Site-Directed Mutagenesis of Glu-269 L-Arabinose Isomerase from *Geobacillus stearothermophilus* Isolated from Tanjung Api Poso, Indonesia

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Abstract

Industrializing of tagatose requires enzymes that meet to industrial need such as thermophile, slightly acidic and metal independent. Previously, we cloned, sequenced and expressed L-arabinose isomerase from *Geobacillus stearothermophilus* isolated from Tanjung Api, Poso, Indonesia. Based on DNA alignment analysis, the gene had high homology with those of *G. stearothermophilus* T6 (Gene Bank Acc No: AAD45718) which has optimum activity at high temperature and alkaline condition. In this paper, we described site-directed mutagenesis approach to mutate Glu-269 (Q269) to Lys-269 (K269) to decrease the optimum pH of the strain. Sequencing result showed that mutagenesis had been successful to mutate amino acid at position 269 from glutamine (Q) into lysine (K). Expression of mutant Q269 showed protein with molecular mass ~56 kDa.

Keywords: site-directed mutagenesis, Q269K, L-arabinose isomerase, *Geobacillus stearothermophilus*

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Introduction

L-Arabinose isomerase (L-AI) is an intracellular enzyme which catalyzes the conversion of L-arabinose into L-ribulose *in vivo*. It is also referred to as D-galactose isomerase due to its ability to isomerize D-galactose into D-tagatose *in vitro* (Cheetam & Woottom, 1993). Among L-AIs, thermostable L-AI has been intensively isolated and characterized due to several advantages, such as higher conversion yield, faster reaction rate, and decrease viscosity of the substrate in the product stream (Kim *et al*, 2003 & 2005; Jorgensen *et al*, 2004; Jung *et al*, 2005; Lee *et al*, 2005; Rhimi *et al*, 2005). Majority of these thermostable enzymes exhibit an alkaline pH optimum which is introduce undesired effect like browning and unwanted by-product formation (Liu *et al*, 1996). In order to overcome these problems a thermostable L-AIs with acidic pH optimum (pH opt) are desirable for industrial application (Lee *et al*, 2005).

Engineering of proteins have been performed using semi-rational design by amino acid sequence comparison and directed evolution (Lehmann & Wyss, 2001). The semi-rational design methods based on the hypothesis that at a given position in an amino acid se-quence alignment of homologous pro-tein, the non-consensus amino acid ra-ther than the consensus amino acid are responsible for the distinct characteris-tics of each protein (Lehmann *et al*, 2002). Lee *et al* (2005) reported the characterization of an L-AI from the acidophilic *Alicyclobacillus acidocalda-rius* (AAAI) and the key role of K269 in the enzyme acidotolerance. A mutation introduced at the equivalent position D268K in AI from *Bacillus haloduran* (BHAI) decreased the optimum pH of the enzyme from 8.0 to 7.0. Rhimi *et al* (2005), also described a mutation at Q269K in *Bacillus stearothermophilus* US100 AI (BSAI US 100) by rational design could decrease the optimum pH in range 6.0-7.0. By using the technique, they also found the amino acid which has responsibility to temperature optimum 65°C. The results increase the success story of the approach semi-rational design in order to engineer protein through comparison on their amino acid sequences.

We had cloned the *araA* gene enco-ding L-arabinose isomerase from *G. stearo-thermophilus* (GSAI) and expressed it in *Escherichia coli* BL21(DE3)pLysS (Fitriani & Saksono, 2010). Analysis of the sequence revealed that the open reading frame (orf) of *araA* gene consists of 1494 bp nucleotide which encoded a protein of 497 amino acid residues. According to SDS PAGE results GSAI had a molecular mass ~56 kDa.

The amino acid sequence analysis of GSAI showed high homology with those of GSAI T6 (98%), BSAI US100 (97%), and AAAI (97%). GSAI T6 and BSAI US100 have optimum activity at high temperature ($\geq$ 60°C) and alkaline condition (up to 7.0). Moreover we found that amino acid at the position of 269 in GSAI is glutamine (Q). The high sequences homology alignment and the presence of residue Q269, strongly suggested that GSAI may also have optimum activity at alkaline condition. This paper described our effort on engineering of GSAI, by mutation of Q269 into lysin (K) to perform mutant GSAI-Q269K and its expression.

Materials and Methods

Bacterial strain and culture condition. *E. coli* DH5α and *E. coli* BL21(DE3)pLysS were used as host for cloning and expession of mutant GSAI-Q269K. Both strains were grown in Luria Bertani (LB) media containing ampicillin (100 µg/ml) in a rotary shaker at 37°C.

Site-directed mutagenesis of Q269K GSAI. Mutant Q269 of GSAI was created by site-directed mutagenesis using pET-araA as DNA template. Site-directed mutagenesis was generated by PCR using Kafa Hi-Fi DNA polymerase (Kafa Biosystem) and specific primers for mutagenesis. The forward primer was 5'-GCC TTT TTG AAA GAT GGG AAC -3', and reverse primer was 5'-GTT CCC ATC TTT CAA AAA GGC-3' (underlined nucleotides represent those nucleotides changed by point mutation). PCR condition was set up as follows: the initial denaturation was 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 min, annealing at 55°C for 1 min, elongation at 72°C for 5 min, and final elongation at 72°C for 5 min. PCR product was digested with *Dpn*1 and transformed into *E. coli* DH5α com-petent cell. Positive clone was checked by PCR colony amplification and plas-mid isolation. The plasmids were se-quenced to verify the presence of mutation.

Expression of Q269K GSAI. Plasmid har-boring mutant GSAI-Q269K was trans-formed into *E. coli* BL21(DE3)pLysS for ex-pressing the protein. PCR colony ampli-fication was used for checking the positive clones. Colonies of positive clones were grown in LB media containing ampicillin (100 µg/ml) and incubated in rotary shaker at 37°C until OD_{600} value 0.5-1. Cultures we-re induced by 1 mM IPTG (isoprophyl-β-D-thiogalactospyrano-sidase) (Fermentas), grown for 4 h in rotary shaker at 37°C and harvested by centrifugation at 11,000 rpm and 4°C for 15 min. The cell pellets were suspended in 10 mM Tris HCl pH 8.0 and disrupted by sonication. Cells debris were removed by centrifugation at 11,000 rpm and 4°C for 15 min. As puri-fication steps, the supernatants were heated at 60°C for 30 min. Protein expressions were checked by SDS-PAGE electrophoresis using method as described by Laemmli (1970).

Results and Discussion

Bioinformatics study of L-AI homology alignment (as show in Figure 1), exhibited that amino acid at position of 269 in GSAI was glutamine (Q) as well as BSAI US 100, GTAI, GSAI IAM110010 and GSAI T6. All the mentioned AIs have pH optimum up to 7.0. Therefore, muta-tion should be undertaken to reduce the pH optimum by replacing Glutamine (Q) of 269 with Lysine (K) residue.

In order to replace the amino acid, we designed primers and used for mutagenesis based on PCR amplification. PCR product of GSAI-Q269K was showed a band size of 6967 bp in DNA electrophoresis.

Figure 2 showed PCR products and the DNA templates (pET-*ara*A) that were treated with restriction enzyme *Dpn*1. Restriction enzyme *Dpn*1 only degrade the methylated DNA, so that the PCR product was not degraded (lane 1) while the template (a plasmid), was totally degraded (lane 4). The *Dpn*1 treatment also purified the PCR product from the template. This step is important to avoid transformation of wild-type GSAI that

remained in the PCR solution. The *Dpn*1 treated PCR fragment was transformed into *E. coli* DH5α competent cell. The positive clones were selected using colony PCR amplification using a pairs of *araA* specific primers. Plasmid harboring *ara*A-Q269K then was isolated, purified and sequenced.

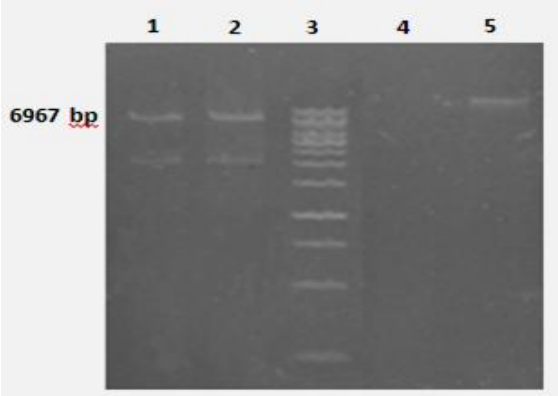

Figure 2. PCR product of Q269K and plasmid pET-*araA* treated with *Dpn*1. Lane 1 and 2, PCR product of Q269K uncut and cut with *Dpn*1 respectively; lane 3 DNA marker 1 kb DNA ladder; lane 4 and 5 were plasmid of pET-*araA* uncut and cut with *Dpn*1 respectively (due to the circular form of the plasmid, the size in gel is not able to be fixed).

As shown in Figure 3, nucleotide CAG (code of Q) in wild type GSAI were changed to AAA (code of K). Homology amino acids sequence of GSAI, also confirmed that the mutations only occurred at the target amino acid. Thus, as shown in Figure 4, a single mutation at position of 269 was confirmed.

Expression of the protein was performed by transformation of plasmid harboring gene *ara*A-Q269K into *E. coli* BL21(DE3)pLysS. As shown in Figure 5, GSAI-Q269K was expressed with molecular mass of ~56 kDa (right side). The expression level of GSAI-Q269K was same with that of the wild-type, indicated that mutation did not allow the differences in expression system on the host.

Conclusion

We had successfully demonstrated the mutagenesis of GSAI through site-directed mutagenesis and expressed the mutant. Further investigation, especially on its bioproperties is important to declare the useful of the enzyme towards industriali-zation of tagatose in Indonesia.

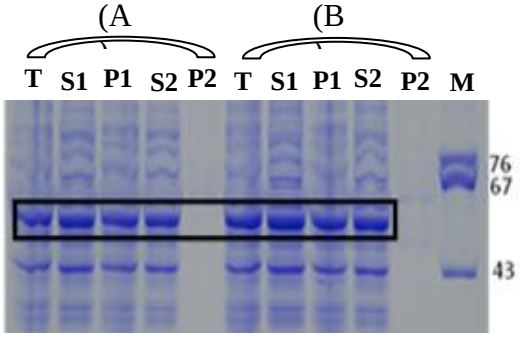

Figure 5. Expression of wild-type GSAI (A) and mutant Q269K (B). Black box showed the protein targets with molecular mass ~56 kDa. T (total protein), S1 (supernatant after freezing thawing), P1 (pellet after freezing thawing), S2 (supernatant after heat treatment), P2 (pellet after heat treatment).

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G. stearothermophilus (GSAI)	RESIREQARIELGLKAFLQDGNFTA
G. stearothermophilus T6 (GSAI T6)	RESIREQARIELGLKAFLQDGNFTA
B. stearothermophilus US100 (BSAI US100)	RESIREQARIELGLKAFLQDGNFTA
Alicyclobacillus acidocaldarius (AAAI)	RESIREQARIELGLKAFLKDGNFAA
Thermus sp. IM6501	RESIREQARIELGLKAFLQDGNFTA
G. stearothermophilus IAM11001(GSAI IAM11001)	RESIREQARIELGLKAFLQDGNFTA
Geobacillus thermodenitrifican (GTAI)	RESIREQARIELGLKAFLQDGNFTA
B. halodurans C-125 (BHAI)	KAAVLEQAKMELALKEFLEEGGYTA
E. coli str. K-12 (ECAI)	RQNVLEAARIELGMKRFLEQGGFHA

Figure 1. Homology of L-AI from some bacteria. Box showed amino acid at position 269.

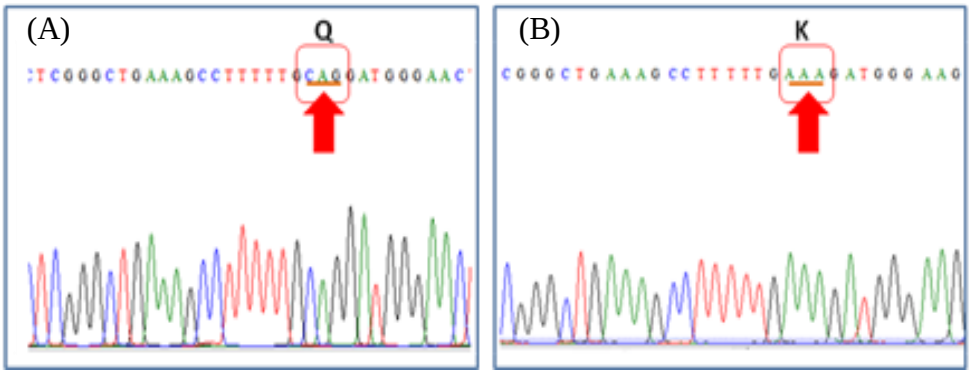

Figure 3. The changed nucleotide of wild-type GSAI (A) and GSAI-Q269K (B). Nucleotide CAG encoding glutamine (Q) of GSAI changed into AAA encoding lysine (K) in GSAI-Q269K.

mutant Q269K	MMLSLRPYEFWFVTGSQHLYGEEALRQVEEHSMMIVNELNQDSVFPFPLVFKSVVTTPEE
wild-type GSAI	MMLSLRPYEFWFVTGSQHLYGEEALRQVEEHSMMIVNELNQDSVFPFPLVFKSVVTTPEE
mutant Q269K	IRRVCLEANASEQCAGVITWMHTFSPAKMWIGGLLELRKPLLHLHTQFNRDIPWDSIDMD
wild-type GSAI	IRRVCLEANASEQCAGVITWMHTFSPAKMWIGGLLELRKPLLHLHTQFNRDIPWDSIDMD
mutant Q269K	FMNLNQSAHGDREYGFIGARMGVARKVVVGHWEDPEVRERLAKWMRTAVAFAESRNLKVA
wild-type GSAI	FMNLNQSAHGDREYGFIGARMGVARKVVVGHWEDPEVRERLAKWMRTAVAFAESRNLKVA
mutant Q269K	RFGDNMREVAVTEGDKVGAQIQFGWSVSGYGIGDLVQYIRDVSEQKVNELLDEYEELYDI
wild-type GSAI	RFGDNMREVAVTEGDKVGAQIQFGWSVSGYGIGDLVQYIRDVSEQKVNELLDEYEELYDI
mutant Q269K	VPAGRQEGPVRESIREQARIELGLKAFIKDGNFTAFTTTFEDLHGMKQLPGLAVQRLMAE
wild-type GSAI	VPAGRQEGPVRESIREQARIELGLKAFIQDGNFTAFTTTFEDLHGMKQLPGLAVQRLMAE
mutant Q269K	GYGFGGEGDWKTAALVRLMKVMADGKGTSFMEDYTYHFEPGNELILGAHMLEVCPTIAAT
wild-type GSAI	GYGFGGEGDWKTAALVRLMKVMADGKGTSFMEDYTYHFEPGNELILGAHMLEVCPTIAAT
mutant Q269K	KPRIEVHPLSIGGKEDPARLVFDGGEGAAVNASLIDLGHRFRLIVNEVDAVKPEHDMPKL
wild-type GSAI	KPRIEVHPLSIGGKEDPARLVFDGGEGAAVNASLIDLGHRFRLIVNEVDAVKPEHDMPKL
mutant Q269K	PVARILWKPRPSLRDSAEAWILAGGAHHTCFSFAVTTEQLQDFAEMAGIECVVINEHTSV
wild-type GSAI	PVARILWKPRPSLRDSAEAWILAGGAHHTCFSFAVTTEQLQDFAEMAGIECVVINEHTSV
mutant Q269K	PSFKNELRWNEVFWRGRY
wild-type GSAI	PSFKNELRWNEVFWRGRY

Figure 4. Homology of mutant Q269K and wild-type GSAI. Box showed the mutated amino acid at position 269.

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