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Original Article

Cloning and nucleotide sequence of a lipase gene from a soil isolate

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Abstract

The present study was aimed to isolate and characterize the lipolytic enzyme producing bacteria from soil samples of regions around Zayande-rood river of Isfahan, Iran. Soil samples were collected from 15 cm depth of soil surface. Based on morphology, distinct colonies were isolated and purified through streak culture on to standard agar plates. Isolated colonies were examined for lipase activity using egg-yolk agar medium. Total of 15 isolates developed clear zones around their growth area which were considered as lipase positive. Preliminary identification of lipolytic active isolates revealed a gram-positive, rod-shaped, endospore-forming and catalase positive bacteria, characteristics indicative of the genus *bacillus*. The gene coding for an extracellular lipase was cloned using PCR techniques. The gene was identified to be 639 bp in length and encoded a peptide of 212 amino acids with calculated molecular mass of 19353 Da, and pI 9.28. The DNA sequence and deduced amino acid sequence of the hypothetical lipase showed striking similarities to lipases from *B. subtilis* strains.

Keywords: Lipolytic bacteria; Lipase gene; Soil

INTRODUCTION

Lipases (triacylglycerol acyl ester hydrolases; EC 3.1.1.3) are biocatalysts that hydrolysis long chain triglycerides at the water/oil interphase to yield free fatty acids, monoglycerides, diglycerides and glycerols (1). These enzymes have been isolated from plants, animals, and microorganisms. The lipase from microorganisms attracted more attention, because of their great versatility, the possible high yields, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations, and rapid growth of microorganisms on inexpensive media (2).

Lipases of different microorganisms are used in various fields such as food, dairy, pharmaceutical, and laundry industry, and in synthesis of fine chemicals, new polymeric materials, and environment industry (1,2). For each of these applications a variety of lipases with different properties with respect to specificity, stability, temperature, pH and catalytic activity in organic solvents are available (3-5). Due to their interesting biological properties, bacteria of the genus *Bacillus* are attractive candidates for a number of industrial applications (6). Several species of this group are non-pathogenic, simple to cultivate and secrete enzymes such as proteases, amylases, and lipases. Enzymes from these microorganisms developed distinctive structure function properties of high thermostability and optimal activity under alkaline conditions.

Bacterial lipases have been classified into 6 families based on sequence homology and protein molecular size (7). Within this classification, *bacillus* lipases belonged to either subfamily I.4 or subfamily I.5. All *Bacillus* lipases share a conserved Ala-X-Ser-X-Gly pentapeptide that contains the catalytic serine residue. In the current study, soil from regions around Zayande-rood river of Isfahan,

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Iran was screened for lipolytic enzyme producing bacteria. Lipase producing isolates were further analysed using biochemical and molecular methods.

MATERIALS AND METHODS

Materials

Tryptone and yeast extract were purchased from Gibco (England). Trypton Soy Broth was obtained from Oxoid, Nutrient agar, nutrient broth, Simmon citrate agar, agar, Voges-Proskauer medium, acrylamide, bis acrylamid, TEMED, ammonium persulfate, EDTA, bromophenol blue, glacial acetic acid, and NaCl from Merck (Germany). Ethidium bromide and loading buffer were purchased from Cinnagen (Iran). Lysozyme, ampicillin, Isopropyl β-D-thiogalactoside (IPTG), agarose from Gibco. oligonucleotide primers, deoxy-nucleotides and Taq polymerase were purchased from Bioron (Germany). Restriction reactions and ligations were performed with enzymes from Fermentas (St Leon-Rot, Germany) under conditions recommended by the manufacturer. Unless mentioned otherwise, all reagents were of analytical or molecular biology grade.

Bacteria, plasmids and media

E. coli cells XL1 blue, HB101 and TOP10 (Promega, Madison, USA) were used for cloning experiments. *B. subtilis* form the Persian Type Culture Collection (PTCC 1254) was used as control in genomic DNA isolation, PCR amplification, cloning and sequencing experiments. Plasmid pTZ57R (Promega, Madison, USA) was used for cloning and subsequently sequencing procedures. The soil isolates were grown at 37 °C in Luria-Bertani (LB) broth overnight for the genomic DNA preparation. The *E. coli* hosts carrying plasmids were grown in LB medium containing 100 µg ampicillin/ml.

Isolation of soil microorganisms

Soil samples were collected from Zayande-rood river in Isfahan, Iran. Surface soil (10 to 20 cm depth) was sieved through a 2 mm mesh to remove plant debris and particulate matter larger than 2 mm and then dried for 24 h at room temperature and stored at 4 °C until use. Ten gram of soil was suspended in 90 ml of sterile 0.9% NaCl and homogenized for 30 min at room temperature. The soil suspension was then treated for 10 min at 80 °C to isolate only spore forming bacteria. After cooling for 10-15 min, ten-fold serial dilutions were made in a dilution tube containing 9 ml of sterile 0.9% NaCl. Aliquots of 100 µl were spread on to nutrient agar or standard plate count agar and incubated for at least 24-48 h at room temperature (28 ± 2 °C) and 37 °C. Bacteria were isolated from plates that contained well separated colonies. Plate from each dilution with well isolated colonies was further streaked on fresh nutrient agar medium to achieve pure culture of each represented grew colonies for further analysis.

Lipolytic activity

Isolates were grown on egg-yolk agar medium in order to screen for lipase activity. The medium contained (per 400 ml) 12 g of dehydrated Trypton Soy Broth, 6 g of agar, 50 ml of egg yolk suspension in 0.9% NaCl at pH 7.4. The isolated pure colonies obtained in the previous section were streaked on this medium and incubated for at least 24-48 h at its respective temperature. Clones conferring lipolytic activity were identified by the formation of clear zone around the edges of their colony. Strains with this ability were selected for further analysis.

Characterization of microorganism with lipolytic activity

Isolates with lipolytic activity were further examined using selected biochemical tests described elsewhere (8). Isolates were inspected with respect to the following characteristics: Gram's stain reaction, spore position and shape, swelling of sporangia, aerobic or anaerobic growth, Vogues-Proskauer test, oxidase, catalase, use of citrate, nitrate reduction, and lecitinase reaction. Tests were performed in duplicate or triplicate and differential characteristics were recorded as positive or negative response. All biochemical characteristics were investigated for at least 24 h. For staining procedures, an overnight culture of soil isolate on nutrient agar was

used. Staining was performed according to available procedures (8). Cell morphology was examined by light microscopy (Nikon, Japan). For most isolates spore position and shape were determined on both Gram stained and Malachite green stained slides.

DNA preparation and transformation

Genomic DNA was prepared by high pure PCR template preparation kit as described by the manufacturer (Roche Applied Sciences, Germany). Plasmid DNAs were isolated using Aurum Plasmid Miniprep column spin format kit. Transformation of *E. coli* strains with recombinant plasmids was performed using standard $CaCl_2$ procedures (9). General DNA manipulations and recombinant DNA techniques were performed as described elsewhere (9).

PCR amplification

Known lipase nucleotide sequences from *B. subtilis* (M74010 and D78508), *B. pumilus* (A34992), *B. licheniformis* (AJ297356), and *Bacillus sp*. (AF232707), were aligned and used to design a set of detection primers.

The purified genome from isolates was screened by PCR using a pair of primers as forward L1 5'-atggttcacggtattggagg-3' and reverse L2 5'-ctgctgtaaatggatgtgta-3'. The aim was to amplify a fragment internal to lipase-coding regions of aligned sequences. Isolate that produced PCR product of expected size using above primer pair was further screened using a second pair of primers designed on the basis of known coding region for lipases of *B. subtilis* strains. The current pair was designed to cover full length of lipase coding region. The primers were upstream LG1 primer 5'-ggaattccatatgaaatttgtaaaaagaagg-3' and the downstream LG2 primer 5'-cgcggatccattaattcgtattctggcc-3'. The restriction sites for *Nde*I and *Bam*HI were incorporated into the forward and reverse primer sequence, respectively.

Taq polymerase was used to perform PCR with soil bacterial isolates genomic DNA as the template. The PCR conditions were as follows: one initial denaturation step at 94 °C for 5 min, 35 cycles at 94 °C for 1 min, annealing at 60 °C for 2 min, extension at 72 °C for 3 min, and a final extension for 5 min. The PCR products were electrophoretically resolved on a 0.8% w/v agarose gel and visualized under UV irradiation.

Cloning and sequencing of the lipase gene

The appropriate PCR products recovered with an agarose gel DNA extraction kit (Qiaquick gel extraction kit, Qiagen). The purified DNA fragments were ligated into the cloning vector pTZ57R, according to the manufacturers' instructions. Recombinant vector was then transformed into *E. coli* competent cells, and plated on LB agar plates containing 100 µg/ml ampicillin. The clone containing the PCR product amplified by internal primer was named pTZ-371 and the one containing the PCR product amplified using outer primer was named pTZ-639. The nucleotide sequences of cloned PCR products in cloning vectors were determined by Fazabiotech (Tehran, Iran).

Sequence analysis

Nucleotide sequences and oligonucleotide primers were investigated using BLASTN at the National Center for Biotechnology Information (10). Multiple sequence alignments were performed with the program MegAlign of the software package DNAstar (Lasergene). Nucleotide Sequence data in this study was translated to amino acid sequences using translation table 1 at NCBI. BLASTX at NCBI was used for amino acid sequence comparison. Theoretical pI and molecular weight was calculated using tools at ExPASy.

RESULTS

Lipolytic activity screening

From the soil sample employed in this study, 15 bacterial colonies were isolated in pure cultures with the ability to develop clear zone around their growth area following incubation on egg-yolk agar plates. Preliminary characterization of lipolytic active strains was performed using microscopic and selected biochemical examinations as described in methods. Following gram staining, isolates that were gram-positive and rod-shaped had short or long chains or single or pairs

appearance. Cells were observed to be endospore-forming bacteria. Central, subterminal or terminal, ellipsoidal or spherical endospores were also observed through light microscopy in swollen or non-swollen sporangia following Malachite green staining. However, spores were observed more frequently at subterminal position inside the cell. Isolates showed optimum growth at 37 °C and relatively poor growth at 25 °C. The organisms were also shown to be aerobic or facultative anaerobic and catalase positive bacteria. From the total of 15 isolates examined for lecithinase activity, 6 isolates were found to be positive while others appeared negative in this respect. Except for isolate 2 which remained negative for nitrate examination, other 14 gave a positive results in this test. With respect to Voges-Proskauer test, from 15 isolates investigated, 10 isolates (3, 5-8, 11-15) were found to be positive. Most isolates changed citrate medium to blue color following incubation, except for isolates 9 and 10. In the case of Maltose test, 6 isolates (4,5,7,10,14,15) turned out to be able to degrade maltose during their growth. The results of biochemical tests are summarized in Table 1. The obtained results particularly those for gram and Malachite staining, and catalase test were characteristics which had been described for the genus *Bacillus* (8). Therefore, lipolytic isolates were considered as *bacillus* species. However, due to disagreement of some test results, assignment to a particular species was not possible. The entire lipolytic isolates were employed in the following investigations.

First PCR amplification

Lipolytic isolates were further investigated using molecular methods. The aim was to detect and subsequently clone the complete sequence of the detected lipases in the plate assay examination. Using oligonucleotide primers (L1 and L2 described under methods) genomic DNA from lipolytic active isolates were screened. From the total of 15 isolates investigated, only isolate no 6 produced a band following examination of PCR products on agarose gel electrophoresis. Other examined isolates did not produce any band under applied conditions (data not shown).

Cloning and sequencing of the first PCR product

The amplified PCR product was ligated into the cloning site of the cloning vector. The recombinant vector was submitted for sequencing using T7 promoter primer. The sequenced data revealed that the amplified product using L1 and L2 primers was 371 bp in length (data not shown). The 371 bp fragment was searched for homology in NCBI.

Table 1. Biochemical characteristics of soil isolates.

Sample number	Matlose	Citrate	Voges-Proskauer	Nitrate	Lecitinase	Catalase
1	-	+	-	+	+	+
2	-	+	-	-	+	+
3	-	+*	+	+	-	+
4	+	+	-	+	-	+
5	+	+	+	+	-	+
6	-	+	+	+	-	+
7	+	+	+	+	-	+
8	-	+*	+	+	-	+
9	-	-	-	-	+	+
10	+	-	-	+	+	+
11	-	+	+	+	+	+
12	-	+	+	+	+	+
13	-	+	+	+	-	+
14	+	+	+	+	-	+
15	+	+	+	+	-	+

* Faster response to citrate test as compared to samples 6 and 13

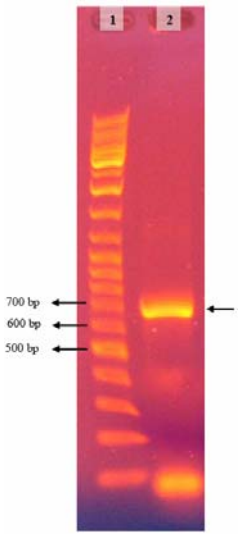

Fig. 1. Agarose gel electrophoresis of PCR product using LG1 & LG2 primers. Lane 1: DNA ladder, lane 2: genomic DNA from isolate clone No 6.

The results showed maximum similarity with group I.4 of *bacillus* lipases with the most coverage value for lipase from *B. subtilis* strains.

Second PCR amplification

To amplify the complete sequence of the detected target gene in the previous step using L1 and L2 primers, a second pair of oligonuceotide primers (LG1 and LG2 described under methods and materials) were designed. Using the current pair, genomic DNA from isolate 6 was investigated which revealed to produce a single band following examination on agarose gel electrophoresis (Fig. 1, lane 2).

Cloning and sequencing of the second PCR product

The amplified product from the second round of PCR was extracted from the gel and ligated into the cloning vector. The recombinant vector was designated as pTZ-GHlip and submitted to sequencing using T7 promoter primer. The resulting complete lipase gene sequence was deposited into Genbank sequence databases at NCBI under the accession number of FJ481899. The cloned sequence (designated as GHlip) was found to be 639 bp in length (Fig. 2). The statistics of this sequence with respect to counts of nucleotide residues are summarized and compared with that of lipase A (LipA) from *B. subtilis* (Table 2). The deduced amino acid sequence of GHlip was also created which revealed to be a preprotein consists of 212 amino acid residues including a segment of 31 amino acid residues as its putative signal sequence at N-terminus (data not shown). The count and frequency of each amino acid residue with respect to that of lipase A protein (the most similar protein sequence in NCBI), was also generated. On homology search, the

ATGAAATTTGTAAAAAGAAGGATCATTGCACTTGTAACAATTTTGATGCTGTCTGTTACGTCGCTGTTTGC
GTTGCAGCCGTCAGCAAAAGCCGCTGAACACAATCCAGTCGTTATGGTTCACGGTATTGGCGGGGCATCA
TTCAATTTTGCGGGAATTAAGAGCTATCTCGTACCTCAGGGCTGGTCGCGGGACAAGCTGTATGCAGTTG
ATTTTTGGGACAAGACAGGCACAAATTATAACAATGGACCGGTATTATCACGATTTGTGCAAAAGGTTTT
AGATGAAACGGGTGCGAAAAAAGTGGATATTGTCGCTCACAGCATGGGGGGCGCGAATACACTTTACTA
CATAAAAAATCTGGACGGCGGAAATAAAATTGCAAACGTCGTAACGCTTGGCGGCGCCAACCGTTTGAC
GACAGGCAAGGCGCTTCCGGGAACAGATCCAAATCAAAAGATTTTATACACATCCATTTACAGCAGTGCC
GATATGATTGTCATGAATTACTTATCAAGATTAGATGGTGCTAGAAACGTTCAAATCCATGGCGTTGGAC
ACATCGGTTTATTGATGAGCAGCCAAGTCAACAGCCTGATCAAAGAAGGGCTGAACGGCGGGGGCCAGA
ATACGAATTAA

Fig. 2. Nucleotide sequence of the GHlip lipase (accession number FJ481899).

Table 2. Comparison of nucleotide counts of cloned lipase gene (GHlip) and LipA from *B.subtilis*.

Nucleotide	GHlip	LipA
Adenine (A)	194	193
Cytosine (C)	124	124
Guanine (G)	162	164
Thymine (T)	159	158
C+G	286	288
A+T	353	351

primary structure of the GHlip protein demonstrated significant homology with those of lipases from *B. subtilis* strains especially with that of LipA. However, little homology were found with those lipase sequences from other *bacillus* species (data not shown).

DISCUSSION

The aim of this study was the isolation of lipolytic bacteria from the local soil sample. In general, cultivation has been successfully used to screen single microbes or consortia with diverse catabolic capabilities, including the ability to degrade lipid substances (11,12). Isolated colonies considered as different on the basis of their morphology. Bacterial colonies were obtained in pure cultures and screened for lipolytic activity. Isolates showing clear zone around their colonies were considered as lipolytic active strain and further analysed using biochemical and microscopic characteristics. The soil isolates in this study were found to be aerobic, gram-positive, endospore-forming, rod-shaped bacteria, the characteristics described earlier for *bacillus* species (13). These bacteria (*bacillus* species) have been commonly found in soil, water sources or associated with plants (14). They are distinguished from the other endospore-forming bacteria on the basis of being a strict or facultative aerobe, rod-shaped, and (usually) catalase-positive.

Since preliminary findings from bio-chemical and microscopic examinations supported the isolation of *bacillus* species from collected soil, screening for *bacillus* lipases came into focus at first attempts. From available lipase sequences of *bacillus* species (described under methods and materials) a pair of primer was designed to cover the coding region of lipase sequences. Genomic DNA from lipolytic isolates was first screened using a pair of internal detection primer. In this attempt among 15 soil isolates identified as being lipolytic active, only clone isolate designated as no 6 from the bank of the Zayande-rood river was found to produce a single band under deployed PCR conditions. Positive isolates in this step were further examined using a pair of outer primer (LG1and LG2 described under methods). As sequencing of the PCR product using internal primers showed that the target sequence in soil isolate belonged to *B. subtilis*, in the second attempt the oligonucleotide primers were designed based on lipases from *B. subtilis* strains. The nucleotide sequence of GHlip was compared to available lipase sequences at NCBI. The results revealed maximum similarity with lipases from group I.4 of *bacillus* lipases especially those of *B. subtilis* strains (data not shown). The sequence was compared to its most similar sequence from *B. subtilis* lipase (LipA). From this comparison (Table 2) it was found that adenine and thymine nucleotides were more frequent in GHlip compared to that of LipA. In contrary, guanine and cytosine nucleotides were found to occur more frequently in LipA compared to that of GHlip. The nucleotide sequence of the GHlip was also aligned with that of LipA and it was found that GHlip differ from LipA in 16 nucleotide residues. The sequence was also found to encode a polypeptide of 212 amino acid residues following theoretical translation with calculated molecular mass and pI of 19353.96.Da and pI 9.28 respectively. The deduced amino acid sequence of GHlip was compared to available protein sequences at NCBI. The results showed about 98% identity with lipases from *B. subtilis* strains (data not shown) and 68% identity with the esterase from *B. subtilis* (15,16). The GHlip was also exhibits approximately 75% identity with lipases from *B. pumilus* (17) and 72% with that of *B. licheniformis* (18). However some variations were also observed with respect to different strains of the mentioned species obtained in different studies. In general sequence alignments indicated that GHlip was most similar to the *Bacillus* lipases of family I.4 especially with high levels of similarity to

B. subtilis 168 LipA, while it showed little similarity to other bacillus lipases (family I.5. data not shown). The statistics of the deduced amino acid sequence of GHlip was also generated and compared to that of LipA. In this respect, GHlip was observed to have more isolucine, methionine and praline residues compared to that of LipA. While serine, valine and tyrosine were found more frequently in LipA compared to that of GHlip (data not shown).

With respect to its most similar sequence (LipA), the GHlip was found to differ at 3 or 4 amino acid residue from LipA. Amino acid at position 59 (numbered based on LipA precursor from *B. subtilis* 168) in LipA *B. subtilis* (the most similar sequence at NCBI) was found to be changed from S to P in isolated sequence in this study. This change occurred in a helical region of protein. Amino acid at position 127 was found to be changed from V in LipA *B. subtilis* 168 to I in isolated sequence in this study. This was found to be in a β-strand region. Amino acid at position 192 LipA *B. subtilis* 168 was found to be changed from Y to M in isolated sequence in this study which is in a helical region in the protein. Another change was found to be at position 133 which was amino acid L in isolated sequence in this study and V for LipA *B. subtilis* 168 in some studies (19).

The active site of many α/β-hydrolases consists of a catalytic triad formed by a nucleophilic residue followed by a catalytic acid, which is not directly involved in catalysis, and consists of a histidine. In lipases, this active site usually consists of a serine, an aspartate or glutamate, and a histidine (20). Analysis of the deduced amino acid sequence showed that GHlip has the lipase catalytic triad, Ser, Asp, and His located at positions 108, 164, and 187, respectively. The positions of the catalytic triad residues of GHlip were proposed based on a sequence alignment of the closely related lipases from *B. pumilus* and *B. subtilis* (data not shown).

Collectively, with regard to presented data in this study, it can be concluded that the soil isolate in this study is a bacteria of the genus *bacillus* origin and more precisely it is a strain of *B. subtilis* and finally the cloned fragment designated in this study as GHlip is a complete sequence of a lipase gene. In other words it can be considered a variant form of the lipase of *B. subtilis*. Further investigations are in progress to identify the isolated strain in detail, and verify its lipolytic enzyme and its potential biotechnological application. To investigate the effect(s) of amino acid changes on activity and/or stability of cloned lipase in this study, expression attempts of the recombinant protein is under way.

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