Synonymous codon mutation improved the expression of laccase CotA isolated from Bacillus licheniformis

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Abstract

The codon bias could affect the expression of the target gene. The ribosome moves faster on the rich codons than that of the rare codons, but the expression was not consistent with the rate of the ribosome migration. In this study, some conserved rare codons were detected in the laccase cotA isolated from Bacillus licheniformis. As the codon usage bias between Bacillus licheniformis and E.coli is different, the rare codon in Bacillus licheniformis has become to the rich or common codon in E.coli. Therefore, some synonymous codon mutations were constructed and the results indicated that the substitution of the codons in residues 44, 294 and 455 to the rare codons in E.coli could increase the laccase yield to 1.9, 5.1 and 7.6 folds, respectively. The expression of the double mutations on the residue 44, 294 and 455 was higher than that of the wild type, but lower than that of the single point mutant on the residue 455. In addition, a red fluorescence protein was inserted into the C-terminal of the laccase CotA to indicate the expression of the fusion protein. The results showed that the fluorescence intensity was in keeping with the output of the fusion protein. All of the results indicated that the non-optimal codons were also functional to the protein expression and useful to design the highly expression of the target gene in the heterologous expression system.

Keywords

Laccase; Synonymous mutation; Expression.

1. Background

All of genes were encoded by multiple synonymous codons. The codons usage bias has been identified as the most important factor in prokaryotic gene expression[1]. Many reports have shown that the codons usage of N-terminal could significantly affect the expression of the target gene. The nucleotide sequences surrounding the N-terminal region of the protein appear particularly sensitive, both to the presence of rare codons[2, 3] and to the identities of the codons immediately adjacent to the initiation AUG[4, 5]. Random substitute the first ten codons with synonymous codons of the exocellulase CelEdx-SE301, the synonymous codon variant fusion proteins exhibited 35- to 530-fold increases in functional expression compared with wild-type controls[6]. Rare codons are enriched at the N terminus of genes in most organisms, Daniel[7] measured expression from >14,000 synthetic reporters in *Escherichia coli* and showed that using N-terminal rare codons instead of common ones increased expression by ~14-fold (median 4-fold).

Non-optimal codons can reduce the rate of translation, which could make the polypeptide folding accurately. Non-optimal codon usage of *frq* is essential for its circadian clock function, because codons optimization not only increase FRQ levels but also result in conformational changes in FRQ protein, altered FRQ phosphorylation profile and stability, and impaired functions in the circadian feedback loops[8]. Introduced more non-optimal codons into FibHsp could increase the production and secretion levels of luciferase[9]. Reports have shown that the silent mutations affect *in vivo* protein folding in *Escherichia coli*. Replaced five non-optimal codons of the *Echinococcus granulosus* fatty acid binding protein 1 gene with their synonymous codons, one of the silent mutation markedly

decreased the solubility of the protein when expressed in *Escherichia coli*. For the altered region corresponds to a turn between two short alpha helices[10]. Consecutive rare codons in chloramphenicol acetyltransferase (CAT) gene were substituted by frequent ones, the silent mutagenesis reduced the pauses in translation of CAT in *E.coli* S30 extract cell-free system and led to the acceleration of overall rate of CAT protein synthesis and the silently mutated protein synthesized in the *E.coli* S30 extract system was shown to possess 20% lower specific activity[11].

In this study, we analyzed the codon usage conservation of the laccase gene *cotA* from *Bacillus licheniformis.* As the codon usage bias between *Bacillus licheniformis* and *E.coli* is different, the rare codon in *Bacillus licheniformis* has become to the rich or common codon in *E.coli*. The common codons of laccase gene *cotA* were replaced by rare codons to investigate the effect of rare codons on the expression of laccase in the heterologous expression host *E.coli*. The study will help us to understand of the function of the rare codon and design the new gene with improving the expression of the protein.

2. Material and Methods

2.1 Chemical and media

Auto induction medium was purchased from Biolab. DNA polymerase was purchased from Qiangen. The T4 DNA ligase and the restriction enzyme were purchased from New England Biolabs. The ABTS was obtained from Sigma-Aldrich.

2.2 Bacterial Strains and Plasmids

E.coli BL21(DE3) was used for analyzing the expression of synonymous variant genes, it was purchased from Qiangen. The vector pET30a(+) was conserved in Chinese Academy of Agricultural Sciences.

2.3 Synonymous codon mutation construction

The laccase gene was digested with *Nco*I and *Sac*I restriction enzymes and the red fluorescence protein gene was digested with *Sac*I and *Xho*I restriction enzymes, then ligated into the pET30a(+). The red fluorescence protein was used as a reporter to indicate the expression of the variants. Synonymous codons were introduced by the overlap PCR with the primers in Table S1.

2.4 Protein expression and purification

The laccase genes were cloned into a pET30a(+) expression vector and overexpressed in the BL21(DE3) *E. coli* strain. A single colony of the transformed *E. coli* carrying the laccase gene was cultured in auto induction liquid medium containing 50 μg/mL kanamycin and 0.25mmol/L Cu^{2+} at 30 ℃ overnight, The cells were then collected by centrifugation, re-suspended with 20mM Tris-HCl buffer (pH=8.0) and disrupted by sonication on ice. The pellets were re-suspended with 20mM Tris-HCl buffer (pH=8.0). The insoluble fraction of the broken cells were removed by centrifugation at 4 ℃, 13,000rpm for 10 min and the supernatant was stored at 4 ℃. The fusion protein was purified by affinity chromatography and concentrated by PEG8000.

2.5 Screen the variants with high yield

Variants selected based on red fluorescence and the activity of the supernatant extracted by osmotic shock. The clones were cultured in 96-well plates and every well with 1ml auto induction medium at 30℃ for 21h. The cells were collected by centrifugation, re-suspended with buffer 1 (20mM Tris-HCl, 20% sucrose,pH8.0) then set the cells on ice for 30min, centrifugation and discard the supernatant, re-suspended with buffer 2 (20mM Tris-HCl, 20%,pH8.0), put the cells on ice for 1h,after centrifugation the supernatant was used to screen the variants with high expression at 37℃,pH4.5.

2.6 Determination of the laccase activity

Laccase activity in the supernatant was assayed at 60 ℃ using ABTS as the substrate. The assay mixture contained 200ul ABTS (5mM), 50ul of supernatant, 750ul citrate-phosphate (50mM, pH4.5).

The reactions were incubated at 37 ℃ for 3min. Oxidation of ABTS was monitored by determining the increase in A420 (ε420=36,000 M-1cm-1). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute.

The optimum pH was determined by the standard assay in Na_2HPO_4-citrate buffer(pH2.5-8.0). The effects of temperature were examined at pH4.5. Thermostability was evaluated by incubating the enzyme at 60℃ and 70℃.

3. Results

3.1 The codon usage analysis

The dataset of bacterial genomes with full annotation was downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/) on December 8, 2014, which contained 1723 genomes. The codon usage bias was calculated for each genome. The laccase CotA from Bacillus licheniformis was aligned to all of the protein in the genome dataset and alignment result with codon usage bias was shown in Figure 1. As shown in Figure 1, we could choose the low-frequency codon when the color was blue. However when the color was red, this position tended to choose a high-frequency codon. In this figure we can detected that some regions were conserved with the low-frequency codon usage.

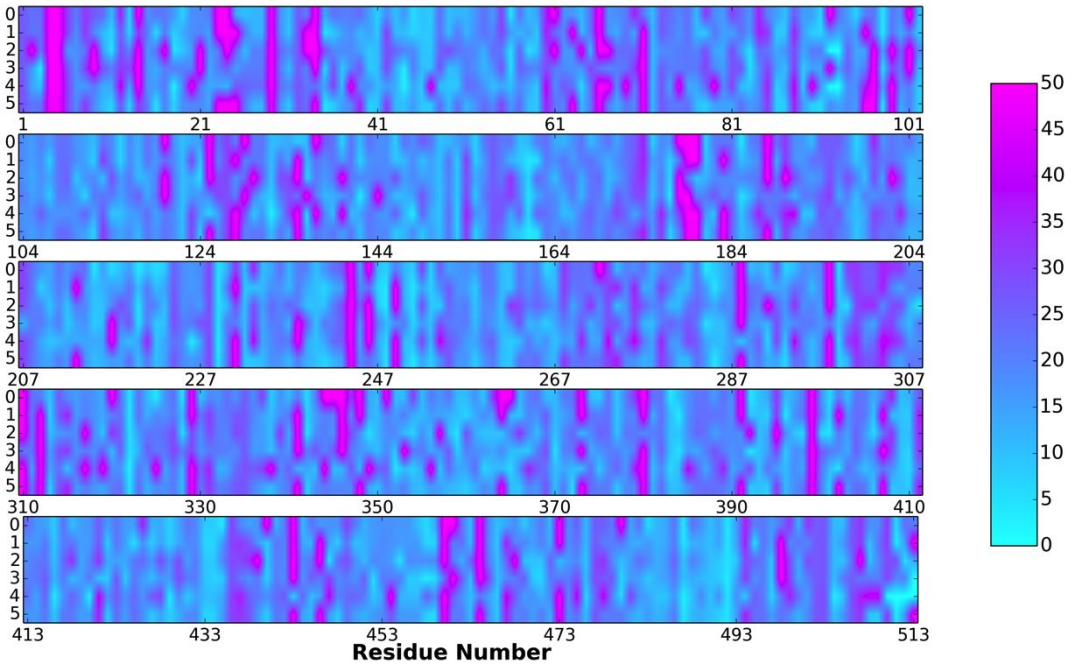

Figure 1. Codon conversation of laccase cotA from Bacillus licheniformis. Every line represents a sequence similar to laccase from Bacillus licheniformis and every column was a codon. The color represents the codon usage frequency of a codon.

Different amino acids were encoded by various synonymous codons and the bacteria have different codons usage bias. However, the folds between the frequencies of the high- and low-frequency codon were similar. As shown in Table 1, the rate of high-frequency codon of the arginine was about 13.94-fold of the rare codons. In addition, the codons such as leucine, isoleucine and proline also show large difference between the high- and low-frequency synonymous codons. So if these amino acids were substituted by synonymous codons, the translation rate will be affected greatly. Based on the codon usage conservation and the amino acid composition, there are three interesting regions (43-45, 294-294 and 454-457) of the laccase CotA, which are enriched as the low-frequencey codons in the native host and amino acids with large folds between the frequencies of the high- and low-frequency.

Table 1. The folds of the synonymous codon usage between the high- and low-frequency codon

AA	Average folds	AA	Average folds
R	13.94 ± 6.13	Q	4.36 ± 3.91
L	12.76 ± 6.85	K	3.91 ± 3.51
I	8.72 ± 6.37	C	3.42 ± 3.15
P	7.99 ± 5.50	D	3.39 ± 2.95
S	7.70 ± 5.77	N	3.29 ± 3.41
T	7.19 ± 5.89	Y	3.18 ± 3.17
V	6.87 ± 5.83	E	3.02 ± 2.66
A	6.04 ± 4.94	H	2.87 ± 2.80
G	5.83 ± 4.41	M	1.00 ± 0.00
F	4.51 ± 4.60	W	1.00 ± 0.00

3.2 The expression of the synonymous codon variants

The synonymous codon mutations at (43, 44, 45, 293, 294, 454, 455, 456 and 457) were constructed by the method of overlapping PCR. The wild type and mutants were expressed in the *E.coli*. The activity was measured with ABTS at pH4.5, 37℃. The reaction was in 96-well plates for 3 min and the osmotic shock cannot break the cells thoroughly. As shown in figure 2, we found that the expression amounts of three mutants with synonymous mutation in these positions at 44, 294 and 455 were higher than the wild type, which were about 1.9-, 5.1- and 7.6- folds higher than that of the wild type. However the other mutants substituted by synonymous codons were not shown the improved expression amount than that of the wild type. As the N-terminal of the laccase was inserted into a red fluorescence protein, the cell with the expressed protein will display as the red color. As shown in Figure 3, we could find the obvious red color in the mutant 294 and 455, but the wild type. In addition to the enzyme activity and red fluorescence, the SDS-PAGE (Figure 5) was also confirmed that the expression of the mutant 294 and 455 was higher than that of the wild type. As a result, the expression of the laccase CotA could be improved by the synonymous codon mutation at the residue 294 and 455.

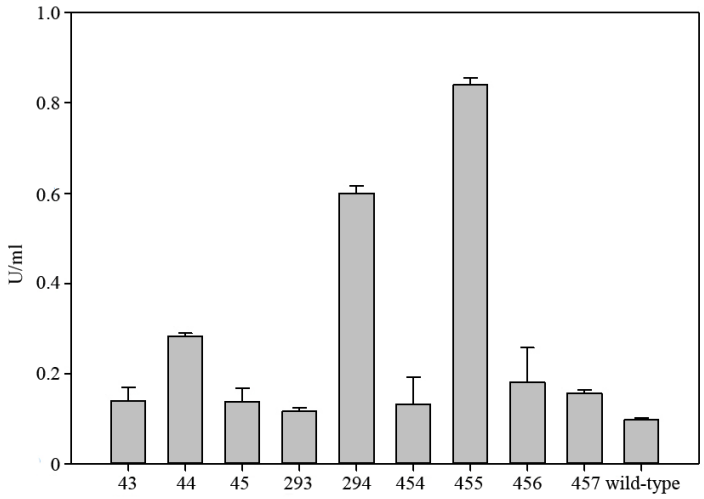

Figure 2. The activity of the supernatant of wild type and its mutants with single point synonymous mutation.

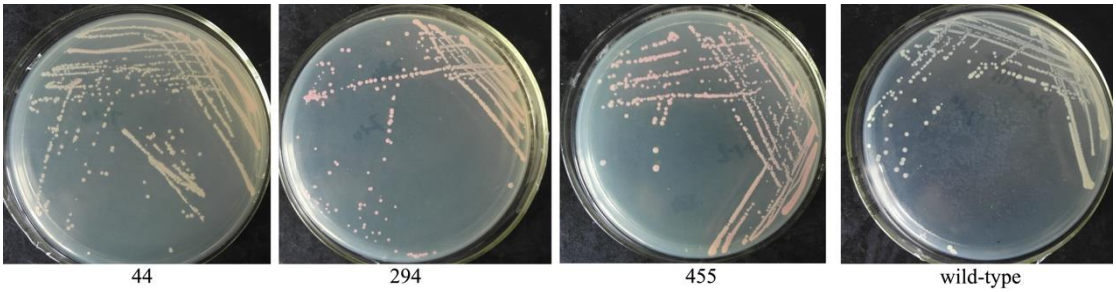

Figure 3. The red fluorescence of the mutants and the wild type.

The color of the red fluorescence depends on the expression of the laccase.

In addition to the single point mutation, the double mutation among the residue 44, 294 and 455 were also constructed. As shown in Figure 4, the expression of the double mutations on the residue 44, 294 and 455 was higher than that of the wild type, but lower than that of the single point mutation on the residue 455. The result also indicated that the suitable number of the rare codon is important to the highly expression of the target gene.

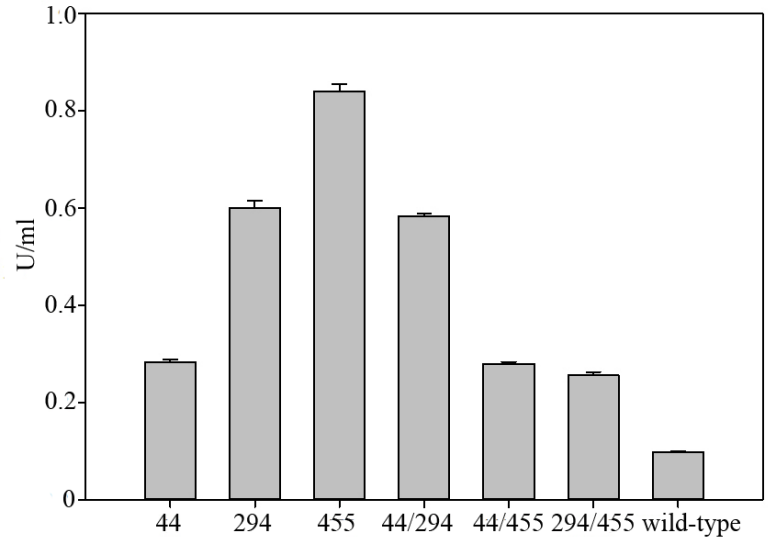

Figure 4. The laccase activity of the supernatant of the wild type and its mutants

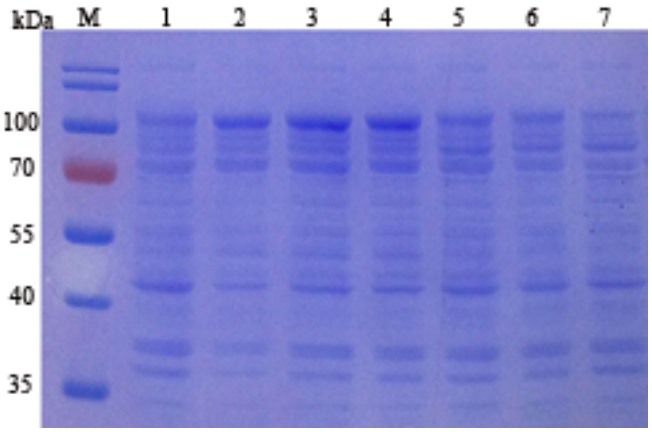

Figure 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant CotA produced in E.coli. Lane M. protein markers. Lane 1.The supernatant of the synonymous codon variant 44. Lane 2. The supernatant of the synonymous codon variant 294. Lane 3.The supernatant of the synonymous codon variant 455. Lane 4 The supernatant of the synonymous codon variant 44/294. Lane 5.The supernatant of the synonymous codon variant 44/455. Lane 6.The supernatant of the synonymous codon variant 294/455. Lane 7.The wild type. The crude enzyme was extracted by the sonication and the target protein is about 105kDa.

4. Discussion

In this study, we found that the synonymous codons could affect the expression of laccase CotA from *Bacillus licheniformis*. The red fluorescence protein was also used as a reporter to indicate the expression of fusion protein. Compared with the other method, we could directly know the yield of the fusion laccase.

Many reports showed that the 5' coding region of a gene transcript are related to protein expression[7]. Because the ribosome encompassed about 15 to 25 nucleotides on the start codon during translation initiation, the 5' coding region of a gene could influence gene expression[12]. In this study, we found that the residues at the N-terminal (residue 44), C-terminal (residue 455) and the middle of the protein (residue 294) could also affect the expression of the target protein. It worth to note that the substitution occurred in 455, the yield was about 7.6-fold higher level than the wild type. Therefore, the position was close to the C-terminal, which also could regulate the expression of the target protein.

The synonymous codon mutation can improve the expression without the change of amino acid. Unlike the mutants with amino acid substitutions, the chemical properties of the mutants with synonymous codon mutation will not be greatly changed. If we just want to improve the expression of the protein but change the character of the protein, this method provides a new idea to design the target protein.

Acknowledgements

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