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Engineering a chimeric acid-stable α-amylase-glucoamylase (Amy-Glu) for one step starch saccharification

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ABSTRACT

For saccharifying starch in one step, a chimeric biocatalyst (Amy-Glu) was generated from engineered α-amylase (*Ba-Gt-amy*) of *Bacillus acidicola* and glucoamylase (*Glu*) gene of *Aspergillus niger*. In order to join two enzymes, a linker peptide of 25 amino acids was used. Chimeric *Amy-Glu* was expressed in *E. coli*. Glu is of 75 kDa, while Amy-Glu is of 145 kDa. Both Amy-Glu and Glu displayed similar pH profile with good activity in the acidic pH range like that of Ba-Gt-amy with optimum at pH 4.0. All three enzymes (Ba-Gt-amy, Amy-Glu and glucoamylase) exhibited activity in the temperature range between 40 and 70 °C with optimum at 60 °C. Amy-Glu and Glu have $T_{1/2}$ of 90 and 70 min at 60 and 70 °C, respectively. The K_m, V_{max} and K_{cat} values of Glu (soluble starch) are 0.34 mg mL^{-1}, 606 μmol mg^{-1} min^{-1} and 727 s^{-1}, while for Amy-Glu are 0.84 mg mL^{-1}, 13,886 μmol mg^{-1} min^{-1} and 4.2×10^4 s^{-1}, respectively. The end product analysis suggested that Amy-Glu retains the activity of both parental enzymes and forms maltodextrins along with glucose as the major products. Amy-Glu saccharifies wheat and corn starches more efficiently than the Ba-Gt-amy and glucoamylase.

1. Introduction

Starch is the most abundant polysaccharide produced by plants in the form of water insoluble granules after cellulose. Hydrolysis of starch is essential for generation of fermentable sugars, which are used in various food and fermentation industries [1]. Enzymatic hydrolysis of starch entails three basic steps: first, 20–40% starch slurry gelatinized in a jet cooker at 100–105 °C for 5–10 min, followed by liquefaction by α-amylase at 95 °C for a maximum of 3 h with adjustment of pH at 6.0–6.5 and saccharification by the action of glucoamylase at 60 °C (pH 4–4.5) to yield glucose [2,3]. This process encounters a few challenges: firstly, the pH of native starch slurry is around 3.0–4.0, whereas the commercial amylases work best at pH 5.0–6.5, thus requiring additional pH adjustment step [3]. Further, the addition of both α-amylase and glucoamylase separately adds to the cost of the process. In case enzymes capable of hydrolyzing α-1, 4- and α-1, 6- linkages in starch at acidic pH, the cost of enzymes in an industrial process can be minimized. Therefore, the acid-stable, thermostable and raw starch degrading enzymes are required for an ideal starch saccharification process.

Besides discovering novel enzymes from novel and unexplored microbial sources, attempts are being made to improve the existing enzymes through molecular approaches as per the needs of the industry. Among various techniques, the generation of virtual enzymes with improved catalytic properties from naturally occurring enzymes is a straight forward approach [4,5]. Several chimeric enzymes have been engineered for improving thermostability and to change the substrate or product specificities [6–8].

In our previous study, we have reported engineering of a chimeric α-amylase (Ba-Gt-amy) by fusing truncated acidic α-amylase gene (*Ba-amy*) of *Bacillus acidicola* (*Ba-amy*) with 5′- and 3′- terminal end portions of α-amylase gene of *Geobacillus thermoleovorans* (Gt-amy) [5]. In this investigation, a chimeric catalyst (Amy-Glu) was generated for one step starch saccharification by linking *Ba-Gt-amy* and *glucomylase* of *Aspergillus niger* and expressed it in *E. coli*. The kinetic characteristics of the chimeric enzyme (Amy-Glu) have been compared with those of parental enzymes and tested its applicability in saccharifying raw wheat and corn starches.

2. Material and methods

2.1. Strains, vectors, enzymes and reagents

Escherichia coli DH5α [F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZ*ΔM15 *Δ(lacZYA-argF)*U169, hsdR17($r_K^- m_K^+$), λ$^-$] and *E. coli* BL21 (DE3) [F$^-$ *ompT hsdS*(rB^- mB^-) *gal dcm* λ(DE3[*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K\text{-}12}$(λ^S)] were used as the host strains for cloning and expression of tar-

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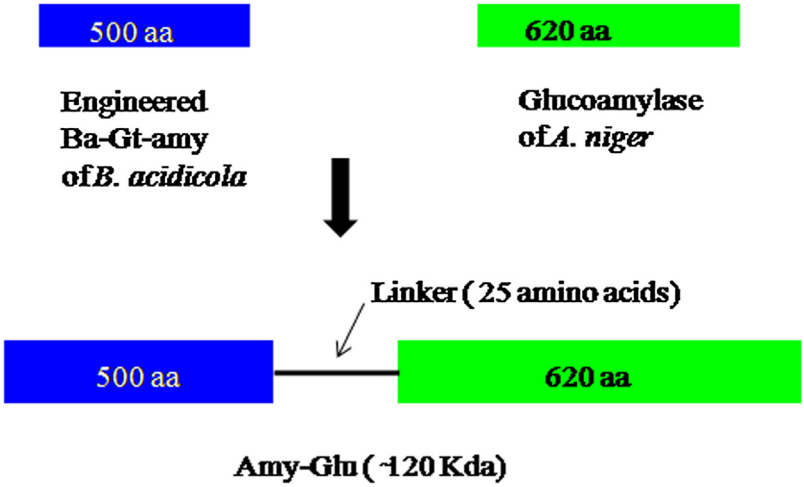

Fig. 1. Strategy for generating Amy-Glu chimeric enzyme. Ba-Gt-amy and Glucoamylase (from *A. niger*) were fused through a linker peptide of 25 amino acids [(Gly-Gly-Thr-Gly-Ser)$_5$].

get gene. *E. coli* clone harbouring *Ba-Gt-amy* and *A. niger* MTCC 478 were procured from the laboratory culture collection. Vectors pGEMT [Real Biotech Corporation (RBC), Taiwan] and pCold I (Takara, Japan) were used for sequencing and expression of the chimeric gene, respectively. Ni^{2+}- NTA agarose resin was purchased from Qiagen (Hilden, Germany). The restriction enzymes have been purchased from New England Biolabs (Beverly, USA). The primers used in this investigation were purchased from Sigma-Aldrich (USA). Genomic DNA extraction kit was purchased from Zymo Research. Plasmid extraction and gel elution kits were procured from Real Biotech Corporation (RBC), Taiwan. All amplifications were done by using the Herculase DNA polymerase (Agilent).

2.2. Construction of bifunctional fusion enzymes

The pCold-Ba-Gt-amy construct harboring the α-amylase gene was used as the template for PCR amplification of the *Ba-Gt-amy* gene using primers P1 and P2 [5]. *Glucoamylase* (*Glu*) gene was amplified from the genome of *A. niger*. *Glu* consists of five exons and three introns. Exons were amplified separately and ligated together by using the overlap extension PCR. *Ba-Gt-amy* and *Glu* were ligated to the 25 amino acid long linker peptide by using the *Sac* I and *Hin*d III sites, while *Glu* and *Amy-Glu* were cloned in pCold vector by using the *Nde* I and *Xba* I restriction sites. All amplifications were done by using Herculase II fusion DNA polymerase (Agilent) in thermocycler (Bio Rad, USA). The strategy for the construction of the chimeric enzyme (Amy-Glu) is presented in Fig. 1, and the primers used in this study are given in Table 1.

2.3. Bioinformatic analysis

The nucleotide and protein sequences were compared with the NCBI nucleotide/protein database using BLASTN and BLASTP programs, respectively. Homology modeling was done using Swiss model server (http://swissmodel.expasy.org). The structure file in the pdb format was viewed using PyMol. Molecular mass was predicted by ProtPram tool. Stability of secondary structures of the chimeric enzyme was predicted using the PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/).

2.4. α-Amyalse assay

α-Amylase was assayed as described by Parashar and Satyanarayana [5]. To 0.5 mL of 0.5% potato starch (Sigma) prepared in 100 mM sodium acetate buffer (pH 4.0), appropriately diluted 0.5 mL enzyme (0.21 U) was added and incubated for 15 min at 60 °C. The reducing sugars liberated were determined using 3,5-dinitrosalicylic acid (DNSA) reagent [9]. One unit of α-amylase is defined as the amount of enzyme that liberates 1 μmol of reducing sugars as maltose per min under the assay conditions.

2.5. Glucoamylase assay

Glucoamylase was assayed according to Bagheri et al. [10] with some modification. To 0.5 mL of 0.5% potato soluble starch (Sigma) prepared in 100 mM acetate buffer (pH 4.0), 0.5 mL appropriately diluted enzyme (0.25 U) was added and incubated for 15 min at

Table 1
Primers used in the present investigation.

S. no	Product	Position of amplicon	primer sequence
P1	*Exon 1*	1–257	GTGATTTCCAAGCGCGCGACC
P2	*Exon 1*	1–257	GGTGTAGAAGTCAATCAATGAC
P3	*Exon 2*	290–576	GTCATTGATTGACTTCTACACC
P4	*Exon 2*	290–576	TAGCCATTGTCAAGCAGCCATT
P5	*Exon 3*	632–728	AATGGCTGCTTGACAATGGCTA
P6	*Exon 3*	632–728	TTCCCAGAGATCATATCCTGTC
P7	*Exon 4*	790–1424	GACAGGATATGATCTCTGGGAA
P8	*Exon 4*	790–1424	GCGTGAGTTTCCACAATAGAGA
P9	*Exon 5*	1483–2169	TCTCTATTGTGGAAACTCACGC
P10	*Exon 5*	1483–2169	CCGCCAGGTGTCAGTCACC
P11	*Glucoamylase*	1–2169	ACGCGTTCTAGACCGCCAGGTGTCAGTCA
P12	*Glucoamylase*	1–2169	CAAGAAGCTTGTGATTTCCAAGCGC
P13	*Ba-Gt-amy*	1–1584	AGCCATATGATGATGCAGTATTTTG
P14	*Ba-Gt-amy*	1–1584	AGTGAGCTCAGGAACCCAAACCGA

Note: Number denotes the nucleotide position in the ORF of the gene