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Cloning and Sequence Analysis of Lipase Gene from DMS3 Isolate

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Genes encoding a lipase from DMS3, isolated from Kawah Domas hotspring, Indonesia was cloned and characterised. The lipase gene was cloned based on PCR amplification from genomic DNA. Two set of primers was designed to amplify the lipase gene. An open reading frame of 1248 bps encoding polypeptide of 416 amino acid residues, has been amplified and sequenced. The sequence of amplicon showed high homology (99%) with lipase from *Geobacillus thermoleovorans*. Detail comparison among three highest homology of lipase showed that there are some variation of amino acid residues. However the subtitution of amino acid residue in lipase ITB3.1, especially at amino acid residue 194 from Asp to Asn is predicted to showh no significant different for the characteristic of lipase ITB3.1 compared to that the other lipase from family 1.5.

Key words: Lipase, Kawah Domas Hot Spring, PCR Cloning, *Geobacillus thermoleovoran*.

Lipases (triacyglycerol acylhydrolases, EC 3.1.1.3) catalyze the hydrolysis of the ester bonds of long-chain triglycerides into fatty acids and glycerol. These enzymes are particular importance in biotechnology because of diverse applications such as on detergents, food production, pharmaceuticals and synthesis of fine chemical industries[1,2,3,4].

Lipase from thermophiles have became objects of interest for understanding of basic properties and industrial applications since they exhibit higher thermodynamic stability at elevated temperatures and in organic solvents[5]. Enzyme from an organism usually has temperatur or pH optimum at or close to its growth condition as consequence of adaptation of the corresponding microorganism to its enviroment[5,6]. Some thermostable lipase have been isolated from *Bacillus* and *Geobacillus*. Most of them showed optimum activity at temperature above 55°C and stable in various organic solvent[7,8,9]. The immobilized lipase from *Bacillus stearothermophilus* MC7 even exhibits good operational thermostability with half-life of 50 days at 60°C in a solvent-free system[10].

Previously, we reported several thermophilic microorganisms from various sources in Indonesia, including some hot springs and thermogenic compost[11,12,13,14]. Some of the microorganisms showed lipolytic activity, and most of them were identified as member of genera *Bacillus* and *Geobacillus* based on its 16S rRNA

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gene sequences[15]. Among them, DMS3 isolate has the highest lipolytic activity. In this report we describe cloning and characterisation of lipase gene from DMS3 isolate. The cloning was carried out based on PCR methods and the characterisation of the gene was based on its nucleotide sequences.

MATERIALS AND METHODS

Materials

Peptone, yeast extract, NaCl, Tris base, EDTA and potassium acetate were supplied from Biobasic. Lysozyme and proteinase were purchased from Fermentas. HCl, acetic acid glacial, chloroform, isoamyl alcohol and ethanol were purchased from Sigma. All other reagents were analytical grade unless otherwise stated. Primer Flip1, Flip2, Rlip1 and Rlip2 were used to amplify the gene (Table 1). DMS3 isolate was taken from microbe collections in our laboratory. DMS3 originally was isolated from Kawah Domas Hot Springs, West Java, Indonesia.

Cultivation of Microbes

The bacterial cultures were cultivated in media ½ T (pepton 0.4%, yeast extract 0.2%, NaCl 0.1%) and incubated at 70°C for 18 hours. The pellet cell was collected by centrifugation at 6000 g for 10 minutes.

Table 1. Primers used for amplification of lipase gene

Primer	Sequences
Flip1	5'- CACCCATCGTGCTTCTCCAT -3'
Flip2	5'-ATGTGAGGGGAGGAGAAGG-3'
Rlip1	5'- CCCTTGGCTGTGGGCGA -3'
Rlip2	5'- GAGCCATCCGATCGAGATG -3'

Isolation of Chromosomal DNA

The pellet cells were suspended in 10 mM Tris HCl buffer (pH 8.0) containing 8 mg/ml of lysozyme and incubated at 37 °C for 1 h. The cells were lysed by adding lysis buffer containing 2% SDS, 0.8 mg/ml proteinase K and 200 mM EDTA pH 8.0. The lysis process was carried out by incubation at 50 °C for 30 min. Ice cold potassium acetate and acetic acid glacial mixed solution were added. The denatured proteins were precipitated by centrifugation at 8000 g for 15 min. Supernatants were mixed with an equal volume of chloroform isoamylalcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids were obtained by centrifugation at 16.000 g for 20 min at room temperature, washed twice with cold 70% ethanol, and re-suspended in sterile deionized water.

Amplification of lipase gene

Amplification of lipase gene was carried out by PCR method. Flip1 and Rlip1 were used as internal primers, while Flip2 and Rlip2 were used as external primers. A total of 50 µL reaction mixture consists of 5 ng DNA template, 10 pmol of primers, 200 µM dNTP mixture, 5 µL 10x PCR buffer, 1,25 U *Taq* Polymerase, was used for PCR reaction. The PCR were carried out using i-Cycler (Bio-Rad). The reaction conditions used were: 1 cycle (95°C for 5 min), 30 cycles (95°C for 1 min, 50°C 1 min, and 72°C for 2 min), and final cycle of 72°C 10 min.

Homological analysis of Lipase Gen Sequence

Sequences of the full length PCR amplicon were obtained by primer walking strategy and assembling of the partial sequences using the tool SeqManTM of the software packet DNA-Star. Sequence homology analysis of the lipase was carried out by comparing the nucleotide sequence of local lipase genes with nucleotide sequence from the Gene Bank database at NCBI (National Centre of Biotechnological Information) through web site http://www.ncbi.nlm.nih.gov using BLAST program for screening of sequence similarity. Sequences alignments were performed by ClustalX program[16] and visualized using GenDoc program.

Structure modelling

The initial coordinate of Lipase ITB1.2 was obtained from homology structure modelling using Swiss-Model[17]. Three dimensional structure visualization of Lipase ITB3.1 was generated using Visual Molecular Dynamics (VMD) software version 1.9[18].

Nucleotide sequence accession numbers

Nucleotida sequence of lipase gene for DMS3 isolate has been deposited in the GenBank database under accession number HQ398859.

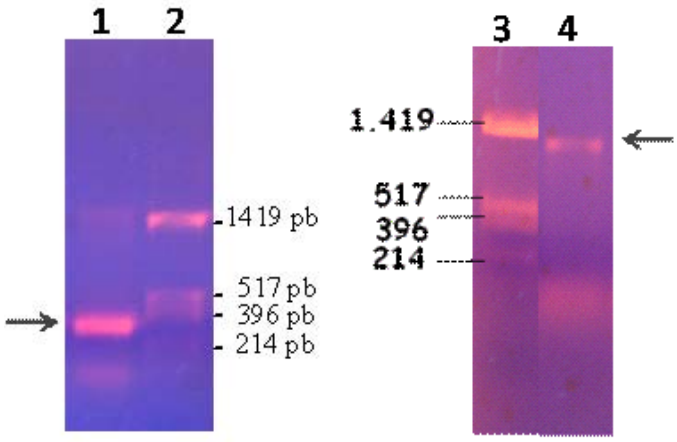

Fig 1. Agarose gel electrophoregram of PCR (A) Fragment of lipase gene as result of amplification using internal primer (lane 1). (B) Lipase gene of DMS3 isolate (lane 4). Lane 2 & 3 Marker PUC19/H*inf* I

RESULTS

Cloning of Lipase ITB3.1

The culture of DMS3 showed high lipase activity[15]. The lipase gene from DMS3 isolate was cloned based on PCR method directly from genomic DNA. The critical step in the amplification of gene from the genomic DNA is on designing and choosing the primers, especially if the genes have only few conserved of amino acids in its sequences[19]. Two conserved region in lipase gene are often used in designing primers[19], one region was at around one of three lipase's catalytic residues, the amino acid serin. This region is known as conserved pentapeptide which always in form of Gly-X-Ser-X-Gly[20]. Another region is the oxyanion hole region which located as 60-108 aa upstream of the conserved pentapeptide[19]. The region is recognized by the presence of short hydrophobic region (6 aa) upstream of moderately conserved His-Gly (HG) dipeptide. In this study, a set of internal primers were designed based on sequence in these two conserved regions. The primers were used to amplify fragment of lipase gene from DMS3 isolate with length of approximately 300 bp (Fig. 1A). The nucleotide sequence of thisfragment, aligned with sequence data in GenBank, showed high homology with all sequence of lipase gene from *Geobacillus*, indicating that DMS3 isolate was belong to genus *Geobacillus*. The homology result was in agreement with identification of the DMS3 isolate based on its 16S rRNA gene sequence[15]. In order to amplify the whole gene of lipase, another pair of primer, external primers, were further designed. External primers were designed based on conserved region upstream and downstream of coding region. 6 lipase genes which showed closest homology with the DMS3 lipase were used for designing external primer. The external primer amplify polinucleotide with length of approximetly 1300 bp (Fig. 1B). The gene was further named as lipase ITB3.1.

Homological analysis of Lipase ITB3.1 sequences

The complete sequence of lipase ITB3.1 consists of 1248 nucleotide sequences (gene bank accession number HQ398859). Homological analisis of 416 deduced amino acid sequences using blastN program through website http:// ncbi.nlm.nih.gov showed that lipase ITB3.1 has high homology with lipase from *Geobacillus*. The result suggested that lipase ITB3.1 is one of the member of bacterial lipase group family 1.5. Table 2 shows 50 lipases which were close to lipase ITB3.1. The highest homology of lipase ITB3.1 was lipase from *G. thermoleovorans* (accession number AF134840) with 3 aa differences. Detail comparison of lipase ITB3.1 to the three best homologs (*G. thermoleovorans*, *G. stearothermophilus* and *Geobacillus sp.* lipases with accession number AF134840.1, AAX11388.1, ADU94368.1 respectively) showed that there are few variation of amino acid residues among them (Table 3).

Table 2. Homology analysis of lipase ITB3.1 with lipase from various microorganism

No	Accession No.	Description	% homology	Score
1	AF134840_1	*Geobacillus thermoleovorans*	99	858
2	AAX11388.1	*Geobacillus stearothermophilus*	99	855
3	ADU94368.1	*Geobacillus sp.* Y412MC61	99	855
4	ADI26534.1	*Geobacillus sp.* C56-T3	98	822
5	AAO92067.2	*Geobacillus zalihae*	98	821
6	JC8061	*Geobacillus sp.* T1	97	820
7	AAW47928.1	*Bacillus sp.* L2	98	820
8	AAV35102.1	*Bacillus sp.* 42	98	819
9	AAY82869.1	*Geobacillus sp.* SF1	96	810
10	AAM21775.1	*Bacillus sp.* Tosh	96	809
11	ABC48693.1	*Geobacillus thermoleovorans*	95	807
12	AAF40217.1	*Geobacillus stearothermophilus*	96	805
13	CAL36912.1	*Geobacillus thermoleovorans*	98	804
14	AAC12257.1	*Geobacillus stearothermophilus*	92	801
15	AAM21774.1	*Geobacillus thermoleovorans*	95	800
16	ACJ07039.1	uncultured bacterium	92	798
17	BAH28804.1	*Geobacillus sp.* SBS-4S	99	796
18	BAD76271.1	*Geobacillus kaustophilus* HTA426	95	796
19	AEB71527.1	*Geobacillus sp.* MNK	93	782
20	CAA64621.1	*Geobacillus thermocatenulatus*	95	781
21	ACN79581.1	*Geobacillus sp.* NTU 03	92	777
22	AF429311_1	*Geobacillus stearothermophilus*	95	776
23	ABK34427.1	*Geobacillus stearothermophilus*	95	773
24	AF141874_1	*Bacillus sp.* TP10A.1	93	759
25	ACS93141.1	*Geobacillus sp.* RD-2	95	748

Table 3. Comparation of amino acid residues between Lipase ITB3.1 and *G. thermoleovorans*, *G. stearothermophilus* and *Geobacillus sp* lipases with accession number AF134840.1, AAX11388.1, ADU94368.1 respectively

No residues	DMS3	G. *thermoleovorans*	*G. stearothermophilus*	*Geobacillus sp.*
3	Cys	Cys	Gly	cys
12	Gly	Gly	Gly	Gln
23	Pro	Ser	Ser	Pro
31	Val	Ala	Thr	Ala
194	Asn	Asp	Asp	Asp
225	val	val	Val	Ala
370	Ala	Ala	Ala	Val
416	Arg	Arg	Gln	gln

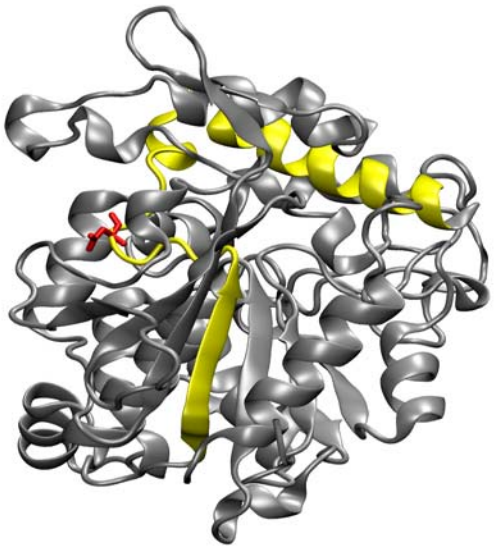

Fig 2. 3D Structure of Lipase ITB3.1. b-6 and a-6 are colored in yellow. Residue Asn194 is drawn as red sticks.

The three dimensional structure of Lipase ITB1.2 was constructed by homology modelling with Swiss-Model and the result showed highest homology (98,7%) with Lipase T1 (2DSN.PDB) from *G. zalihae*.

DISCUSSION

Lipase ITB3.1 showed highest homology to lipase from *G. thermoleovorans* (accession number AF134840.1). Based on 16S rRNA gene sequence previously reported, DMS3 isolate was closely related to *G. kaustophillus* (accession number EU652092) and *G. thermoleovorans* (acession number AY074879), both of them located in the same branch. However, homological analysis of lipase ITB3.1 with lipase from *G. kaustophillus* HTA426 was only 95% compared to that homology of the gene to lipase from *G. thermoleovorans* which was 99%. From the homology analysis of lipase ITB3.1 and 16S rRNA gene sequences of DMS3 isolate, suggested that DMS3 isolate show closer to *G. thermoleovorans* compared to that the *G. kaustophillus.*

Lipase ITB3.1 shows 99% homology with three published lipases and at least 92% homology with other lipases from sub-family 1.5 (Table 2). The lipase has same catalytic residues in the same position with other lipases from sub-family 1.5. There are no differences in amino acid residues observed in oxyanion hole as well as the lid. Lipase ITB3.1 has 8 amino acid differences compared to three closest related lipase from *G. thermoleovorans*, *G. stearothermophilus* and *Geobacillus sp.*with accession number AF134840.1, AAX11388.1, ADU94368.1 respectively. Among them, one residue (Asn194) which is conserved among three other lipases but substituted on lipase ITB3.1 (Table 3). The residue of Asn194 was replacing residue Asp194 on other lipases. Alignment analysis of amino acid sequences of lipase ITB3.1 and other lipases showed residue Asn194 probably located in a turn between β-6 and α-6[21]. The α-6 and α-7 were identified as lid in lipase sub-family 1.5[22]. Asn194 residue probably located in the surface of the enzyme, which has contact with solvent, as shown in Fig 2. Therefore the substitution of Asp to Asn which is a negatively to neutrally charged amino acid may not exhibit significant effect to the characteristic of lipase ITB3.1.

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