OVEREXPRESSION OF RECOMBINANT LIPASE FROM *BURKHOLDERIA CEPACIA* IN *ESCHERICHIA COLI*

M. RAFTARI[1], S. GHAFOURIAN[2,3], N. SADEGHIFARD[3], F. ABU BAKAR[1], N. SAARI[1] and Z. SEKAWI[2]

[1]Faculty of Food Science and Technology, University Putra Malaysia, Serdang, Selangor, Malaysia; [2]Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, University Putra Malaysia, Serdang, Selangor, Malaysia; [3]Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran

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This study attempts to clone and express the extracellular lipase from *Burkholderia cepacia* in *Escherichia coli* using pET system as well as to determine the enzyme activity of recombinant lipase. The extracted DNA from *B. cepacia* was used as a template for amplifying lipase gene, and then the lipase gene was subcloned into pET-32a and subsequently transformed into *E. coli* BL21. Media assay and SDS-PAGE were carried out to analyse the results. Nucleotide sequencing of the DNA insert from the clone revealed that the lipase activity corresponded to an open reading frame consisting of 1092 bp coding for a 37.5-kDa protein. The successful expression of lipase was confirmed by obtaining blue color colonies on Nile Blue Sulphate Agar and big band at 37.5-kD size on SDS-PAGE. The enzyme activity assay also showed the high lipase activity around 590 μg lipase ml^{-1} culture 30 min^{-1} of recombinant *E. coli* BL21. The specific lipolytic activity of the recombinant lipase was 185 U/mL which is around 35-fold higher than the native baseline. The findings suggest that the crude recombinant lipase has potential application in digestion of lipids and fatty acids. In conclusion, the results of the current study showed a lipase gene encoding an enzyme with non-specific hydrolysis activity, which could be applied as lipase biosensor for digestion of lipids in food and medicine as well as oil-contamination treatment.

Lipases are a class of enzymes able to hydrolyze ester bonds in triacylglycerides to form fatty acids and glycerol, or catalyze the reaction reversibly under certain conditions (1). These properties make lipases widely useful for the production of free fatty acids, inter-esterification of fats, synthesis of esters, etc (2).

Lipase activity generally depends on the availability of large surface area and requires extreme mild conditions. Several studies on lipases have provided clues to the understanding of hydrolytic activity, interfacial activation and stereoselectivity (3). A survey by Linko and Wu on major commercial lipases revealed that *Aspergillus* lipases were highly selective for short-chain acids and alcohols; *C. rugosa* lipases for propionic acid, butyric acid, butanol, pentanol and hexanol; and *M. miehei* and *R. arrhizus* lipases for long-chain acids and acetates (4). This information on enzyme catalyses has been valuable for the production of flavour esters (5). Increase in lipase activity depends

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Mailing address: Fatimah Abu Bakar,
Faculty of Food Science and Technology,
University Putra Malaysia,
UPM Serdang, 43400,
Serdang, Selangor, Malaysia
Tel.: +60 03 89468368 Fax: +60 03 89423552
e-mail: fatimah_upm_fst@yahoo.com

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on the concentration of ammonium sulphate solution used during the purification process.

A large number of fat clearing enzymatic lipases are produced on an industrial scale. Most of the commercial lipases produced are utilized for flavor development in dairy products and processing of other foods, such as meat, vegetables, fruit, baked foods, milk product and beer (6). Lipase immobilized on silica and microemulsion based organels were widely applied for ester synthesis. Lipases are extensively used in the dairy industry for the hydrolysis of milk fat. The dairy industry uses lipases to modify the fatty acid chain lengths, to enhance the flavours of various cheeses. Current applications also include the acceleration of cheese ripening and the lipolysis of butter, fat and cream (7).

B. cepacia lipase which was formerly known as *Pseudomonas cepacia* lipase has been shown to be a useful enzyme which catalyzes a broad range of different reactions in water and non-polar solvents under mild conditions. *B. cepacia* lipase is a highly selective catalyst for a broad range of substrates (8), including the kinetic resolution of racemic mixtures of secondary alcohols by hydrolysis in water or esterification in organic solvents (9, 10 and 11).

In the present study, overexpression of extracellular lipase from *B. cepacia* ATCC21808 in *Escherichia coli* BL21 using pET system studied and characterized. Molecular cloning and sequence analysis of lipase encoding gene was carried out to explore its expression in *E. coli* BL21 which it can be used for future application.

MATERIALS AND METHODS

Strains, plasmids, and culture medium

B. cepacia ATCC21808 was purchase from ATCC. The *E. coli* XL10-gold and *E. coli* BL21 were used for gene cloning and expression, respectively. All *E. coli* strains were cultivated in Luria-Bertani (LB; 1.0% tryptone, 0.5% yeast extract, and 1.0% NaCl) medium at 37°C. Ampicillin was added when necessary at a final concentration of 100 μg/mL.

Preparation of genomic DNA from B. cepacia

B. cepacia was grown at 37°C in M9 medium containing 0.5% yeast extract and 0.5% olive oil (M9YO) for 24 h with reciprocal shaking (180 r/min). The cells were harvested by centrifugation at 12,000 r/min for 15 min.

Genomic DNA was extracted using DNA extraction Kit (GenAll, Korea) according to the manufacturer's instructions. The purity of genomic DNA was confirmed by agarose gel electrophoresis and the ratio of OD260 and OD280 of DNA.

Cloning and sequencing of the lipase gene

Lipase gene from *B. cepacia* was cloned and expressed by the standard protocol of Sambrook and Russell (12). Briefly, after extraction of DNA, based on the consensus amino acid sequences of the lipase gene acquired from the NCBI nucleotide database, a pair of primers (Forward: 5-GGATCCATGGCCAAATCGATGCGTTCC-3 and Reverse: 5- AAGCTTTTACACGCCCGCGAGCTTCA -3) were designed and used for the amplification of lipase gene. BamHI endonuclease site was included at the forward primer, while HindIII endonuclease site and two strong stop codons TAA were incorporated at the reverse primer. The PCR-amplified products were directly cloned into pJET1vector (Fermentas, USA) according to the manufacturer's instructions.

The DNA sequencing of the lipase gene was carried out. The nucleotide and amino acid sequences were analyzed and compared with entries in protein databases provided by GenBank™ and EMBL by use of the FASTA and BLAST programs.

Expression and purification of the lipase

To clone and express lipase gene, the pET32a was double digested with BamHI and HindIII; after that the lipase gene was ligated in it using T4 DNA ligase (Fermantas, Lithuania) resultant the recombinant expression vector pET32a-lip. The recombinant expression vector was transformed into *E. coli* BL21 competent cells by chemical CaCls method. Positive clones were identified by colony PCR and double restriction digestion. Detailed protocols were carried out according to Sambrook and Russell (12).

Expression of lipase gene in E.coli BL21

A transforming of *E. coli* BL21 harboring pET32a-lip was cultured with vigorous shaking at 37°C overnight in 3 mL LB broth containing 100 μg/mL ampicilin. The culture was transferred using 1% inoculums to 100 ml fresh LB broth containing the same concentration of ampicilin; the culture was incubated with shaking at 37°C until the OD600 reached 0.6–0.8; IPTG was then added to a final concentration of 0.1 mM. Cells were collected 2, 4 and 6 h after IPTG addition for protein detection by SDS-PAGE. The negative control was prepared by the same method using cells harboring the non-cloned vector.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular mass of the recombinant enzyme

was determined using SDS-PAGE (12%). A volume of 15 µl of protein sample was added into each lane of gel. After electrophoresis the gel was stained with Coomassie Brilliant Blue, and distained by washing with mixture of acid-methanol-water (10:25:65, v/v/v).

Lipase activity

Agar plate assay is the most frequently used procedure for screening of lipase existent on petri-dishes. In this assay, the changing color of colony shows the enzyme activity (13).

Liberated free fatty acids were determined by calorimetric method using olive oil as a substrate (19). An equal volume of olive oil (Merck, Germany) and 50 mM phosphate buffer (pH 7) was mixed to prepare the emulsion. One milliliter of enzyme was added to 2.5 mL of the emulsion plus 20 µL of 0.02 M CaCl2 and was shaken at 200 rpm for 30 min at 37°C. One milliliter of 6 N HCl and 5 mL of isooctane were then added to stop the enzyme reaction. This was followed by a vigorous mixing for 30 s with a vortex mixer. Four milliliters of the upper isooctane layer was transferred to a test tube containing 1 mL of copper reagent. The copper reagent was prepared by 5% (w/v) copper (II) acetate-1-hydrate and the pH was adjusted to 6.1 with pyridine. The absorbance of the upper layer which contained the liberated fatty acids was read at 715 nm. Lipase activity was determined by measuring the amount of free fatty acid released by referring to the standard curves of free fatty acids. One unit of lipase activity was defined as the rate of 1 µmol of fatty acid released Per minute.

RESULTS

Cloning and sequencing of the lipase gene

PCR fragments of the expected size (1092 bp) were obtained using degenerated primers, subcloned and sequenced.

Nucleotide sequence analysis revealed an open reading frame (ORF) starting at bp 1 with an ATG codon and ending at a TAA at bp 1092, thereby encoding a protein of 365 amino acids lipases are known to have an active-site consensus sequence Gly-X-Ser-X-Gly and to form a catalytic triad consisting of Ser, Asp and His residues. LipA had the Gly-X-Ser-X-Gly sequence at positions 106-110, and Ser, Asp and His residues at 208, 315 and 278, respectively. In addition, two Asp residues, known Ca+2-binding sites, were found at positions 240 and 279, and two Cys residues forming a disulfide bond at positions 210 and 261 were also conserved.

Overexpression of the lipase gene in E. coli

The lipase gene was inserted in double digested site of pET32a by BamHI and HindIII, and then the recombinant vector transformed into *E. coli* BL21. The positive cloned cells were collected from LB agar which contains 100 ug/ ml of ampicillin. The results showed 1092 bp increasing in the size of plasmid after screened on 1% agarose gel. To study the lipase expression the recombinant *E. coli* BL21 containing pET32a-lip, *E. coli* BL21 containing pET32a was cultured on Nile Blue Agar. The results showed no lipase activity in *E. coli* BL21 containing pET32a as control while the blue color of recombinant cells confirm the expression of lipase (results not shown). In the next step, the protein expression was monitored and analyzed using SDS-PAGE technique. SDS-PAGE analysis revealed the presence of a new protein in the supernatant obtained after sonication treatment. High activity was observed in all supernatants treated with IPTG but the highest was attained after 6 h with 0.1 mM IPTG (Fig. 1). The new protein had an approximate molecular weight of 37 kDa and was not observed in the control sample. The size of the expressed protein agreed well with the predicted size of the lipase (37.5 kDa).

Screening the results for elastase activity showed high lipase activity of recombinant *E. coli* around 590 µg elastase ml^{-1} culture 30 min^{-1}. The specific lipolytic activity of the recombinant lipase was 185 U/mL which is around 35-fold higher than the native.

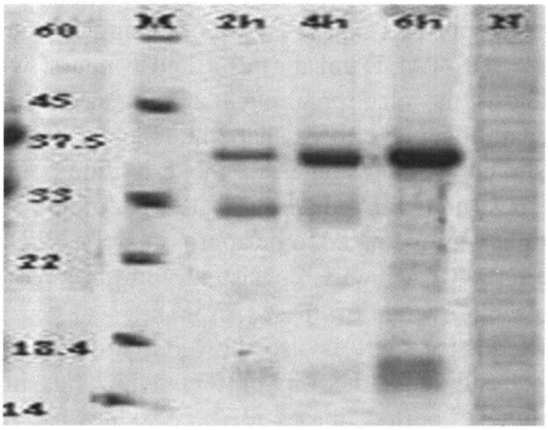

Fig. 1. *SDS–PAGE and activity analysis of the recombinant lipase after induction by IPTG at different time-points.*

DISCUSSION

Enzymes were used widely in our life in many applications including powder detergents, but nowadays are seldom found. Their wide applications cause accumulation of their chemical components. Detergents were considered as a hidden constant source of pollution. Lipases have been commonly used in the production of a variety of products, ranging from fruit juices to vegetable fermentation. Lipases facilitate the removal of fat from meat and fish products (14). Cao et al. reported a lipase-catalyzed solid phase synthesis of sugar fatty acid esters (15). Pandey et al. (3) reviewed the direct conversion of alkane diols into their monoesters and diesters, which has strong implications for the food and pharmaceutical industries, because the products have non-ionic surfactant activity and can be used as monomeric units in cross-linking in polymerization. The processing of sausages with microbial lipases is an emerging use of meat technology (14).

The aim of this study was to clone and express a lipase with a high hydrolytic activity to degrade fatty acids. Meta-genomic library was then constructed and used to screen lipase gene (3). The lipase gene encoding an extracellular lipase cloned from the meta-genomic library was composed of 1092 bp in length. The predicted protein consisted of 364 amino acids with a calculated molecular mass of 37.5-kDa. Nucleotide sequence alignment showed that lipase gene has 100% identity with *B. cepaciae* lipase and the lowest identity was observed with *Vibrio vulnificus* lipase.

The purified lipase showed a single band in 12% SDS–PAGE gel during 4 and 6 hours, which suggests the electrophoretical homogeneity of the preparation. Nevertheless, at 2 hours more band was observed, which suggested the 6-h time-point as the best hour for induction by IPTG. Its molecular weight was estimated to be approximately 37kDa by SDS–PAGE, which is consistent with the predicted molecular weight of lipase (37.5kDa).

Sequence analysis also showed that there is only one cysteine in the Lip protein, which is similar to other reported lipases (16). Cysteines are often involved in the formation of disulfide bonds in proteins, and proteins without cysteines are generally more flexible because of the lack of disulfide bonds (17). This characteristic is important for protein secretion of extracellular bacterial proteins, which also readily allow the conformational change that accompanies interfacial activation. The data demonstrated that lipase was mainly produced in BSL2 strain with the form of extracellular enzyme.

In conclusion, we reported a lipase gene encoding an enzyme with non-specific hydrolysis activity. It could be applied as lipase biosensor for digestion of lipids in the food and medicine, and for oil-contamination treatment.

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