarentolae* as a host, exhibited equal expression and activity level for the wild-type and mutant His103Leu and its activity was comparable with another eukaryotic system, *P. pastoris* (Table 2A). Specific activities of these proteins in eukaryotic hosts were not much different for *P. pastoris*, *L. tarentolae* and genes with no 6×His-tag and with C-terminal 6×His-tag in the wheat germ translation system (Table 2B).

In principal, functionally active proteins of different origins can be synthesized successfully in cell-free systems using either bacterial or eukaryotic extracts. Unsuccessful attempts to synthesize some mammalian proteins in active form using a bacterial extract were hypothesized to result from non-optimized folding

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conditions, particularly a translation elongation rate that might be too fast [16]. However, recent measurements by Underwood et al., [22] indicate that even in prokaryotic cell-free systems, ribosomes do add 1–2 amino acids per second, which is significantly slower than living prokaryotic (20 amino acids per second) and eukaryotic cells (4–9 amino acids per second). The Pure system synthesis rate is about 1.8 peptide bonds per ribosome per second compared to a rate of about 20 *in vivo* [23]. Table 3 provides comparison of main characteristics of the five expression systems employed in this research in the terms of cloning, selection, genetics, expression and main culture conditions in details.

One of reasons for similar heterologous expression profile for both the wild-type and the mutant His103Leu in eukaryotic expression systems could be the favorable protein synthesis and folding machinery in these host systems. Investigation of expressibility of the wild-type enzyme and mutant His103Leu and observation of the same expression profiles in the studied eukaryotic expression systems and significant difference among those of the prokaryotic systems are the most important findings of this study. Besides, presenting expression of a wild-type and mutant eukaryotic gene in such a broad range of expression systems would be another novelty of this research and attractive to the readers.

Conclusion

The interesting phenomenon of *in vivo* protein solubility enhancement was originally observed in *E. coli* using directed evolution. It is uniquely specific to the prokaryotic systems, while the gene and its mutant exhibited similar activity, specific activity and expression profiles in the eukaryotic expression systems, probably because of presence of chaperones or other folding assistants. The mutant His103Leu or His103Met have the advantage of correct *in vivo* folding after the mutation in the complex environment of the cytoplasm of *E. coli*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2010.12.010.

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