



AU9478557

(12) PATENT ABRIDGMENT (11) Document No AU-B-78557/94
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No 671392

(54) Title
LIPASE-LABELLED PROBE

International Patent Classification(s)
(51) G01N 033/535 C12Q 001/34 C12Q 001/68 G01N 033/58

(21) Application No 78557/94 (22) Application Date : 13.10.94

(87) PCT Publication Number : WO95/10775

(30) Priority Data

(31) Number (32) Date (33) Country
2071/93 15.10.93 AT AUSTRIA

(43) Publication Date 04.05.95

(44) Publication Date of Accepted Application : 22.08.96

(71) Applicant(s)
BOEHRINGER MANNHEIM GMBH

(72) Inventor(s)
FRITZ PITTLNER; THOMAS SCHALKHAMMER; BERNHARD ECKER; EVA KYNCLOVA; WERNER WAKOLBINGER

(74) Attorney or Agent
DAVIES COLLISON CAVE , 1 Little Collins Street, MELBOURNE VIC 3000

(56) Prior Art Documents
WO 92/21778
EP 350808

(57) Claim

1. Enzyme-labelled probe or test substance, wherein the enzyme is a lipase from plants, a corresponding isoenzyme or a structural derivative having an amino acid homology of at least 70 % and lipase activity.
2. Enzyme-labelled probe or test substance as claimed in claim 1, wherein the enzyme having lipase activity is obtainable from *Candida rugosa* DSM 2031.
3. Enzyme-labelled probe or test substance as claimed in claim 1 or 2, wherein the lipase is conjugated with biotin, avidin, lecithin, protein A, protein G, antibody-binding proteins, antibodies, antigens, viral protein antigens, bacterial surface proteins, digoxigenin, DNA, RNA, oligonucleotides or synthetic analogues, metal colloids or plastic microparticles (< 5 μ m).



(51) Internationale Patentklassifikation ⁶ :

G01N 33/535, 33/58, C12Q 1/68, 1/34

A1

(11) Internationale Veröffentlichungsnummer: WO 95/10775

(43) Internationales
Veröffentlichungsdatum:

20. April 1995 (20.04.95)

(21) Internationales Aktenzeichen: PCT/EP94/03379

(22) Internationales Anmeldedatum: 13. Oktober 1994 (13.10.94)

(30) Prioritätsdaten:

A 2071/93 15. Oktober 1993 (15.10.93) AT

(71) Anmelder (für alle Bestimmungsstaaten ausser US):
BOEHRINGER MANNHEIM GMBH [DE/DE]; D-
68298 Mannheim (DE).

(72) Erfinder; und

(75) Erfinder/Anmelder (nur für US): PITTNER, Fritz [AT/AT];
Khekgasse 40-42/11, A-1235 Wien (AT). SCHALKHAM-
MER, Thomas [AT/AT]; A-3072 Kasten 105 (AT).
ECKER, Bernhard [AT/AT]; Hintenbergerstrasse 130a,
A-2551 Enzesfeld (AT). KYNCLOVA, Eva [CZ/AT];
Linzerstrasse 83/11, A-1140 Wien (AT). WAKOLBINGER,
Werner [AT/AT]; Im Bäckerfeld 1, A-4061 Pasching (AT).

(74) Anwälte: FOUQUET, Herbert usw.; Boehringer Mannheim
GmbH, D-68298 Mannheim (DE).

(81) Bestimmungsstaaten: AU, CA, JP, NZ, US, europäisches
Patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT,
LU, MC, NL, PT, SE).

Veröffentlicht

Mit internationalem Recherchenbericht.
Vor Ablauf der für Änderungen der Ansprüche zugelassenen
Frist. Veröffentlichung wird wiederholt falls Änderungen
eintreffen.

67 1392

(54) Title: LIPASE-LABELLED PROBE

(54) Bezeichnung: LIPASE-MARKIERTE PROBE

(57) Abstract

In order to improve the thermal and chemical stability of an enzyme-labelled probe, a lipase that is preferably extracted from *Candida rugosa* and whose isoenzymes or structural analogues have at least 70 % aminoacid homology and lipase activity is used as the enzyme.

(57) Zusammenfassung

Zur Verbesserung der thermischen und chemischen Stabilität einer mit Enzymmarkierten Probe wird der Einsatz von Lipase, welche bevorzugt aus *Candida rugosa* erhältlich ist, deren Isoenzymen oder strukturellen Analoga mit einer Aminosäurehomologie von wenigstens 70 % und Lipaseaktivität als Enzym verwendet.

LIPASE-LABELLED PROBE

The invention concerns a probe or test substance labelled with lipase and the use of lipase for the determination of biological material.

It has become known that markers and test substances can be developed for the detection of biologically relevant groups, certain genes, antigens, antibodies, bacterial surface proteins or such like which are intended to facilitate the detection of the substances that are being searched for.

In particular it is known in the field of genetic engineering that gene probes can be radioactively labelled and such a gene probe can be used to search for complementary nucleic acids. In the case of test substances labelled with enzymes, peroxidase or alkaline phosphatase have been previously used as the enzyme. Both enzymes are characterized by a relatively good sensitivity and a relatively easy detectability which underscores their suitability as a marker or as a test substance. However, these special enzymes do have disadvantages. Thus for example certain general conditions have to be adhered to which make the use of such enzymes for the detection of certain substances impossible. Moreover, the shelf-life and thermostability of such enzymes is limited. In addition such enzymes require that the work is carried out in an exactly defined medium which greatly limits the scope of their application. Finally the presence of metal ions, which



is for example required when using alkaline phosphatase, creates difficulties for a number of tests and substances which complex metal ions such as EDTA not only make it impossible to use phosphatase but at the same time result in the risk of a denaturation and thus of a considerable worsening of the detection limits also in the case of peroxidases.

The object of the invention is therefore to create a probe or test substance labelled with an enzyme which is characterized by a considerably improved stability and a considerably broader application spectrum and in this manner enabling the analytical determination of substances for which it was previously not possible at all to use enzyme-labelled probes or test substances.

This object is essentially achieved by using as the enzyme a lipase from plants, isoenzymes thereof or structural analogues with an amino acid homology of at least 70 % and lipase activity. *Candida rugosa* which has proven to be particularly suitable as a source and which is also described in the literature as *Candida Cylindracea* contains a lipase whose structure has been extensively determined. The cloning and analysis of the lipase sequence is described in detail in particular in "Gene" 124:1 (February 14, 1993, pages 45-55). The enzyme is described in this publication as a 57 kDa protein with 534 amino acids, 5 isoenzymes with an amino acid homology of 80 % having already been structurally analysed. In any case it is a lipase from plants, in particular from fungi. A lipase from *Candida rugosa* DSM 2031 is particularly preferred. The use of this lipase results in a substantial improvement in the product properties compared to all previously known enzymes and also compared to other known lipases, in particular



animal lipases. In particular it was found that when using the lipase suggested according to the invention, a thermostability of up to at least 60°C is ensured which can be achieved neither with alkaline phosphatase nor with peroxidase. Particularly for hybridization tests in which the use of an increased temperature is an important prerequisite for an acceptable reaction rate, the thermostability achieved in this manner creates the prerequisite for being able to carry out such tests within a reasonable time period.

A further advantage of the lipase suggested according to the invention is that it requires no metal ions at all for its activity. In the case of alkaline phosphatase magnesium and zinc ions are required for enzymatic activity and exactly these metal ions would for example activate nucleases which consequently prevents the use of alkaline phosphatase for gene probes.

In order to be certain to prevent the activation of nucleases, particularly when using gene probes labelled with enzyme, complexing substances such as for example EDTA are often used. EDTA not only complexes metal ions and thus no longer allows the use of alkaline phosphatase, but also appropriately large amounts of EDTA also have the tendency to denature proteins. The lipase suggested according to the invention is not only distinguished by a substantially higher thermostability than known enzymes but is also distinguished by a complete stability towards EDTA. A further significant superiority of the lipases used according to the invention compared to known enzymes is that urea and corresponding derivatives also do not impair the function of the lipase at all. When lipase is used, EDTA can be used to protect SH groups which was not possible



at all with previous probes or test substances labelled with enzyme.

The lipases used according to the invention are also distinguished by a considerably improved tolerance towards organic solvents. In particular the lipases suggested according to the invention can also be used in toluene which considerably increases the scope of the analytical application of the probes or test substances according to the invention.

The high stability enables the probes and test substances according to the invention to be used in a pH range between 2.5 and 9 and the insensitivity towards ureas enables the detection of highly hydrophobic substances. The use of large amounts of for example urea which would not have been permissible with other probes or test substances labelled with an enzyme, additionally enables the secondary structure of a DNA to be expanded to such an extent that the reliability of gene probes is considerably improved.

Due to the considerably higher stability of the lipases suggested according to the invention there are also hardly any limits with regard to the type of coupling to reactive substances and in particular to biorecognitive groups.

Due to their high stability it is possible to dry lipase conjugates and to store them for years at room temperature without loss of activity if desired in a lyophilized form or only dried in the air. Overall the conversion rate is of the same order of magnitude compared to known probes labelled with enzymes but with



a concurrent improved sensitivity which is due to the improved detectability of lipase.

Whereas for example in a comparative experiment with alkaline phosphatase the detection limit is at about 250 to 100 pg, experiments have shown that lipase can still be determined exactly in a range down to about 5 pg whereby, in addition to a purely qualitative analysis, a relatively simple estimation is even possible down into the range of 10 ng.

The determination of lipase can be carried out in the usual manner in which for example esters of phenols or their derivatives can be used as substrates. The colour reaction achieved in this manner enables a direct and simple analysis. Appropriate methods are known to a person skilled in the art.

The stability advantages of the lipase according to the invention are particularly significant especially in connection with oligonucleotide probes.

According to a preferred embodiment of the invention the probe or test substance is characterized in that the lipase is conjugated with biotin, avidin, lecithin, protein A, protein G, antibody-binding proteins, antibodies, antigens, viral protein antigens, bacterial surface proteins, digoxigenin, DNA, RNA, oligonucleotides or synthetic analogues, metal colloids or plastic microparticles ($< 5 \mu\text{m}$). Such conjugates have specific reactive groups for a broad range of applications and in particular they have biorecognitive groups which thus provides an extremely large field of applications for probes or test substances labelled in



this manner.

The invention in addition concerns the use of lipases from plants in particular from *Candida rugosa* DSM 2031, isoenzymes thereof or structural analogues with an amino acid homology of at least 70 % and lipase activity for the production of analytical probes or test substances in which the lipase is used in a form conjugated with biorecognitive groups and in which such test substances are particularly suitable for bioassays, test strips, biosensors or as gene probes. In this connection classical oligonucleotide probes can in particular be used as gene probes which, due to the favourable thermostability of the lipase, enable a selective hybridization under stringent conditions.

The superiority of the lipases from plants suggested according to the invention, in particular from *Candida rugosa* or isoenzymes thereof and the structural analogues mentioned above, is elucidated in more detail by the following figures and examples.

Fig. 1 shows the thermostability of the lipase from *Candida* species. It can be seen that the activity remains almost constant over time at a pH value of 5 at temperatures of 50°C and that at a pH value of 7 it still retains over 75 % of its initial activity even after one hour. Even at temperatures of 60°C and a pH value of 5 the values remain at about 80 % of the original activity after one hour.

Fig. 2 shows the detection limits of colour reactions of lipase and alkaline phosphatase. It can be seen that in the case of alkaline phosphatase the limit of detection



is almost reached at about 250 pg whereas in the original even at 5 pg a spot could be detected as a colour reaction in the case of lipase. The diameters of the spots substantially correlate with the actual amount down into a range of 10 ng in the case of lipase whereas a simple quantitative estimation is no longer easily possible in the case of alkaline phosphatase beyond about 500 pg.

Fig. 3 illustrates schematically in a flow diagram the use of lipase for an oligonucleotide gene probe. In this procedure a structural gene 2 is immobilized to a microtitre plate 1 via a biotin-avidin coupling. An oligonucleotide probe 4 is linked to lipase 3, the lipase being immobilized to the microtitre plate in the case of an analyte 5 when this analyte recombines with the gene which is immobilized on the microtitre plate 1 as well as with the oligonucleotide 4. Overall the lipase 3 is immobilized on the microtitre plate 1 by the hybridization reaction when a particular biological substance 5 to be detected is present. The qualitative detection is then possible by conventional methods, for example by a colour reaction of the lipase with an appropriate substrate as reagent.

Fig. 4 shows a comparison of the optimal pH values of lipase, peroxidase and alkaline phosphatase.

Fig. 5 shows a comparison of dye precipitation assays for lipase and alkaline phosphatase.



Examples

General procedures

Horseradish peroxidase activity assay using ABTS

Principle:

Horseradish peroxidase was detected spectrophotometrically at 23°C by monitoring the oxidation rate of ABTS (1.49 mM) in the presence of hydrogen peroxide (0.045 %) in 0.1 M citrate/phosphate buffer, pH 4.5 at a wavelength of 420 nm.

Description of the method:

A substrate mixture of 0.5 ml ABTS (20 mg/10 ml water), 1 ml hydrogen peroxide (0.6 % in water) and 9.5 ml 0.1 M citrate/phosphate buffer, pH 4.5 was prepared.

50 μ l of an enzyme solution was mixed with 500 μ l of the aforementioned substrate solution and the kinetics of the enzymatic reaction were monitored at a wavelength of 420 nm over a time period of one minute. The concentration of the oxidized substrate was calculated using the extinction coefficient for ABTS. The concentration of the enzyme solution was adjusted in such a way that a linear time course of the absorbance curve was ensured under the working conditions.



Determination of the activity of alkaline phosphatase using p-nitrophenyl phosphate as the substrate.

Principle:

Alkaline phosphatase was determined spectrophotometrically at 23°C by monitoring the hydrolysis of 0.1 mM p-nitrophenyl phosphate in 0.1 M TRIS, 0.1 M NaCl and 50 mM MgCl₂, pH 8.5 at a wavelength of 405 nm [Lazdunski, C. and Lazdunski, M. (1966) BBA 113, 551-566].

Description of the method:

5 µl of an enzyme solution was mixed with 500 µl 0.1 M TRIS, 0.1 M NaCl, 50 mM MgCl₂, pH 8.5. After adding 100 µl of a 0.6 mM p-nitrophenyl phosphate solution, the reaction time course was monitored kinetically at 405 nm over a time period of one minute. The concentration of the enzyme solution was adjusted in such a way that a linear time course of the absorbance curve was ensured under the working conditions. The concentration of the hydrolysed substrate was determined using a p-nitrophenol calibration curve at pH = 8.5.

Determination of lipase activity using p-nitrophenyl butyrate

Principle:

Lipase was determined spectrophotometrically at 23°C by monitoring the hydrolysis of 0.1 mM p-nitrophenyl butyrate in 0.1 M phosphate buffer, pH 7.0 at a wavelength of 405 nm.

Description of the method:

A stock solution of p-nitrophenyl butyrate (PNPB) was prepared by mixing 23 µl PNPB with 5 ml 1 % polyvinyl



alcohol in water while shaking vigorously. The phase containing undissolved pNPB was separated by centrifugation (4°C, 1500 g, 7 min).

The concentration of the remaining aqueous solution of pNPB was determined with the aid of a calibration curve of p-nitrophenol at pH = 7.0.

5 μ l enzyme solution was mixed with 500 μ l 0.1 M phosphate buffer, pH 7.0. After adding 100 μ l of the 0.6 mM p-nitrophenyl butyrate solution, the reaction kinetics were monitored at a wavelength of 405 nm over a time period of one minute. The concentration of the enzyme solution was adjusted in such a way that a linear time course of the absorbance curve was ensured under the working conditions. The concentration of the hydrolysed substrate was determined using a p-nitrophenol calibration curve at pH = 7.0.

Pretreatment of the lipase:

X-ray crystallographic studies of various lipases showed that the active centre is shaped like a groove which is covered in a cap-like manner by a helical protein part [Schrag, J.D. and Cygler, M., J. Mol. Biol. 230, 575-591 (1993); Derewenda, U., Brzozowski, A.M., Lawson, D.M. and Derewenda, Z.S., Biochemistry 31, 1532-1541 (1992)]. Various effectors are able to cause an extensive spatial restructuring thus making the active centre more freely accessible to the substrate.

Lipase (at a protein concentration of 0.09 mg/ml; determined by means of the Bradford method) was incubated for 1 to 60 min with a 20 to 44 % (v/v) effector solution (e.g. tetrahydrofuran, 2-methyl-



pentanediol, 2-isopropanol...) in 0.1 M acetate buffer, 0.1 % BSA, pH 5.0 at room temperature. Subsequently the enzyme solution was diluted to a measurable concentration with 0.1 M acetate buffer, pH 5.0, 0.1 % BSA, or 0.2 % (v/v) Tween 20 in 0.1 M acetate buffer, pH 5.0, 0.1 % BSA. The samples were stored at 4°C. The activity was determined using the pNPB assay.

Determination of lipase activity using hydroquinone monobutyrate

Principle:

Lipase was determined spectrophotometrically at 23°C by monitoring the hydrolysis of 0.38 mM hydroquinone monobutyrate in 0.1 M buffer, pH 1.5 to 9.0 at a wavelength of 295 nm.

Description of the method:

A stock solution of hydroquinone monobutyrate (HMB) was prepared by mixing 10 mg HMB in 6 ml of a 1 % aqueous polyvinyl alcohol solution while shaking vigorously. The concentration of the HMB solution was determined with the aid of a hydroquinone calibration curve and was 3.08 mmol/l.

20 µl of an enzyme solution was mixed with 700 µl 0.1 M buffer which had been adjusted to the desired pH value. After adding 100 µl of a 3.08 mM HMB solution, the kinetics of the enzymatic reaction were monitored at a wavelength of 295 nm over a time period of 2 minutes. The concentration of the enzyme solution was adjusted in such a way that a linear time course of the absorbance curve was ensured under the working conditions. The concentration of the hydrolysed substrate was determined with the aid of a hydroquinone calibration curve.



Example 1:

Comparison of the specific activities of lipase and alkaline phosphatase with the aid of p-nitrophenyl butyrate or p-nitrophenyl phosphate

1. purified horseradish peroxidase 1050 $\mu\text{mol}/\text{min} \cdot \text{mg}$
(ABTS, pH = 4.5, 23°C)
2. purified alkaline phosphatase 900 $\mu\text{mol}/\text{min} \cdot \text{mg}$
(p-nitrophenyl phosphate, pH = 8.5, 23°C)
3. crude preparation of a lipase from *Candida rugosa*
2700 $\mu\text{mol}/\text{min} \cdot \text{mg}$ (p-nitrophenyl butyrate,
pH = 7.0, 23°C)

The activity was related to the total protein concentration of the enzymatic crude preparation.

In the case of purified lipase isoenzymes the specific activity is expected to significantly increase.

Example 2:

Determination and comparison of the pH optima of lipase, horseradish peroxidase and alkaline phosphatase

Lipase activity was determined at different pH values with the aid of the hydroquinone assay. The enzyme lipase has a very broad pH optimum (between pH 3 - 8) and is thus suitable for linked enzyme assays (e.g. with



peoxidase). Alkaline phosphatase is active at pH values of more than 7, horseradish peroxidase has a pH optimum of about pH = 5 when using ABTS as the substrate (Fig. 4).

Example 3:

Stability of lipase, horseradish peroxidase and alkaline phosphatase towards EDTA

All the above-mentioned enzymes were incubated at 4°C in the presence of 50 mM EDTA. All measured enzyme concentrations were adjusted to a protein content of 20 nmol.

- Horseradish peroxidase was determined using 0.1 M phosphate buffer, pH 7.5, containing 0.05 % BSA.
- Alkaline phosphatase was diluted with 50 mM TRIS buffer, pH 7.6, containing 1 mM MgCl₂ and 0.1 mM ZnCl₂, 0.05 % BSA.
- Lipase was diluted with 0.1 M acetate buffer, pH 5.0 containing 0.05 % BSA.

Table 1

	t = 0 min	t = 30 min	t = 16 h
Lipase	100 %	100 %	100 %
Alkaline phosphatase	100 %	< 14 %	< 4 %
Peroxidase	100 %	100 %	100 %



Example 4:

Thermostability of lipase, horseradish peroxidase and alkaline phosphatase at 50°C

The thermostability of the above-mentioned enzymes was compared at a protein concentration of 0.2 mg/ml by incubation at 50°C during a time period of 120 min.

- Horseradish peroxidase was incubated in 0.1 M phosphate buffer, pH 7.5.
- Alkaline phosphatase was incubated in 50 mM TRIS, 1 mM MgCl₂ and 0.1 mM ZnCl₂, pH 7.6.
- Lipase was diluted with 0.1 M acetate buffer, pH 5.0 containing 0.5 % BSA.

Table 2

	t=0 min	t=30 min	t=90 min	t=120 min
Lipase	100 %	100 %	100 %	89 %
Alkaline phosphatase	100 %	100 %	96 %	87 %
Peroxidase	100 %	90 %	82 %	75 %



Example 5:

Stability of lipase, horseradish peroxidase (HRP) and alkaline phosphatase in the presence of azide ions

All the above-mentioned enzymes were incubated in the presence of 0.05 % sodium azide at 25°C and 4°C. In order to compare these enzymes with one another, equivalent molar protein concentrations of 20 nmol/ml were used.

- Horseradish peroxidase (HRP) was diluted with 0.1 M phosphate buffer, 0.5 % BSA, pH 7.5.
- Alkaline phosphatase was diluted with 50 mM TRIS, 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, 0.05 % BSA, pH 7.6.
- Lipase was diluted with 0.1 M acetate buffer, 0.05 % BSA, pH 5.0.

Azides can be used to preserve all the above-mentioned enzymes. With regard to alkaline phosphatase a slight decrease in the enzymatic activity (10 - 20 % per day) was observed. In addition azide leads to a lowering of the peroxidase activity by inactivating peroxidase (HRP) in the presence of hydrogen peroxide (during the assay).



Example 6:

Stability of lipase, horseradish peroxidase and alkaline phosphatase in the presence of 7 M urea solutions

All the above-mentioned enzymes were incubated with 7 M urea at 25°C and 4°C. In order to compare these enzymes, protein concentrations of 20 nmol/ml were used.

- Peroxidase (HRP) was diluted with 0.1 M phosphate buffer (pH 7.5), 0.05 % BSA.
- Alkaline phosphatase was diluted with 50 mM TRIS (pH 7.6), 1 mM MgCl₂ and 0.1 mM ZnCl₂, 0.05 % BSA.
- Lipase was diluted with 0.1 M acetate buffer (pH 5.0), 0.05 % BSA.

At 4°C, 7 M urea solution only leads to a slight denaturation of all the above-mentioned enzymes (10 % decrease in the activity per day). The activity of these enzymes decreases by 50 % at 25°C.

Example 7:

Introduction of sulfhydryl groups (SH) in lipase (for coupling to reporter molecules containing maleimide or iodoacetate groups)

The introduction of thiol groups in lipase was carried out in 0.1 M EDTA, 0.1 M phosphate buffer, pH 7.5 at a final protein concentration of 3 mg/ml using a ten-fold



excess of 2-iminothiolane.

After incubating for 20 min at room temperature, excess 2-iminothiolane was separated from modified enzyme by means of ultrafiltration using Centricon 30 tubes. The extent of modification was determined using Ellman's reagent [5,5'-dithio-Bis-(2-nitrobenzoic acid)]. The protein modified with SH groups was stored at 4°C in 0.1 M EDTA, 0.1 M acetate buffer, pH 5.

Example 8:

Introduction of maleimide residues into lipase by means of heterobifunctional cross-linkers (for coupling to reporter molecules containing thiol groups)

(Sulfo)succinimidyl-4-(N-maleimidomethylcyclohexane); (PIERCE 22322X) m-maleimidobenzoyl-N-hydroxysuccinimide ester; (PIERCE 22312X) (sulfo)succinimidyl-4-(p-maleimidophenyl)butyrate; (PIERCE 22317X) (sulfo)-succinimidyl-(4-iodoacetyl)aminobenzoate; (PIERCE 22327X)

The above-mentioned and commercially available heterobifunctional cross-linkers which have at least two different reactive groups, allow sequential conjugations and minimize an undesired polymerization or self conjugation. These cross linkers were tested as follows:

Lipase was modified with the above-mentioned cross linkers in 50 mM borate buffer, 0.1 M EDTA, pH = 7, using a 50 molar excess of heterobifunctional cross linker. The protein concentration of the lipase fraction was determined by the Bradford test and was 3 mg/ml. The



lipase showed no significant loss of enzyme activity.

Example 9:

Coupling of lipase and polyethylene glycol

Principle:

The carboxyl groups of lipase were modified in the presence of polyethylene glycol, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS).

Description of the method:

Modified lipase with isoelectric points $pI = 5.5$ to $pI = 7.0$

A stock solution of 0,0'-Bis-(2-aminopropyl)polyethylene glycol 1900 (M.W: 2000) (DiaminoPEG) was prepared at a concentration of 0.59 g/ml in water and adjusted to pH = 4.6 with dilute hydrochloric acid.

A stock solution of EDC/NHS was prepared by mixing an aqueous solution of EDC with a solution of NHS in dimethylformamide (DMF) in a molar ratio of 15:1. This stock solution was used immediately.

Lipase (384 μ g; 6.7 nmol) was mixed with a solution of DiaminoPEG (12.2 mg, 6.4 μ mol). Afterwards a mixture of EDC/NHS (EDC: 0.15 mg, 780 nmol; NHS: 0.006 mg, 52 nmol) was added and the mixture was incubated for 16 hours at 4°C in the dark (the pH of the reaction mixture was 6.8). The final protein concentration was 5 mg/ml. The reaction was stopped with 500 mM acetate buffer, pH 5 and the excess DiaminoPEG was separated by means of



electrodialysis against 50 mM acetate buffer, pH 5.0. The reaction products were analysed on a native polyacrylamide gel and detected by staining with indolyl acetate/nitro blue tetrazolium salt (NBT).

Modified lipase with isoelectric points of pI = 7 to pI = 9

A stock solution of 0,0'-Bis-(2-aminopropyl)polyethylene glycol 1900 (M.W. 2000) (DiaminoPEG) was prepared at a concentration of 0.59 g/ml in water and adjusted to pH = 4.6 using dilute hydrochloric acid.

A stock solution of EDC/NHS was prepared by mixing an aqueous solution of EDC with a solution of NHS in dimethyl formamide (DMF) in a molar ratio of 15:1. This stock solution was used immediately.

Lipase (384 μ g; 6.7 nmol) was mixed with a solution of DiaminoPEG (12.2 mg, 6.4 μ mol). Afterwards a mixture of EDC/NHS (EDC: 0.61 mg, 3.2 μ mol; NHS: 0.024 mg, 0.21 μ mol) was added and the mixture was incubated for 16 hours at 4°C in the dark (the pH of the reaction mixture was 6.8). The final protein concentration was 5 mg/ml. The reaction was stopped with 500 mM acetate buffer, pH 5 and the excess DiaminoPEG was separated by means of electrodialysis against 50 mM acetate buffer, pH 5.0. The reaction products were analysed on a native polyacrylamide gel and detected by staining with indolyl acetate/nitro blue tetrazolium salt (NBT).

Modified lipase with isoelectric points of pI = 7 to pI = 9

A stock solution of 0,0'-Bis-(2-aminopropyl)polyethylene glycol 1900 (M.W. 2000) (DiaminoPEG) was prepared at a



concentration of 0.59 g/ml in water and adjusted to pH = 4.6 with dilute hydrochloric acid.

A stock solution of EDC/NHS was prepared by mixing an aqueous solution of EDC with a solution of NHS in dimethyl formamide (DMF) in a molar ratio of 15:1. This stock solution was used immediately.

Lipase (384 μ g; 6.7 nmol) was mixed with a solution of DiaminoPEG (12.2 mg, 6.4 μ mol). Afterwards a mixture of EDC/NHS (EDC: 0.61 mg, 3.2 μ mol; NHS: 0.024 mg, 0.21 μ mol) was added and the mixture was incubated for 16 hours at 4°C in the dark (the pH of the reaction mixture was 6.8). The final protein concentration was 5 mg/ml. The reaction was stopped with 500 mM acetate buffer, pH 5 and the excess DiaminoPEG was separated by means of electrodialysis against 50 mM acetate buffer, pH 5.0. The reaction products were analysed on a native polyacrylamide gel and detected by staining with indolyl acetate/nitro blue tetrazolium salt (NBT).

Example 10:

Coupling lipase to deoxyoligonucleotides

Modification of lipase (modified with polyethylene glycol) using SMCC:

PEG lipase was modified in 20 mM phosphate buffer, 150 mM NaCl, 1 mM EDTA, pH 7.4 at a final protein concentration of 10 mg/ml using a 20-fold molar excess of SMCC (with regard to five amino groups to be modified). After incubating for 30 min at room temperature and in the dark, excess cross-linker was separated on a Sephadex-10 column which had previously



been equilibrated with 20 mM phosphate buffer + 2 mM EDTA (pH 6.5).

The desired fractions having lipase activity were concentrated with the aid of Centricon-30 tubes at a room temperature of 4°C and subsequently lyophilized. The modified lipase was stored at -20°C and used directly for coupling to oligonucleotides.

Preparation of SH-oligonucleotides:

The oligonucleotides were synthesized using the phosphoramidite method. The terminal sulfhydryl group at the 5' end was introduced during the last synthesis step using C6-thiol modifiers. After purifying on reverse phase HPLC (0.1 M triethylammonium acetate pH 7 and methanol as the mobile phase), the trityl group was cleaved off using silver nitrate and it was stored at -20°C in DTT [working instructions by Clontech].

Coupling SMCC lipase and deoxyoligonucleotides:

5 µg of trityl-free oligonucleotide in a volume of 50 µl was desalted on a Sephadex G-25 column and directly mixed with the lyophilized enzyme.

After the enzyme had dissolved, the reaction was firstly carried out for one hour at room temperature and subsequently for 16 hours at 4°C to complete the reaction.



Example 11:

Immobilizing lipase and antigen on metal colloids

The synthesis of colloidal gold was carried out according to Frens, G. et al., [Nature Lond. Phys. Sci. 241, 20 (1973)]. Lipase (10 μ l, protein concentration of 1 mg/ml) in 0.1 M phosphate buffer and 6 μ l viral antigen (0.8 mg/ml) were mixed with one millilitre of colloidal gold solution and incubated for 30 minutes at room temperature.

Unbound proteins were removed by a 15 min. centrifugation at 1600 g. The supernatant was removed by pipette and the precipitate was dissolved in 1000 μ l 10 % polyethylene glycol 20000 (PEG 20000) (Polyscience). Each step in the dissolution required 50 μ l 10 % SDS and the use of ultrasound for several minutes (Bandelin SONOREX Sper RK510II). This procedure was repeated three times.

After the last centrifugation the precipitate was dissolved in 50 μ l 1 % PEG 20000 and 200 μ l PBST-3 % BSA. The supernatants and the dissolved precipitates were examined for lipase activity and antigen activity. Only the first supernatant exhibited lipase activity and thus represents unbound proteins. No enzyme activity could be found in the supernatants after the other centrifugation steps. In contrast the dissolved precipitates exhibited lipase activity, as expected.



Example 12:

Comparison of a dye precipitation assay for lipase and alkaline phosphatase

Precipitation assay with lipase:

Substrate stock solution: 50 mg indolyl acetate in 1 ml 100 % dimethylformamide (DMF); stock solution of nitro blue tetrazolium chloride (NBT); 50 mg NBT dissolved in 1 ml 70 % dimethylformamide. The substrate solution was prepared by mixing 66 μ l NBT stock solution in 10 ml 0.1 M phosphate buffer pH 6.5. The mixture was used immediately.

Precipitation assay with alkaline phosphatase:

Substrate stock solution: 50 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) dissolved in 1 ml 100 % dimethylformamide (DMF); NBT stock solution: 50 mg NBT dissolved in 1 ml 70 % dimethylformamide (DMF). The substrate solution was prepared by mixing 33 μ l substrate stock solution and 66 μ l NBT stock solution in 10 ml 0.1 M substrate buffer (0.1 M TRIS, 0.1 M NaCl, 50 mM $MgCl_2$, pH 8.5). The mixture was used immediately.

In any case better the resolution is improved at high concentrations due to the low solubility of the lipase reaction product (Fig. 5).

The lipase which was used in this case was not optimally purified. Therefore a better detection limit can be achieved using a pre-purified and pretreated lipase.



- C l a i m s -

1. Enzyme-labelled probe or test substance, wherein the enzyme is a lipase from plants, a corresponding isoenzyme or a structural derivative having an amino acid homology of at least 70 % and lipase activity.
2. Enzyme-labelled probe or test substance as claimed in claim 1, wherein the enzyme having lipase activity is obtainable from *Candida rugosa* DSM 2031.
3. Enzyme-labelled probe or test substance as claimed in claim 1 or 2, wherein the lipase is conjugated with biotin, avidin, lecithin, protein A, protein G, antibody-binding proteins, antibodies, antigens, viral protein antigens, bacterial surface proteins, digoxigenin, DNA, RNA, oligonucleotides or synthetic analogues, metal colloids or plastic microparticles ($< 5 \mu\text{m}$).
4. Use of lipase from plants, isoenzymes or structural analogues thereof having an amino acid homology of at least 70 % and lipase activity for the production of analytical probes or test substances, wherein the lipase is used ^{as a label} conjugated with bio-recognitive groups.
5. Use as claimed in claim 4, wherein the lipase is obtainable from *Candida rugosa* DSM 2031.



6. Use as claimed in claim 4 or 5 for the production of test substances for bioassays, test strips, biosensors or gene probes.

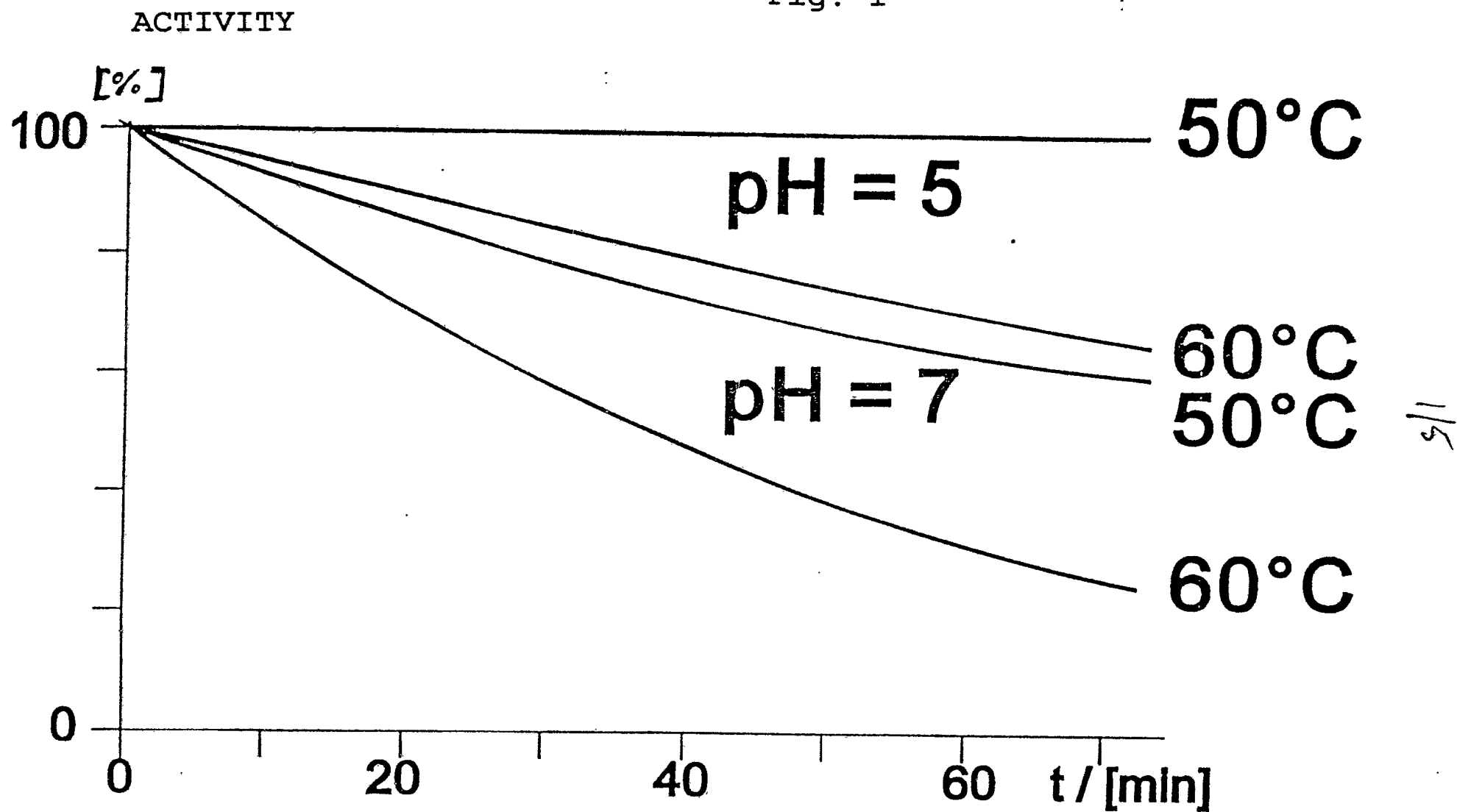


Abstract

In order to improve the thermal and chemical stability of an enzyme-labelled probe, lipase which can preferably be obtained from *Candida rugosa*, isoenzymes or structural analogues thereof having an amino acid homology of at least 70 % and lipase activity is used as the enzyme.



Fig. 1



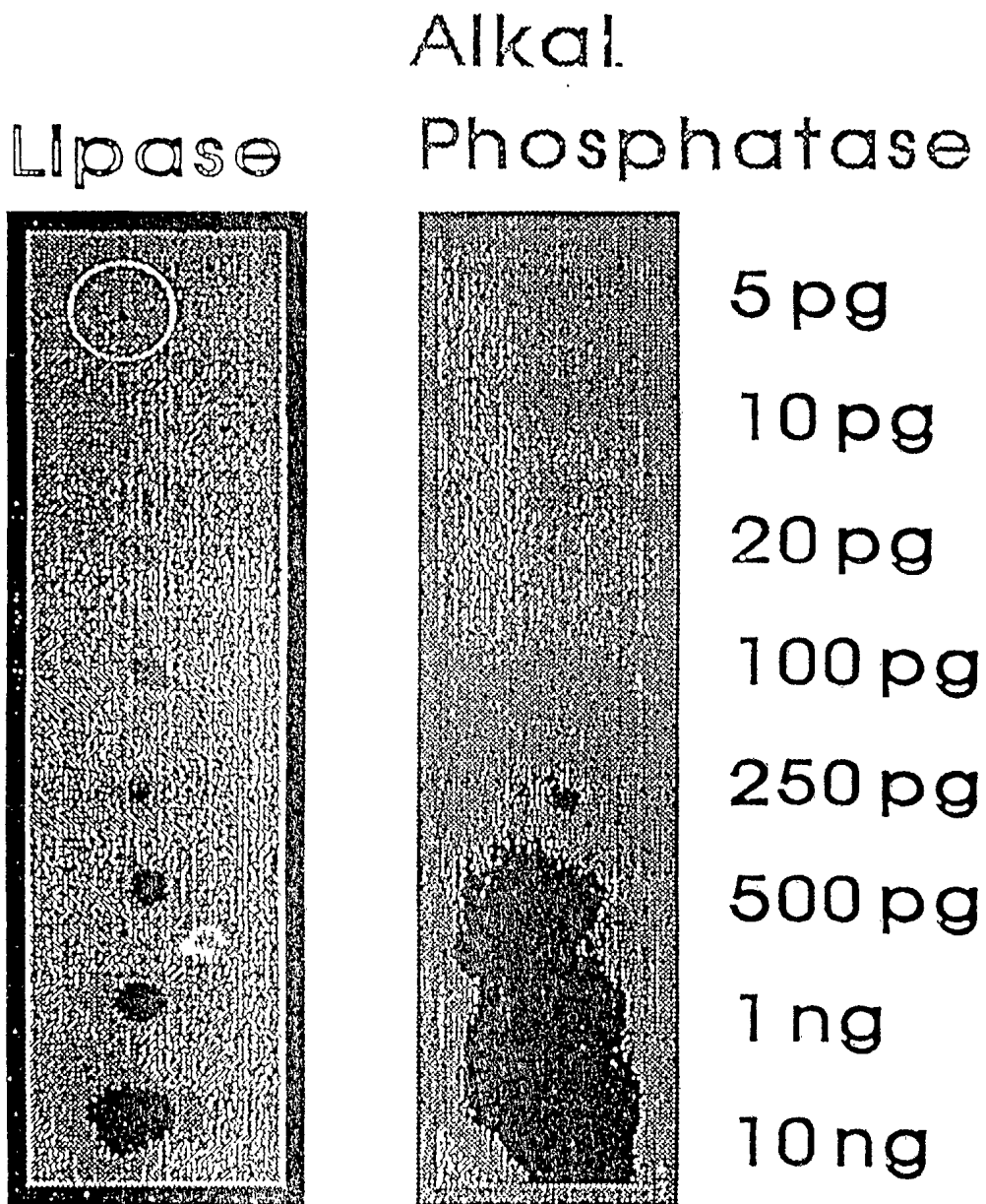


Fig. 3

DNA
Biotin
Avidin

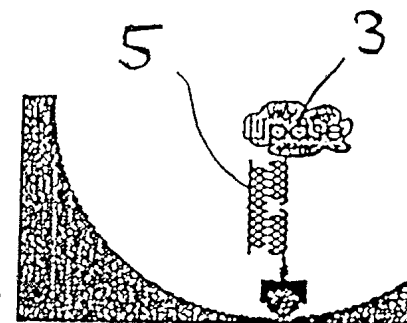
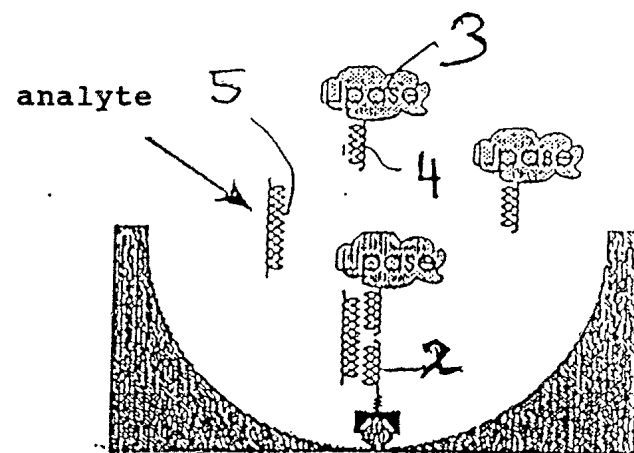
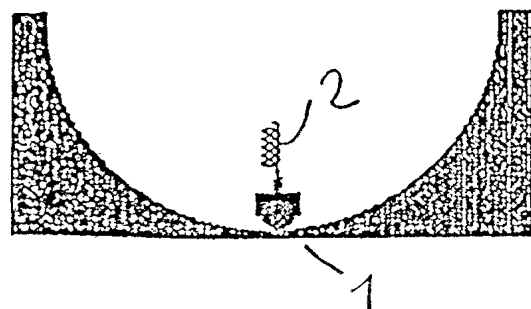
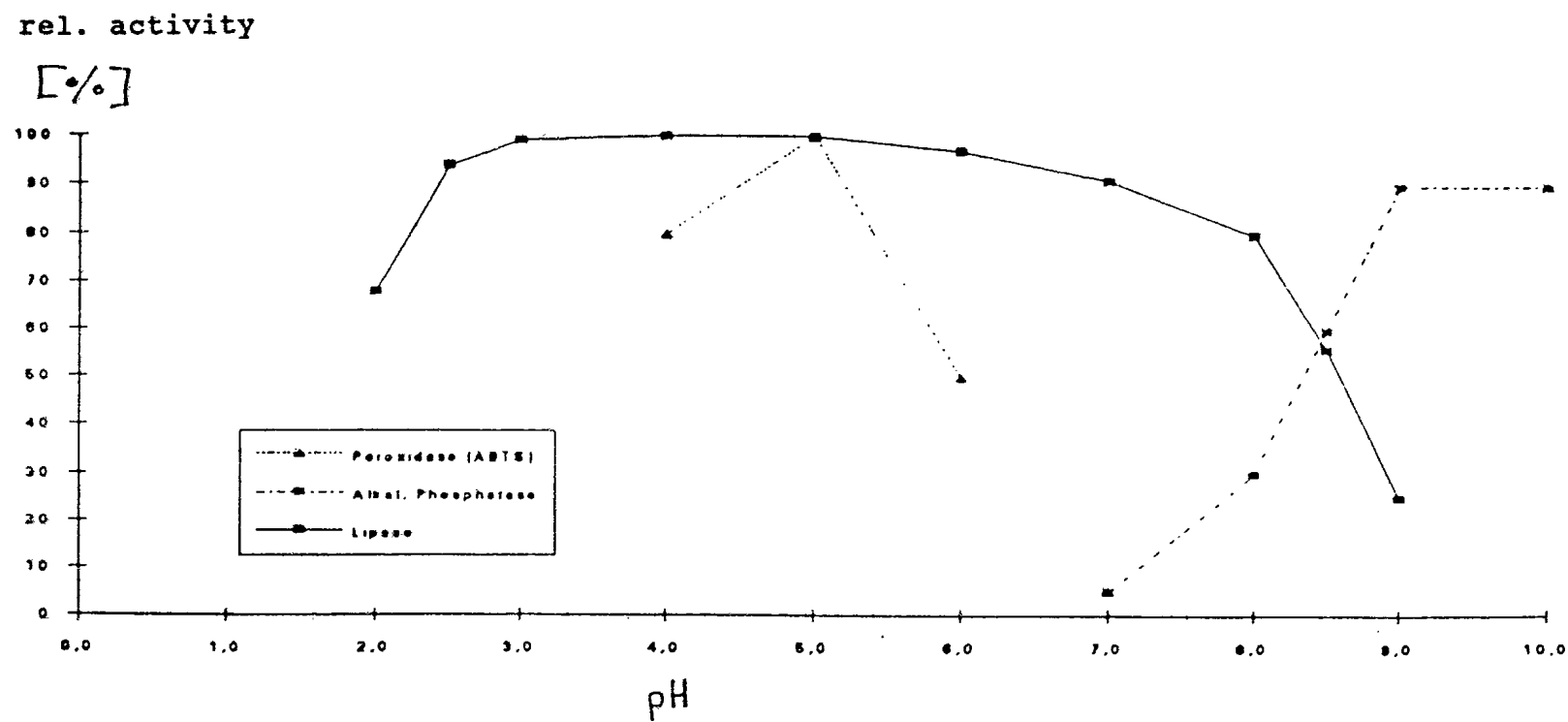


Fig. 4

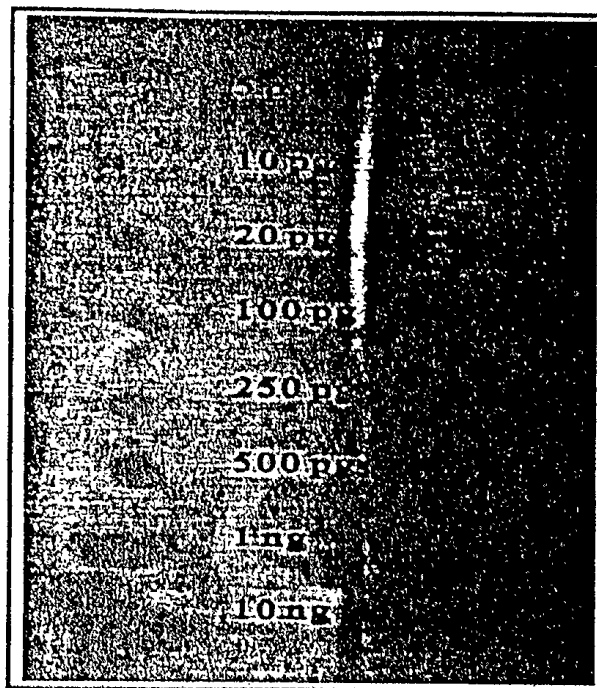


4/5

5/5

Fig. 5

Lipase Alkal.
 Phosphatase



INTERNATIONAL SEARCH REPORT

 Intern al Application No
 PCT/EP 94/03379

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 6	G01N33/535	G01N33/58 C12Q1/68 C12Q1/34
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 6 G01N C12Q C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,92 21778 (LIFE TECHNOLOGIES, INC.) 10 December 1992 see page 3, line 16 - line 32 see page 8, line 4 - page 9, line 31 ---	1-6
Y	GENE, vol.124, no.1, 14 February 1993, AMSTERDAM NL pages 45 - 55 MARINA LOTTI ET AL. 'Cloning and analysis of Candida cylindracea lipase sequences' cited in the application see the whole document --- -/--	1-6
☒ Further documents are listed in the continuation of box C. ☒ Patent family members are listed in annex.		
* Special categories of cited documents : 'A' document defining the general state of the art which is not considered to be of particular relevance 'E' earlier document but published on or after the international filing date 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) 'O' document referring to an oral disclosure, use, exhibition or other means 'P' document published prior to the international filing date but later than the priority date claimed 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. '&' document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
22 February 1995		13.03.95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (- 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (- 31-70) 340-3016		Authorized officer Döpfer, K-P

INTERNATIONAL SEARCH REPORT

Intern. d. Application No.

PCT/EP 94/03379

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, vol.54, no.5, May 1990, TOKYO JP pages 1253 - 1258 TARO IIZUMI; KOICHI NAKAMURA AND TETSURO FUKASE 'Purification of a Thermostable Lipase from Newly Isolated Pseudomonas sp. KWI-56' see the whole document in particular page 1257, right-hand column, lines 28-44	1,3,5,6
Y	EP,A,0 384 717 (MICHIGAN BIOTECHNOLOGY INSTITUTE) 29 August 1990 see page 2, line 1 - line 11 ---	1,3,4,6
Y	WO,A,86 03774 (CHR. HANSEN'S LABORATORIUM A/S) 3 July 1986 see claims 33-37 -----	1,3,4,6

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES

IPK 6 G01N33/535 G01N33/58 C12Q1/68 C12Q1/34

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierte Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

IPK 6 G01N C12Q C12N

Recherchierte aber nicht zum Mindestprüfstoff gehorende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
Y	WO,A,92 21778 (LIFE TECHNOLOGIES, INC.) 10. Dezember 1992 siehe Seite 3, Zeile 16 - Zeile 32 siehe Seite 8, Zeile 4 - Seite 9, Zeile 31 ---	1-6
Y	GENE, Bd.124, Nr.1, 14. Februar 1993, AMSTERDAM NL Seiten 45 - 55 MARINA LOTTI ET AL. 'Cloning and analysis of Candida cylindracea lipase sequences' in der Anmeldung erwähnt siehe das ganze Dokument --- -/--	1-6

☒ Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen☒ Siehe Anhang Patentfamilie

* Besondere Kategorien von angegebenen Veröffentlichungen :

A Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

E älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

L Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

O Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

P Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

T Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

X Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderscher Tätigkeit beruhend betrachtet werden

Y Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderscher Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

Z Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche

22. Februar 1995

Absenddatum des internationalen Recherchenberichts

13.03.95

Name und Postanschrift der Internationalen Recherchenbehörde
Europäisches Patentamt, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Bevollmächtigter Bediensteter

Döpfer, K-P

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.

PCT/EP 94/03379

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9221778	10-12-92	US-A- 5248618	28-09-93

EP-A-0384717	29-08-90	US-A- 5093256	03-03-92
		JP-A- 2273178	07-11-90
		US-A- 5166069	24-11-92

WO-A-8603774	03-07-86	DE-A- 3585624	16-04-92
		EP-A, B 0205506	30-12-86

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
Y	AGRICULTURAL AND BIOLOGICAL CHEMISTRY, Bd.54, Nr.5, Mai 1990, TOKYO JP Seiten 1253 - 1258 TARO IIZUMI; KOICHI NAKAMURA AND TETSURO FUKASE 'Purification of a Thermostable Lipase from Newly Isolated Pseudomonas sp. KWI-56' siehe das ganze Dokument siehe insbesondere Seite 1257, rechte Spalte, Zeilen 28-44 ---	1,3,5,6
Y	EP,A,0 384 717 (MICHIGAN BIOTECHNOLOGY INSTITUTE) 29. August 1990 siehe Seite 2, Zeile 1 - Zeile 11 ---	1,3,4,6
Y	WO,A,86 03774 (CHR. HANSEN'S LABORATORIUM A/S) 3. Juli 1986 siehe Ansprüche 33-37 -----	1,3,4,6

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichung, die zur selben Patentfamilie gehören

Internationales Aktenzeichen

PCT/EP 94/03379

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
WO-A-9221778	10-12-92	US-A- 5248618	28-09-93
EP-A-0384717	29-08-90	US-A- 5093256	03-03-92
		JP-A- 2273178	07-11-90
		US-A- 5166069	24-11-92
WO-A-8603774	03-07-86	DE-A- 3585624	16-04-92
		EP-A, B 0205506	30-12-86