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Improving enzyme characteristics by gene shuffling; application to β-glucosidase

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Abstract

The genes of family 3 β-glucosidase enzymes consist of five distinct regions; the N-terminal residues, an N-terminal catalytic domain, a nonhomologous region, a C-terminal domain of unknown function and the C-terminal residues. The β-glucosidase genes derived from *Cellvibrio gilvus* (CG) and *Agrobacterium tumefaciens* (AT) have been subjected to gene deletion, truncation and shuffling. The folding information was found to be distributed unevenly across the different regions based on the gene manipulation results. Chimeric enzymes with improved enzyme characteristics were obtained only by gene shuffling at the C-terminal domain.

Keywords: β-Glucosidase; Gene shuffling; Chimeric enzyme; Folding; GroEL/ES

1. Introduction

Several enzymes are utilized on an industrial scale and are essential in maintaining modern life. However, improvement or modification of the individual characteristics of specific enzymes is extremely difficult. When an enzyme with a different characteristic is required, screening to search for a new enzyme with that characteristic has to be undertaken. Gene shuffling provides an alternative way of obtaining enzymes with improved properties [1,2].

We have successfully applied gene shuffling techniques to β-glucosidase [3,4], xylanase [5], aminopeptidase [6] and cell wall hydrolysing enzymes. In this report, we describe the results of gene shuffling on the family 3 β-glucosidases produced by *Cellvibrio gilvus* (CG) and *Agrobacterium tumefaciens* (AT).

β-glucosidases of CG and AT share 40% amino acid sequence homology. However, despite this high degree of similarity, their enzymatic properties are quite distinct. The heat stability of CG is 41°C while that of AT is 67°C. No significant *trans*-glycosylation activity was observed in CG whereas a high level of *trans*-glycosylation activity is evident in AT [7,8].

Based on the amino acid sequence of the β-glucosidase, it was found that the gene is constructed of five regions: the N-terminal residues, an N-terminal catalytic domain, a nonomologous region between

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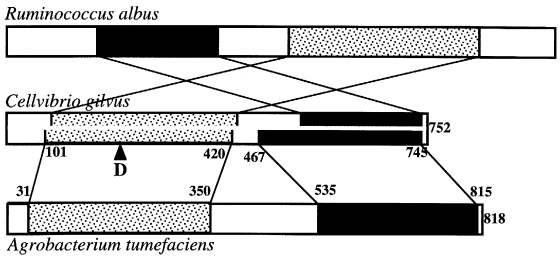

Fig. 1. Homologous regions in amino acid sequence of β-glucosidases.

the N- and C-terminal domains, a C-terminal domain of unknown function and the C-terminal residues. The N-terminal catalytic domain and the C-terminal domain of unknown function from CG showed homology to the C-terminal and N-terminal domains from *Ruminococcus albus*, respectively, as shown in Fig 1. This inversion of the two domains has only been observed previously in two enzymes produced by *Butyrivibrio fibrisolvens* [9] and *R. albus* [10].

Gene deletion and shuffling was applied in the β-glucosidase genes from CG and AT in an attempt to improve the properties of the enzymes. Additionally, it was speculated that the folding information for the enzymes was unevenly distributed across their respective genes.

2. Experimental

2.1. Construction of plasmids

The coding regions of the β-glucosidases of CG and AT were amplified by PCR and cloned into *pET-28a* plasmids (Novagen). DNA sequences of the constructed plasmids were confirmed using a DNA sequencer (Applied Biosystems, model 377).

2.2. Expression and preparation of the target protein

Escherichia coli BL21(DE3) was transformed with the above vectors. The transformants were grown at 25°C in LB medium containing kanamycin (30 μg/ml) or tetracycline (12.5 μg/ml) to an O.D. of 0.6 at 660 nm and the target protein was then induced by addition of 0.4 mM IPTG and incubation for 16 h. After induction, the cells were harvested by centrifugation and subjected to sonication. The chimeric enzymes in an active form were obtained from the supernatant and those in an inactive form were recovered from the insoluble fraction. The histidine-tagged chimeric protein was purified using Ni-NTA agarose (Qiagen).

2.3. Refolding by co-expression with GroEL / ES

The plasmid of *pKY206* [11] carrying the GroEL/ES genes was derived from *pACYC184* and can be stably maintained in cells carrying the *pET-28a* chimeric plasmids and are suitable for the overproduction of the chaperone GroEL/ES with the β-glucosidase [12].

2.4. Refolding by slow dialysis

Protein was dissolved in 8 M urea solution containing 60 mM Tris–HCl buffer, pH 8.6 and then reduced for 2 h at 40°C by addition of 0.5% 2-mercaptoethanol. The reduced solution was then oxidized by the addition of glutathione (oxidized form) at a concentration of 1.5%. The reduced and oxidized solution was then dialyzed at 4°C against 100 mM Tris–HCl buffer, pH 8.0 containing 1 mM EDTA, 0.16% oxidized glutathione, 0.5% mercaptoethanol and 8 M urea, decreasing the urea concentration from 8 to 0 M over 4 days [13]. Refolding of the enzymes was monitored in the supernatant by UV absorbance at 280 nm and was then confirmed by obtaining the CD spectra.

2.5. Assay for β-glucosidase activity

β-Glucosidase activity was measured in 25 mM MOPS buffer, pH 6.5 containing 1 mM *p*-nitrophenyl-β-glucoside as the substrate. A 100-fold improvement in the accuracy of the analysis over the previously reported assay [14] was achieved using a kinetic analysis, monitoring the increase in absorbance at 405 nm at 20-s intervals for 10 min at 30°C using a spectrophotometer (DU-600, Beckman, USA).

3. Results and discussion

3.1. Shuffling at the N-terminal catalytic domain

Two sets of unique restriction enzyme sites, *Bam*HI–*Sac*I and *Bam*HI–*Eco*RI, were selected for gene shuffling at the N-terminal catalytic domain. The *Bam*HI–*Eco*RI site includes a catalytic amino acid aspartate residue, as indicated in Fig. 2.

The amino acid homology in the N-terminal domain of the CG and AT β-glucosidases is 36.4% and that of the shuffling sites of *Bam*HI–*Sac*I (35 amino acid residues) and *Bam*HI–*Eco*RI (132 amino acid residues) are 37% and 39%, respectively. Despite the similarities in the amino acid sequences, when the chimeric genes of the *Bam–Sac* and *Bam–Eco* glucosidases, constructed on a *pET-28a* vector, were over-expressed in *E. coli* BL21(DE3), the chimeric enzymes were obtained as inclusion bodies and were inactive, as shown in Fig. 3.

The molecular chaperone, GroEL/ES has been used to solubilize over-expressed proteins under in vivo conditions [11]. The co-expression of GroEL/ES with the native β-glucosidase genes of AT and CG [12], or a five amino acid deleted mutant of CG [15], was successful in the solubilization of otherwise insoluble proteins. However, solubilizing the over-expressed chimeric β-glucosidases was not successful with co-expression of GroEL/ES, even employing low growth temperatures (20–25°C).

Subsequently, a slow dialysis system was applied and it was confirmed that both parental enzymes successfully refolded into an active form. However, the chimeric enzymes of *Bam–Sac* and *Bam–Eco* showed very unstable activities; activity was less than 2% of the native enzymes and varied from batch to batch. It was found that the presence of 2 M urea in the reaction mixture was the most suitable environment for the expression of enzyme activity in these chimeric enzymes; suggesting that the enzyme conformation is disturbed by gene shuffling.

Another method of obtaining the chimeric enzymes with higher reproducibility has been established; the chimeric enzyme possessing a HisTag peptide was trapped onto a His-Trap column (Pharmacia; 1 ml) and subjected to a urea gradient from 8 to 1 M at a slow flow rate; 0.2–0.4 ml/min (total:

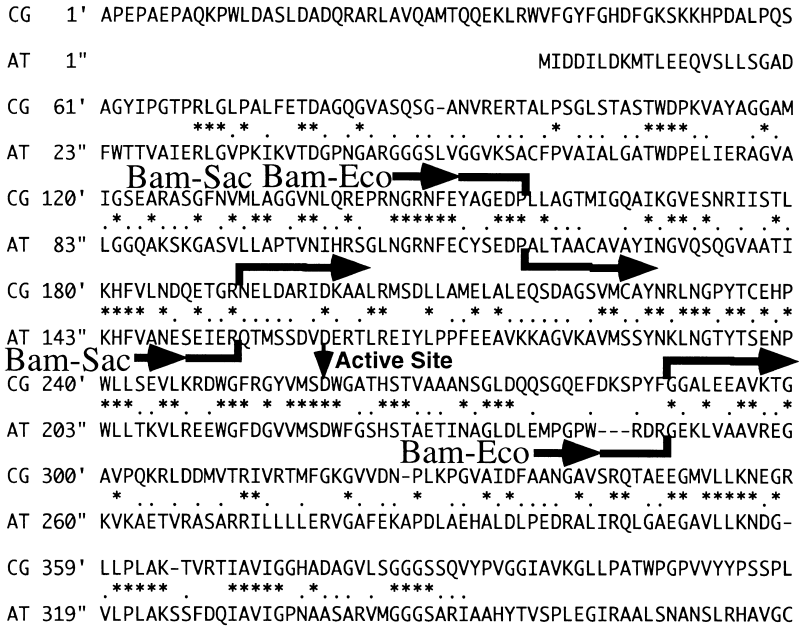

Fig. 2. Construction of two CG-based chimeric β-glucosidase genes.

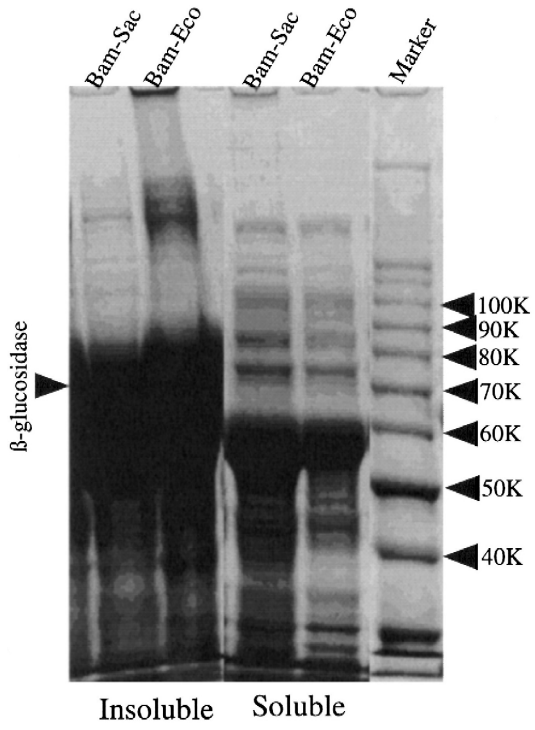

Fig. 3. SDS-PAGE showing the effect of GroEL/ES on the solubilization of chimeric β-glucosidase of *Bam–Sac* and *Bam–Eco*.

30–40 column volumes) using FPLC. The folded proteins were then eluted with 0.5 M imidazole. However, the result was still the same; the native enzymes refolded whereas none of the chimeric enzymes refolded stably.

It appears that the folding information at the N-terminal catalytic domain was disturbed by gene shuffling.

3.2. Truncation of the nonhomologous region between the N- and C-terminal domains

The length of the nonhomologous region between the N- and C-terminal domains is very different for CG and AT; CG possesses only five amino acid residues whereas AT has 162 residues in this region. No homologous sequences for the 162 residues in AT were recognised using data base searching. Mutant enzymes of AT truncated by 4, 21, 62 and 89 (PD-4) amino acid residues were constructed. All of the truncated mutants were expressed in an active form. The enzyme properties of PD-4 were further investigated; *trans*-glycosidation activity present in the native AT enzyme was lost in PD-4. The heat stability of PD-4 was reduced by 10–15°C and the Km value for *p*-nitrophenyl-β-glucoside increased from 0.012 to 0.36 mM. Despite these modifications to the enzyme properties, it is worth noting that all four truncated mutant proteins folded into active forms.

3.3. Shuffling at the C-terminal domain of unknown function

We have previously had success shuffling genes to improve the heat stability of β-glucosidases (1, 2). Chimeric enzyme genes were constructed by replacing varying lengths (39%, 30%, 22% and 8%) of the gene in the C-terminal region of CG with the corresponding AT regions, as shown in Fig. 4. After expressing the shuffled genes in *E. coli*, the kinetic parameters and the heat and pH stabilities of the chimeric enzymes were investigated. While the chromatographic behavior of the chimeric enzymes was the same as that of CG, their enzymatic properties were a mixture of the two parental enzymes. The heat stability of CG is 41°C while that of AT is 67°C. The heat stability of the chimeric enzymes increased by 6–16°C relative to CG as the degree of

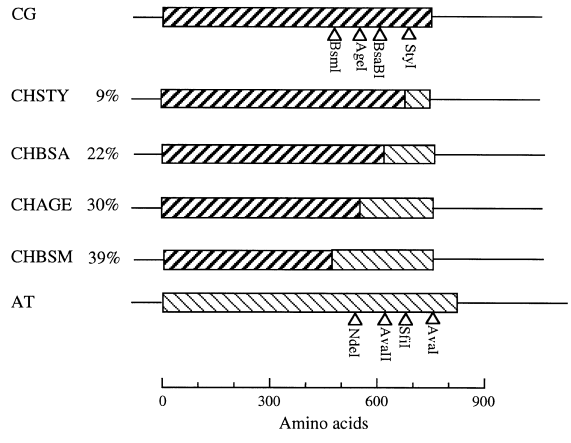

Fig. 4. Preparation of chimeric enzyme genes.

AT incorporation increased from 8% to 39%, as shown in Fig. 5. Interestingly, the yield of the chimeric enzyme with 8.0% AT incorporation was the lowest, probably due to difficulties encountered in protein folding.

3.4. Deletion of C-terminal residues

The extremely low yields observed for the chimeric enzyme shuffled by only 8.0% (60 amino acid residues) in the C-terminal domain indicates the importance of the C-terminal amino acid residues in determining the folding of the enzyme. The deletion of RGRAR residues at the C-terminus resulted in the deleted mutant enzyme denoted as ΔRGRAR. Overproduced ΔRGRAR accumulated in an insoluble form. Lowering the cultivation temperature and IPTG concentration, which slowed down protein synthesis and inhibited the aggregation of the synthesized protein [16], partly improved the solubilization of the native enzyme of CG but did not improve it for ΔRGRAR.

However, co-expression with the molecular chaperone GroEL/ES was quite effective in obtaining ΔRGRAR in a soluble form. The β-glucosidase activity in the soluble fraction of the cells harboring both *pET-dRGRAR* and *pKY206* plasmids reached 835 U/ml after an 8-h induction period. However, the activity for the cells harboring only the *pET-ΔRGRAR* plasmid reached only 52.4 U/ml. These results suggest that co-expression of GroEL/ES is quite effective in folding the deleted mutant protein, resulting in a 16-fold enhancement in the observed enzyme activity.

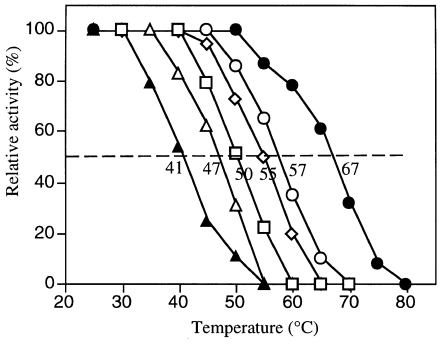

Fig. 5. Construction of four chimeric β-glucosidase genes by shuffling varying length of CG in C-terminal region with AT and heat stability of chimeric enzymes. ▲, CG; △, CHSTY; □, CHBSA; ◇, CHAGE; ○, CHBSM; ●, AT.

Arginine residues are often responsible for forming salt bridges with acidic amino acid residues [17]. The three basic amino acid residues of RGRAR in the C-terminal region may play an important role, as has been reported for the arginine triplet in the last domain of the ATP/ADP carrier protein [18].

No significant differences in the kinetic parameters were observed between the native enzyme and the deleted mutant; the Km values for the *p*-nitrophenyl-β-glucoside of CG and ΔRGRAR were 1.8 and 0.92 mM, respectively, and those for *p*-nitrophenyl-β-xyloside were 6.2 and 6.1 mM, respectively [16].

4. Conclusions

The β-glucosidases produced by CG and AT belong to family 3 of the glycosidases. Based on the amino acid sequence alignments, these enzymes were considered to be constructed of five regions; the N-terminal residues, an N-terminal catalytic domain, a nonhomologous region, a C-terminal domain of unknown function and the C-terminal residues. The folding information was found to be unevenly distributed across these regions based on the results of gene manipulations. In increasing order, the impotence of each region in determining enzyme folding is considered to be as follows; the nonhomologous region (the four truncated AT mutants resulted in active enzymes with only slight character modification), the C-terminal unknown functional domain (all four chimeric enzymes formed active enzymes), the C-terminal residues (the removal of five amino acid residues (RGRAR) required the co-expression with the molecular chaperone GroEL/ES to produce an active enzyme) and the N-terminal catalytic domain (the two shuffled chimeric enzymes were obtained as inclusion bodies even though they were expressed with GroEL/ES and refolding of these proteins by slow dialysis was only partly successful).

Acknowledgements

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