



(22) Date de dépôt/Filing Date: 2006/12/08

(41) Mise à la disp. pub./Open to Public Insp.: 2007/06/14

(45) Date de délivrance/Issue Date: 2015/04/07

(62) Demande originale/Original Application: 2 633 847

(30) Priorité/Priority: 2005/12/09 (JP2005-356936)

(51) Cl.Int./Int.Cl. *C12N 15/55* (2006.01),
C12N 1/21 (2006.01), *C12N 15/63* (2006.01),
C12N 9/18 (2006.01), *C12P 7/62* (2006.01)

(72) Inventeurs/Inventors:
NAKAO, MASAHIRO, JP;
KANAMORI, MASAKI, JP;
FUKAMI, HARUKAZU, JP;
KASAI, HIROAKI, JP;
OCHIAI, MISA, JP

(73) Propriétaire/Owner:
SUNTORY HOLDINGS LIMITED, JP

(74) Agent: NORTON ROSE FULBRIGHT CANADA
LLP/S.E.N.C.R.L., S.R.L.

(54) Titre : NOUVELLE LIPASE DE 40 KDA DU GENRE TETRASPHERA

(54) Title: A NOVEL 40 KDA LIPASE FROM TETRASPHERA

(57) **Abrégé/Abstract:**

The present invention provides a novel lipase with a molecular weight of about 32 kDa, which is produced by a strain belonging to the genus *Tetrasphaera*, as well as a gene encoding the same. This lipase has the ability to recognize a medium-chain fatty acid as a substrate. The present invention also provides a novel lipase with a molecular weight of about 40 kDa, which is produced by a strain belonging to the genus *Tetrasphaera* and has the ability to recognize both a medium-chain fatty acid and a long-chain fatty acid as substrates, as well as a polynucleotide encoding the same. The present invention further provides *Tetrasphaera* sp. strain NITE P-154. The lipase of the present invention can be used as an immobilized enzyme and is useful in fields such as production of digestants and/or flavorings, production of clinical laboratory reagents, detergent enzymes and/or fats, as well as production of optically active intermediates for agricultural chemicals and pharmaceutical preparations.

ABSTRACT

The present invention provides a novel lipase with a molecular weight of about 32 kDa, which is produced by a strain belonging to the genus *Tetrasphaera*, as well as a gene encoding the same. This lipase has the ability to recognize a medium-chain fatty acid as a substrate. The present invention also provides a novel lipase with a molecular weight of about 40 kDa, which is produced by a strain belonging to the genus *Tetrasphaera* and has the ability to recognize both a medium-chain fatty acid and a long-chain fatty acid as substrates, as well as a polynucleotide encoding the same. The present invention further provides *Tetrasphaera* sp. strain NITE P-154. The lipase of the present invention can be used as an immobilized enzyme and is useful in fields such as production of digestants and/or flavorings, production of clinical laboratory reagents, detergent enzymes and/or fats, as well as production of optically active intermediates for agricultural chemicals and pharmaceutical preparations.

- 1 -

A NOVEL 40 KDA LIPASE FROM TETRASPHERA

TECHNICAL FIELD

[0001] The present invention relates to a novel lipase. This lipase is produced by cells of a new *Tetrasphaera* sp. strain. This lipase has the ability to recognize a medium-chain fatty acid and/or a long-chain fatty acid as a substrate. This lipase can be immobilized on an anion exchange resin or a hydrophobic resin through adsorption, and can be used as an immobilized enzyme. This lipase is useful in fields such as production of digestants and/or flavorings, production of clinical laboratory reagents, detergent enzymes and/or fats, as well as production of optically active intermediates for agricultural chemicals and pharmaceutical preparations.

BACKGROUND ART

[0002] Lipases have been demonstrated to be excellent biocatalysts for synthesis and decomposition of various esters, transesterification, and optical resolution of racemic mixtures. In fact, lipases have been used for production of digestants and/or flavorings, production of clinical laboratory reagents, detergent enzymes and/or fats, as well as production of optically active intermediates for agricultural chemicals and pharmaceutical preparations.

[0003] Among lipases, animal pancreatic lipases are well known, but it is primarily microbial lipases that are often used industrially. For most of these lipases, their genes have been cloned and the amino acid sequences thereof are also

- 2 -

known (*Candida rugosa*: Non-patent Document 1; *Rhizopus delemar*: Non-patent Document 2; *Bacillus subtilis*: Non-patent Document 3; *Staphylococcus aureus*: Non-patent Document 4; and *Pseudomonas aeruginosa*: Non-patent Document 5).

[0004] Lipases produced by filamentous fungi or bacteria such as *Bacillus* spp., *Staphylococcus* spp. or *Pseudomonas* spp. are used for industrial purposes. These lipases principally target a higher fatty acid (containing 16 or more carbon atoms) as a substrate, while those targeting a short-chain fatty acid (containing 6 or less carbon atoms) as a substrate are called esterases. No lipase is known which successfully recognizes a medium-chain fatty acid and shows not only hydrolytic activity, but also esterification activity.

[0005] Triglycerides having medium-chain fatty acids are hydrolyzed by the action of not only pancreatic lipases, but also gastric lipases, indicating that lipases are also advantageous in digestion and/or absorption of triglycerides (Non-patent Document 6). Moreover, the absorbed medium-chain fatty acids are less likely to be resynthesized into triglycerides in intestinal tract cells of the small intestine. They are transported through the portal vein to the liver and burned as energy. In contrast, higher fatty acids are resynthesized in intestinal tract cells, absorbed through the lymph and transported to the liver, and in some cases may be accumulated as fat. This means that higher fatty acids are accumulative in the body, whereas medium-chain fatty acids are not accumulative. For this reason, healthy fats and oils are produced by transesterification between medium-chain fatty

- 3 -

acid triglycerides and common edible fats and oils.

Non-patent Document 1: Kawaguchi et al., Nature, 341, 164-166 (1989)

Non-patent Document 2: Haas et al., Gene, 109, 107-113 (1991)

Non-patent Document 3: Dartois et al., B. B. A., 1131, 253-260 (1992)

Non-patent Document 4: Lee et al., J. Bacteriol., 164, 288-293 (1985)

Non-patent Document 5: Wohlfarth et al., J. General Microbiology, 138, 1325-1335, (1992)

Non-patent Document 6: I. Ikeda, Y. Tomari, M. Sugano, S. Watanabe, and J. Nagata: Lipids, 26, 369-373 (1991)

DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0006] However, medium-chain fatty acid esters other than triglycerides have not been produced with lipases on an industrial scale. Since medium-chain fatty acids do not cause fat accumulation, food materials esterified with medium-chain fatty acids may be materials with reduced problems such as obesity.

MEANS FOR SOLVING THE PROBLEMS

[0007] The inventors of the present invention have made extensive and intensive efforts to study lipases. As a result, the inventors have isolated and purified a novel lipase with a molecular weight of about 32 kDa or about 40 kDa from the culture supernatant of cells of a new *Tetrasphaera* sp. strain, and have further found that these lipases are novel ones

- 4 -

successfully recognizing a medium-chain fatty acid as a substrate, thereby completing the present invention.

[0008] I. Novel lipase

The present invention provides a polynucleotide or a homolog thereof, which encodes a lipase with a molecular weight of about 32 kDa produced by a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154), i.e., a polynucleotide comprising (A₁), (B₁), (C₁), (D₁), (E₁), (F₁) or (G₁) shown below:

(A₁) a polynucleotide which consists of all of the nucleotide sequence shown in SEQ ID NO: 10 or a part thereof covering at least nucleotides 25-801;

(B₁) a polynucleotide which is hybridizable under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide shown in (A₁) and which encodes a protein having lipase activity;

(C₁) a polynucleotide which consists of a nucleotide sequence comprising substitution, deletion, insertion and/or addition of one or several nucleotides in the nucleotide sequence of the polynucleotide shown in (A₁) and which encodes a protein having lipase activity;

(D₁) a polynucleotide which shares an identity of at least 80% or more with the nucleotide sequence of the polynucleotide shown in (A₁) and which encodes a protein having lipase activity;

- 5 -

(E₁) a polynucleotide which encodes a protein consisting of the amino acid sequence shown in SEQ ID NO: 11;

(F₁) a polynucleotide which encodes a protein consisting of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 11 and having lipase activity; or

(G₁) a polynucleotide which encodes a protein consisting of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence shown in SEQ ID NO: 11 and having lipase activity.

[0009] SEQ ID NO: 10 shows the nucleotide sequence of a polynucleotide which encodes a lipase with a molecular weight of about 32 kDa produced by *Tetrasphaera* sp. strain NITE P-154, while SEQ ID NO: 11 shows the amino acid sequence of the lipase. Likewise, SEQ ID NO: 15 shows the nucleotide sequence of genomic DNA which contains a polynucleotide encoding the lipase, while SEQ ID NO: 16 shows an amino acid sequence including a sequence located upstream of SEQ ID NO: 11.

[0010] As used herein, "nucleotides 25-801" in relation to SEQ ID NO: 10 can be interchanged with "nucleotides 388-1164" in relation to SEQ ID NO: 15, unless otherwise specified. As used herein, "amino acid sequence of SEQ ID NO: 11" can be interchanged with "amino acid sequence covering amino acids 48-306 of SEQ ID NO: 16," unless otherwise specified. In the present invention, a polynucleotide consisting of a part of the nucleotide sequence shown in SEQ ID NO: 10 which covers at least nucleotides 25-801 can be replaced with a polynucleotide

- 6 -

consisting of a part of the nucleotide sequence shown in SEQ ID NO: 15 which covers at least nucleotides 388-1164 (e.g., a polynucleotide consisting of a part of the nucleotide sequence shown in SEQ ID NO: 15 which covers nucleotides 247-1167). Likewise, a protein consisting of the amino acid sequence shown in SEQ ID NO: 11 (or a polynucleotide encoding the same) can be replaced with a protein consisting of all or part of the amino sequence shown in SEQ ID NO: 16 (preferably a protein consisting of amino acids 32-306, more preferably amino acids 48-306) (or a polynucleotide encoding the same). Such polynucleotides and proteins also fall within the scope of the present invention. Amino acids 1-47 of SEQ ID NO: 16 appear to constitute a pre-pro sequence. Amino acids 1-31 serve as a pre-sequence which is a secretion signal, while amino acids 32-47 is deduced as a pro-sequence which may be cleaved after secretion probably by the action of another protein. Thus, a mature protein essential for serving as a lipase lies in the sequence downstream of amino acid 48; it would be suitable to remove the pre-pro sequence if the lipase is expressed in a heterologous expression system such as *E. coli*.

[0011] Preferred examples of the polynucleotide of the present invention or a homolog thereof, which encodes a lipase with a molecular weight of about 32 kDa, are as follows.

A polynucleotide selected from (A₁'), (B₁'), (C₁'), (D₁'), (E₁'), (F₁') or (G₁') shown below:

(A₁') a polynucleotide which consists of all of the nucleotide sequence shown in SEQ ID NO: 10 or a part thereof

- 7 -

covering nucleotides 25-801;

(B₁') a polynucleotide which is hybridizable under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide shown in (A₁') and which encodes a protein having lipase activity;

(C₁') a polynucleotide which consists of a nucleotide sequence comprising substitution, deletion, insertion and/or addition of one or several nucleotides in the nucleotide sequence of the polynucleotide shown in (A₁') and which encodes a protein having lipase activity;

(D₁') a polynucleotide which shares an identity of at least 80% or more with the nucleotide sequence of the polynucleotide shown in (A₁') and which encodes a protein having lipase activity;

(E₁') a polynucleotide which encodes a protein consisting of the amino acid sequence shown in SEQ ID NO: 11;

(F₁') a polynucleotide which encodes a protein consisting of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 11 and having lipase activity; or

(G₁') a polynucleotide which encodes a protein consisting of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence shown in SEQ ID NO: 11 and having lipase activity.

A polynucleotide comprising (A₂), (B₂), (C₂), (D₂), (E₂), (F₂) or (G₂) shown below:

- 8 -

(A₂) a polynucleotide which consists of all of the nucleotide sequence shown in SEQ ID NO: 15 or a part thereof covering at least nucleotides 247-1167 or 388-1164;

(B₂) a polynucleotide which is hybridizable under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide shown in (A₂) and which encodes a protein having lipase activity;

(C₂) a polynucleotide which consists of a nucleotide sequence comprising substitution, deletion, insertion and/or addition of one or several nucleotides in the nucleotide sequence of the polynucleotide shown in (A₂) and which encodes a protein having lipase activity;

(D₂) a polynucleotide which shares an identity of at least 80% or more with the nucleotide sequence of the polynucleotide shown in (A₂) and which encodes a protein having lipase activity;

(E₂) a polynucleotide which encodes a protein consisting of all of the amino acid sequence shown in SEQ ID NO: 16 or a part thereof covering at least amino acids 48-306;

(F₂) a polynucleotide which encodes a protein consisting of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence of the protein shown in (E₂) and having lipase activity; or

(G₂) a polynucleotide which encodes a protein consisting of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in

- 9 -

(E₂) and having lipase activity.

A polynucleotide selected from (A₂'), (B₂'), (C₂'), (D₂'), (E₂'), (F₂') or (G₂') shown below:

(A₂') a polynucleotide which consists of all of the nucleotide sequence shown in SEQ ID NO: 15 or a part thereof covering nucleotides 247-1167 or 388-1164;

(B₂') a polynucleotide which is hybridizable under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide shown in (A₂') and which encodes a protein having lipase activity;

(C₂') a polynucleotide which consists of a nucleotide sequence comprising substitution, deletion, insertion and/or addition of one or several nucleotides in the nucleotide sequence of the polynucleotide shown in (A₂') and which encodes a protein having lipase activity;

(D₂') a polynucleotide which shares an identity of at least 80% or more with the nucleotide sequence of the polynucleotide shown in (A₂') and which encodes a protein having lipase activity;

(E₂') a polynucleotide which encodes a protein consisting of all of the amino acid sequence shown in SEQ ID NO: 16 or a part thereof covering at least amino acids 48-306;

(F₂') a polynucleotide which encodes a protein consisting of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence of the protein shown in (E₂') and having lipase activity; or

- 10 -

(G₂') a polynucleotide which encodes a protein consisting of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (E₂') and having lipase activity.

[0012] The present invention also provides a gene or a homolog thereof, which encodes a lipase with a molecular weight of about 40 kDa produced by a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154), i.e., a polynucleotide comprising (H₂), (I₂), (J₂), (K₂), (L₂), (M₂) or (N₂) shown below:

(H₂) a polynucleotide which consists of all of the nucleotide sequence shown in SEQ ID NO: 28 or a part thereof covering at least nucleotides 414-1688 or 498-1685;

(I₂) a polynucleotide which is hybridizable under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide shown in (H₂) and which encodes a protein having lipase activity;

(J₂) a polynucleotide which consists of a nucleotide sequence comprising substitution, deletion, insertion and/or addition of one or several nucleotides in the nucleotide sequence of the polynucleotide shown in (H₂) and which encodes a protein having lipase activity;

(K₂) a polynucleotide which shares an identity of at least 80% or more with the nucleotide sequence of the polynucleotide shown in (H₂) and which encodes a protein

- 11 -

having lipase activity;

(L₂) a polynucleotide which encodes a protein consisting of all of the amino acid sequence shown in SEQ ID NO: 29 or a part thereof covering at least amino acids 29-424;

(M₂) a polynucleotide which encodes a protein consisting of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence of the protein shown in (L₂) and having lipase activity; or

(N₂) a polynucleotide which encodes a protein consisting of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (L₂) and having lipase activity.

[0013] SEQ ID NO: 28 shows the nucleotide sequence of a gene which encodes a lipase with a molecular weight of about 40 kDa produced by *Tetrasphaera* sp. strain NITE P-154, while SEQ ID NO: 29 shows the amino acid sequence of the lipase.

[0014] Preferred examples of the polynucleotide of the present invention or a homolog thereof, which encodes a lipase with a molecular weight of about 40 kDa, are as follows.

A polynucleotide selected from (H₂'), (I₂'), (J₂'), (K₂'), (L₂'), (M₂') or (N₂') shown below:

(H₂') a polynucleotide which consists of all of the nucleotide sequence shown in SEQ ID NO: 28 or a part thereof covering nucleotides 414-1688 or 498-1685;

(I₂') a polynucleotide which is hybridizable under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence

- 12 -

of the polynucleotide shown in (H₂') and which encodes a protein having lipase activity;

(J₂') a polynucleotide which consists of a nucleotide sequence comprising substitution, deletion, insertion and/or addition of one or several nucleotides in the nucleotide sequence of the polynucleotide shown in (H₂') and which encodes a protein having lipase activity;

(K₂') a polynucleotide which shares an identity of at least 80% or more with the nucleotide sequence of the polynucleotide shown in (H₂') and which encodes a protein having lipase activity;

(L₂') a polynucleotide which encodes a protein consisting of all of the amino acid sequence shown in SEQ ID NO: 29 or a part thereof covering amino acids 29-424;

(M₂') a polynucleotide which encodes a protein consisting of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence of the protein shown in (L₂') and having lipase activity; or

(N₂') a polynucleotide which encodes a protein consisting of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (L₂') and having lipase activity.

[0015] As used herein, the term "lipase" is intended to mean an enzyme that hydrolyzes glycerol esters to release fatty acids, unless otherwise specified. As used herein to describe a protein, the phrase "having lipase activity" (or the term "lipase") is intended to mean that the protein (or

- 13 -

lipase) at least has the ability to hydrolyze esters of glycerol with fatty acids, preferably the ability to hydrolyze esters with medium-chain fatty acids and/or esters with long-chain fatty acids, and more preferably the ability to hydrolyze both esters with medium-chain fatty acids and esters with long-chain fatty acids, unless otherwise specified. Such a protein having lipase activity (or lipase) preferably has the ability to catalyze the transfer reaction (esterification or transesterification) of fatty acids, and more preferably has the ability to catalyze the transfer reaction of medium-chain fatty acids.

[0016] To evaluate whether a protein has the ability to hydrolyze esters of glycerol with medium-chain or long-chain fatty acids, the protein may be subjected to a test for measuring the ability to hydrolyze 4-methyl unberryferylcapyrate (MU-C8) or 4-methyl unberryferyloleate (MU-C18), for example, as shown in the Example section described later. Likewise, to evaluate whether a protein has the ability to catalyze the transfer reaction of medium-chain fatty acids, the protein may be subjected to an experiment for esterification between 3-phenyl-1-propanol or 1-phenyl-2-propanol and tricaprillin (MCT), for example, as shown in the Example section described later.

[0017] When a protein (or lipase) has the ability to hydrolyze esters of fatty acids or the ability to catalyze the transfer reaction of fatty acids, such a protein (or lipase) can also be expressed herein as having the ability to recognize the fatty acid as a substrate, unless otherwise

- 14 -

specified.

[0018] The protein of the present invention with a molecular weight of about 32 kDa or about 40 kDa, which is produced by a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154), at least has the ability to hydrolyze 4-methyl unberryferylcapyrylate (MU-C8). The latter protein further has the ability to hydrolyze 4-methyl unberryferyloleate (MU-C18) and is also confirmed to have the ability to generate a corresponding caprylic acid ester from tricaprilin and 3-phenyl-1-propanol or 1-phenyl-2-propanol (see Example 1). Thus, both proteins can be regarded as having lipase activity. More specifically, the former can be regarded as having the ability to hydrolyze esters with medium-chain fatty acids (i.e., the ability to recognize a medium-chain fatty acid as a substrate), while the latter can be regarded as having the ability to hydrolyze esters with medium-chain fatty acids and with long-chain fatty acids (i.e., the ability to recognize both a medium-chain fatty acid and a long-chain fatty acid as substrates) and also as having the ability to catalyze the transfer reaction (particularly esterification) of medium-chain fatty acids.

[0019] As used herein, the term "medium-chain fatty acid" refers to a saturated or unsaturated fatty acid containing 7 to 15 carbon atoms, unless otherwise specified. Examples of a medium-chain fatty acid include caprylic acid, capric acid and lauric acid.

- 15 -

[0020] As used herein, the term "long-chain fatty acid" refers to a saturated or unsaturated fatty acid containing 16 or more carbon atoms, unless otherwise specified. Examples of a long-chain fatty acid include palmitic acid, stearic acid, palmitoleic acid, oleic acid, linolic acid, α -linolenic acid and γ -linolenic acid.

[0021] When used herein in relation to glycerol and fatty acids, the term "ester" may be used to mean not only a triacylglycerol, but also a diacylglycerol or a monoacylglycerol (the latter two may also be collectively referred to as a partial acylglycerol), unless otherwise specified.

[0022] As used herein, the term "stringent conditions" refers to conditions of 6 M urea, 0.4% SDS and 0.5 $\times$ SSC, or hybridization conditions equivalent thereto, unless otherwise specified. If necessary, more stringent conditions (e.g., 6 M urea, 0.4% SDS and 0.1 $\times$ SSC) or hybridization conditions equivalent thereto may be applied in the present invention. Under each of these conditions, the temperature may be set to about 40°C or higher. When more stringent conditions are required, the temperature may be set to a higher value, for example about 50°C and more particularly about 65°C.

[0023] Moreover, the expression "nucleotide sequence comprising substitution, deletion, insertion and/or addition of one or several nucleotides" as used herein does not provide any limitation on the number of nucleotides to be substituted, deleted, inserted and/or added, as long as a protein encoded by a polynucleotide consisting of such a nucleotide sequence

- 16 -

has desired functions. The number of such nucleotides is around 1 to 9 or around 1 to 4, or alternatively, a larger number of nucleotides may be substituted, deleted, inserted and/or added as long as such a mutation allows encoding of the same or a functionally similar amino acid sequence. Likewise, the expression "amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids" as used herein does not provide any limitation on the number of amino acids to be substituted, deleted, inserted and/or added, as long as a protein having such an amino acid sequence has desired functions. The number of such amino acids is around 1 to 9 or around 1 to 4, or alternatively, a larger number of amino acids may be substituted, deleted, inserted and/or added as long as such a mutation provides a functionally similar amino acid. Means for preparing a polynucleotide which has such a nucleotide sequence or encodes such an amino acid sequence are well known to those skilled in the art.

[0024] As used herein to describe a nucleotide sequence, the term "high identity" refers to a sequence identity of at least 50% or more, preferably 70% or more, more preferably 80% or more, even more preferably 90% or more, and most preferably 95% or more.

[0025] As used herein to describe an amino acid sequence, the term "high identity" refers to a sequence identity of at least 50% or more, preferably 70% or more, more preferably 80% or more, even more preferably 90% or more, and most preferably 95% or more. The identity of the amino acid sequence shown in

- 17 -

SEQ ID NO: 11 with the amino acid sequences of known lipases is shown in Table 4 in the Example section. Likewise, the identity of the amino acid sequence shown in SEQ ID NO: 28 with a known sequence is described in Example 8.

[0026] Search and analysis for identity between nucleotide or amino acid sequences may be accomplished by using any algorithm or program (e.g., BLASTN, BLASTP, BLASTX, ClustalW) well known to those skilled in the art. In the case of using a program, parameters may be set as required by those skilled in the art, or alternatively, default parameters specific for each program may be used. Detailed procedures for such analysis are also well known to those skilled in the art.

[0027] To describe the molecular weight of a protein or lipase, a value determined by SDS-PAGE is used herein, unless otherwise specified (see Example 1, Figure 2).

[0028] The polynucleotide of the present invention can be obtained from natural products by using techniques such as hybridization and polymerase chain reaction (PCR).

[0029] More specifically, genomic DNA (gDNA) is prepared in a routine manner (e.g., DNeasy Tissue Kit (QIAGEN)) from a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154). Alternatively, total RNA is prepared in a routine manner (e.g., RNeasy Plant isolation Kit (QIAGEN)) from cells of the above strain, followed by reverse transcription (1st strand cDNA synthesis) using the total RNA as a template in a routine manner (e.g., SuperScript II Reverse Transcriptase

- 18 -

(Invitrogen)).

[0030] To obtain a desired polynucleotide, a 5'-primer is designed based on the N-terminal sequence of the 32 kDa lipase protein. Further, a 3'-primer is designed based on a sequence conserved among putative proteins which are derived from relatively closely related *Streptomyces* spp. and share identity with the above N-terminal amino acid residues. A lip32 gene fragment is amplified by degenerate PCR with the above primer set using Strain #375 cDNA as a template to determine a partial nucleotide sequence. After digestion with an appropriate restriction enzyme(s), the cyclized Strain #375 gDNA is further used as a template to obtain the nucleotide sequence information of neighboring regions by inverse PCR with primers designed in the outward direction on the template sequence. In this way, a nucleotide sequence of about 900 bp in total is determined for the lip32 region gDNA.

[0031] The polynucleotide of the present invention encompasses DNA, including genomic DNA, cDNA and chemically synthesized DNA. These DNAs may be either single-stranded or double-stranded.

[0032] Preferred examples of the polynucleotide of the present invention are those derived from the genus *Tetrasphaera*.

[0033] The present invention also provides a recombinant vector carrying the polynucleotide of the present invention, as well as a transformant (e.g., a transformed *E. coli*, yeast or insect cell) transformed with the recombinant vector. The present invention further provides a transformation method

- 19 -

comprising the step of transforming a host (e.g., an *E. coli*, yeast or insect cell) by using the polynucleotide of the present invention (e.g., the step of transforming a host with the recombinant vector of the present invention).

[0034] There is no particular limitation on the vector into which the polynucleotide of the present invention is inserted, as long as it allows expression of the insert in a host. Such a vector generally has a promoter sequence, a terminator sequence, a sequence for inducible expression of an insert in response to external stimulation, a sequence recognized by a restriction enzyme for insertion of a target gene, and a sequence encoding a marker for transformant selection. To create such a recombinant vector and to effect transformation with such a recombinant vector, techniques well known to those skilled in the art may be applied.

[0035] The present invention also provides a lipase with a molecular weight of about 32 kDa or a homolog thereof, which is produced by a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154), i.e., a protein comprising (e₁), (f₁) or (g₁) shown below:

(e₁) a protein which consists of the amino acid sequence shown in SEQ ID NO: 11;

(f₁) a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 11 and which has lipase activity; or

- 20 -

(g₁) a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence shown in SEQ ID NO: 11 and which has lipase activity.

[0036] Preferred examples of the lipase of the present invention with a molecular weight of about 32 kDa or a homolog thereof are the following proteins.

A protein selected from (e₁'), (f₁') or (g₁') shown below:

(e₁') a protein which consists of the amino acid sequence shown in SEQ ID NO: 11;

(f₁') a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 11 and which has lipase activity; or

(g₁') a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence shown in SEQ ID NO: 11 and which has lipase activity.

A protein comprising (e₂), (f₂) or (g₂) shown below:

(e₂) a protein which consists of all of the amino acid sequence shown in SEQ ID NO: 16 or a part thereof covering at least amino acids 48-306;

(f₂) a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence of the protein shown in (e₂) and which has lipase activity; or

(g₂) a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino

- 21 -

acid sequence of the protein shown in (e₂) and which has lipase activity.

A protein selected from (e₂'), (f₂') or (g₂') shown below:

(e₂') a protein which consists of all of the amino acid sequence shown in SEQ ID NO: 16 or a part thereof covering amino acids 48-306;

(f₂') a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence of the protein shown in (e₂') and which has lipase activity; or

(g₂') a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (e₂') and which has lipase activity.

[0037] This lipase with a molecular weight of about 32 kDa has the ability to recognize a medium-chain fatty acid as a substrate. Under the conditions shown in Example 11, this lipase has an optimum temperature of about 40°C and an optimum pH of around 7.0.

[0038] The present invention also provides a lipase with a molecular weight of about 40 kDa or a homolog thereof, which is produced by a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154), i.e., a protein comprising (l₁), (m₁) or (n₁) shown below:

(l₁) a protein which consists of the amino acid sequence shown in SEQ ID NO: 35;

- 22 -

(m₁) a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence shown in SEQ ID NO: 35 and which has lipase activity; or

(n₁) a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence shown in SEQ ID NO: 35 and which has lipase activity.

[0039] SEQ ID NO: 35 and Figure 16 each show the mature protein amino acid sequence of a lipase with a molecular weight of about 40 kDa produced by *Tetrasphaera* sp. strain NITE P-154.

[0040] Preferred examples of the lipase of the present invention with a molecular weight of about 40 kDa or a homolog thereof are the following proteins.

A protein selected from (l₁), (m₁) or (n₁) shown above.

A protein comprising (l₂), (m₂) or (n₂) shown below:

(l₂) a protein which consists of all of the amino acid sequence shown in SEQ ID NO: 29 or a part thereof covering at least amino acids 29-424;

(m₂) a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence of the protein shown in (l₂) and which has lipase activity; or

(n₂) a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (l₂) and which has lipase activity.

- 23 -

A protein selected from (l_2'), (m_2') or (n_2') shown below:

(l_2') a protein which consists of all of the amino acid sequence shown in SEQ ID NO: 29 or a part thereof covering amino acids 29-424;

(m_2') a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one or several amino acids in the amino acid sequence of the protein shown in (l_2') and which has lipase activity; or

(n_2') a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (l_2') and which has lipase activity.

[0041] This lipase with a molecular weight of about 40 kDa has the ability to recognize both a medium-chain fatty acid and a long-chain fatty acid as substrates. Under the conditions shown in Example 11, this lipase has an optimum temperature of about 45°C to 50°C and an optimum pH of around 7.0.

[0042] The above protein or lipase may be isolated and purified from a culture supernatant obtained by culturing a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154) in a commercially available medium (e.g., Marine broth 2216 (Difco); Marine broth is a liquid medium characterized by having a salt content of about 2%).

[0043] Details are as follows. First, the cell suspension is inoculated at 0.1% to 5% into the medium and cultured at

- 24 -

10°C to 40°C for 2 to 10 days. The culture supernatant is subjected to column chromatography using 0.01 to 1 part of a hydrophobic gel (e.g., ϕ -Sepharose) equilibrated with Tris-HCl buffer containing calcium chloride and magnesium chloride. After washing with the above buffer, the column is eluted with the above buffer supplemented with 1% nonionic surfactant (e.g., Triton X-100) to obtain a fraction having lipase activity. This lipase fraction is diluted with 10 parts of the above buffer and then adsorbed onto 0.001 to 1 part of an anion exchange gel (e.g., Q-Sepharose). After washing with the above buffer supplemented with 0.1% amphoteric surfactant (e.g., 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)), the gel is eluted with a concentration gradient of aqueous sodium chloride to obtain a lipase active fraction. This lipase fraction is subjected to high performance liquid chromatography (HPLC) on an anion exchange column (e.g., HiTrapQ), followed by gradient elution with a concentration gradient of aqueous sodium chloride to obtain a fraction which has lipase activity and is confirmed for the presence of a protein with a molecular weight of about 32 kDa or about 40 kDa. For more detailed procedures, reference may be made to Example 1 described herein later.

[0044] The above protein or lipase may be obtained as a recombinant protein from a transformant (e.g., a transformed *E. coli* cell) transformed with a recombinant vector carrying the polynucleotide of the present invention.

[0045] Details are as follows. First, the nucleotide sequence of a gene to be used for *E. coli* expression systems

- 25 -

is amplified by PCR with an appropriate primer set, followed by cloning and sequencing to confirm the nucleotide sequence. The cloned DNA fragment is extracted by digestion with an appropriate restriction enzyme(s) and then integrated into an *E. coli* protein expression vector. This vector is used to transform *E. coli* cells to thereby induce protein expression. *E. coli* cells expressing the desired gene are homogenized, and the supernatant is purified on a column to obtain a desired protein of about 32 kDa or about 40 kDa. For more detailed procedures, reference may be made to Example 3 or 8 described herein later.

[0046] The present invention also provides a lipase with a molecular weight of about 40 kDa, which has the ability to recognize both a medium-chain fatty acid and a long-chain fatty acid as substrates and which is produced by a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154), or alternatively, a lipase with a molecular weight of about 40 kDa, which can be isolated by a production method comprising the following steps:

[0047] applying the culture supernatant of a strain belonging to the genus *Tetrasphaera* onto a hydrophobic resin column to elute the product adsorbed on the hydrophobic resin with a buffer containing 1% nonionic surfactant;

applying the eluate to an anion exchange resin to elute the product adsorbed on the anion exchange resin with a 0.5 M NaCl solution; and

- 26 -

applying the eluate, after dialysis, onto an anion exchange column to elute the same with a NaCl gradient solution.

[0048] More specifically, the production method comprises steps 1) to 3) shown below:

1) applying the culture supernatant of a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154) onto a hydrophobic resin column to elute the product adsorbed on the hydrophobic resin with a buffer containing 1% Triton X-100, if necessary after washing with a buffer containing 0.5% nonionic surfactant (e.g., Triton X-100), etc.;

2) applying the eluate to an anion exchange resin to elute the product adsorbed on the anion exchange resin with a 0.5 M NaCl solution, if necessary after washing with a buffer containing 0.1% amphoteric surfactant (e.g., 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)), etc.; and

3) applying the eluate, after dialysis, onto an anion exchange column to elute the same with a NaCl gradient solution. For example, the dialyzed eluate is applied onto an anion exchange column of 1 ml volume and eluted with a NaCl gradient solution of 0 to 0.75 M. The eluate is fractionated into 0.5 ml volumes.

[0049] The above 40 kDa lipase may be isolated and purified in the same manner as described for the 32 kDa lipase from a culture supernatant obtained by culturing a strain belonging

- 27 -

to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154) in a commercially available medium (see Example 1).

[0050] Preferred examples of the above 40 kDa lipase are those having the amino acid sequence shown in SEQ ID NO: 3 and/or SEQ ID NO: 14, more specifically those having the amino acid sequences shown in SEQ ID NOs: 3 and 14.

[0051] According to the present invention and the information provided herein, it is possible to obtain the sequence information of a polynucleotide encoding the 40 kDa lipase and such a polynucleotide per se encoding the 40 kDa lipase, for example, by using all or part of the above 40 kDa lipase, the amino acid sequence shown in SEQ ID NO: 3 or SEQ ID NO: 14, a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154) or genomic DNA obtained from such a strain, if appropriate in combination with the various procedures disclosed herein for the 32 kDa lipase. More specifically, genomic DNA obtained from a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154) is completely digested with an appropriate restriction enzyme(s) and ligated with a cassette corresponding to the restriction enzyme(s) to prepare template DNA. On the other hand, amino acid sequence information obtained from a part of the 40 kDa lipase (e.g.,

- 28 -

SEQ ID NO: 3 (N-terminal amino acid sequence), SEQ ID NO: 14 (internal amino acid sequence)) is used to design an appropriate primer. PCR is performed using this primer and a cassette primer to obtain a genomic DNA sequence around the 40 kD lipase gene. The nucleotide sequence thus obtained and others may be used to predict the ORF of the gene encoding the 40 kDa lipase. For amplification, cloning and nucleotide sequencing of the predicted ORF and functional confirmation of the encoded protein, it is possible to adapt the procedures described herein which are used for the 32 kDa lipase for the same purposes.

[0052] Namely, the present invention also provides a method for producing the nucleotide sequence information of a polynucleotide encoding a 40 kDa lipase, which comprises using the amino acid sequence shown in SEQ ID NO: 3 or SEQ ID NO: 14, all or part of the 40 kDa lipase (i.e., the lipase per se or a fragment thereof) or a strain belonging to the genus *Tetrasphaera* (preferably belonging to the same species as *Tetrasphaera* sp. strain NITE P-154, more preferably being *Tetrasphaera* sp. strain NITE P-154).

[0053] II. *Tetrasphaera* sp. strain NITE P-154

The present invention also provides *Tetrasphaera* sp. strain NITE P-154 capable of producing the lipase of the present invention.

[0054] *Tetrasphaera* sp. strain NITE P-154 was selected from a novel marine microorganism library belonging to Marine Biotechnology Institute Co., Ltd. (Japan), depending on whether halo formation occurs on agar plates containing fats

- 29 -

of medium-chain fatty acids (MCT).

[0055] [Microbiological properties of *Tetrasphaera* sp.
strain NITE P-154]

Tetrasphaera sp. strain NITE P-154 (herein also referred to as "#375") is morphologically seen in coccal form ($0.86 \times 0.86 \mu\text{m}$) and has no motility. In the physiological aspect, it is Gram-positive and is grown at a temperature ranging from 15°C to 45°C , and can use D-fructose, D-glucose, D-mannitol, raffinose and sucrose as carbon sources, but cannot use L-arabinose, inositol, L-rhamnose and D-xylose. In the chemotaxonomical aspect, its isoprenoid quinone is MK-8(H4) and the nucleotide composition of DNA is 70.5%.

- 30 -

[0056] [Table 1]

a. Morphological properties

- 1) Cell shape and size :coccal, $0.86 \times 0.86 \mu\text{m}$
- 2) Presence or absence of motility :absence

b. Culture properties

- 1) ISP medium No.2 plate culture :forming round, hemispherical, entire and glossy white colonies
- 2) ISP medium No.4 plate culture :forming round, hemispherical, entire and glossy white colonies
- 3) ISP medium No.5 plate culture :forming white colonies with less growth
- 4) ISP medium No.6 plate culture :forming round, hemispherical, entire and glossy yellowish-white colonies
- 5) ISP medium No.7 plate culture :forming white colonies with less growth
- 6) Marine Agar plate culture :forming round, hemispherical, entire and glossy white colonies

c. Physiological properties

- 1) Gram staining :positive
- 2) Growth temperature range :15-45°C
- 3) Alkaline phosphatase activity :negative
- 4) Esterase (C4) activity :positive
- 5) Esterase lipase (C8) activity :positive
- 6) Lipase (C4) activity :positive
- 7) Leucine arylamidase activity :positive
- 8) Valine arylamidase activity :positive
- 9) Cystine arylamidase activity :negative
- 10) Trypsin activity :negative
- 11) Chymotrypsin activity :negative
- 12) Acid phosphatase activity :positive
- 13) Naphthol-AS-BI-phosphohydrolase activity :positive
- 14) α -Galactosidase activity :negative
- 15) β -Galactosidase activity :positive
- 16) β -Glucuronidase activity :negative
- 17) α -Glucosidase activity :positive
- 18) β -Glucosidase activity :positive
- 19) N-Acetyl- β -glucosaminidase activity :negative
- 20) α -Mannosidase activity :negative
- 21) α -Fucosidase activity :negative
- 22) Assimilation of carbon sources
 - L-Arabinose :-
 - D-Fructose :+
 - D-Glucose :+
 - Inositol :-
 - D-Mannitol :+
 - L-Rhamnose :-
 - Raffinose :+
 - Sucrose :+
 - D-Xylose :-

using BIOLOG SFP2 microplates

d. Chemotaxonomical properties

- 1) Isoprenoid quinine :MK-8(H4)
- 2) Nucleotide composition of DNA :70.5%

[0057] When cultured in Marine broth 2216 (Difco),

Tetrasphaera sp. strain NITE P-154 secretes the lipase of the present invention into the extracellular environment.

- 31 -

[0058] The present invention also provides a strain or a mutant thereof, which has the same microbiological properties as *Tetrasphaera* sp. strain NITE P-154, as well as a strain or a mutant thereof, which has a 16S rRNA gene consisting of a nucleotide sequence sharing high identity (e.g., more than 98% identity, preferably 98.5% identity, more preferably 99% identity, even more preferably 99.5% identity) with the 16S rRNA gene of *Tetrasphaera* sp. strain NITE P-154 (SEQ ID NO: 1). According to the studies made by the inventors of the present invention, the highest identity was 98% with known strains.

[0059] The strain of the present invention and a mutant strain thereof can be used for production of the protein and lipase of the present invention.

[0060] The present invention also provides a polynucleotide consisting of the nucleotide sequence shown in SEQ ID NO: 1.

BRIEF DESCRIPTION OF THE DRAWINGS

[0061] [Figure 1] Figure 1 shows the nucleotide sequence of the 16S rRNA gene of *Tetrasphaera* sp. NITE P-154 (#375).

[Figure 2] Figure 2 is a photograph showing SDS-PAGE of each fraction fractionated by HPLC. MW represents a molecular weight marker, while 21 to 35 represent lanes in which fractions #21 to #35 were electrophoresed, respectively.

[Figure 3] Figure 3 shows the alignment results between *Streptomyces* sp. putative secretion protein and *Streptomyces rimosus*-derived GDSL-lipase. The N-terminal 15 amino acid residues of the 32 kDa protein produced by Strain #375 were found to share high identity with a putative secretion protein from relatively closely related *Streptomyces* spp., while this

- 32 -

Streptomyces sp. putative secretion protein was found to share identity with the amino acid sequences of several lipases including *Streptomyces rimosus*-derived GDSL-lipase.

[Figure 4] Figure 4 shows an amino acid sequence corresponding to the nucleotide sequence of a gene expected to encode the 32 kDa protein (lip32 gene). The lip32 gene is composed of at least 777 bp and appears to encode a protein composed of 259 amino acid residues.

[Figure 5] Figure 5 shows the construction of a LIP32 protein expression vector (pET22b::lip32Nc).

[Figure 6] Figure 6 is a photograph showing SDS-PAGE of the column-purified LIP32 protein. M represents a marker, while 2 and 3 represent lanes in which fractions #2 and #3 were electrophoresed, respectively.

[Figure 7] Figure 7 is a graph showing the Bradford assay results of the column-purified LIP32 protein.

[Figure 8] Figure 8 shows the nucleotide sequence of genomic DNA containing a gene encoding LIP32, along with the amino acid sequence of LIP32. The shaded part represents a sequence identical to the N-terminal amino acid sequence of purified LIP32.

[Figure 9] Figure 9 shows putative pre- and pro-sequences of LIP32, as well as pETLIP32-F, pETLIP32-M and pETLIP32-S vectors.

[Figure 10] Figure 10 shows the nucleotide sequence of genomic DNA containing a gene encoding LIP40, along with the amino acid sequence of LIP40. The underlined part represents a secretion signal sequence, while the shaded parts each

- 33 -

represent a sequence identical to a partial amino acid sequence of purified LIP40. The double-underlined part represents a conserved region among lipases.

[Figure 11] Figure 11 shows the construction of a LIP40 protein expression vector (pETLIP40HP).

[Figure 12] Figure 12 is graphs showing the optimum temperatures for LIP32PH and LIP40HP.

[Figure 13] Figure 13 is graphs showing the optimum pH for LIP32PH and LIP40HP.

[Figure 14] Figure 14 shows HPLC analysis charts of the reaction solution when using immobilized LIP40HP or a control (pET22b) to effect fatty acid transfer to astaxanthin.

[Figure 15] Figure 15 shows HPLC analysis charts of the reaction solution when using LIP40HP or a control (pET22b) to effect fatty acid transfer to catechin.

[Figure 16] Figure 16 shows the amino acid sequence of the mature LIP40 protein.

PREFERRED MODE FOR CARRYING OUT THE INVENTION

[0062] The 32 kDa or 40 kDa lipase of the present invention can be immobilized on an appropriate carrier for use as an immobilized enzyme.

[0063] As a carrier, any conventional resin used for the same purpose may be used, including basic resins (e.g., MARATHON WBA (Dow Chemical), resins of SA series, WA series or FP series (Mitsubishi Chemical Corporation, Japan), and Amberlite IRA series (Organo)), as well as hydrophobic resins (e.g., FPHA (Diaion, Mitsubishi Chemical Corporation, Japan), HP series (Mitsubishi Chemical Corporation, Japan), and

- 34 -

Amberlite XAD7 (Organo)).

[0064] Likewise, any conventional technique used for the same purpose may be used to immobilize the lipase onto a carrier. For example, relative to 1 part of the above resin carrier, 10 parts of #375 culture supernatant may be added and then directly dried in vacuo, or alternatively, may be adsorbed to remove the supernatant before drying.

[0065] Such an immobilized lipase is industrially useful. Namely, when filled into a column, the immobilized enzyme allows a continuous reaction in which source materials are passed through the column. Moreover, the immobilized enzyme can be readily removed from the reaction solution for reuse.

[0066] The lipase or immobilized lipase of the present invention can be used in the transfer reaction of a medium-chain fatty acid and/or a long-chain fatty acid, as well as in the production of a medium-chain fatty acid ester and/or a long-chain fatty acid ester. Such an ester encompasses esters of carotenoids (including carotenes and xanthophylls) such as astaxanthin with fatty acids, and esters of polyphenols such as catechin with fatty acids.

[0067] As used herein, the term "transfer reaction" is intended to mean esterification or transesterification, unless otherwise specified.

[0068] Transfer reaction is useful in producing various fatty acid esters. For example, as in the case of conventional lipases used in industrial practice such as transesterification between triglycerides, production of sterol esters, and production of fatty acid methyl esters, the

- 35 -

lipase of the present invention can also be used in these instances. Among esters which can be produced by the lipase of the present invention, the following can be presented as particularly useful examples: sterol esters (e.g., β -sitosterol caprylic acid ester), astaxanthin caprylic acid ester, catechin caprylic acid ester, etc.

[0069] The present invention also provides the sequence information and a part thereof shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 14, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 28 or SEQ ID NO: 29, which has not been provided until the present invention, as well as use of the sequence information and a part thereof.

EXAMPLES

[0070] The present invention will now be described in more detail by way of the following examples, which are not intended to limit the scope of the invention.

[0071] [Example 1] Isolation and purification of lipase proteins from Strain #375 culture supernatant, as well as sequencing of their N-terminal 15 residues:

Into Marine broth 2216 (Difco) liquid medium, Strain #375 was inoculated at 1% and cultured with shaking at 25°C for 4 days. The culture supernatant (200 ml) was adsorbed onto a hydrophobic resin Phenyl Sepharose CL4B (2 ml, Amersham Biosciences), which had been equilibrated with Buffer A (50 mM Tris-HCl buffer (pH 8.0)/2 mM CaCl_2 /2 mM MgCl_2), and then subjected to column chromatography. After washing with Buffer A, the column was washed with 10 ml of Buffer A containing 0.5% Triton X-100 to remove substances other than lipase

- 36 -

proteins, and then eluted with 10 ml of Buffer A containing 1% Triton X-100 to obtain a lipase active fraction.

[0072] This lipase fraction (10 ml) was diluted with 10 volumes of Buffer A and then adsorbed onto an anion exchange resin Q-Sepharose (0.4 ml, Amersham Biosciences). The resin was fully washed with Buffer A containing 0.1% CHAPS to replace the surfactant Triton X-100 with CHAPS, followed by stepwise elution with 0.5 M and 1 M NaCl. The 0.5 M NaCl-eluted fraction (2 ml) showing lipase activity was fully dialyzed against Buffer A containing 0.1% CHAPS, and then eluted by HPLC on an anion exchange column HiTrapQ (1 ml, Amersham Biosciences) with a NaCl gradient of 0 to 0.75 M. The eluate was fractionated into 0.5 ml volumes. The fractions were each tested for their lipase activity and analyzed by SDS-PAGE to detect bands. Table 2 shows lipase activity and transfer activity, while Figure 2 shows the results of SDS-PAGE.

[0073] Lipase activity was measured using MU-C8 or MU-C18 as a substrate in the same manner as detailed in Example 3 below, and expressed as "Unit" (1 Unit (1 MU) = the ability of 1 L sample to release 1 μ mol MU for 1 minute through hydrolysis).

[0074] Likewise, transfer activity was measured as follows. To a mixture of 3-phenyl-1-propanol (10 μ l) or 1-phenyl-2-propanol (10 μ l) and tricaprilin (150 μ l), an enzyme solution (100 μ l) was added and reacted while vigorously stirring at 45°C for 3 days. The reaction solution was centrifuged to collect the upper layer (50 μ l). Acetonitrile (50 μ l) was

- 37 -

added to this layer, 10 µl of which was then analyzed by HPLC. Analysis conditions were set as follows: column: Develosil C30-UG-5 (4.6 x 150 mm) (Nomura chemical, Aichi, Japan); mobile phase: 90% acetonitrile/0.08% TFA; flow rate: 1 ml/minute; and temperature: room temperature. The results were expressed as formation rate of individual caprylic acid esters.

[0075] [Table 2]

Table 2. Lipase activity

	Q Fr22	Q Fr23	Q Fr24	Q Fr25	Q Fr26	Q Fr27	Q Fr28	Q Fr29	Q Fr30	Q Fr31	Q Fr32	Q Fr33	Q Fr34	Q Fr35	Q Fr36
C8	48.50	64.75	49.24	33.62	16.49	9.95	6.07	3.94	2.97	2.47	7.78	33.97	13.94	2.69	2.17
C18	10.67	13.46	10.58	7.58	4.73	2.88	1.70	1.24	0.90	0.76	0.74	0.69	0.57	0.46	0.43
3P1	15.15	22.31	20.89	13.47	5.85	3.09	2.44	2.62	+	+	+	+	+	+	+
1P2	6.24	8.28	8.57	3.97	+	+	+	+	+	0.00	0.00	0.00	0.00	0.00	0.00

[0076] Two lipases (32 kDa, 40 kDa) were sequenced with a protein sequencer to determine the amino acid sequences of their N-terminal 15 residues. The results obtained are shown in Table 3 and the Sequence Listing (SEQ ID NO: 2, SEQ ID NO: 3).

[0077] [Table 3]

Table 3. N-terminal amino acid sequences of lipases

	1	5	10	15
Lip32k	G D A P A	Y E R Y	V A L G D	s
Lip40k	G P D S V	P G T A	G A T T V	T

[0078] With respect to the lipase with a molecular weight of 40 kDa, the corresponding band was further excised from SDS-PAGE and digested with trypsin, followed by reversed-phase HPLC to separate fragment peptides. The 12-residue amino acid

- 38 -

sequence LSTNVGPTHGG (SEQ ID NO: 14) was determined from the N-terminal end of a peptide at any peak.

[0079] [Example 2] Cloning of 32 kDa lipase gene:

[Preparation of DNA and RNA from #375]

For preparation of genomic DNA (gDNA) and total RNA from this strain, a DNeasy Tissue Kit (QIAGEN) and an RNeasy Plant isolation Kit (QIAGEN) were used, respectively, according to the protocol of each kit. Reverse transcription (1st strand cDNA synthesis) using the total RNA as a template was accomplished by using SuperScript II Reverse Transcriptase (Invitrogen) according to its protocol.

[0080] For ligation of a PCR product into a vector, a TOPO TA-cloning Kit (Invitrogen) was used, and the vector was then transformed into *E. coli* cells in a standard manner. The nucleotide sequence of the cloned DNA fragment was determined by primer walking using primers designed outside the MCS of the cloning vector (e.g., M13 primer M4, RV) or primers designed on known sequences. Sequencing samples were prepared with ABI PRISM BigDye Terminator v3.1 (Applied Biosystems) according to its protocol. The sequencer used was an ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems), and the data analysis software used was Vector-NTI 9.0 (InforMax).

[0081] [Obtaining of lip32 gene]

To obtain a gene expected to encode a 32 kDa protein of Strain #375 (hereinafter referred to as the lip32 gene), the sequence of N-terminal 15 amino acid residues of this protein (GDAPAYERYVALGDS) was searched by blastp against the genbank amino acid sequence database. As a result, high identity was

- 39 -

observed with a putative secretion protein from *Streptomyces* spp. (Accession No. CAC42140) which were relatively closely related to Strain #375. This *Streptomyces* sp. putative secretion protein was found to share identity with some amino acid sequences including lipases. Among them, *Streptomyces rimosus*-derived GDSL-lipase (Accession No. AAK84028) was aligned by ClustalW with this putative protein, and the results obtained are shown in Figure 3. Among sequences conserved between these proteins, two underlined sequences VALGDSYS (SEQ ID NO: 4) and IGGNDS (SEQ ID NO: 5) were used to design a sense degenerate primer 375-dg-F3 (5'-TGGCCCTCGGCGACTCSTAC -'3) (SEQ ID NO: 6) and an antisense degenerate primer 375-dg-R3 (5'- CGTCGTTGCCNCCGATG -3') (SEQ ID NO: 7). Strain #375 cDNA was used as a template to perform DNA amplification by PCR with the primers 375-dg-F3 and 375-dg-R3 using ExTaq (Takara Bio Inc., Japan) under the following conditions: 98°C for 2 minutes, (98°C for 20 seconds, 55°C for 30 seconds, 72°C for 1 minute) x 35 cycles, and 72°C for 5 minutes. The nucleotide sequence was determined for the resulting DNA fragment to obtain a partial nucleotide sequence covering nucleotides 73-290 of SEQ ID NO: 10. Next, primers 375-IPC32-F1 (5'- CGGCGCGGACACGACGGACATGACG -3') (SEQ ID NO: 8) and 375-IPC32-R1 (5'- GGTAGCAGCCGCCCGCGATGTCGAG -3') (SEQ ID NO: 9) were designed in the outward direction on the resulting sequence. Strain #375 gDNA was digested with a restriction enzyme PstI or NotI and then cyclized by self-ligation. This was used as a template to perform PCR (inverse PCR) with the primers 375-IPC32-F1 and 375-IPC32-R1 using

- 40 -

LA Taq (Takara Bio Inc., Japan) under the following conditions: 98°C for 20 seconds, 68°C for 15 minutes (+10 seconds/cycle) x 35 cycles, whereby a neighboring sequence was amplified. The resulting DNA fragment was cloned to determine its partial nucleotide sequence. Taken together with the partial nucleotide sequence obtained earlier, a nucleotide sequence of about 900 bp in total was determined for the lip32 region gDNA. The amino acid sequence of LIP32 protein was deduced from the N-terminal amino acid sequence determined in Example 1 (SEQ ID NO: 2). The DNA sequence and deduced amino acid sequence of the lip32 gene region in this strain are shown in Figure 4 and the Sequence Listing (SEQ ID NO: 10, SEQ ID NO: 11). The N-terminal amino acid sequence determined in Example 1 (SEQ ID NO: 2) was identical to the N-terminal amino acid sequence shown in Figure 4. In view of these results, the mature LIP32 protein appeared to be a protein composed of 259 amino acid residues.

[0082] Moreover, the deduced LIP32 protein was analyzed by ClustalW to determine its identity with known lipase protein amino acid sequences. Table 4 shows identity with the amino acid sequence of each protein.

[0083] [Table 4]

Table 4. Alignment analysis on LIP32

	<i>Streptomyces rimosus</i> GDSL-ipase	<i>Candida cylindracea</i> lipase 1	<i>Geo. candidum</i> GCL1 (LIP1)	<i>Geo. candidum</i> GCL2 (LIP2)	<i>Pseudomonas</i> sp. lipase	<i>Rhizopus</i> <i>niveus</i> lipase
#375 LIP32	27.5	16.3	13.4	14.4	21.4	14.5

[0084] [Example 3] Introduction of 32 kDa lipase gene into

- 41 -

E. coli cells and lipase activity:

[Preparation of LIP32 protein (LIP32PH) by *E. coli* expression system]

For use in *E. coli* expression systems, the cDNA sequence of the Strain #375 lip32 gene ORF was amplified by RT-PCR with primers lip32-Nc-F (5'- CCATGGGCGACGCACCGGCATACGAACGC -3') (SEQ ID NO: 12) and lip32-Xh-R1 (5'- CTCGAGGGTGAGCTCGTCGATGAGCAGGTG -3') (SEQ ID NO: 13), followed by cloning and sequencing to conform its nucleotide sequence. The cloned cDNA fragment was extracted by digestion with restriction enzymes Nco I and Xho I, and then integrated between recognition sites for these restriction enzymes in an *E. coli* protein expression vector pET22b(+) (Novagen). The resulting vector was designated as pET22b::lip32Nc vector (Figure 5). *E. coli* strain BL21 (DE3) was transformed with the pET22b::lip32Nc vector and used for protein expression.

[0085] A single colony of *E. coli* cells transformed with the LIP32 protein expression vector (pET22b::lip32Nc) was inoculated into 2 ml LB medium (supplemented with 100 µg/ml ampicillin) and pre-cultured (>150 rpm, 12 hr, 37°C). The pre-cultured cell suspension was inoculated into 50 ml Enriched medium (2% trypton, 1% yeast extract, 0.5% NaCl, 0.2% (v/v) glycerol, 50 mM KH₂PO₄, pH 7.2; supplemented with 100 µg/ml ampicillin) and cultured with shaking (150 rpm, 25°C). At the time point when the cell suspension reached OD₆₀₀ = 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1.0 mM to induce LIP32 protein expression. The cells were further cultured with shaking (150

- 42 -

rpm, up to 4 hr, 25°C) to produce a large amount of LIP32 protein. The *E. coli* cells were collected by centrifugation (6000 rpm, 10 min, 4°C) and washed twice with Storage buffer (20 mM β -mercaptoethanol (β -ME), 50 mM Tris-HCl, pH 8.0). After being suspended again in 40 ml of ice-cold Lysis buffer (500 mM NaCl, 5 mM imidazole, 20 mM β -ME, 10% (v/v) glycerol, 25 mM Tris-HCl, pH 8.0), the cells were homogenized ultrasonically. The homogenate was centrifuged (10000 rpm, 60 min, 4°C) and then passed through a filter of pore size ϕ 0.22 μ m to remove cell debris and insolubles. The resulting solution was used as a crud protein extract. The crud protein extract (10 ml) was passed through a HiTrap Chelating HP column (1.0 ml bed volume) which had been loaded with 1.0 ml of 0.1 M NiSO₄ and equilibrated with 10 ml of Equilibration buffer (500 mM NaCl, 20 mM β -ME, 10% (v/v) glycerol, 25 mM Tris-HCl, pH 8.0). To remove proteins non-specifically bound to the column, the column was washed by sequentially passing 10 ml Wash-1 buffer (500 mM NaCl, 0.8 mM imidazole, 20 mM β -ME, 10% (v/v) glycerol, 25 mM Tris-HCl, pH 8.0) and 1.0 ml Wash-2 buffer (500 mM NaCl, 40 mM imidazole, 20 mM β -ME, 10% (v/v) glycerol, 25 mM Tris-HCl, pH 8.0). The LIP32 protein was eluted from the column with 5 ml Elution buffer (500 mM NaCl, 250 mM imidazole, 20 mM β -ME, 10% (v/v) glycerol, 25 mM Tris-HCl, pH 8.0), and the eluate was fractionated into 500 μ l volumes. The purity and concentration of the protein contained in each fraction were confirmed by SDS-PAGE (Figure 6) and Bradford assay (Figure 7). As a result of SDS-PAGE, a band of about 30 kDa corresponding to the LIP32 protein was

- 43 -

clearly observed in fractions #2 and #3. Moreover, each fraction was measured for its lipase activity using MU-C8 as a substrate (in the same manner as used in lipase activity measurement described below). As a result, fraction #3 was found to have strong lipase activity. Thus, fraction #3 was used as an enzyme solution of LIP32 protein (about 530 ng/ μ l) in the subsequent studies.

[0086] [Lipase activity of LIP32 protein (LIP32PH)]

To determine the lipase activity of LIP32 protein, 20 μ l of a diluted *E. coli* culture solution (suspension) and 180 μ l of a substrate solution (0.1 mM MU-C8, 50 mM potassium phosphate buffer (pH 7.0), 1% DMF) were mixed to prepare a reaction solution (200 μ l), followed by measuring changes in fluorescence intensity at 37°C for 20 minutes with SPECTRAMax GEMINI XS (Molecular Devices). 1 Unit (1 MU) was defined to be the ability of 1 L sample to release 1 μ mol MU for 1 minute through hydrolysis.

[0087] Lipase activity was measured by using an *E. coli* suspension induced to express LIP32 protein, indicating that LIP32 had high lipase activity (high ability to hydrolyze MU-C8) (Table 5).

[0088] [Table 5]

Table 5. Lipase activity of LIP32-expressing *E. coli* suspensions

proteins	MU(μ M/min/l)
	MU-C8
1 μ g/ml of OF	250.52
pET22b(+)	1.75
pET22b(+):Lip32 Nc	54358.00

OF: *Candida rugosa*-derived lipase

[0089] [Example 4] Immobilization of Strain #375 culture

- 44 -

supernatant onto anion exchange resin and transfer activity:

To 500 mg of a strong anion exchange resin (MARATHON WBA, Dow Chemical), a #375 culture solution (5 ml) was added and dried in vacuo at room temperature to obtain an immobilized enzyme. The immobilized enzyme (50 mg) was added to a mixture of 3-phenyl-1-propanol (25 μ l) and tricaprilin (375 μ l), and further supplemented with water (20 μ l), followed by stirring at 40°C for 3 days. The reaction solution was analyzed by HPLC, indicating that the formation rate was 75% for caprylic acid ester of 3-phenyl-1-propanol.

[0090] [Example 5] Immobilization of Strain #375 culture supernatant onto hydrophobic resin and transfer activity:

The same procedure as shown in Example 4 was repeated using 500 mg of FPHA (Diaion, Mitsubishi Chemical Corporation, Japan) to obtain an immobilized enzyme. This immobilized enzyme (50 mg) was used to perform the same reaction as in Example 4. In this case, the ester formation rate was 47%.

[0091] [Example 6] Determination of full-length lip32 gene sequence:

Strain #375 gDNA was digested with a restriction enzyme PstI or NotI and then cyclized by self-ligation. This was used as a template to perform PCR (inverse PCR) with the primers 375-IPC32-F1 and 375-IPC32-R1 using LATaq (Takara Bio Inc., Japan) under the following conditions: 98°C for 20 seconds, 68°C for 15 minutes (+10 seconds/cycle) x 35 cycles, whereby a neighboring sequence was amplified. The resulting DNA fragment was cloned to determine its partial nucleotide sequence and then ligated to the nucleotide sequence obtained

- 45 -

earlier (SEQ ID NO: 10) to obtain a genomic DNA sequence containing a LIP32-encoding ORF (SEQ ID NO: 15, Figure 8) and its deduced amino acid sequence (SEQ ID NO: 16, Figure 8).

[0092] [Example 7] Studies on putative pre- and pro-sequences of LIP32 protein:

For amplification of LIP32-F which encodes the full-length amino acid sequence of LIP32 protein, the following primers were synthesized: primers LIP32-Full-Nde-F (5'-CATATGAGCTCGTCACGTCGTACCGTCCGCACC -3') (SEQ ID NO: 17) and LIP32stop-Xho-Rv (5'-CTCGAGTCAGGTGAGCTCGTCGATGAGCAGGTC -3') (SEQ ID NO: 18). Likewise, for amplification of LIP32-M which encodes an amino acid sequence free from the pre-sequence, primers LIP32-Mid-Nco-F (5'-CCATGGCGACCGAGCGGGCGTCGGCGCCCACG -3') (SEQ ID NO: 19) and LIP32stop-Xho-Rv were synthesized, while for amplification of LIP32-S which encodes an amino acid sequence free from the pre-pro-sequence, primers LIP32-sht-Nco-F (5'-CCATGGGCGACGCACCGGCATACGAACGCTAT -3') (SEQ ID NO: 20) and LIP32stop-Xho-Rv were synthesized. Using Strain #375 gDNA as a template, PCR was performed with each primer set to amplify a DNA fragment, which was then cloned into a pCR4Blunt-TOPO vector (Invitrogen) and confirmed for its nucleotide sequence. Each lip32 gene fragment was excised with restriction enzymes NdeI & XhoI (LIP32-F) or NcoI & XhoI (LIP32-M, -S) and then integrated between recognition sites for these restriction enzymes in an *E. coli* expression vector pET22b(+) (Novagen). The resulting vectors were designated as pETLIP32-F, pETLIP32-M and pETLIP32-S vectors, respectively (Figure 9).

- 46 -

[0093] Each *E. coli* transformant was cultured with shaking in LB medium until OD₆₀₀ reached about 0.6, followed by addition of IPTG at a final concentration of 1 mM. Shaking culture was continued for an additional 3 hours to induce LIP32 protein expression. These *E. coli* suspensions were measured for their lipase activity as follows.

Namely, 20 µl of a diluted *E. coli* suspension and 180 µl of a substrate solution (0.1 mM MU-C8, 50 mM potassium phosphate buffer (pH 7.0), 1% DMF) were mixed to prepare a reaction solution (200 µl), followed by measuring changes in fluorescence intensity at 37°C for 20 minutes with SPECTRAMax GEMINI XS (Molecular Devices) to determine lipase activity. 1 Unit (1 MU) was defined to be the ability of 1 L sample to release 1 µmol MU for 1 minute through hydrolysis. The analysis results are shown in the table below.

[0094] [Table 6]

Table 6. Lipase activity of LIP32-expressing *E. coli* suspensions

Proteins	MU (µmol/min/l)
	MU-C8
pET22b(+)	0.71
pETLIP32-F	10.47
pETLIP32-M	23.54
pETLIP32-S	173.21

[0095] This result suggested that cleavage of the putative pre- and pro-sequences was required to allow LIP32 protein to exert its activity.

[0096] [Example 8] Cloning of lip40 gene:

With respect to the lipase with a molecular weight of 40 kDa, the corresponding band was excised from SDS-PAGE and digested with trypsin, followed by reversed-phase HPLC to

- 47 -

separate fragment peptides. In addition to the amino acid sequences obtained earlier (SEQ ID NO: 3, SEQ ID NO: 14), the following partial amino acid sequences were obtained from peptides at any peak.

[0097] GPDSVPGTAGATTVT (N-terminal) (SEQ ID NO: 3) see Example 1

LSTNVGPTHGG (SEQ ID NO: 14) see Example 1

APWFGLGAR (SEQ ID NO: 21)

QLAESVTEYE (SEQ ID NO: 22)

GYAVAFTDYQ (SEQ ID NO: 23)

Among the partial amino acid sequences, SEQ ID NO: 23 and amino acids 3-12 of SEQ ID NO: 14 were used to synthesize a sense degenerate primer LIP40-9 and an antisense degenerate primer LIP40-5, respectively:

primer LIP40-9 (GGNTAYGCNGTNGCNTTYACNGAYTAYCA) (SEQ ID NO: 24); and

primer LIP40-5 (CCNCCNARRTGNGTNGGNCCNACRTTNGT) (SEQ ID NO: 25).

[0098] #375 genomic DNA (100 ng) was used as a template to perform PCR using LA Taq with GC buffer (Takara Bio Inc., Japan) in a total volume of 20 μ l by using GC buffer II and by adding the primers LIP40-9 and LIP40-5 (each at a final concentration of 10 mM) and LA Taq (0.2 units), under the following conditions: 94°C for 1 minute, (94°C for 30 seconds, 50°C for 30 seconds, 72°C for 2 minutes) $\times$ 40 cycles, and 72°C for 5 minutes. The PCR products were analyzed by agarose gel electrophoresis, confirming a DNA fragment of approximately 0.7 kb. Then, this fragment was excised from the gel,

- 48 -

purified with a GFX kit (Amersham) and cloned with a TOPO-TA cloning kit (Invitrogen). The nucleotide sequence was determined for the cloned DNA to obtain a partial nucleotide sequence (covering nucleotides 932-1571 of SEQ ID NO: 28). Next, for full-length cloning of the lip40 gene, inverse PCR was performed. #375 genomic DNA was completely digested with a restriction enzyme NotI and then self-ligated. This was used as a template to perform PCR with primers LIP40-13 (gacgcggttcatgtaggtgtgcgtcc) (SEQ ID NO: 26) and LIP40-14 (gtgcgccaagggcgccaacgtccgcc) (SEQ ID NO: 27) using LA Taq with GC buffer (Takara Bio Inc., Japan).

[0099] The resulting 7 kb DNA fragment was cloned with a TOPO-TA cloning Kit (Invitrogen) to determine its nucleotide sequence from both ends. The resulting nucleotide sequence was ligated to the nucleotide sequence obtained earlier to obtain the nucleotide sequence of SEQ ID NO: 28. In view of ORF analysis and partial amino acid sequences of LIP40, LIP40 appeared to be encoded by an ORF located between 414 and 1688 bp. This ORF was found to encode a protein composed of 424 amino acid residues (SEQ ID NO: 29, LIP40 amino acid sequence). The N-terminal amino acid sequence (SEQ ID NO: 3) of the purified protein was identical to the sequence downstream of amino acid 29 in SEQ ID NO: 29 (LIP40 amino acid sequence), so that a peptide composed of amino acids 1-28 of SEQ ID NO: 29 appeared to be a secretion signal (Figure 10).

[0100] The LIP40 amino acid sequence was found to share 72.9% identity with a *Janibacter* sp. HTCC2649-derived hypothetical protein (gi#84498087).

- 49 -

[0101] [Example 9] Introduction of 40 kDa lipase gene into *E. coli* cells and lipase activity:

[Preparation of LIP40 protein by *E. coli* expression system]

#375 genomic DNA was used as a template to perform PCR with primer L40EcoRI-F1 (GAATTCGGGACCGGACTCCGTGCCCCGGCAC) (SEQ ID NO: 30) or L40NdeI-F3 (CATATGACGTCAGCACTGCTCCGACGAGCCCTCGC) (SEQ ID NO: 31) and primer L40HindIII-R1 (AAGCTTCTAGACGGCCCAGCAGTTGCTGAG) (SEQ ID NO: 32) using LA Taq with GC buffer. The amplified DNA fragments of approximately 1.2 kbp were each cloned with a TOPO-TA cloning Kit and confirmed for their nucleotide sequences to obtain plasmids pCR-375LIP40P and pCR-375LIP40S. The plasmid pCR-375LIP40P was digested with EcoRI and HindIII, while the plasmid pCR-375LIP40S was digested with NdeI and HindIII. Then, the resulting fragments were each ligated to an EcoRI- and HindIII-digested or NdeI- and HindIII-digested *E. coli* expression vector pET22b(+) (Novagen) using ligation high (Toyobo Co., Ltd., Japan) to thereby obtain plasmids pET375L40P and pET375LP40S.

[0102] A single colony of *E. coli* cells transformed with the LIP40 protein expression vector (plasmid pET375L40P or pET375L40S) was inoculated into 2 ml LB medium supplemented with 100 µg/ml ampicillin and pre-cultured (>150 rpm, 12 hr, 37°C). The pre-cultured cell suspension was inoculated into 50 ml Enriched medium (2% trypton, 1% yeast extract, 0.5% NaCl, 0.2% (v/v) glycerol, 50 mM KH₂PO₄, pH 7.2) supplemented with 100 µg/ml ampicillin and cultured with shaking (150 rpm, 25°C). At the time point when the cell suspension reached OD₆₀₀ = 0.6,

- 50 -

isopropyl- β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 1.0 mM to induce LIP40 protein expression. The cells were further cultured with shaking (150 rpm, up to 4 hr, 25°C).

[0103] [Lipase activity of LIP40]

To determine lipase activity, 20 μ l of a diluted *E. coli* culture solution (suspension) and 180 μ l of a substrate solution (0.1 mM MU-C8 or MU-C18, 50 mM potassium phosphate buffer (pH 7.0), 1% DMF) were mixed to prepare a reaction solution (200 μ l), followed by measuring changes in fluorescence intensity at 37°C for 20 minutes with SPECTRAMax GEMINI XS (Molecular Devices). 1 Unit (1 MU) was defined to be the ability of 1 L sample to release 1 μ mol MU for 1 minute through hydrolysis.

[0104] The results obtained are shown in the table below.

[0105] [Table 7]

Table 7. Lipase activity of <i>E. coli</i> culture solutions		
	MU/L (μ mol/min/l)	
	C8	C18
pET22b(+)	0.15	0
pET375L40S	191.02	23.14
pET375L40P	5255.73	151.06

[0106] Transfer activity was measured as follows. To a mixture of 3-phenyl-1-propanol (10 μ l) or 1-phenyl-2-propanol (10 μ l) and tricaprilin (150 μ l), an *E. coli* culture solution (100 μ l) was added and reacted while vigorously stirring at 45°C for 3 days. The reaction solution was centrifuged to collect the upper layer (50 μ l). Acetonitrile (50 μ l) was added to this layer, 10 μ l of which was then analyzed by HPLC.

- 51 -

Analysis conditions were set as follows: column: Develosil C30-UG-5 (4.6 x 150 mm) (Nomura chemical, Aichi, Japan); mobile phase: 90% acetonitrile/0.8% TFA; flow rate: 1 ml/minute; and temperature: room temperature. The results were expressed as formation rate of individual caprylic acid esters.

[0107] The results obtained are shown in the table below.

[0108] [Table 8]

Table 8. Transfer activity of *E. coli* culture solutions

	1P2	3P1
pET22b(+)	-	-
pET375L40S	13.86	47.54
pET375L40P	33.84	79.14

[0109] [Example 10] Preparation of LIP40 protein (LIP40HP) by *E. coli* expression system:

To express a 6-His-tagged Strain #375 LIP40 protein in *E. coli* cells, a vector was constructed as follows. Strain MBI375 genomic DNA was used as a template to perform amplification by PCR with primers 375L40EcoRI-His-F (5'-GAATTCGCACCACCACCACCACGGACCGGACTCCGTGCCCCGGCAC -3') (SEQ ID NO: 33) and 375L40HindIII-R1 (5'-AAGCTTCTAGACGGCCCAGCAGTTGCTGAG -3') (SEQ ID NO: 34). The amplified fragment was cloned into a pCR2.1TOPO vector, confirmed for its nucleotide sequence, and then extracted by digestion with restriction enzymes EcoRI and HindIII. The Lip40 gene fragment thus extracted was integrated between recognition sites for these restriction enzymes in an *E. coli* expression vector pET22b(+) (Novagen). The resulting vector was designated as pETLIP40HP vector (Figure 11). *E. coli*

- 52 -

strain BL21 (DE3) was transformed with the pETLIP40HP vector and used for protein expression.

[0110] The same procedure as used for the LIP32 protein was repeated to express a LIP40HP protein in *E. coli* cells, except that the culture period after IPTG induction was changed to 12 hours. The LIP40HP protein was purified by affinity purification through the 6-His-tag fused to the N-terminal end. The procedure used was the same as shown in Example 3 for the LIP32 protein, with the following minor modifications: (1) Lysis buffer, Equilibration buffer and Wash-1 buffer were each replaced with a common buffer (500 mM NaCl, 5 mM imidazole, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM Tris-HCl, pH 8.0); and (2) Elution buffer was replaced with 500 mM NaCl, 250 mM imidazole, 2 mM CaCl₂, 2 mM MgCl₂, 25 mM Tris-HCl, pH 8.0.

[0111] [Example 11] Characterization of LIP32PH and LIP40HP:

[Optimum temperatures for LIP32PH and LIP40HP]

Reaction was initiated by mixing 20 µl of a diluted enzyme solution purified from *E. coli* cells with 180 µl of a substrate solution (0.1 mM MU-C8, 1% DMF, 50 mM Tris-HCl, pH 7.0) which had been maintained at a test temperature (10, 20, 25, 30, 35, 40, 45, 50 or 60°C). For activity determination, changes in the intensity of MU fluorescence were measured over time during reaction at each test temperature.

[0112] The results of activity measurement indicated that LIP32PH (obtained in Example 3) had an optimum temperature of 40°C, while LIP40HP had an optimum temperature of 45°C to 50°C (Figure 12).

- 53 -

[0113] [Optimum pH for LIP32PH and LIP40HP]

Reaction was initiated by mixing 20 μ l of a diluted enzyme solution purified from *E. coli* cells with 180 μ l of a substrate solution (0.1 mM MU-C8, 1% DMF, 50 mM buffer of different pH values) which had been maintained at 37°C. For activity determination, the intensity of MU fluorescence was measured over time during reaction at 37°C for 20 minutes. The following buffers of different pH values were used: 50 mM Sodium acetate-acetic acid (pH 4.0-6.0), 50 mM MES-NaOH (pH 5.5-7.0) and 50 mM Tris-HCl (pH 6.5-9.0).

[0114] The results of activity measurement indicated that LIP32PH and LIP40HP each had an optimum pH of around 7.0 (Figure 13).

[0115] [Example 12] Immobilized LIP40HP enzyme-catalyzed fatty acid transfer reaction to astaxanthin:

For LIP40HP-catalyzed fatty acid transfer reaction from tricaprilin (MCT) to astaxanthin, an immobilized LIP40HP enzyme was used. The immobilized LIP40HP enzyme (WBA-LIP40HP) was prepared as follows. To 50 mg of a strong anion exchange resin (MARATHON WBA, Dow Chemical), 500 μ l of LIP40HP (about 3.65 ng/ μ l) was added and stirred for 2 hours (1000 rpm, 10°C), followed by vacuum drying at room temperature.

[0116] The transfer reaction was performed by sequentially adding WBA-LIP40HP (50 mg) and H₂O (6.25 μ l) to a mixture of astaxanthin (1.25 mg, Wako Pure Chemical Industries, Ltd., Japan) and MCT (125 μ l), and then allowing the mixture to stand at 45°C for 3 days. To the reaction solution, 100 μ l acetone was added and stirred, followed by centrifugation to

- 54 -

collect the upper layer (100 μ l), 20 μ l of which was then analyzed by HPLC. Analysis conditions were set as follows: column: Develosil C30-UG-5 (Nomura Chemical Co., Ltd., Japan, 4.6 x 150 mm); mobile phase: 75%-100% acetone, gradient elution for 1-15 minutes at a flow rate of 1.0 ml/min; analysis temperature: room temperature; and detection wavelength: 480 nm. Transfer activity was determined as formation rate of astaxanthin ester at a retention time of 16.4 minutes. The analysis indicated that the formation rate was 2.25% for caprylic acid ester of astaxanthin (Figure 14). On the other hand, there was no formation of caprylic acid ester for astaxanthin during reaction using WBA-pET22b prepared as a control in the same manner (Figure 14).

[0117] [Example 13] LIP40HP-catalyzed fatty acid transfer reaction to catechin

LIP40-catalyzed fatty acid transfer reaction from tricaprilin (MCT) to catechin was performed by adding 50 μ l LIP40HP enzyme solution (1.0 μ g/ μ l) to a mixture of 100 μ l catechin solution (0.01 mg/ μ l) and 150 μ l MCT, followed by stirring at 45°C for 2 days. The reaction solution was centrifuged to collect the oil layer, and an equal volume of acetonitrile was added thereto, 10 μ l of which was then analyzed by HPLC. Analysis conditions were set as follows: column: Develosil C30-UG-5 (Nomura Chemical Co., Ltd., Japan, 4.6 x 150 mm), mobile phase: (A) 0.1% TFA and (B) 90% acetonitrile/0.08% TFA under gradient conditions of 5% to 100% Eluent B/5 minutes at a flow rate of 1.0 ml/min; analysis temperature: room temperature; and detection wavelength: 280

- 55 -

nm. Transfer activity was determined as formation rate of catechin ester at a retention time of 8.4 minutes. The analysis indicated that the formation rate was 38.70% for caprylic acid ester of catechin (Figure 15). On the other hand, there was no formation of caprylic acid ester for catechin during reaction using pET22b prepared as a control in the same manner (Figure 15).

- 56 -

Claims

1. A polynucleotide comprising (H₂), (I₂), (J₂), (K₂), (L₂), (M₂) or (N₂) shown below:

(H₂) a polynucleotide which consists of all of the nucleotide sequence shown in SEQ ID NO: 28 or a part thereof covering at least nucleotides 414-1688 or 498-1685;

(I₂) a polynucleotide which hybridizes under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide shown in (H₂) and which encodes a protein having lipase activity;

(J₂) a polynucleotide which consists of a nucleotide sequence comprising substitution, deletion, insertion and/or addition of one to nine nucleotides in the nucleotide sequence of the polynucleotide shown in (H₂) and which encodes a protein having lipase activity;

(K₂) a polynucleotide which shares an identity of at least 80% or more with the nucleotide sequence of the polynucleotide shown in (H₂) and which encodes a protein having lipase activity;

(L₂) a polynucleotide which encodes a protein consisting of all of the amino acid sequence shown in SEQ ID NO: 29 or a part thereof covering at least amino acids 29-424;

(M₂) a polynucleotide which encodes a protein consisting of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one to nine amino acids in the amino acid sequence of the protein shown in (L₂) and having

- 57 -

lipase activity; or

(N₂) a polynucleotide which encodes a protein consisting of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (L₂) and having lipase activity,

wherein the stringent condition consists of hybridization at 65°C in a solution containing 6M urea, 0.4% sodium dodecyl sulfate (SDS), and 0.1Xsaline sodium citrate (SSC).

2. The polynucleotide of claim 1, which is selected from (H₂'), (I₂'), (J₂'), (K₂'), (L₂'), (M₂') or (N₂') shown below:

(H₂') a polynucleotide which consists of all of the nucleotide sequence shown in SEQ ID NO: 28 or a part thereof covering nucleotides 414-1688 or 498-1685;

(I₂') a polynucleotide which hybridizes under stringent conditions with a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide shown in (H₂') and which encodes a protein having lipase activity;

(J₂') a polynucleotide which consists of a nucleotide sequence comprising substitution, deletion, insertion and/or addition of one to nine nucleotides in the nucleotide sequence of the polynucleotide shown in (H₂') and which encodes a protein having lipase activity;

(K₂') a polynucleotide which shares an identity of at least 80% or more with the nucleotide sequence of the polynucleotide shown in (H₂') and which encodes a protein

- 58 -

having lipase activity;

(L₂') a polynucleotide which encodes a protein consisting of all of the amino acid sequence shown in SEQ ID NO: 29 or a part thereof covering amino acids 29-424;

(M₂') a polynucleotide which encodes a protein consisting of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one to nine amino acids in the amino acid sequence of the protein shown in (L₂') and having lipase activity; or

(N₂') a polynucleotide which encodes a protein consisting of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (L₂') and having lipase activity,

wherein the stringent condition consists of hybridization at 65°C in a solution containing 6M urea, 0.4% sodium dodecyl sulfate (SDS), and 0.1Xsaline sodium citrate (SSC) .

3. The polynucleotide of claim 1 or 2, which is derived from the genus *Tetrasphaera*.

4. A vector carrying the polynucleotide of any one of claims 1 to 3.

5. A transformed cell transformed with the vector of claim 4.

6. A protein comprising (l₁), (m₁) or (n₁) shown below:

- 59 -

(l₁) a protein which consists of the amino acid sequence shown in SEQ ID NO: 35;

(m₁) a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one to nine amino acids in the amino acid sequence shown in SEQ ID NO: 35 and which has lipase activity; or

(n₁) a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence shown in SEQ ID NO: 35 and which has lipase activity.

7. The protein of claim 6, which is selected from (l₁), (m₁) or (n₁).

8. The protein of claim 6, which comprises (l₂), (m₂) or (n₂) shown below:

(l₂) a protein which consists of all of the amino acid sequence shown in SEQ ID NO: 29 or a part thereof covering at least amino acids 29-424;

(m₂) a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one to nine amino acids in the amino acid sequence of the protein shown in (l₂) and which has lipase activity; or

(n₂) a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (l₂) and which has lipase activity.

- 60 -

9. The protein of claim 8, which is selected from (l_2'), (m_2') or (n_2') shown below:

(l_2') a protein which consists of all of the amino acid sequence shown in SEQ ID NO: 29 or a part thereof covering amino acids 29-424;

(m_2') a protein which consists of an amino acid sequence comprising substitution, deletion, insertion and/or addition of one to nine amino acids in the amino acid sequence of the protein shown in (l_2') and which has lipase activity; or

(n_2') a protein which consists of an amino acid sequence sharing an identity of at least 80% or more with the amino acid sequence of the protein shown in (l_2') and which has lipase activity.

10. The protein of any one of claims 6 to 9, which is immobilized on a resin.

11. Use of a protein of any one of claims 6 to 10 for esterification or transesterification of a medium-chain fatty acid.

12. Use of the protein of any one of claims 6 to 10 for producing a medium-chain fatty acid ester.

13. The use of claim 12, which is for producing an ester between polyphenol and medium-chain fatty acid or an ester between carotenoid and medium-chain fatty acid.

Figure 1

TAACACATGCAAGTCGAACGGTGACCTCGAGAGCTTGCTCTCGAGCGATCAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCAGACTCTGGAATAACCCCGGGA
AACCGGAGCTAATACCGGATACGAGACGAAGCTGCATGGCTATCGTCTGGAAAGTTTTTCGGTCTGGGATGGACTCGCGGCCTATCAGCTTGTGGTGAGGTAAACGGCTCA
CCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGC
AAGCCTGATGCAGCGACGCCGCTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCACC GGCTAACT
ACGTGCCAGCAGCCGGTAATACGTAGGGTGGGAGCCTTGTCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTCCGTCTGCTGTGAAAATCCGGGGCTCAAC
CCCGGACTTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTACGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAG
GTCTCTGGGCCACTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGTTGGGCGCTAGGTGTGGGACTCATTCC
ACGAGTTCCGTGCCGAGCTAACGCATTAAGCGCCCCCCTGGGGAGTACGGCCGCAAGGCTAAACTCAAAGGAATTGACGGGGGGCCGACAAGCGGGCGGAGCATGTGG
ATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAACACCTGGAGACAGGTGCCCCGCAAGGTGGTATACAGGTGGTGCATGGTTGTGTCAG
CTCGTGTGTCGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCGGTTATGGCGGGGACTCATAGAAGACTGCCGGGGTCAACTCGGAGG
AAGGTGGGGATGAGGTCAAATCATCATGCCCTTATGTCTTGGGCTTCACACATGCTACAATGGCCGTTACAAAGGGCTGCGAAACCGCGAGGTGGAGCGAATCCCAAAA
ACCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTTGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACAC
CGCCCGTCAAGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCTTGTGGAGGGAGCCGTGGAAGGTGGGACTGGCGATTGGGACTAAGTCGTAACAAGGTAA

2/12

Figure 2

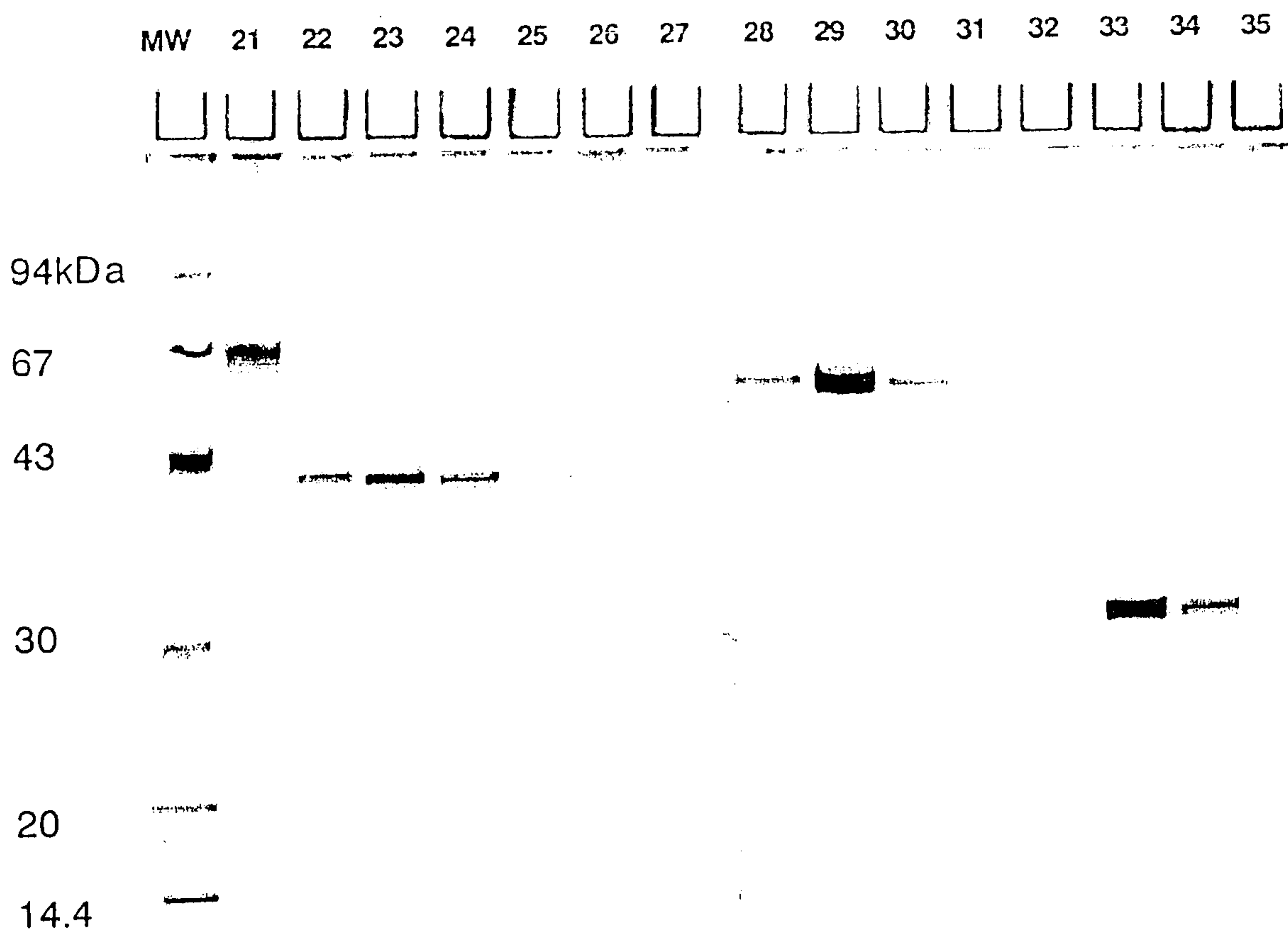


Figure 3

```

putative secreted hydrolase : MPKPALRRVNTATVAAVCTLLALCLTDATAHAAPAQATPTLDYVALGDSYSAGSGVLPVDPANLLCLRSTANYPHVIADT- : 79
GDSL-lipase : ---NRLSRRRAATASALLLTPALALFGASAAVS-APRIQAIDYVALGDSYSAGSGVAGSYDSSSGSCKRSTKSYPALMAASH : 76
                                     _____

putative secreted hydrolase : TGA RLTDVTGGAACQADFTTRAQYGVAPQLDALGTGTDLVTLTIGGNDNSTFINAITACGTAGVLSGGKSPCKDRHGTS : 159
GDSL-lipase : TGTRFNFTACSGARTGADV-----LAKQLTPVNSGTDLVSIITIGGND-AGFADTNTTCN----LQGESACLARIAKARA : 144
                                     _____

putative secreted hydrolase : FDDEIEANTYFALKEALLGVRAARAPHARVAAALGYPMITPATADPSCFLKLPLAAGDVPVYLRRIQAHNLNDAVRRADPEETCA : 239
GDSL-lipase : YIQQTLP---AQLDQVYDAIDSRAPAAQVVVVLGYPRFYKLGGS----CAVGLSEKSRRAINRAADDINAVTAKRAADHGF : 217

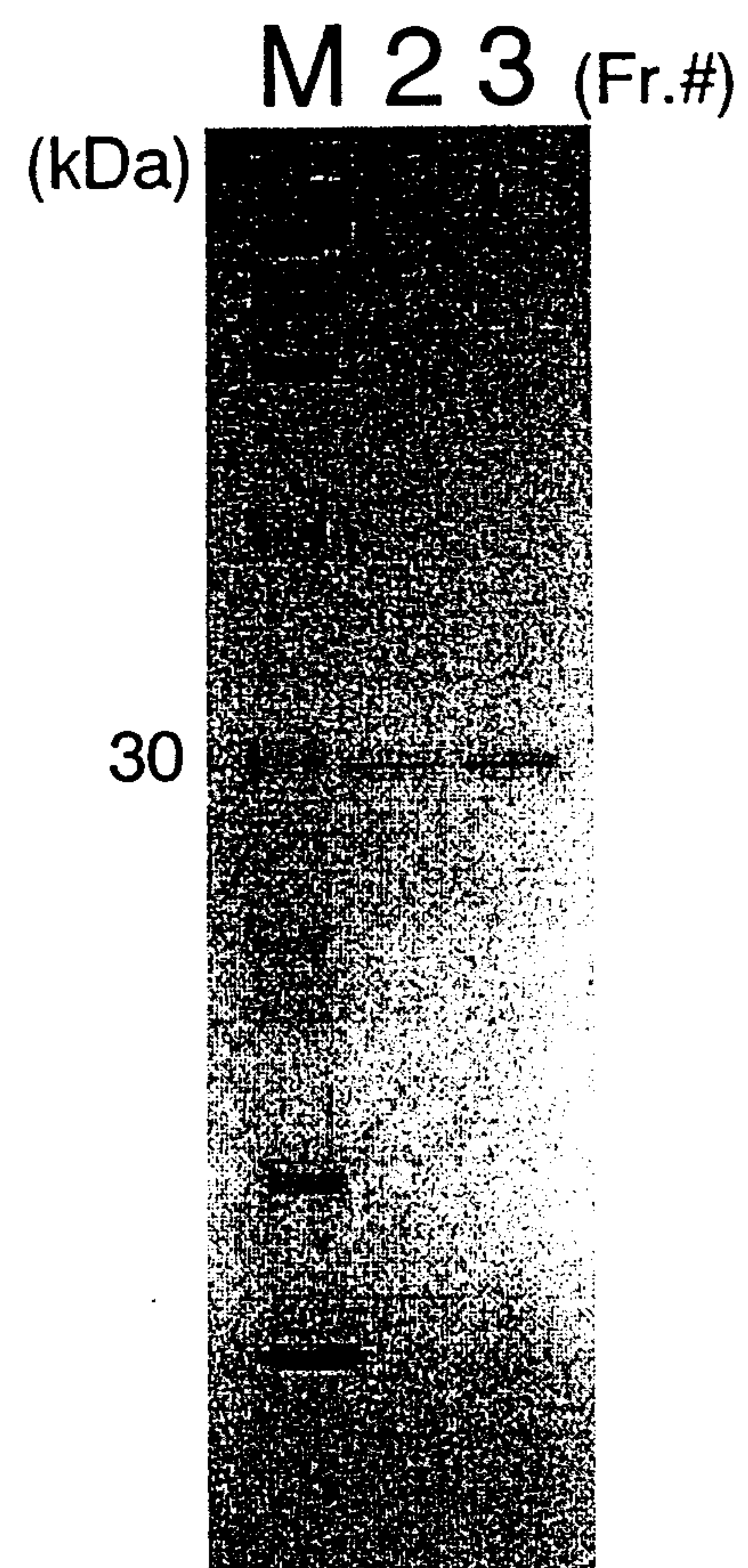
putative secreted hydrolase : TYVDFSGVSDGHDACEAPGTRMIEPLLFCHSLVPVHPNNALGERMAEHTMDVLGLD : 295
GDSL-lipase : AFGDVNTTFAGHELCSG---APQLHSVTLPEVENS-YHPTNNNGCSKGYLPVLNSAT-- : 268

```

ACGCAGGCCGCGCCCGCCCCGGACGGCGACGCACCGGCATACGAACGCTATGTCGCCCTC	60
G D A P A Y E R Y V A L	
GGCGACTCCTACACGGCGGCGCCGCTCGTGCCGAACCTCGACATCGCGGGCGGGCTGCTAC	120
G D S Y T A A P L V P N L D I A G G C Y	
CGCTCGACGAACAATTACCCGAGCCTGCTCGCGCGCGAGCTCGGTGTGACGACGTTTCGTC	180
R S T N N Y P S L L A R E L G V T T F V	
GACGCGAGCTGCTCCGGCGCGGACACGACGGACATGACGCAGAGCCAGCTCGCCGGCGTC	240
D A S C S G A D T T D M T Q S Q L A G V	
GCACCGCAGCTGGACAACCTCACCCCCGACACCGACCTCGTCACGCTGAGCATCGGCGGC	300
A P Q L D N L T P D T D L V T L S I G G	
AACGACTTCAACGTCTTCGGCACCCCTCGTCGGCTATTGCACGACGCTGCGGGCGAGCGAC	360
N D F N V F G T L V G Y C T T L R A S D	
CCGACGGGCAGCCCGTGCCGGGACGAGATGCGCAGCGACGGGCAGGACCGGCTGCTCGCC	420
P T G S P C R D E M R S D G Q D R L L A	
GCCGTCAAAGAGACCCGGGCACGCATCGACGCGGTCATCGCCGAGATCGAGGAGCGCTCA	480
A V K E T R A R I D A V I A E I E E R S	
CCGGACGCGCGCATCCTCGTCGTCGGGTACCCGCAGATCGCGCCGCGTCAGGGCACCTGC	540
P D A R I L V V G Y P Q I A P R Q G T C	
CCCGACCTGCTGCCGCTCGCCGACGGTGACGTGTCGTATGCCGTGCAGGTCAACAAGCGC	600
P D L L P L A D G D V S Y A V Q V N K R	
CTCACCGACGCGCTGCGGCAGGCGGCCAAGAGCAACCAAGTGGAGTACGTGACGTGTGG	660
L T D A L R Q A A K S N Q V E Y V D V W	
AAGGCGAGCCAGGGGCACGACATCTGCTCCGGCGACCCGTGGGTCAACGGGCAGGTCACC	720
K A S Q G H D I C S G D P W V N G Q V T	
GACATCACCCGGGCGCAGAACTACCACCCGTTGCGGAACGAGCAGCGCGCCATCGCCGAC	780
D I T R A Q N Y H P F A N E Q R A I A D	
CTGCTCATCGACGAGCTCACCTGAGGACCACCACCGGGGCATCACGATGTGCACTGCCGA	840
L L I D E L T *	

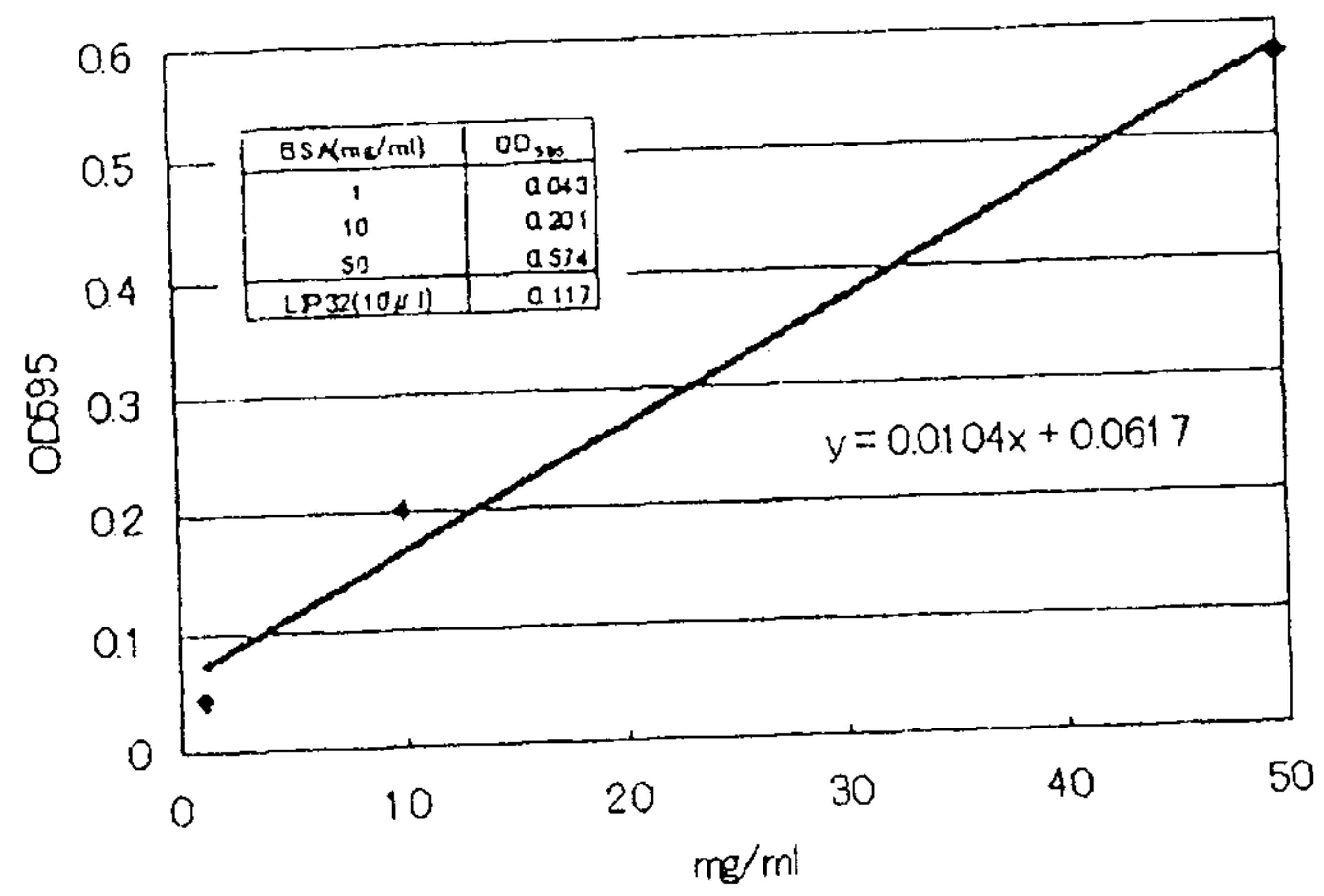
The diagram illustrates the lip32Nc expression vector. It is a circular plasmid with the following components in sequence: a T7 promoter (represented by a circle), a lac operator (represented by a rectangle), a pelB leader (represented by a stippled rectangle), the lip32Nc gene (represented by a large rectangle), a His tag (represented by a small rectangle), and a T7 terminator (represented by a circle). Restriction sites are marked above the plasmid: Nde I and Nco I are located within the pelB leader, and Xho I is located within the His tag.

Figure 6



6/12

Figure 7



7/12

Figure 8

```

1   CGCGGTTTCATCCAGCAGATCGCACGGCGGGTCCACGGGCGCGTGGCGCGCGGAGCTCG
61  ACGACCTCGGGCCCGCGTGGTCCGACGCTATCTCGGGTCTCGGGCTCGCGCGGTCTTG
121 GCGGTGGTCTTACCGTACCACTTCGGGACCTGTTCCGCTCGCGGGTCTTCTCGCGCG
181 GCTGACCGCAGCAGCGCGCGCGTATCGTGGGAGGACGACCGCGCATGAAGGAGC

241 TCACGGATGAGCTCGTCACGTCTACCGTCCGACCGCGCGTGGCGGTGGTGGCTGCT
    M S S S R R T V R T A V A A A W A A

301 GCGGTGCTCGTGGCGGTGCGCGTGGCAGTGGATGGCGCGACCGAGCGCGGTGGCGG
    A L L V A V P G A Q S M A A T E R A S A

361 CCCACGAGCGCGCGCGCGCGCGGACGGCGACCGACCGCATACGAACGCTATGTCGGC
    P T Q A A P A P D G D A P A Y E R Y V A

421 CTCGGGACTCCTACACGGCGCGCGCGTGGTGGCGAACCTCGACATCGCGGGCGGTGG
    L G D S Y T A A P L V P N L D I A G G C

481 TACCGCTCGACGAACAATTACCGAGCTGCTGGCGCGGAGCTCGGTGTGACGAGCTTC
    Y R S T N N Y P S L L A R E L G V T T F

541 GTCCAGCGGAGCTGCTCGCGCGGACACGACGACATGACGACAGCCAGCTCGCGCGG
    V D A S C S G A D T T D M T Q S Q L A G

601 GTCCACCGCAGCTGGACAACCTCACCCCGACACCGACCTCGTCACGCTGAGCATCGGC
    V A P Q L D N L T P D T D L V T L S I G

661 GGCAACGACTTCAACGCTCTCGGACCCCTCGTGGCTATTGCACGACGCTCGCGCGGAGC
    G N D F N V F G T L V G Y C T T L R A S

721 GACCCGACGGGACCGCGTCCCGGACGAGATGGCAGCGACGGGACGACCGCGCTGCTC
    D P T G S P C R D E M R S D G Q D R L L

781 GCCCGCGTCAAGAGACCGCGGACGATCGACCGGTGATCGCGGAGATCGAGAGCGC
    A A V K E T R A R I D A V I A E I E E R

841 TCACCGGACCGCGCATCCTCGTGGTACCGGAGATCGCGCGGTGACGGCACC
    S P D A R I L V V G Y P Q I A P R Q G T

901 TGCCCGACCTGCTGGCGTCCCGACGGTGACGTGTGCTATGCCGTGACGTCAACAAG
    C P D L L P L A D G D V S Y A V Q V N K

961 CGCCTACCGACCGCTCGCGGACGGGCAAGAGCAACCAAGTGGAGTACGTGACGTG
    R L T D A L R Q A A K S N Q V E Y V D V

1021 TGGAAGCGAGCGAGGGGACGACATCTGCTCCGGGACCGGTGGTCAACGGGACGTC
    W K A S Q G H D I C S G D P W V N G Q V

1081 ACCGACATCACCGCGCGGAGAACTACACCGGTTCGGAACGAGGAGCGCGCATCGGC
    T D I T R A Q N Y H P F A N E Q R A I A

1141 GACCTGCTCATCGACGAGCTCACCTGAGGACCACCGGGGATCAGGATGTGCACTGC
    D L L I D E L T *

1201 CGA

```

Figure 9

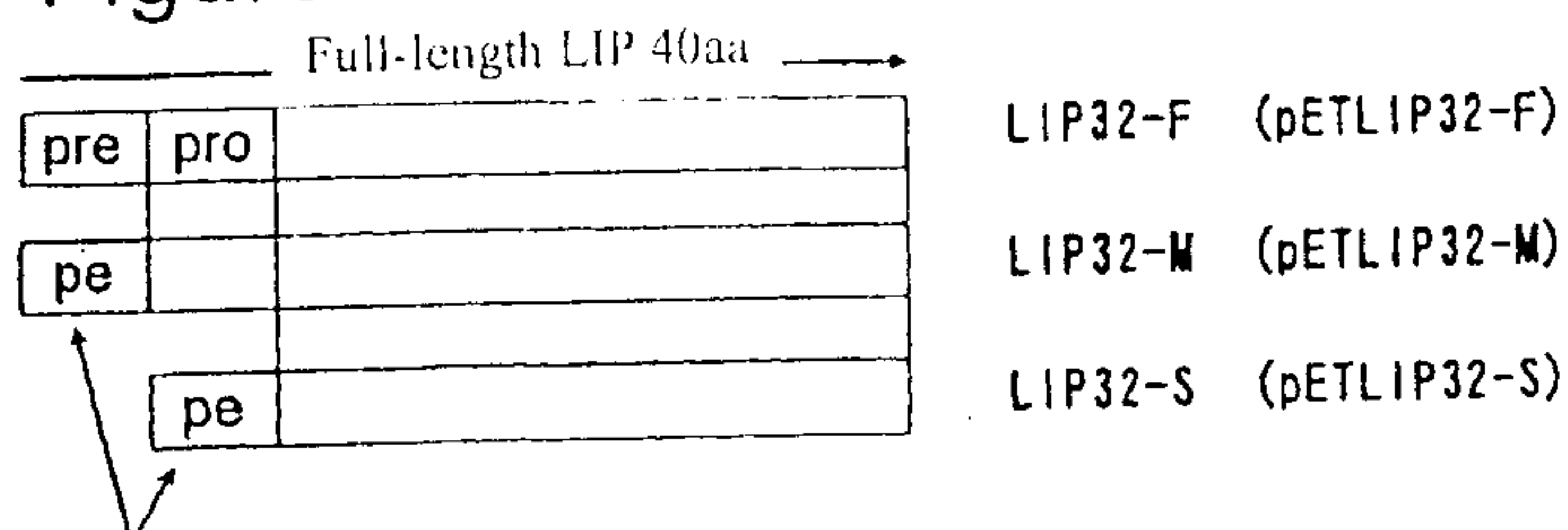
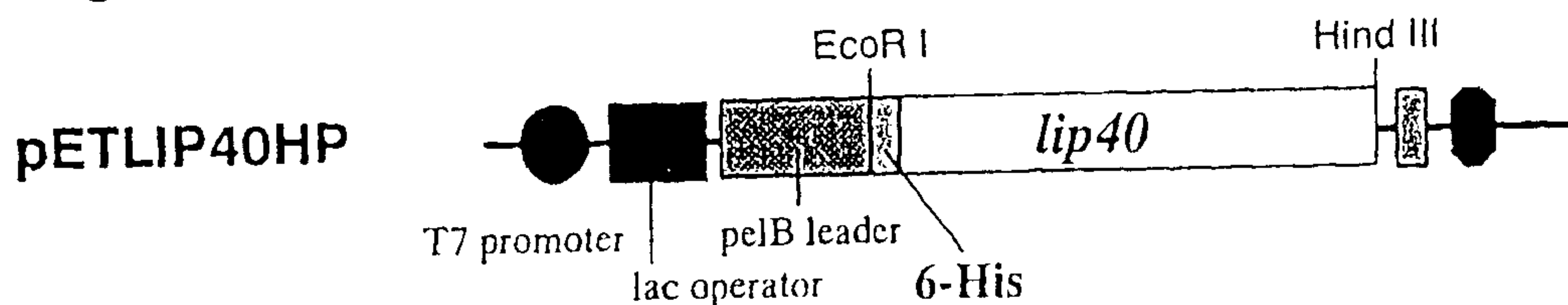


Figure 10

1 GTGAACAGCA CGAGCCGAC GGAGAGCCG AGGATCGGA CCGGTTCCG GCGATGTCG ATGAGCCAGG TGTTCAGGC CCGGCATAG ACCAGCCGG
 101 GCAACAGCCG GAGCAGGACG ATGTCGGCT CGAGCTCGG GTCCCGATG AACGGCAGG ACAGCCGAC GATGCCACG CCGACGAGG CGATGGCGA
 201 CCGGAGGCGG AACGGGCGG ACAGCCGCG GACGACGATG ACCGTCACG CGATCCCGG GAGGTCAGG GCGAGTCCA CCGGCACATT GTCCGTCAAC
 301 GCGGAGGCGG GTCCCGCGG CTCCGGTTC CCGGACATT ACCCGCTGT AGCGTGAAG TCGCGCGTA TCCCGAGAG CTCCGTGAC ACCCGTCGG
 401 AAGTCTTCG ACAATGACG CAGCACTGT CCGAGGAGC CTCCCGCTG CCTCCCGCT CCGTCTCGG GTCACCTCG CCGACCCCG GTCCGCGGA
 M T S A L L R R A L A P A L A L G L A V T L G A P A S A G
 501 CCGGACTCG TCGCCGAC CCGCGTGGG AGGACGTGA CCGACCCCG CAGCCCGCT CCGCCCGCT TCTACGAGC GCGGCGAGG ATCCCGCGA
 P D S V P G T A G A T T V T D T P E P P R P A F Y E P P A T I P G T
 601 CACCCGCGC GGTATCGG ACCGAGTCG CAGCTACCT CCTCGACCG CTCCCGCTGT CCGAGACGT CGTGACGTC ACCCGGTCA TGTACTCTC
 P G T V I R T E S A T Y L L D P L G L S Q T V V T S T R V M Y S S
 701 GTCGACCGG CAGGCGCGG CCATCGCGT CACCGCAGG ATCCTGAGC CGAGCGCGG GTGGTTCGG CTCCGTGCG GACCGTCAT CTCGTATCG
 L D R Q G R P I A V T G T I L E P K A P W F G L G A R P L I S Y A
 801 GTGGCAGCC AGGCATGGG TGACCGTGC GCGCCGTCG GCGAGTCG CAGTCCGTG ACCGAGTAC AAGCCGGTT CATCTCGGG CTCCGTACG
 V G T Q G M G D R C A P S R Q L A E S V T E Y E A G F I S G L V T R
 901 GCGGTACCG CGTTGCTTC ACCGACTAC AGGTCTCTC GAGCCCGGG AGGCACACT ACATGAACG CGTCTCCAG GAGCCCGCN TCCTGACAT
 G Y A V A F T D Y Q G L S T P G T H T Y M N R V V Q G R A X L D M
 1001 GGCAGAGCA GCGTCCCGG GCAACGGAC CAGCTGACT GCGAGACTC CCGTCCGAT CTACCGCTAC TCGAGCGCG CCGCGCGAG TCGTCCGG
 A R A A L R R N G T T L T A T T P V G I Y G Y S Q G G G A S A S A
 1101 GCGAGCTCA CCGGACGTA TCGCCGAG CTCCGGTCA AGGTGCCCT CCGCGTGGG GTTCCCGCG ACCTCAAGG GGTGCGCAG AACCTCGAT
 A E L T A T Y A P E L R V K G A L A G A V P A D L K A V A Q N L D G
 1201 GCTCGTGTG TCGGAGTTC CTCAACTTC CGTCTCGG CCGTCCGGG GGTACGGCA TCGACCTAA CTCCTACCTC AACGAGCGG GCGAGCGGT
 S L Y A E F L N F A L L G L S A G Y G I D L N S Y L N E R G Q A V
 1301 GCGCGCGAC ACCGAGAAC ACTGCGTAC CAGCTCGCG AAGCGCGCT TCCAGCAGT GTCAGCGTG ACCCGGAGG GTCGCGTCT GCTGACTAC
 A A D T E N H C V T D L P K A A F Q Q S S T L T R D G R G L L D Y
 1401 CTCGAGAGG AGCGTTCCG GTCGGTATC GCGGACAAC GCATCGGAC GATCAAGCG TCGTCCCGG TCCTCATCTC GACTCGGTC GCGAGGAG
 L D E E P F A S V I A D N R I G T I K P S V P V L I S H S V A D D V
 1501 TCATCCCGTA CCGGTGGCG AAGCAGTCG CCGCGACTG GTCCGCAAG GCGGCAACG TCGCCTCTC GACGAACGT GCGCGGAGC ACCTCGCGG
 I P Y A V G K Q L A R D W C A K G A N V R L S T N V G P T H L G G
 1601 GCGCTCGCG TCGCGCGCG AGAGTACG GTTCTTCAG CCGCGTTCG CCGCGTGGC CAGCTCAGC AACTCTGGG CCGTCTAGT GACTCGGG
 A L P S A A E S Y A F F E A R F A G V P Q L S N C W A V *
 1701 GCGAGTTGG TGACAGTAG CCGTTGACG CAGGAGAAC CTCGGGTGA GGTGTGATT GTGGAAGTC CAACCGCCG GAGGTCTCG TGTCCACGG
 1801 TAACGCTCG CTGAATGTC ATGTCGTCG TCTGTTGTC GACCGGTC CCGGCAAGG GTCCGACTG GTCATGCGG CGAAGCGAT GGGATCTCG
 1901 CGTCAGTCG CTCACAGTG GGTGTCAGG TTCGACCGG AGGTGAGGC CCGGTTGTC GACCGGCTC CCGCGCGCA CTCCAGCCT CCGCGACCG
 2001 CGACATCGT CAGGACCGG GTGTGCGT CCGCGCGGA GACCGCGT GGTACGACT GATCCGGCG TGAATGGT GTCCCGGAC GCACGTCAG
 2101 CCGAATCCT CCGCGCATG ACCTGCCCT CCGCGCAT TCGGACCGG TACCGGGA CCGTATCGG GCTCGAAGA CCACCGCGT CCGGTATCA
 2201 CCGGACCGG CCGCGAGCT GTTCACGTT GATGTAAGA AGATCGGCG CATCCCGAC GGTGCGGGT GGAGAGCCA CCGCGCGAG ATCGGCTCG
 2301 CCGCGGAGG GAAGAAAGC CGATCGGTG AGACTACGT GACTCCATG GTCGATGAC ACTCCGGCT CCGTACAGC CAGATCTCC CCGAGGAG
 2401 CCGCGGAGG TCGCGCGGT TCATCTCG CCGCGCGAA CACTTCGCG CCGCGCAT CCGCGCATC GAACGGTCA TCACCGCAA CCACTTCAG
 2501 TACCGCAAAA GCAACGAGT CAGGAGCGG ATGACCGCA TGGGCGCAC GCACAAGTC ATCGCGCGG ACTGCGG

Figure 11



9/12

Figure 12

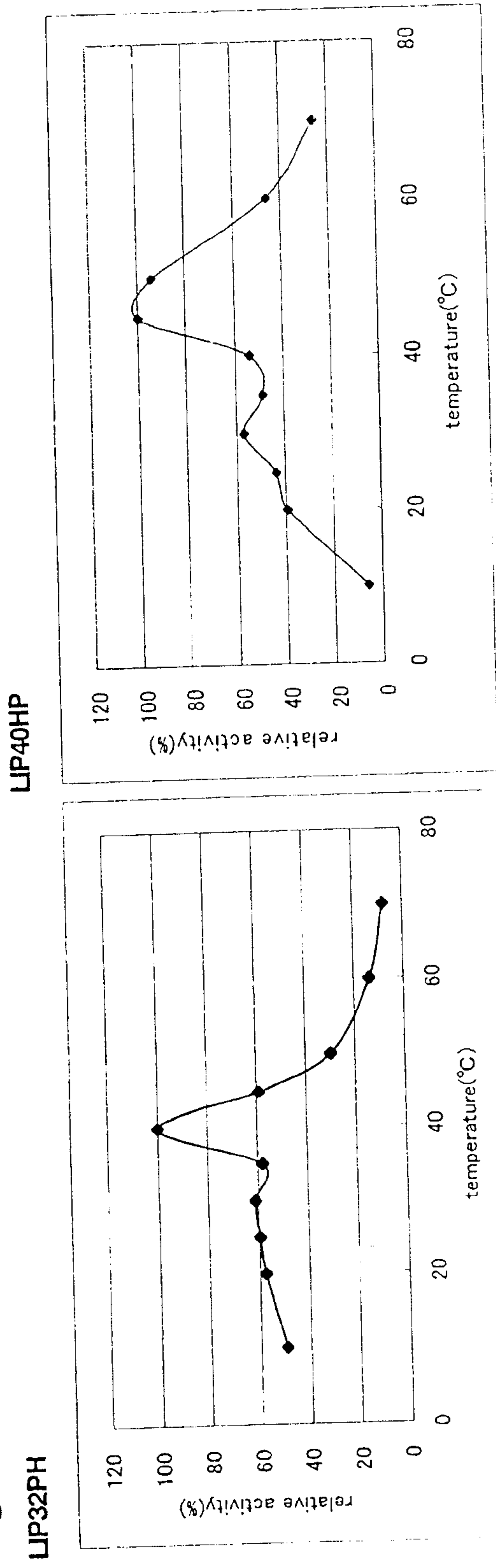
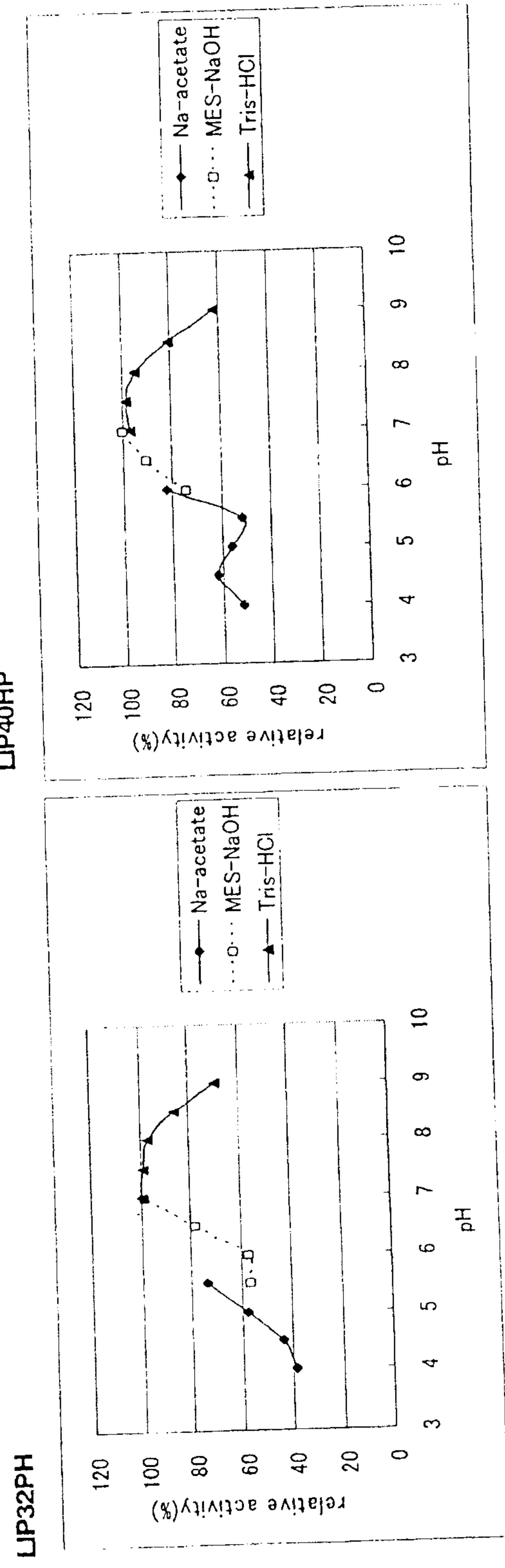
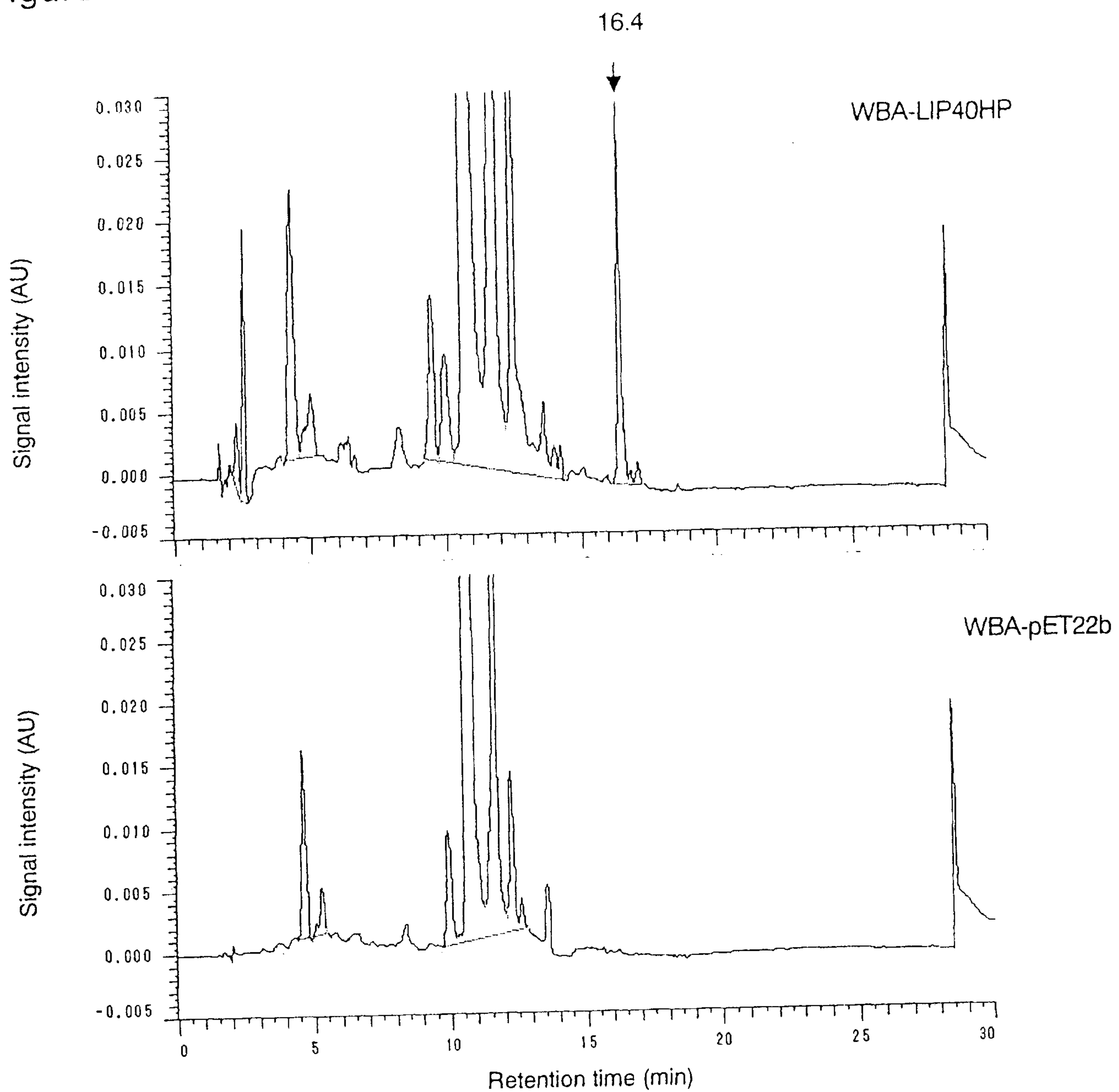


Figure 13



10/12

Figure 14



11/12

Figure 15

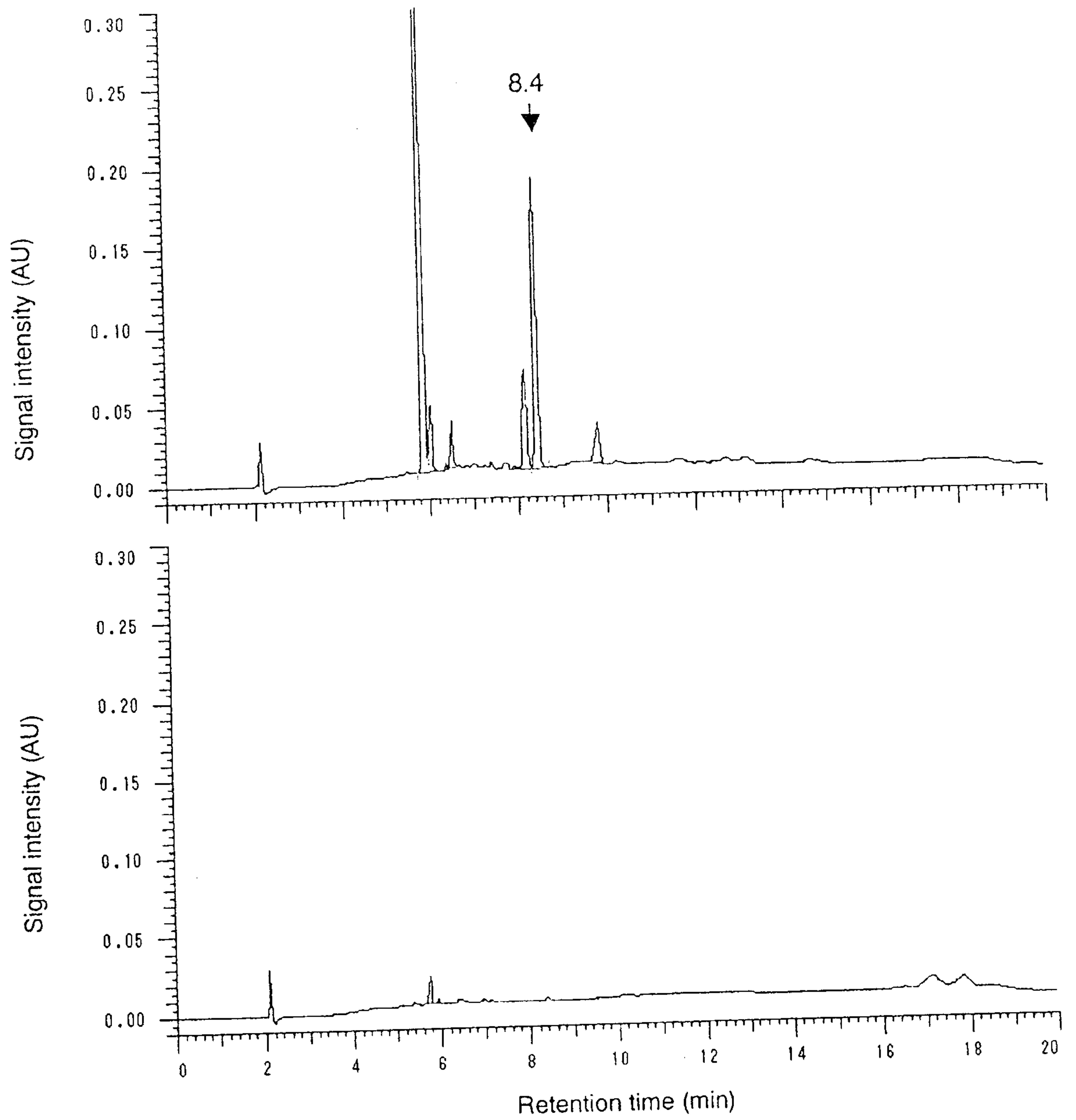


Figure 16

GPDSVPGTAGATTVTDTPPPRPAFYEPPTIPGTPGTVIRTESATYLLDPLGLSQTVVTSTRVMYSSLD
RQGRPIAVTGTILEPKAPWFGLGARPLISYAVGTQGMQDRCAPSRQLAESVTEYEAGFISGLVTRGYAVA
FTDYQGLSTPGHTYMNRRVQGRAXLDMARAALRRNGTTLTATTPVGITYGSQGGASASAAELTATYAP
ELRVKGALAGAVPADLKAVAQNLGSLYAEFLNFALLGLSAGYGIDLNSYLNERGQAVAADTENHCVTDL
PKAAFQQSSTLTRDGRGLLDYLDEEPFASVIADNRIGTIKPSVPVLI SHSVADDVIPYAVGKQLARDWCA
KGANVRLSTNVGPTHLGALPSAAESYAFFEARFAGVPQLSNCWAV