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Research Article

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Construction of a chimeric CotA protein bearing glutathione S-transferase for improved enzyme activity

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ABSTRACT

Chimeric enzymes with novel catalytic combinations have been created for various purposes, especially to improve kinetic properties and catalytic performances of the parental enzymes. Herein, a bifunctional enzyme was constructed by fusing catalytic conserved region of CotA from Bacillus subtilis to glutathione S-transferase (GST) to produce a chimera exhibiting both laccase and GST activities. Enzyme assays revealed that the chimeric GST-CotA protein, which was expressed in Escherichia coli BL21 harboring recombinant pGEX-4T-2 cloning vector, preserved both parental catalytic properties. Furthermore, GST activity of GST-CotA was about 12% higher than that of the parental GST, suggesting improved enzyme activity. The proteomic profile analysis by SDS-PAGE technique confirmed that a 36 kDa-protein corresponding molecular weight of GST-CotA was efficiently produced by E. coli. Considering the versatility of laccase and GST, this chimeric enzyme could be potentially used for various biotechnological applications, particularly for detoxification and bioremediation of a broad range of environmental pollutants.

Key words: *Bacillus subtilis*, Chimeric protein, CotA, Glutathione S-teransferase, laccase

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INTRODUCTION

The spore coat protein of *Bacillus subtilis*, CotA, displays similarities with multicopper oxidases. This protein is a 65 kDalaccase, whose catalytic mechanism has been well characterized [1]. Laccases are versatile multicopper oxidases, catalyzing the reduction of molecular oxygen to water by the transfer of electrons derived from the oxidation of a broad range of substrates, in particular phenolic compounds. Moreover, these enzymes are considerable biotechnological enzymes due to their applications in bioremediation of toxic compounds, biomedicine and biocatalysis[2, 3].

Glutathione S-transferases (GST) are known for their ability to catalyze the conjugation of the reduced form of glutathione (via a sulfhydryl group) to different xenobiotic substrates. This reaction makes toxic compounds more accessible and water-soluble for the purpose of detoxification [4]. Furthermore, GST can bind with high affinity to glutathione coupled to a sepharose column [5]. Regarding to biotechnological applications of laccase and GST, it is desirable to develop an efficient process for improved enzyme activity. In addition, considering the versatility of laccase and GST, synergistic effect of these enzymes while express together is predictable.

There are two ways to fuse different genes for construction of novel chimeric protein with improved catalytic performances. One way is insertion fusion of one gene within the other. Another rout is 'end-to-end' connection, in which the C-terminal of one enzyme is linked to the N-terminal of the other enzyme. The end-to-end fusion method is frequently used to design multifunctional chimeric enzymes [6-8]. Chimeric enzymes are hybrid proteins that can be created by fusion of two or more different genes to improve enzyme stability, specificity and affinity. These constructed enzymes frequently have an improved performance as compared with the activities of each separate enzyme. The construction of chimeric proteins has been shown to be a useful strategy for the improvement of proteins involved in biodegradation, biosensor, and vaccine production [9-11].

In this study, the active site of CotA was connected to GST using end-to-end fusion method to construct a chimeric enzyme exhibiting both laccase and GST activities. On the other hand, this GST-tagged chimeric protein could be potentially purified with an affinity chromatography.

EXPERIMENTAL SECTION

Bacterial strains and culture conditions

The conserved sequence of *cotA* gene, which encodes copper oxidase (CuO), was selected and amplified from genomic DNA of *Bacillus subtilis*ATCC6051. This bacterium was cultivated overnight in nutrient broth at 37 °C before its use for DNA isolation and PCR amplification. To construct and amplify the GST-CotA expressing vector, *Escherichia coli* DH5α was used and inoculated in ampicillin-containing LB (Luria-Bertani) broth, and then incubated overnight at 37 °C. *E. coli* BL21 grown in LB broth was used for expression of the chimeric protein.

Amplification of the *cotA* gene

The genomic DNA of *B. subtilis* was extracted by standard methods and used as a template [12]. PCR amplification was performed by a pair of primers that were designed to amplify the Cu oxidase region of *cotA* gene (250 bp). The forward primer was CuO-F (5'-CGGATCCAAACACCAAAAGTCGGCA-3'), and the reverse primer was CuO-R(5'-GGAATTCCATGCGCTTGAATGGTGT-3'). Recognition sites for *BamHI* (forward) and *EcoRI*(reverse) are underlined.

The PCR program was started with an initial denaturation step (5 minutes at 95 °C), followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 1 minute, and a final extension step at 72 °C (7 minutes). The PCR product was purified using a PCR purification kit (Bioneer).

Vector construction, cloning and expression of the chimeric protein

The 250-bp PCR product was digested with *BamHI* and *EcoRI*(both from Fermentas), and then cloned into an expression vector, pGEX-4T-2 (GE Healthcare, 28-9545-50), which contains glutathione S-transferasegene at upstream of its cloning site to yield *gst-cotA* chimeric gene. The recombinant plasmid was transformed into *E. coli* DH5α competent cells [12]. After incubation overnight on LB agar plates (containing 100 mg ampicillin per mL), the construction of the hybrid enzyme was verified by colony PCR using CuO-F and CuO-R primers. The recombinant plasmids were extracted from *E. coli* using plasmid extraction kit (Sinagen), and then sequenced. The chimeric GST-CotA protein was expressed in *E. coli* BL21 following induction by IPTG (100 mM). To extract and analyze the recombinant protein, the bacterial cells were harvested by centrifugation (8000 g for 5 minute at 4°C), and then the pellet was sonicated in PBS buffer. After centrifugation for 5 minutes, the supernatant was used for enzyme assay and SDS-PAGE analysis[12].

Enzyme activity assays

To measurelaccase (Copper oxidase) activity, 200 µL of supernatant (chimeric enzyme) was added tothe reaction mixture containing 75mM catechol as a substrate in 50mM sodium phosphate buffer, pH5. The mixture was incubated at 40 °C, and then the laccase activity was determined by the rate of catechol oxidation, which was monitored at 440 nm[13]. Furthermore, GST activity of the chimeric protein was determined based on the reaction between the reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The reaction mixture was 100 µL KH_2PO_4, 10 µL GSH, 10 µL CDNB, in 880 µL distillated water. The supernatant (30 µL) was added to the reaction mixture, and then the formation of GSH-CDNB due to GST activity was monitored at 340 nm[14].

Protein analysis

Protein concentrations were determined by the Bradford method at 595 nm using bovine serum albumin as a standard[12]. To analyze total proteins, the supernatant that contained the chimeric enzyme was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Ten microliters of the sample was mixed with 10 µL of 2X loading buffer (125 mMTris-HCl [pH 6.8], 10 % [w/v] glycerol, 4 % [w/v] SDS, 10 % [v/v] 2-mercaptoethanol, and 0.05 % [w/v] bromophenol blue), and then heated in a boiling water bath for 10 minutes. The total proteins of GST-CotA transformant were separated on a 10 % (w/v) SDS-PAGE gel, and compared with protein profile of *E. coli* BL21 harboring non-recombinant vector. Finally, the protein bands were stained with Coomassie brilliant blue G-250 [12].

RESULTS AND DISCUSSION

Construction of the gst–cotA Chimeric Gene

Like other detoxification enzymes, laccase and GST have been known as versatile enzymes that have broad substrate specificities and low catalytic requirements. These enzymes are of particular interest due to their potential applications as biocatalysts in various industrial processes [2- 5]. However, for efficient biotechnological application, enzymes usually need to be adjusted by protein engineering to improve their activities. In the present work, our aim was to produce a chimeric enzyme that exhibited both GST and laccase activities. In this regard, catalytic conserved region of CotA (copper oxidase) gene from *B. subtilis* was inserted into a GST encoding vector, pGEX-4T-2, to produce GST-CotA chimera. As shown in Fig 1, copper oxidase region of *cotA*was fused downstream of glutathione S-teransferase gene of pGEX-4T-2 cloning vector, in which the C-terminal of GST was linked to the N-terminal of CotAlaccase in the final product. The end-to-end fusion strategy was performed to insert the sequence encoding CotAlaccase from *B. subtilis* into the GST encoding vector for production of a chimeric protein exhibiting both enzymatic activities. Bioinformmatic analysis of the sequence obtained by CuO-F and CuO-R primers confirmed that this conserved domain of laccase is similar to multicopper oxidases.

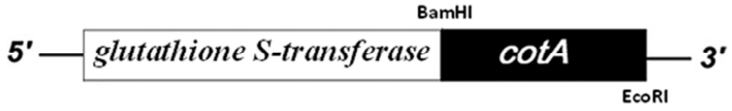

Figure 1.Design and construction of the bifunctional enzyme gene. The C-terminal of glutathione S-transferase was linked to the N-terminal of copper oxidase domain of CotAlaccase

Catalytic Activities of the Chimeric Enzyme

To confirm the vector construction procedures and the chimeric enzyme activity, GST and laccase activities were assayed after expression of GST-CotA protein in *E. coli*. The catalytic property of GST was investigated following the formation of GSH-CDNB conjugate in the reaction mixture. Based on the results presented in Fig 2, the cell extract containing GST-CotA hybrid protein showed a higher GST activity than the parental enzyme.

As shown in Fig 3, the constructed chimeric enzyme displayed a significant laccase activity in the presence of catechol as a substrate. Enzyme assays revealed that the chimeric enzyme displayed both GST and laccase catalytic activities.

The end-to-end method has been frequently used to design multifunctional chimeric enzymes [7, 8]. These constructed enzymes mainly displayed an improved performance as compared with the activities of each parental enzyme [15]. The most important aspect for efficient production of multifunctional enzymes is to retain or improve their catalytic functions. Moreover, the physicochemical compatibility between two catalytic activities is a key factor for creation of enzyme chimeras [11]. Herein, we successfully designed and produced the GST-CotA chimera that exhibited both parental catalytic activities (Fig 2 and 3). In addition, a synergistic effect for GST was observed in the chimeric enzyme, as GST activity of GST-CotA was about 12% higher than that of the parental GST. These results are in agreement with previous studies showing that the hybrid laccase-xylanase and laccase-glucanase enzymes were more active than each separate parental enzyme, however, they used insertion fusion method to construct the chimeras [11, 16].

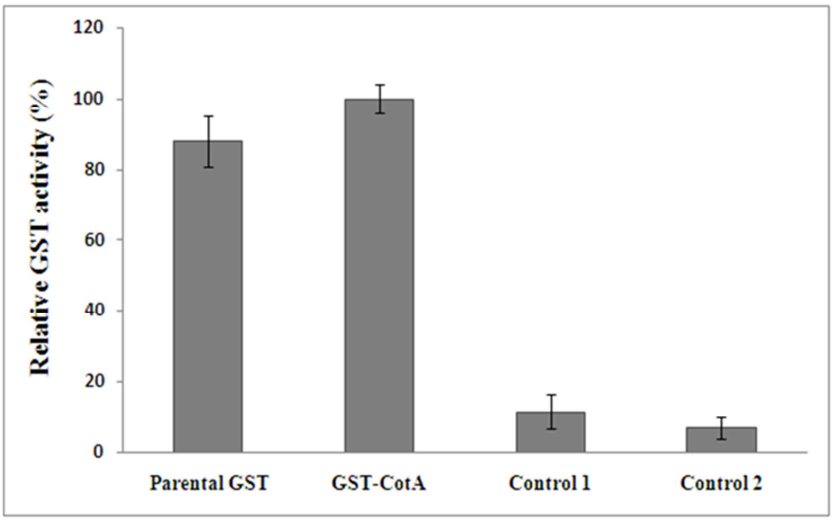

Figure 2.Relative glutathione S-transferase activity in the presence of CDNB as a substrate. Parental GST: produced by the transformant harboring non-recombinant pGEX-4T-2 (*gst*); GST-CotA: produced by the transformant harboring recombinant pGEX-4T-2 (*gst-cotA*); control 1: supernatant of non-transformant *E. coli* BL21; control 2: the reaction mixture without the enzymes

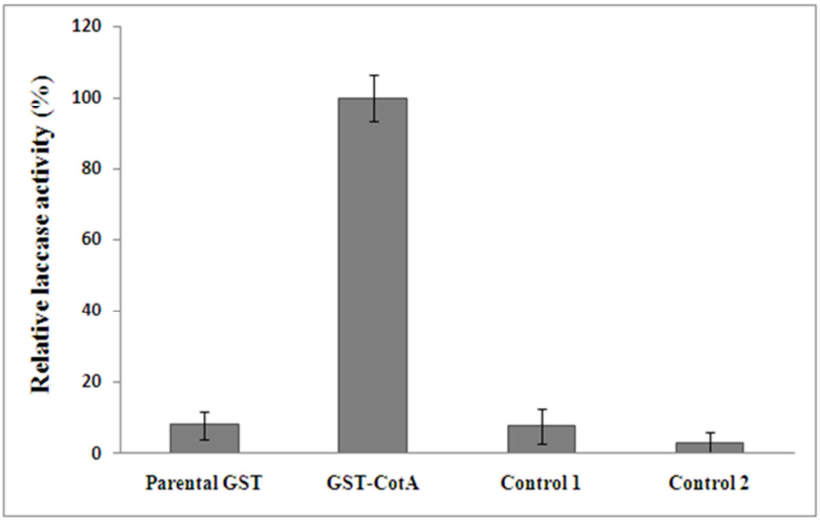

Figure 3.Relative laccase activity in the presence of catechol as a substrate. Parental GST: produced by *E. coli* BL21 harboring non-recombinant pGEX-4T-2 (gst); GST-CotA: produced by *E. coli* BL21 harboring recombinant pGEX-4T-2 (gst-cotA); control 1: supernatant of non-transformant *E. coli* BL21; control 2: the reaction mixture without the enzymes

Protein Profile of GST-CotA Transformant

Molecular mass of parental GST and CuO conserved domain of laccase were estimated 26 and 10 kDa, respectively. As shown in Fig 4, total proteins in the supernatant of *E. coli* were analyzed by SDS-PAGE. A major band with a molecular mass close to 36 kDa was detected for GST-CotA transformant. This band, which was not observed in protein profile of *E. coli* harboring non-recombinant plasmid, verified the successful production of GST-CotA chimeric protein in the bacterial host. This GST-tagged chimeric protein could be potentially purified with an affinity chromatography using glutathione coupled to a sepharose matrix.

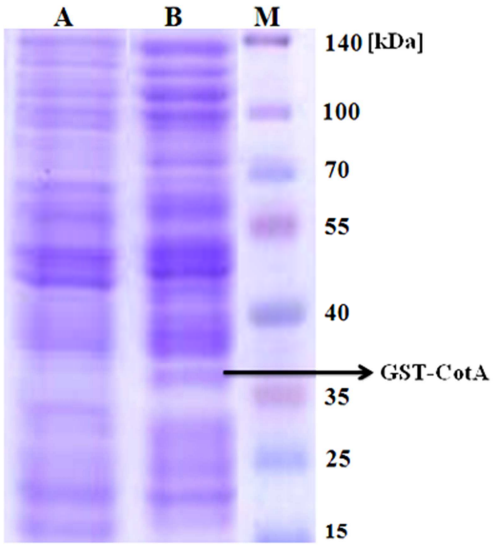

Figure 4. The SDS-PAGE analysis of total proteins produced by *E. coli* BL21transformants on a 10% polyacrylamide gel. Lane A: the total protein from *E. coli* BL21 harboring non-recombinant pGEX-4T-2 (*gst*); lane B: the total protein from *E. coli* BL21 harboring recombinant pGEX-4T-2 (*gst-cotA*); M: molecular size standard

CONCLUSION

In conclusion, the end-to-end strategy used in this study was successful in the construction of GST-CotA chimera. The resulting hybrid protein displayed altered enzymatic activities, which not only the catalytic properties of parental GST and laccase enzymes preserved but also an evidence of improvement was observed in the final product. This chimeric protein could be potentially used for various biotechnological applications, particularly for detoxification and bioremediation of a broad range of environmental pollutants.

Acknowledgements
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