Special Feature: Biotechnology

Research Report

Engineering of Biomass-degrading Enzymes Using a Cell-free Expression System

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ABSTRACT We attempted to increase the catalytic activity of biomass-degrading enzymes, with the aim of developing low-cost enzymatic saccharification systems for biorefinery applications or biofuel production. We have already developed a novel protein library construction and screening system named SIMPLEX (single-molecule-PCR-linked *in vitro* expression). In this study, in order to solve the problem of misfolding enzymes in cell-free synthesis, a functional expression system was developed using chaperone co-expressed *E. coli* extract in which redox conditions were optimized. As a result, homo-dimeric beta-glucosidases (BGL), disulfide-containing cellobiohydrolases, endoglucanases, and lignin-degrading heme protein were successfully synthesized in their active forms. Our novel expression system allows high-throughput construction and screening of a large diversity of mutant enzymes, and makes it possible to improve enzymatic function efficiently. For instance, here we improved beta-glucosidase activity under acidic conditions using novel evolutionary protein engineering approaches.

KEYWORDS Biomass-degrading Enzyme, Protein Engineering, Beta-glucosidase, Enzymatic Saccharification, Biorefinery, Biofuel

1. Introduction

Cell-free protein expression systems allow for the rapid production of proteins directly from plasmids or polymerase chain reaction (PCR)-amplified DNA templates. Cell-free expression can be completed within a few hours. Easy modification of the composition of the reaction mixtures is another advantage of the system. Cell-free expression systems can be applied to protein engineering with the benefits described above. Recently, we developed a new evolutionary protein engineering approach called SIMPLEX (single-molecule-PCR-linked *in vitro* expression) for the purpose of changing protein function and stability.(1,2) A schematic of SIMPLEX is shown in **Fig. 1**. A pool of mutated genes is diluted to one molecule per well in 384-well plates and amplified individually by PCR. The mutated gene library is used as a template for *E. coli* cell-free expression, resulting in a mutated protein library. Subsequently, the protein library is screened with the desired conditions. A portion of the mutated gene library is used as a template for cell-free expression, resulting in a mutated protein library. Linkage of the genotype (PCR

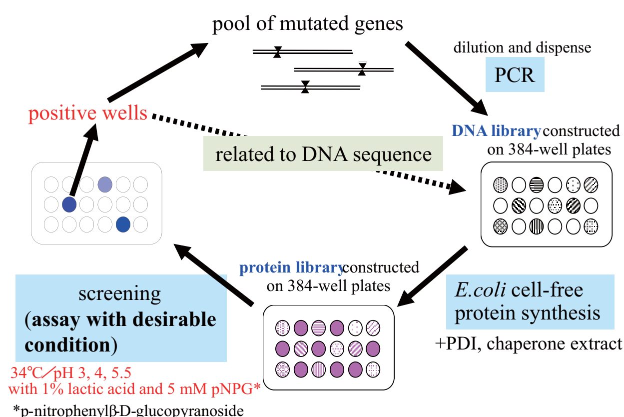

Fig. 1 Schematic showing simple steps for protein library construction using SIMPLEX.
The pool of mutated genes is diluted to one molecule per well in 384-well plates and amplified individually by PCR. The mutated gene library is used as a template for cell-free expression, resulting in a mutated protein library. Various markings represent various proteins in the library. Subsequently, the protein library is screened for desired properties.

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products) with phenotype (*in vitro* synthesized protein) by the position of wells on a plate makes it possible to screen a large diversity of mutant proteins without using living cells. However, misfolding is a serious problem when expressing heterologous proteins in an expression system.

In our recent work, L-lactic acid was produced by metabolically engineered yeast that expressed exogenous L-lactate dehydrogenase. Additionally, the beta-glucosidase (BGL) enzyme was expressed on the cell surface of a lactic-acid-producing *Saccharomyces cerevisiae* strain to enable lactic acid fermentation with cellobiose.[3,4] Due to enzymatic saccharification, cellobiose is a main hydrolysate from lignocellulosic biomass. BGL-displaying yeast can convert cellobiose into glucose as a substrate for lactic acid fermentation.[5] In such cases, BGL is likely to be exposed in the culture medium in the presence of lactic acid. Optimization of BGL for appropriate culture conditions (pH 3.0-5.0 and 30-35ºC) is necessary.

To address these issues, we first focused on the establishment of a functional cell-free expression system. We then aimed to improve *Thermotoga maritima* BGL activity under both acidic pH and low temperature conditions using an improved SIMPLEX method.[6]

2. Experimental

2. 1 Construction of Chaperone Co-expressed *E. coli* Strain

The full-lengths of the GroES-GroEL operon, the DnaK-DnaJ operon, and GrepE genes were amplified from *E. coli* genomic DNA. The plasmid pBAD-SLEKJ, which expresses these chaperon genes under the control of the arabinose promoter, was constructed as follows. First, the GroES-GroEL operon was amplified using primers GroE-NcoN (CATACCCAT GGGCAATATTCGTCCATTGCATGAT) and GroE-Xba-Xho-Hind-PmeC (GGAGACCGTTTAAACAA GCTTCTCGAGTCTAGATCACATCATGCCGCCC ATGCCACCCA), and cloned into the NcoI/PmeI sites of the pBAD-TOPO (Invitrogen) plasmid. The resulting plasmid, pBAD-SL, possesses the arabinose promoter, rbs, the GroES-GroEL operon, and a XbaI-XhoI-HindIII-PmeI MCS. Second, the GrepE gene was amplified using primers grpE-XbaN (ATATT CTAGAATACGAATTTAAGGAATAAAGATAATG AGTAGTAAAGAACAGAAAACGCCTGAGGGGC AA) and grpE-XhoC (GCGCCTCGAGTTAAGCTTT TGCTTTCGCTACAGTAACCAT), and cloned into the XhoI/XbaI sites of pBAD-SL. Finally, the DnaK-DnaJ operon was amplified using primers DnaKJ-RBS-F2 (GGAGACCAAGCTTAATAATTTTGTTTAACTTT AAGAAGGAGATATACATACCCATGGGTAAAAT AATTGGTATCGACCTGGGT) and DnaKJ-PmeC (GGAGACCGTTTAAACTCAGCGGGTCAGGTCG TCAAAAAACTTCTTCA), and cloned into the HindIII/PmeI sites of pBAD-SLE. The plasmid pBAD-SLEKJ was transformed into an *E. coli* strain.

2. 2 *In vitro* Coupled Transcription/translation

The 1,4-beta-glucosidase (EC3.2.1.21, BGL) genes (Genebank: X74163 and AE001690), which belong to the 1 and 3 GHF (Glycosyl Hydrolase Family) classification, were amplified from *Thermotoga maritima* genomic DNA (ATCC 43589). Each gene was cloned into NdeI/Hind II of the pET23b(+) (Novagen) plasmid. The resulting plasmid possesses a T7 promoter, rbs (ribosome-binding site), and T7 terminator. The plasmids were amplified from the T7 promoter to the T7 terminator, and then used as cell-free expression templates. Similarly, GHF1 BGL genes from *Bacillus circulans* (M96979), *Bacillus polymyxa* (M60210), and *Streptomyces sp.* (Z29625), and GHF3 BGL genes from *Aspergillus aculeatus* (D64088) and *Phanerochaete chrysosporium* (AF036873), were cloned into the pET23b(+) plasmid. In addition, we collected fourteen cellobiohydrolase (CBH) genes from five microorganisms, *Trichoderma reesei*, *Phanerochaete chrysosporium, Aspergillus aculeatus*, *Aspergillus oryzae*, and *Clostridium thermocellum*, which belong to the 5, 6, 7, 9 GHF classification. Also, we collected twenty endoglucanase (EG) genes from six microorganisms, *Trichoderma reesei, Phanerochaete chrysosporium*, *Aspergillus niger*, *Humicola insolens*, *Chaetomium globosum*, and *Clostridium thermocellum,* which belong to 5, 6, 8, 9, 12, 45, 61 GHF classification. Lignin-degrading heme protein, MnP (manganese peroxidase), and lignin peroxidase from *Phanerochaete chrysosporium* were also prepared.

BGLs were synthesized by an *E. coli* cell-free protein expression system using an S30 extract, which was prepared from chaperone co-expressed *E. coli* cells described above. 50 μg/ml DNA template was added into a 10 μl transcription/translation reaction mixture (56.4 mM Tris-acetate, pH 7.4; 1.2 mM ATP; 1 mM each of GTP, CTP, UTP; 40 mM creatine

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phosphate; 0.7 mM each 20 kinds of amino acids; 4.1 %(w/w) polyethylene glycol 6000; 35 µg/ml folinic acid; 0.2 mg/ml *E. coli* tRNAs; 36 mM ammonium acetate; 10 mM $Mg(OAc)_2$; 100 mM KOAc; 0.15 mg/ml creatine kinase; 10 µg/ml rifampicin; 25 units T7 RNA polymerase (TaKaRa, Japan); 28.3% (v/v) S30 extract; 1.0 mM GSH; 0.1 mM GSSG, and 0.5 µM fungal PDI from *Humicola insolens* KASI[7]), followed by incubation at 26ºC for 180 min.

2. 3 Screening of the BGL Library under Both Acidic and Low Temperature Conditions

Compared with standard PCR, error-prone PCR was performed using the full-length *Thermotoga maritima* BGL gene (X74163, GHF1) as a template to introduce random mutations, modified by 1.0 mM $MnCl_2$, 7.0 mM $MgCl_2$, 1.0 mM dCTP and dTTP, and 5 U/100 µl Taq DNA polymerase. The reaction was allowed to proceed for 25 cycles of 94ºC for 30 sec, 50ºC for 30 sec, and 72ºC for 1.5 min.

The pool of mutated BGL genes was amplified using primers F1-TmBGL2 (GCGATGAGGCAGTCAGCCTAAAAATCTCGATCCCGCGAAATTAATACG and R1-TmBGL2 (GCGATGAGGCAGTCAGCCTAAAATCCGGATATAGTTCCTCCTTTCAG), both of which contained a 5' homo-tail sequence (underlined) of 23 bases. Homo-tailed PCR products were precipitated with ethanol and used for the PCR. A single primer (GCGATGAGGCAGTCAGCCTAAAA) was used to avoid formation of primer-dimers.[8,9] The homo-tailed templates were diluted to one molecule/µl in 0.1% blue dextran 2000/TE buffer. The mixture for PCR was comprised of one molecule of template, 0.02 units of LA Taq polymerase (TaKaRa, Japan), 0.2 mM each dNTP, and 0.5 µM of the primer at 7 µl/well in a 384-well plate. The reaction was allowed to proceed at 94ºC for 2 min, followed by 65 cycles of 96ºC for 10 sec, 62ºC for 5 sec, and 72ºC for 100 sec. A 3 µl portion of the amplified products (containing ~500 ng) was directly transferred to 37 µl of the transcription/translation reaction mixture, followed by incubation for 180 min at 26ºC. All screening steps were carried out in 384-well plates. Then a 1 µl volume of the synthesized reaction mixture was added to 79 µl of BGL assay buffer comprising 2 mM pNPG (p-nitrophenyl beta-D-glucopyranoside, Sigma-Aldrich), 100 mM sodium lactate buffer, pH 3.0, 4.0, and 5.5. After incubation at 35ºC for 30 min, 20 µl volume of 1N sodium hydrate was added to stop the reaction, and the absorbance was measured at 405 nm.

3. Results and Discussion

3. 1 Improved Cell-free Expression System

An important issue is whether proteins are expressed in their active forms in the screening system. First, we attempted to express the heme-containing protein MnP with four disufide bridges.[2] In the preparation of *E.coli* S30 extract for MnP synthesis, a reducing agent, dithiothreitol (DTT), was omitted to maintain oxidizing conditions for the formation of the disulfide bridges of the protein, because functional MnP was not formed in the presence of a strong reducing agent, 1.7 mM DTT (data not shown). Also, hemin was added to the cell-free expression mixture to supply heme for MnP. To improve the MnP production system, the conditions for the reaction were optimized. To enhance the yield of functional MnP, reduced and oxidized glutathione (1.0 mM GSH and 0.1 mM GSSG), 0.5 µM protein disulfide isomerase (PDI), and chaperones (1.0 µM DnaK, 0.4 µM DnaJ, 1.25 µM GroE, and 0.4 µM GrpE) were added to the *in vitro* expression mixture (**Fig. 2**). When fungal PDI was present in the reaction mixture, the MnP activity increased up to 4-fold compared with the case of a

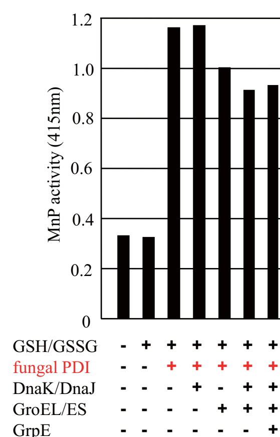

Fig. 2 Improvement of the MnP activity of the transcription/translation product synthesized with various kinds of molecular chaperones and PDI.
Chaperones and PDI were added to the reaction mixture as described in the experimental section (+, added; -, not added). 200 µl of the product was concentrated with 40 µl of His-tagged beads, and then the activity of MnP on the beads was measured.[2]

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control mixture without PDI or chaperones. The addition of chaperones (DnaK/J, GroES/EL, and GrpE) hardly changed the MnP activity. PDI is known as a catalyst for the formation of native disulfide bonds of nascent peptides located in the endoplasmic reticulum of eukaryotic cells. PDI has both isomerase and chaperone activities *in vitro*[10] and it assists the folding of denatured and reduced disulfide-containing proteins with broad protein substrate specificity. Therefore, PDI should facilitate the correct formation of disulfide bonds, resulting in a high MnP activity. Eventually the enzymatic activity of MnP produced in the reaction mixture was increased sufficiently to be measured directly without concentration in the screening system.

Secondly, we tried to express homo-dimeric BGL from *Streptomyces sp*. (SpBGL). Although it does not contain disulfide bridges, functional SpBGL was not formed. Therefore, the effect of chaperones on the synthesis of SpBGL was examined. S30 extract from heat-treated *E. coli* or chaperone co-expressed *E. coli* were evaluated in terms of the functional cell-free expression system. Heat treatment was performed by incubating cells at 42 ºC for 30 min to induce endogenous heat shock proteins. Chaperone co-expressed cells contain the plasmid pBAD-SLEKJ which has GroES-GroEL, DnaK-DnaJ, and GrepE genes. A SpBGL gene was used for the template of cell-free protein expression using S30 extracts described above. Results are shown in **Fig. 3**. In the case of using extract from untreated cells, the BGL activity slightly increased with incubation time. In the case of using heat-treated extract, the BGL activity was twice that of the untreated one. In contrast, chaperone co-expressed extract showed much higher activity, 16-fold over the control value.

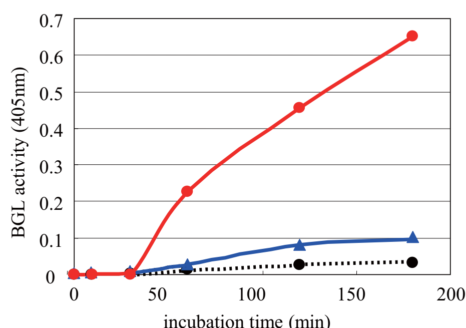

Fig. 3 Effect of S30 extracts on a BGL activity. *Streptomyces* BGL, a homodimeric protein, was used for the template of cell-free expression using three kinds of S30 extracts. BGL activities were measured using chaperone co-expressed (red), heat treated (black), and untreated (blue) S30 extracts.

Moreover, using the improved cell-free expression system described above, disulfide containing proteins and unfolded proteins were successfully folded. In this system, disulfide-containing CBHs, EGs, homo-dimeric BGLs, and the lignin-degrading heme protein MnP were successfully synthesized in their active forms. We found that 39 out of 43 enzymes were functionally expressed.

3. 2 SIMPLEX Screening for the BGL Library

About 10^4 of randomly mutated *Thermotoga maritima* BGLs which were expressed in 384-well plates independently were screened by measuring the BGL activity in the presence of 100 mM sodium lactate buffer, pH 3.0, 4.0, and 5.5. Twenty-three wells were selected as having improved activities compared with the wild type. The PCR products in the twenty-three positive wells were directly cloned into the pT7-Blue3 blunt vector (Novagen), and each clone was amplified in triplicate again to prepare individual templates for cell-free expression. BGL activity studies were carried out, and among them, 16 clones showed improved BGL activity under acidic conditions (**Fig. 4**a). The clones were classified into two groups. The BGL activity of clones 1-53, 1-64, 1-89, and 1-91 were two times higher than that of the wild type at pH 3.0, pH 4.0, and pH 5.5. On the other hand, clones 1-32, 1-33, and 1-35 showed higher BGL activity at acidic pH, but lower activity at pH 5.5. Therefore, two typical clones, 1-91 and 1-33, were selected and used to amplify the templates for the next round of a screening. Error-prone PCR was performed to introduce random mutations in order to construct the 2nd BGL library. About 10^4 samples were screened in the same manner as the 1st screening. As a result, eleven clones showed improved BGL activity under acidic conditions compared with the wild type (**Fig. 4**b).

3. 3 pH-dependent Activities of Mutant Enzymes

Among the eleven clones shown in Fig. 4b, the top two clones, 2-12 and 2-93, were selected and were purified by absorption on His-tagged beads (Qiagen). Then pH dependence studies were carried out (**Fig. 5**).

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The amino acid substitutions of the clones are summarized in **Table 1**. BGL activities of clones 2-12 and 2-93 at pH 4.0 were four times higher than that of the wild type. These two clones have common mutation sites of L130Q/V169D. Clone 2-12 has two other mutations V205A and A385T; in fact, this suggests that 2-12 is derived from clone 1-33. Also, the A382T substitution of clone 2-93 indicates that it is derived from clone 1-91. Even though clones 2-12 and 2-93 have common mutations of L130Q/V169D, these clones were selected from different sources of the library. On the other hand, clones 2-12, 2-93, 2-26, and 1-33 have a common substitution at position 169. V169 was changed to either D or A. These clones showed an optimum pH at 5.0, which is 0.5 pH units lower than that of the wild type. Clones 2-12 and 2-93 showed not only altered enzymatic activity, but a shift in pH optimum. These data suggest that the V169D substitution plays an important role in BGL activity at an acidic pH. Since the L130Q/V169D substitutions had converged from different sources of the library as a result of selection, this high-throughput screening system is likely to work well.

Clones 2-3, 2-30, and 1-91 showed increased activity over a pH range of 3.0-6.5, but the same pH specificity as wild-type BGL. These clones have common mutations of L130Q/A382T. We assume that the L130Q/A382T substitutions are important to alter the BGL activity over a broad range of pHs.

4. Conclusions

We have optimized an *E. coli in vitro* coupled transcription/translation system to express proteins functionally, which made it possible to apply enzymes to SIMPLEX. Hence, we have successfully increased the catalytic activities and acid stabilities of BGL using

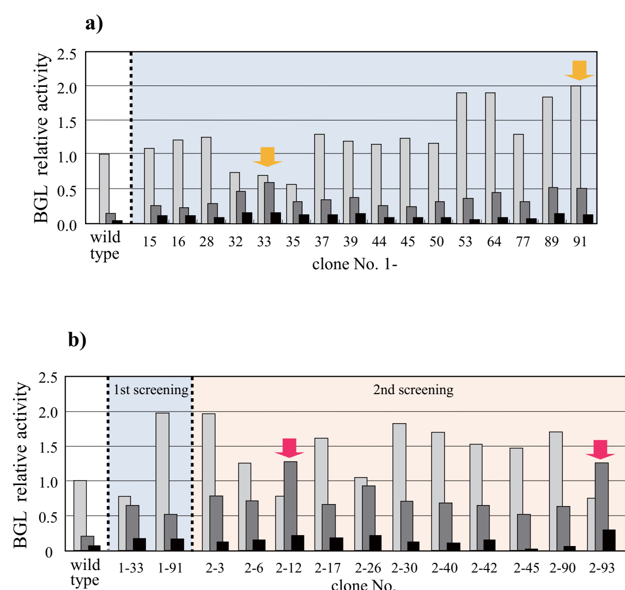

Fig. 4 BGL activity of positive clones.
a) BGL activity of positive clones after the 1st screening.
BGL activities of wild type and clones were assayed at pH 3.0 (black), pH 4.0 (gray), and pH 5.5 (light gray). Orange arrows show two typical clones.
b) BGL activity of the top 10 clones after the 2nd screening.
We screened randomly mutated 1-33 and 1-91 mixture gene libraries in the same manner as the 1st screening. BGL activities of wild type and clones were assayed at pH 3.0 (black), pH 4.0 (gray), and pH 5.5 (light gray). Pink arrows show the top two clones at pH 4.0.

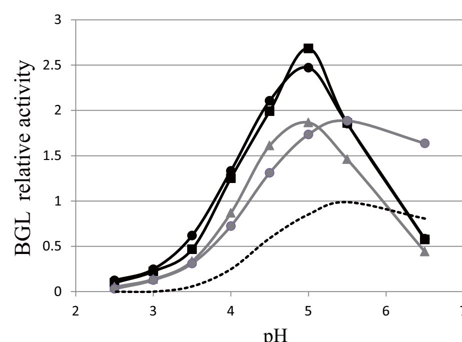

Fig. 5 pH dependence of positive clones.
BGL activities of wild type (broken line, black), clone 1-33 (closed triangles, gray), and 1-91 (closed circles, gray) from the 1st library, and 2-12 (closed squares, black) and 2-93 (closed circles, black) from the 2nd library were assayed over pH 2.5-6.5.

Table 1 Amino acid substitutions of mutants.

library	source of the Lib	clone No.	130	169	172	187	203	205	213	238	382	385	425	444
wild type	-	-	L	V	I	I	R	V	K	A	A	A	K	L
1st Lib	wild type	1-33	.	D	.	.	.	A	.	.	.	T	.	.
	wild type	1-91	Q	.	.	.	.	.	.	.	T	.	.	.
2nd Lib	1-33	2-12	Q	D	T	.	.	A	E	.	.	T	.	.
	1-91	2-93	Q	D	.	V	.	.	.	.	T	.	.	.
	1-91	2-26	Q	A	.	.	.	.	.	.	T	.	.	.
	1-91	2-30	Q	.	.	.	.	.	.	S	T	.	E	.
	1-91	2-3	Q	.	.	.	G	.	.	.	T	.	.	P

(Columns 130–444: amino acid No.)

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the improved SIMPLEX method. In other instances, we have increased the H_2O_2 stability of MnP in a manner similar to BGL screening.[2] A mutant MnP library containing three randomized amino acid residues located in the H_2O_2-binding pocket was designed and constructed in 384-well plates using SIMPLEX. The screening of 10^4 samples independently expressed for improved H_2O_2 stability led to four positive mutants, the H_2O_2 stability of which were nine times higher than that of the wild type.

In addition, in the case of using cellulase-displaying yeast for simultaneous saccharification and fermentation, optimization of enzyme properties for the culture conditions (e.g. acidic pH and growth temperature) is necessary. Improvement of the stability of an enzyme expressed on the cell surface of yeast is required because it is likely to be exposed in the culture medium in the presence of lactic acid. In this study, we succeeded in improving the catalytic activities and acid stabilities of BGL. Thus, the use of acid-resistant BGL-displaying yeasts allows for fermentation from cellulose in acidic conditions. One expected advantage is the prevention of contamination of bacteria in the culture medium because of the acidic pH.

In conclusion, the SIMPLEX method has various benefits as described above. Most importantly, it allows for screening of protein libraries with desired properties. For example, in this study, BGL activities were measured at different pHs simultaneously in the screening. Screening under various conditions enables the selection of various kinds of clones. Our data show that screening with various selective pressures is an important tool for the efficient development of new proteins. SIMPLEX will be a powerful tool that can be widely applied to various kinds of protein screenings with desired properties.

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Fig. 3 and Table 1
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