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Engineering of small sized DNAs by error-prone multiply-primed rolling circle amplification for introduction of random point mutations

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ABSTRACT

Small sized DNAs *per se* or their encoding peptides play various roles in biological systems and for biocatalyst development thus, engineering of those small sized DNAs/peptides is of great interest. By self-ligation of small sized DNAs, circular small sized DNA templates were prepared for error-prone rolling circle amplification using multiply-primed random hexamers to create tandem repeats of small sized DNAs and simultaneous introduction of random point mutations into those tandem repeats of small sized DNAs. We applied this method to randomize the signal peptide of a glucoamylase in recombinant *Saccharomyces cerevisiae*. Random point mutations were efficiently introduced into small sized DNA encoding the signal peptide of glucoamylase and the resulting recombinant *S. cerevisiae* with beneficial point mutations in its signal peptide was able to secrete ca. 30% more glucoamylase than that with native signal peptide.

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1. Introduction

Small sized DNAs *per se* or their encoding peptides such as bacterial promoters [1], ribosomal binding sites [2], signal peptides, cofactor and substrates binding pockets of enzymes [3], and peptide hormones [4] play important roles in various biological systems. Engineering of those small sized DNAs or their encoding peptides for improved performance is of high interest. Some example are, the fine-tuning of expression level of target proteins/enzymes by modifying the strength of bacterial promoters, the engineering of signal peptides of proteins for high level of secretion, and change of the binding pockets of enzymes for a shift of substrate and cofactor specificity, etc.

Although various methods on biomolecular engineering have been developed [5–8], it is still challenging to engineer small sized DNA or its encoding peptide. Saturation mutagenesis is normally employed to randomize specific positions of DNAs or proteins for improved properties. By randomization using NNN for non-coding DNA or NNS for coding DNA at each position (where N denotes A, T, C, or G; S stands for C or G), a huge library will be created even for a small sized DNA template, which is challenging to screen [5,6]. At present, error-prone PCR (ep-PCR) is a commonly practiced method for randomization of target DNA sequences by taking advantage of the low fidelity of *Taq* DNA polymerase and the ability to efficiently introduce point mutations to DNA templates without any background information needed [7–9]. However, it requires the usage of a thermal cycler and optimizing the thermal cycling.

Recently, rolling circle amplification has emerged as an alternative method for biomolecular engineering due to its advantages over the conventional ep-PCR based evolution methods [10–12]. Rolling circle amplification (RCA) has been previously employed to amplify the whole genome of organisms and circular DNA templates [13,14] and recently explored to directly evolve enzymes (the encoding genes cloned in a plasmid) [10,11]. By taking advantage of the strand displacement DNA synthesis of Phi-29 DNA polymerase, RCA of circular DNA template with multiply-primed random hexamers will generate multiple replication forks. As RCA proceeds and product strands are rolled off the template as tandem copies of the circle. The advantages of Phi-29 DNA polymerase over normal DNA polymerase are its high capacity to perform strand displacement DNA synthesis for more than 70,000 nt without dissociating from the template and its stability for efficient DNA synthesis for many hours [13,15]. Besides that, no specific primers are required in RCA because random hexamers are universal primers for any DNA template. Most importantly, RCA is an isothermal amplification process and can perform at a constant temperature (30 °C), thus no optimization of thermal cycling conditions and thermal cycler are required [10,13].

In light of this, RCA was attempted in this research as an alternative to normal ep-PCR based directed evolution methods to introduce random point mutations to the small sized DNAs. To create tandem repeats of the small sized DNAs, the small sized DNAs

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themselves were initially circularized by self-ligation with T4 DNA ligase, followed by isothermal amplification using random primers and Phi-29 DNA polymerase. By including $MnCl_2$ during RCA, random point mutations were introduced into the tandem repeats of the small sized DNA units. To demonstrate that this strategy works, we applied RCA to the evolution of the signal peptide of glucoamylase from *Saccharomyces fibuligera* [16]. Signal peptides are known to control the entry of virtually all secretory proteins to the secretory pathways in both prokaryotes and eukaryotes [17–20] in which the NH_2-terminus of pre-protein is cleaved off while the mature protein is translocated through the membranes [18].

The pre-protein of mature glucoamylase from *S. fibuligera* which catalyzes a one-step depolymerization of starch to glucose is formed inside cells and contains a signal peptide of 27 amino acid residues encoded by 81 bp DNA. Our hypothesis is that the mutated signal peptide will significantly affect the interaction between the cellular machineries which in turn leads to the different secretion levels of the mature enzyme to the culture media. The glucoamylase gene containing the randomized signal peptide was cloned into *Saccharomyces cerevisiae*. The resulting recombinant *S. cerevisiae* containing glucoamylase gene with beneficial point mutations in its signal peptide secreted more mature glucoamylase into the culture. By standard starch-iodine colorimetric assay using 96-well plates, the beneficial point mutations in the signal peptide of glucoamylase were identified from recombinant *S. cerevisiae*.

2. Materials and methods

2.1. Microorganisms, plasmids and culture medium

Escherichia coli TOP10 (F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G, Invitrogen) was used for plasmid maintenance, propagation and storage. *S. cerevisiae* INVSc1 strain (MATa *his3Δ1 leu2 ade2-1 trp1-289 ura3-52*) from Invitrogen (Carselberg, CA, USA) was employed as host for yeast transformation. Yeast plasmid pRS424 (New England Biolabs, Ipswich, MA) was used as backbone for cloning glucoamylase gene. *E. coli* cells were cultivated in LB broth and appropriate antibiotic was supplied where necessary. Yeast complex medium, YPAD, containing 1% yeast extract, 2% peptone, 0.01% adenine hemisulfate, and 2% dextrose was used to culture yeast cells. After transformation, yeast cells were plated onto proper synthetic complex dropout media or agar plates containing 0.7% Difco nitrogen base, 2% glucose and supplemented with appropriate 0.1% synthetic complete dropout mix without Trp from Sigma (St. Louis, MO).

2.2. Reagents

T4 polynucleotide kinase, T4 DNA ligase and Phi-29 DNA polymerase were from New England Biolabs (Ipswich, MA). The oligos were synthesized by IDT DNA (Coralville, IA) and the random hexamers, 5′-NpNpNpNp^{s}Np^{s}Np-3′, were synthesized by Sigma and thiophosphate-modified to prevent degradation during RCA due to the 3′-5′exonuclease activity of Phi-29 DNA polymerase.

2.3. Design of the synthetic oligos

The long oligo, 5′-aca taa aca aac aaa atg aaa ttc ggt gtt tta ttt tcc gtc ttt gct gct att gtt agt gct tta cct ttg caa gaa ggt cct ttg aac aaa aga gcc tat cct tct ttt g-3′ (underlined sequences are derived from pRS424-GLA1 for later subcloning by PCR into this vector by homologous recombination to create pRS424-sp-GLA1), were synthesized. Besides the flanking sequences underlined, the remaining sequence contains 81 nt encoding 27 amino acid residues of the signal peptide of glucoamylase.

2.4. Phosphorylation of 5′ end of oligos

Oligos were phosphorylated at 5′ ends with T4 polynucleotide kinase to facilitate subsequent self-ligation by following the protocol developed by Fire and Xu [21]. In brief, the phosphorylation reaction contained 10 μM oligos and 2 U polynucleotide kinase in 1× kinase buffer. The mixture was incubated at 37 °C for 2 h. The enzyme was inactivated at 70 °C for 15 min during the post incubation. The reaction was extracted by phenol–chloroform and the resulting oligos were ethanol-precipitated, air-dried and resuspended in 30 μl H_2O.

2.5. Circularization of the phosphorylated oligos

The phosphorylated oligos was circularized by self-ligation using T4 DNA ligase to create the circular templates for RCA. To facilitate self circularization of the phosphorylated oligos, a helper oligo (5′-ttt cat ttt gtt tag aag gat agg c-3′) and dilute annealing method described elsewhere [21] were used in this reaction. The reaction was incubated at 95 °C for 2 min to denature the secondary structure of the long oligos. The denatured oligos were slowly cooled down to 4 °C to facilitate the helper oligos to anneal to the denatured long oligos, followed by adding 2 U T4 DNA ligase and incubating at 16 °C overnight.

2.6. Ep-RCA with Phi-29 DNA polymerase

The ligation reaction was extracted with phenol-chloroform to remove enzymes followed by ethanol precipitation. The resulting circularized oligos were diluted and used as templates for rolling circle amplification using multiply-primed random hexamers. The 10 μl reaction was set up which containing 1 ng of circularized DNA templates, 2 μl of 10 μM random hexamers and 1 mM each dNTPs in 1× reaction buffer (New England Biolabs). The mixture was incubated at 95 °C for 3 min, then slowly cooled down to 4 °C. The reaction was initiated by adding 0.2 μl of 5 U/μl Phi-29 DNA polymerase and 1 μl of 15 mM $MnCl_2$ and incubated at 30 °C for 24 h. Post amplification, 2 μl of reaction was loaded onto 0.8% agarose gel and visualized under UV.

2.7. Subcloning of individual copies of small sized DNA units into yeast overexpression vector

A yeast overexpression vector, pRS424-GLA1, was firstly constructed by subcloning yeast TDH3 promoter and CYC1 terminator together with the GLA1 gene [16] which encodes mature glucoamylase without signal peptide sequence into pRS424 by known homologous recombination in yeast. To prepare cassette TDH3p-GLA1-CYC1t, the endogenous TDH3 promoter and CYC1 terminator were amplified from the genomic DNA of *S. cerevisiae* using oligos TDH3p-for (5′-acg act cac tat agg gcg aat tgg gta ccg gat act agc gtt gaa tgt tag-3′) & rev (5′-tga ata agc ttc aaa aga agg ata ggc gga tcc ttt gtt tgt tta tgt g-3′, *BamH* I underlined) and CYC1t-for (5′-ta agg tca agg ctt tgg ctt aaa cag gcc cct ttt cct ttg tcg-3′) & rev (5′-caa aag ctg gag ctc cac cgc ggt ggc ggc gca aat taa agc ctt cga-3′), respectively, then overlapped with GLA1 (without leading DNA sequence encoding the signal peptide) which was amplified using oligos GLA1-for (5′-aaa cac aca taa aca aac aaa gga tcc gcc tat cct tct ttt ga-3′, *BamH* I site underlined) and rev (5′-gac aaa gga aaa ggg gcc tgt tta agc caa agc ctt gac c-3′) by overlap extension PCR (OE-PCR). For subcloning the gene cassette into pRS424, we employed a method for rapid assembly of DNA fragments into yeast developed previously [22].

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