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(54) Title: A PROCESS OF ISOLATION AND UTILIZATION OF RICE BRAN LIPASE

(57) Abstract: Disclosed herein is a novel isolated from nomocotyledon plant sources such as rice bran having the following properties: a) isolated enzyme is a glycoprotein; b) isolated enzyme is stable and catalytically active at high temperatures; c) isolated enzyme is stable and catalytically active at alkaline pH; d) isolated enzyme has molecular weight range of 9 to 12-kDa; e) isolated enzyme is inhibited by inhibitors of serine proteases such as Diisopropylfluorophosphate; f) Isolated enzyme that can utilize both triacylglycerol and phospholipids. This invention also relates to a process of purifying the lipase described above using the following steps: a) delipidation of the plant extract using organic solvents; b) Filtration of the delipidated extract on a suitable matrix; c) Purification of the enzyme using hydrophobic column matrices; d) characterization of the purified product by known methods.

A PROCESS OF ISOLATION AND UTILIZATION OF RICE BRAN LIPASE

This invention relates to a process for the isolation and utilization of Rice Bran Lipase.

Rice bran proteins were solubilized, and then the lipase was purified to homogeneity and characterized enzymatically and biophysically. The purified enzyme was found to be a glycoprotein of 9 to 12-kDa. Enzyme showed maximum activity at 80 "C and in the pH range of 10-1 1. The protein was found to be stable and biologically active at temperatures as high as 90 °C, as judged by the far- and near-W circular dichroism spectroscopy and enzymatic assays, respectively. Differential scanning calorimetric studies indicate that the transition temperature was 76 °C and ΔH was 1.3x105 Cal/mol at *Tm* 76 "C. The purified enzyme exhibited dual substrate specificity including phospholipase A2 and lipase activities. *Tm* for triolein was higher than that for phosphatidylcholine. Activity analysis on reverse-phase HPLC, isoelectric focussing and photoaffinity labeling of the purified protein with photoanalogues of triolein and phosphatidylcholine demonstrated that a single enzyme catalyzes the hydrolysis of both the substrates. The enzyme preferred hydrolysis at *sn*-2 position of phosphatidylcholine whereas it apparently exhibited no positional specificity towards triacylglycerol. Diisopropyl fluorophosphate, an irreversible inhibitor of serine esterase and lipase, inhibited lipase and phospholipase activities of the purified enzyme. Substrate competition, immunoinhibition and catalytic residue modification studies provide evidences that the enzyme contains a common catalytic site and independent binding sites for neutral lipid and phospholipid substrates.

PRIOR ART

Lipases have been purified from animal, plant and microbial sources. These enzymes catalyze ester hydrolysis in aqueous media and a reverse reaction i.e esterification under non-aqueous phase. Enzymatic skills of lipases have been exploited in various industries.

Microbial lipases have been preferred for commercial applications due to their multifold properties and unlimited supply. The interesting properties for which these lipases are used are their thermal and pH stability, activity in organic solvents. Extracellular lipase of A. *niger, Chromobacterium viscosum* and *Rhizopus* sp. are active at acidic pH. *An* alkaline lipase active at pH 11.0 has been isolated from *P. nitroreducens*. Lipases from *A. niger, R. japonicus*, and C. *viscosum* are stable at 50 °C, and lipases of thermotolerant *H. lanuginosa* and *P. sp. nitroreducens* are stable at 60 °C and 70 °C, respectively (Liu et al., 1973) The chief producers of commercial lipases are *Aspergillus niger, Candida cylinderacea, Humicola lanuginosa, Mucor miehei, Rhizopus arrhizus, R delemar, R Japonicus, R. niveus and R. oryzae* (Godfredson et al., 1990).

In a dairy industry, lipases are used for cheese ripening, production of the so-called enzyme-modified cheese (EMC) and lipolysis of fat in milk, butter and cream (Falch et al., 1991). Addition of lipases release short-chain fatty acids that leads to the development of sharp and tangy flavor whereas release of medium-chain fatty acids initiate the synthesis of flavor ingredients such as methyl ketones, flavor esters, aceto-acetate etc.

Using a range of immobilized lipases for hydrolysis, alcoholysis and glycerolysis have been carried out in the oleo chemical industry as it saves energy and avoids a substantial investment in expensive equipment as well as in expenditure of large amounts of thermal energy (Arbige et. al., 1989; **Hoq** et al., 1985).

Demand for lipases in the detergent industry has increased since the world-wide trend is towards lower laundering temperatures. Novo Nordisk'e lipolase (*Humicola* lipase expressed in *Aspergillus oryzae*) is one of the present formulations (Hoq et al., 1985).

Regioselective and thermally stable lipases have been used for enzymatic synthesis of surfactants which are widely used as industrial detergents and as emulsifiers in food formulations like low-fat spreads, sauces, ice-creams, mayonnaises, etc. (Adelhorst et al., 1990) have carried out esterification of alkyl-glycosides using molten fatty acids and immobilized *Candida antarctica* lipase. **A** biosurfactant has been synthesized by transesterification of sugar alcohols with natural oils, using lipase from *A. terreus* (Yadav et al., 1997). Lipases have also been implicated for the synthesis of amphoteric bio-degradable surfactants (Hills et al., 1990; Kloosterman et al., 1988).

Specificities of various lipases have been useful in the synthesis of lipids with high commercial value. **A** typical example of such a high-value asymmetric triglyceride mixture is cocoa butter (Jandacek et al., **1987).** Same approach has been used for the synthesis of many other triglycerides (Soumanou et al., 1997), preparation of nutritionally important products which generally contain medium-chain fatty acids (Eibl et al., 1990),

modification of oils rich in high-value polyunsaturated fatty acids (Fregapane et al., 1991).

Stereoselectivity of the lipases has been exploited for the synthesis of optically active polymers that are used as absorbents (Margolin et al., 1987). Monomers can be prepared by lipase-catalyzed transesterification of alcohols (Margolin et al., 1991). Another example of the application of lipases in pharmaceuticals and agrochemicals is the resolution of racemic mixtures. Optically pure cardiovascular drug Diltizem is an example of a product obtained using this technology (Bornemann et al., 1989). Lipases from A. *Carneus* and A. *terreus* show chemo- and regiospecificity in the hydrolysis of per-acetates of pharmaceutically important polyphenolic compounds (Parmar et al., 1998). Further, lipases have applications in the synthesis of ingredients for personal care products (Hoq et al., 1985), they have potential in paper manufacture, waste processing of many food industries (West et al., 1987).

SUMMARY OF INVENTION

A novel lipase has been isolated from monocotyledon plant sources e.g. rice bran that has the following properties:

The novel lipase is a glycoprotein of size 9-12-kDa that is stable and catalytically active at 80°C. The enzyme can hydrolyze both triacylglycerol and phospholipid, and the enzyme activity is inhibitable by serine protease inhibitors such as diisopropyl fluoraphospate.

The novel lipase isolated fi-om rice bran has been purifed using the following steps:

a) delipdation of the rice bran extract with 10g/100ml of di-ethyl ether and extraction in 10mM Tris-HCl pH 7.5 and 1.0mM EDTA for 12h;

b) filtration of the extract through fine layers of cheese cloth followed by centrifugation at 3,000Xg for 30 min;

c) purification of the enzyme on octyl-Sepharose pre-equilibrated with 0.01 M Tris-HCl pH7.5 and eluting the pure protein with a linear gradient of 0-40% methanol, and

d) characterization of the purified protein by known methods.

The purification of such a lipase from rice bran is not restricted to this method only. Based on known and established principles of protein purification and the description provided in this specification it is possible for any skilled in the art to device alternative methods of purification of such lipases.

Novel lipase obtained from rice bran as described in this specification can be used in a variety of industrial processes as in dairy industry, oleo chemical industry, detergent industry, synthesis of surfactants, synthesis of nutritionally important lipid products, synthesis of optically active polymers, resolution of racemic mixtures and synthesis of ingredients for personal care products and generate either novel or known commercially important products.

DETAILED DESCRIPTION OF INVENTION

Interesting properties of rice bran lipase include its stability to temperature and alkaline pH. The enzyme does not show positional specificity towards triolein and has a

phospholipase A2 activity. Importantly, the enzyme is extracted from a by-product and its extraction involves a single chromatographic step.

EXAMPLE 1

Rice bran was delipidated with (10 g/100 ml) diethyl ether and stirred for 12 h in 10 mM Tris-HCl, pH 7.5, and 1.0 mM EDTA. The extract was passed through layers of cheesecloth and centrifuged at 3,000 x g for 30 min. The clear supernatant was then loaded onto an octyl-Sepharose column that had been pre-equilibrated with 0.01 M Tris-HCl, pH 7.5 at a flow rate of 2 ml/min. The column was washed with the same buffer until the effluent showed a negligible absorbance at 280 nm. The enzyme was eluted with a linear gradient of 0-40 % methanol and fractions of 10 ml were collected. Upon analysis of the fractions on SDS-PAGE, a single protein band was observed in the later fractions, no protein was obtained in the initial ones. The overall purification was 6.8-fold, with an activity yield of 20 %. Protein concentration was determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as standard. Two-dimensional gel electrophoresis and analytical reverse phase HPLC confirmed purity. Protein was analyzed on two-dimensional gel using a Bio-Rad mini-protean II two-dimensional gel apparatus. The first dimension of the gel was run in the acidic direction using ampholytes with pH range of 3.0-10.0. The run was performed at 500 V for 3 h. The second dimension was 15% acrylamide SDS-gel as described by Laemmli (1976) and the protein was visualized by silver staining. Purified lipase was resuspended in 100 µl of water plus 0.1% trifluoroacetic acid (HPLC grade) and loaded onto a C18 reverse phase column (Vydac reversed phase C18 column, 10-µm particle size, 22-mm

inner diameter, 25-cm length). Prior to loading, the column was pre-equilibrated with water plus 0.1% trifluoroacetic acid. Peptides were eluted from the column using a linear gradient of 0-70% acetonitrile (HPLC grade) plus 0.1% trifluoroacetic acid and a flow rate of 1.0 ml/min. The elution profile was monitored by absorbance at 210 nm. Fractions were collected in lmin intervals and each fraction was evaluated for the presence of enzyme activity after dialyzing the sample. Analysis of the purified protein on the SDS-PAGE showed a band of apparent molecular weight of 10-kDa. Samples were electrophoresed **using** the Laemmli discontinuous buffer system (Laemmli et al., 1976) on 15% SDS-PAGE gels (10 x 10 cm) at 100 V and stained with silver (Nesterenko et al., 1994). Molecular weight was further confirmed by MALDI-TOF and was found to be 9.4 kDa. Lipase purification to apparent homogeneity was achieved in a single chromatographic step.

EXAMPLE 2

Purified lipase was concentrated **using** a Centricon (5-kDa cut-off) and applied onto an analytical Superdex 75 FPLC column fitted with Bio-Rad Biologic low-pressure chromatography system with a buffer consisting of 0.01 M Tris-HCl, pH 7.5 containing 100 mM sodium chloride. Elution was carried out with the same buffer at a flow rate of 1.0 ml/min. Fractions were collected in 1 min intervals. Standard markers were from Amersham Pharmacia Biotech that consisted of blue dextran (2000 kDa), bovine serum albumin (67 kDa), ovalbumin **(43** kDa) carbonic anhydrase (29 kDa) and lysozyme (14 kDa). Two peaks, a void volume and **an** included were obtained and the activity was found to be associated with both the peaks and when run on the SDS-PAGE, the void

volume as well as the included peaks run at the same place, indicating the void volume peak was observed because the enzyme formed large aggregates. Protein showed anomalous behaviour on the SDS-PAGE which is a characteristic of glycosylated proteins therefore we analysed if the protein was post-translationally modified by glycosylation. For carbohydrate analysis by periodic acid-Schiff reagent, purified protein was resolved on SDS-PAGE under reducing conditions. Gel fixed with trichloroacetic acid was treated for 50 min with 1% periodic acid in 3% acetic acid, and washed with several changes of distilled water. The oxidized samples were stained for 50 min in the dark with fresh Schiff's reagent, treated for 30 min with 0.5% sodium meta-bisulfite, washed and stored in the dark (Warren et al., 1958). Concurrent samples were stained for protein.

EXAMPLE 3

Enzyme activity was measured using 3H-labeled triolein, labeled at all the fatty acid chains. Substrate was emulsified with 1 % *gum* Arabic, in a total volume of 100 µl of assay buffer (0.01 M Tris-HCl, pH 7.5, unless otherwise mentioned). The mixture was incubated *at* 37 °C (unless mentioned) for 1h before addition of 400 µl of $CHCl_3/CH_3OH$ (1:2, v/v). Lipids were extracted according to the method of Bligh and Dyer (Bligh et al., 1959). The organic phase was dried, lipids were resuspended in chloroform and resolved on thin-layer silica gel plates (Silica Gel 60 F-254, Merck) using petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v). The lipids were visualized with iodine vapour and spots corresponding to the unreacted substrate and the products were scraped off and quantitated by liquid scintillation counting. Purified lipase showed maximum activity at

SO °C. Enzyme was more stable at lower temperatures but did not loose activity completely even at temperatures as high as 80 °C. The optimal pH was between 10-11. To determine the positional specificity towards triacylglycerols, a time course for the release of possible intermediates i.e free fatty acids, diacylglycerol and monoacylglycerol, by the enzyme's action was performed, Upon quantitation of the radioactivity associated with the unreacted substrate and the products, accumulation of fatty acids was observed. Diacylglycerol was formed but did not accumulate. Lipase activity was found to be enhanced marginally by Ca^{2+} whereas other divalent cations like Mg^{2+}, Zn^{2+}, Cu^{2+} showed inhibitory effect.

EXAMPLE 4

Circular Dichroism spectra were recorded on a Jasco J-720 spectrophotometer equipped with a thermostatted cell holder. Spectra were recorded at a protein concentration of 0.5 mg/ml in a 1-cm path length quartz cuvette sealed with a tefflon stopper. A resolution of 0.1 nm and scanning speed of 20 nm/min with a 2 s response time were employed. For monitoring thermal stability, spectra were recorded at 20 °C, 40 °C, 60 °C and 90 "C. Equilibration time of 5 min was included at each temperature interval. In order to check the reversibility, the sample was cooled to 20 °C and rescanned. Spectra revealed a profile characterized by a sharp negative ellipticity at 232 nm. The results show that the enzyme retained more than 90 % of its secondary structure even at 90 °C and there was no change in the near-UV region, indicating conformational stability. Little denaturation observed was found to be reversible upon rescans of the sample after cooling to 20 V.

DSC measurements were performed on a VP- DSC microcalorimeter (Microcal Inc., Northampton, MA). Sample solutions for DSC measurements were prepared by dialyzing the protein against 0.01 M phosphate buffer at pH 7.0 exhaustively. The protein concentrations were 0.07 mg/ml. Samples and reference buffers were degassed by stirring gently under vacuum prior to measurements. Protein unfolding events were recorded between 20 and 90 °C with a scan rate of 90 °C. To check the reversibility of the observed transitions, rescans were performed after slowly cooling to 20 °C. The scans were analyzed after subtraction of an instrument base line recorded with water in both cells using the software ORIGIN from Microcal. The DSC transition corresponding to thermal denaturation was reversible. The denaturation enthalpy ΔH was 1.3x105 Cal/mol at Tm 76 "C.

EXAMPLE 5

For phosphatidylcholine hydrolysis, phospholipid mixed micelles composed of phosphatidylcholine:triton X-100 (1:20, mol/mol) to give about 250,000 cpm/assay. Phosphatidylcholine was 3H-labelled at *sn*-2 position. For analyzing the hydrolysis of other phospholipids, the phosphatidylcholine was replaced with the lipid to be investigated. Assay was performed as described in example 2. Upon enzymatic activity there was a release of free fatty acids and no other intermediates or products were released from phosphatidylcholine. The enzyme did not accept lysophosphatidylcholine or phosphatidic acid as substrates. This confirmed that the enzyme had phospholipase A2 activity. Phospholipase A2 activity was also confirmed by using a 32P-labeled phosphatidylcholine. The phospholipase activity was protein concentration and time

dependent. It did not require calcium for hydrolysis. That both the activities were associated with the same enzyme was confirmed by colocalizing the activities on HPLC and isoelectric focussing gel with a constant specific activity ratio with both the substrates. Further, photoaffinity analogues of triolein and phosphatidylcholine were synthesized. 12-[(4-Azidosalicyl)amino]-dodecanoic acid (ASD) was synthesized from the *N* -hydroxysuccinimide ester of *p*-azidosalicylic acid (Rajasekharan et al., **1993).** 1,2-dipalmitoyl glycerol (lmmol) was then acylated with the synthesized ASD-anhydride (4 mmol) by stirring the mixture for **30** h at room temperature in dry chloroform. *N,N*-dimethyl-4-aminopyridine (0.5 mmol) was used as catalyst. The reaction flask was flushed with nitrogen and sealed. The residue was redissolved in 2 **ml** of chloroform and loaded onto a silicic acid (20 g) column that had been pre-equilibrated with chloroform. The column was washed with chloroform and then eluted with mixtures of chloroform/methanol (1:1, v/v). the purity was checked by TLC using chloroform/methanol/water **(9820.5,** v/v). The yield was approximately **46%.** The purified 1,2-dipalmitoyl,3-(4-azidosalicyl)-12-amino) dodecanoyl-*sn*-glycerol was iodinated using Na125I and chloramine-T (Shin et al., **1985).** The iodinated product was purified using reverse-phase column chromatography. The efficiency of iodination **was 59-63%.** All operations involving azide were carried out under dim safe light. The synthesis of azido-PC was achieved by direct acylation of $CdCl_2$ complex of glycerophosphocholine with ASD-anhydride (Gupta et al., **1977).** The purified product was iodinated as described earlier, The yield was around 52%. These analogues were used to demonstrate a direct specific interaction between the protein and both the

analogues. The photolabeling experiments were carried out in a final volume of 50 µl containing 10 µg of lipase in 0.01 M Tris-HCl, pH 7.5, 0.1 mM 2-mercaptoethanol and the photoprobe, as described earlier. Mixture was preincubated on ice in the dark for 5 min, in a microfuge tube cap and irradiated for 3 min with a hand-held W-lamp with the filter removed (5000 µW/cm2), model WG-54, UV products) at a distance of 8 cm. Radiolabeled photoprobe (0.5 µCi, 0.5 µM) was used in the presence of increasing concentrations of unlabeled triolein or phosphatidylcholiie to analyze the crosslinking on the gel. Crosslinked protein was run on SDS-PAGE. Autoradiography of the gel showed that both the analogues were crosslinked with the lipase and the unlabelled substrate competed With the photoprobe as there was reduction.

EXAMPLE 6

When provided the lipase with radiolabeled phosphatidylcholine as the substrate, unlabeled triolein was able to chase labeled phosphatidylcholine. Radiolabeled substrate was chased more efficiently by phosphatidylcholine as compared to triolein since the *Km* for triolein is higher than for phosphatidylcholine. Moreover, there was a comparable inhibition of both the activities in the presence of serine modifier DIPF. These experiments indicated that both the substrates were competing €or the same catalytic site and the active site contains a serine residue. However, in the immunoinhibition assays, lipase but not the phospholipase activity was inhibited. This could mean that upon its interaction with the antibody, the amphipathic phospholipid but not the neutral substrate is able to interact with the enzyme. Alternatively, in spite of the steric hindrance and/or any conformational changes posed by the antibody, binding site for the triacylglyceride is

masked but not for the phospholipid. This indicates that both have independent binding sites. Therefore, our results provide biochemical evidences to demonstrate that the lipase has a common catalytic site but independent binding sites for the neutral lipids and phospholipids.

EXAMPLE 7

Amino acid composition of the purified rice bran lipase.

Amino acid	MW	pmol	mol%
ASX	115.08	95.4	7.03
GLX	129.11	98.5	7.26
SER	87.07	86.2	6.35
GLY	57.05	188.7	13.9
HIS	137.14	28.6	2.1
ARG	156.18	42.1	3.
THR	101.1	71.4	5.26
ALA	71.07	181.2	13.35
PRO	97.11	71	5.23
TYR	163.17	36	2.65
VAL	99.13	116	8.55
MET	131.19	22.7	1.67
ILE	113.15	54.1	3.99
LEU	113.15	143.6	10.58
PHE	147.17	49.7	3.66
LYS	128.17	72.1	5.31
Cys/2	103.13		0
AIB*	209		
totals:	1357.3	100	

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CLAIMS:

1. A novel lipase isolated from monocotyledon plant sources.

2. A novel lipase as claimed in claim 1 that has the following properties:

   a. isolated enzyme is a glycoprotein;

   b. isolated enzyme is stable and catalytically active at high temperatures;

   c. isolated enzyme is stable and catalytically active at alkaline pH;

   d. isolated enzyme has molecular weight range of 9 to 12-kDa;

   e. isolated enzyme is inhibited by inhibitors of serine proteases such as Diisopropylfluorophosphate; and

   f. isolated enzyme that can utilize both triacylglycerol and phospholipids.

3. A process of purifying the lipase as claimed in claim 1 and 2 using the following steps:

   a. delipidation of the plant extract using organic solvents;

   b. Filtration of the delipidated extract on a suitable matrix;

   c. Purification of the enzyme using hydrophobic column matrices; and

   d. characterization of the purified product by known methods.

4. A novel lipase from rice bran.

5. A novel lipase from rice bran as claimed in claim 4 that has the following properties:

   a. a glycoprotein of size 9 to 12-kDa;

   b. stable and catalytically active at 80°C;

   c. isolated enzyme is stable and catalytically active even at pH 12.0;

d. enzyme activity is inhibitable by serine protease inhibitors such as diisopropyl fluorophospate; and

e. enzyme that can hydrolyze both triacylglycerol and phospholipids,

6. A process of purifying the lipase as claimed in claim 4 using the following steps:

a. delipidation of the plant extract using organic solvents;

b. Filtration of the delipidated extract on a suitable matrix followed by separation of fine insolubles;

c. Purification of the enzyme using hydrophobic column matrices; and

d. characterization of the purified product by known methods;

7, A process of purifying the lipase as claimed in claim 4 using the following steps :

a. delipdation of the rice bran with 10g/100ml of diethyl ether and extraction in 10mM Tris-HCl pH 7.5 and 1.0mM EDTA for 12h;

b. filtration of the extract through fine layers of cheese cloth followed by centrifugation at 3,000Xg for 30 min;

c. purification of the enzyme on octyl-Sepharose pre-equilibrated with 0.01 M Tris-HCl pH 7.5 and eluting the pure protein with a hear gradient of 0-40% methanol; and

d. characterization of the purified protein by known methods.

8. Use of the novel lipase as claimed in claims 1, 2 and 4 in dairy industry, oleo chemical industry, detergent industry, synthesis of surfactants, synthesis of nutritionally important lipid products, synthesis of optically active polymers, resolution of racemic mixtures and synthesis of ingredients for personal care products.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN 02/00126

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: C12N 9/20

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[7]: C12N 9/20

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 practicable, search terms used)

WPI, CAS, Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RAO, K.S. et al. Structural stability of lipase from wheat germ in alkaline pH. Journal of Protein Chemistry, June 1991, Vol. 10, No. 3, pages 291-299, Medline-abstract [online], [retrieved on 30 August 2002 (30.08.02)]. Retrieved from: EPOQUE Medline Database, AN: NLM1910461 *abstract.*	2
A	US 5753283 A (HAMMOND) 19 May 1998 (19.05.98) *claim 5; figures.*	3,6
	EP 0579928 A1 (THE NISSHIN OIL MILLS, LTD.) 25 May 1992 (25.05.92) page 1, lines *1-5; page 5, lines 3-9.*	2,8

☐ Further documents are listed in the continuation of Box C.

☒ See patent family 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 application or patent 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 refemng 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
30 August 2002 (30.08.2002)	13 September 2002 (13.09.2002)
Name and mailing adress of the ISNAT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer MOSSER R. Telephone No. 1/53424/437

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN 02/00126

Box I Observations where certain claims were found unsearchable (Continuation **of** item **1** of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1,4; claims 2 and 3 partly
because they relate to subject matter not required to be searched by this Authority, namely:

Claims **1** and **4** do not contain any technical features. A meaningfullsearch was not possible for these claims. Claims 2 and 3 were partly searchable: Because the scope of the claims 1,2 and 3 is very brad a meaningful search over the whole of the claimed scope of the claims 2 and 3 was impossible. The subject matter of claims 2 and 3 is not fully supported by the description and examples: the examples concern a lipase from rice bran, Consequently, the search has been carried out for those parts which appear to be supported by the description and the examples.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that **no** meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity **of** invention is lacking (Continuation **of** item **2** of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ **As** all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ **As** all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ **As** only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/IN 02/00126-0

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
EP	A1	579928	26-01-1994	US	A	5445955	29-08-1995
				US	A	5569594	29-10-1996
				JP	A2	6038753	15-02-1994
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				US	A	5292537	08-03-1994
				WO	A1	9412038	09-06-1994
				US	A	5376390	27-12-1994
				US	A	5512307	30-04-1996