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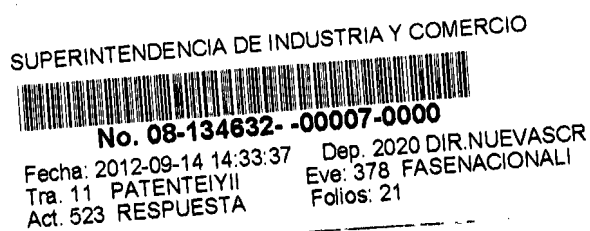
Señor Director

División de Nuevas Creaciones

Superintendencia de Industria y Comercio

Ministerio de Comercio, Industria y Turismo

E. S. D.



Re.: Expediente **No. 08 134632**

Solicitud de Patente de invención para: **“METODO PARA PRODUCIR LIPASA, CELULA DE YARROWIA LIPOLYTICA TRANSFORMADA CAPAZ DE PRODUCIR LA LIPASA”**.

Solicitante: **LABORATOIRES MAYOLY SPINDLER**

RESPUESTA AL EXAMEN DE FONDO

Yo, **Margarita Castellanos Abondano**, identificada como aparece al pie de mi firma, atentamente me permito presentar respuesta al oficio **No.10710** notificado por fijación en lista del 20 de Junio de 2012, estando dentro del término legal, en los siguientes términos:

De la solicitud de patente

Su Despacho concluye que el pliego reivindicatorio de la presente solicitud de patente no cumple con las disposiciones de los artículos 28 y 30 de la Decisión 486. Asimismo, el Examinador sugiere eliminar las reivindicaciones 12 a 14 del pliego reivindicatorio toda vez que las mismas se refieren a usos, los cuales no son patentables en Colombia.

Con el fin de atender las observaciones formuladas a este respecto, y de acuerdo con el artículo 34 de la Decisión 486, se presenta un nuevo capítulo reivindicatorio conformado

por 10 reivindicaciones como sustituto del anteriormente presentado. De tal manera que las objeciones formuladas a este respecto se deben tener por superadas de la siguiente manera:

- La reivindicación 1 ha sido redactada nuevamente incluyendo las características técnicas esenciales de las reivindicaciones 2, 7 y 9. Adicionalmente, la nueva reivindicación 1 ahora especifica que las células usadas en la fermentación son células *Yarrowia lipolytica* transformadas por un vector seleccionado del grupo que consiste de JMP6 y JMP10 expresando la lipasa ácido resistente de levadura LIP2.
- Las reivindicaciones 2, 7, 8, 10, 12 y 14 no han sido incluidas en el nuevo pliego reivindicatorio y se ha corregido la numeración de las reivindicaciones restantes.
- Con respecto a las expresiones "células *Yarrowia lipolytica*", "fuente de carbono de origen lipídico"; "sales inorgánicas", "oligoelementos" y "vitaminas", deseamos poner de presente que las mismas son ampliamente conocidas por una persona versada en la material y por lo tanto, las mismas son claras y precisas.

Así entonces, teniendo en cuenta las modificaciones efectuadas al capítulo reivindicatorio, consideramos que las objeciones que por claridad hizo su Despacho quedan superadas ya que el nuevo capítulo reivindicatorio es claro, conciso y permite determinar el alcance de la protección reivindicada y compararla frente al estado del arte, de acuerdo con las disposiciones de los artículos 28 y 30 de la Decisión 486. Sin embargo, en el evento que el Despacho considere que una modificación adicional sea necesaria, respetuosamente le solicito hacer uso de la facultad que le otorga el artículo 45 inciso 2. de la Decisión 486 de la Comisión de la Comunidad Andina a efecto de requerir nuevamente de nuestra parte cualquier complementación o aclaración.

Adicionalmente, se pone de presente que el título de de la presente solicitud ha sido modificado de la siguiente manera: **"METODO PARA PRODUCIR LIPASA, CELULA DE YARROWIA LIPOLYTICA TRANSFORMADA CAPAZ DE PRODUCIR LA LIPASA"**. Por lo tanto, la objeción formulada a este respecto se debe tener por superada.

Novedad

Su Despacho concluye que los documentos WO0183773 (D1) y WO2006039541 (D2) afectan la novedad de la presente solicitud de patente. En cuanto a las objeciones del examinador deseamos poner de presente lo siguiente:

De acuerdo con el artículo 16 de la Decisión 486:

"Artículo 16. - Una invención es nueva cuando no está comprendida en el estado de la técnica. El estado de la técnica comprenderá todo lo que haya

sido accesible al público, por una descripción escrita u oral, por una utilización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o, en su caso, de la prioridad reconocida”.

De lo anterior, es claro que para determinar si una invención es novedosa o no, el examinador de patentes debe comparar la invención frente al estado de la técnica con el fin de determinar si existe alguna diferencia.

De modo que, si se demuestra que existe al menos una característica técnica de la invención que no ha sido divulgada en el documento citado como el estado de la técnica más cercano se debe tener por superada la objeción por novedad toda vez que para que un documento pueda afectar la novedad de la presente solicitud de patente, dicho documento debe revelar todas las características técnicas reivindicadas en la presente invención.

Ahora bien, D1 describe la producción de la cepa recombinante *Yarrowia lipolytica* denominada MS4 capaz de sobre expresar levadura resistente a los ácidos lipasa. Esta cepa recombinante se obtiene mediante la transformación de una cepa PO1d de *Y. lipolytica* (cepa carente de transposones) con un vector que contiene el gen de la lipasa LIP2.

D1 describe la producción de lipasa por este clon en un medio de cultivo que comprende bactopectona y / o bactotripton y por lo tanto, la preparación de lipasa obtenida contiene impurezas de la mezcla de hidrolizados de proteínas. Además, la cantidad de lipasa producida por el proceso de D1 lleva a una concentración máxima de lipasa de 0,5 g / l (página 17, línea 23 de D1) y un máximo de 1200 U / ml, la cual fue medida usando sustrato de aceite de oliva (página 17, línea 24 de D1).

Sin embargo, D1 no enseña un método para producir preparaciones de lipasas ácido-resistentes como fármaco que comprende las etapas de:

- Un cultivo de dos pasos (precultivo y fermentación), permitiendo una alta tasa de producción de lipasa; y
- Uso de un medio de cultivo de fermentación que está desprovisto de productos de origen animal o mezclas no caracterizadas, tales como hidrolizados de proteínas; estos productos se sustituyen por una fuente de nitrógeno tal como nitrógeno inorgánico, y preferiblemente sulfato de amonio.

Por otro lado, D2 describe métodos para la producción de lipasas, principalmente lipasas procedentes de hongos filamentosos, su uso en la preparación de detergentes y su aplicación en métodos para fregar y hornear. Sin embargo, dicho documento no se refiere a lipasas para aplicación farmacéutica. D2 muestra que las lipasas pueden ser producidas a partir de diversos microorganismos, incluyendo una cepa recombinante de *Yarrowia lipolytica* y recomienda el uso de un medio de cultivo de la técnica anterior que incluyen

fuentes de carbono y nitrógeno y sales inorgánicas (página 47, líneas 14 a 26), tales como triptonas, peptonas, caseína hidrolizada o extractos de levadura (véase la página 62, línea 12, página 63, línea 5).

Sin embargo, D2 no describe la actividad de la lipasa de la preparación o el contenido de lipasas de dichos preparados. Asimismo, a diferencia de la presente solicitud de patente, D2 no describe una preparación de lipasa que se caracteriza por una actividad catalítica a un pH 6 por lo menos de 20000 unidades por ml del cultivo sobrenadante y una concentración de lipasa en dicha preparación comprendida entre 1 a 3 g de lipasa por litro.

Al comparar las características esenciales de la presente invención con respecto a la invención dada a conocer en los documentos D1 y D2, se observa que aunque D1 se refiere a la producción de la cepa recombinante *Yarrowia lipolytica* denominada MS4 y D2 se refiere a un método para la producción de lipasas, dichos documentos no enseñan ni sugieren el procedimiento reivindicado para la producción de forma específica de la lipasa LIP2 ya que no divulgan todas las características técnicas esenciales del mismo, tal como lo hace la presente solicitud de patente. Por lo tanto, las características técnicas esenciales de la invención no se encuentran anticipadas como un todo en dichos documentos y en consecuencia, la objeción formulada por novedad se debe tener por superada.

Nivel inventivo.

Su Despacho concluye que los documentos WO0183773 (D1) y WO2006039541 (D2) afectan el nivel inventivo de la presente solicitud de patente. En cuanto a las objeciones del examinador deseamos poner de presente lo siguiente:

De acuerdo con el artículo 18 de la Decisión 486:

“Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica”.

Así entonces, es claro que en la evaluación de nivel inventivo de la presente invención, los documentos citados D1 y D2 **deberían** revelar sugerencias y enseñanzas claras para el técnico en la materia que lo lleven a obtenerla. Para este fin, el Señor Examinador debe examinar si, de conformidad con dichos documentos (el estado de la técnica más cercano), una persona versada en la materia habría o no propuesto las características técnicas que distinguen la invención reivindicada y que permiten alcanzar los resultados obtenidos por dicha invención.

De conformidad con el anterior artículo, se exponen a continuación las razones por las cuales la presente invención posee nivel inventivo frente a las enseñanzas de los documentos D1 y D2:

La presente invención se refiere al desarrollo de un método para producir preparaciones de lipasas ácido-resistentes que puedan ser empleadas como medicamentos. En efecto, los procesos de producción de lipasa conocidos con anterioridad a la presente solicitud de patente llevaron a preparaciones de lipasa que contienen impurezas que no eran adecuadas para fines médicos.

Para resolver este problema, los inventores han desarrollado un método para la producción de lipasa por las cepas recombinantes de *Yarrowia lipolytica*, que se caracteriza por:

- Un cultivo de dos pasos (precultivo y fermentación), permitiendo una alta tasa de producción de lipasa; y
- Uso de un medio de cultivo de fermentación que está desprovisto de productos de origen animal o mezclas no caracterizadas, tales como proteínas hidrolizadas.

La presente invención también se refiere a una preparación de lipasa que se puede obtener por el método de la invención que se caracteriza por una actividad catalítica de al menos 15.000 unidades por ml de cultivo sobrenadante a un pH 6; esta preparación de lipasa no incluye impurezas resultantes del uso de productos animales o mezclas tales como proteínas hidrolizadas no caracterizadas, y se puede utilizar para fines terapéuticos. Esta invención también se refiere a la cepa de *Yarrowia lipolytica* YL-LIP2-6C.

La nueva reivindicación 1 se encuentra limitada a la utilización de un medio de cultivo de *Yarrowia lipolytica* que comprende:

- Como fuente de nitrógeno: nitrógeno inorgánico;
- Una fuente de carbono seleccionada de fuente de carbono de origen hidrato de carbono, polialcoholes, tales como glicerol, y fuentes de carbono de origen de lípidos tales como ácidos grasos y glicéridos; y
- Sales inorgánicas, oligoelementos y vitaminas.

Específicamente, el medio de cultivo usado en el método de la invención es tal que las fuentes de carbono, nitrógeno, vitaminas y minerales usados comúnmente en los medios de cultivo de microorganismos para la producción de lipasas (proteínas de origen animal o sus digeridos enzimáticos) se sustituyen por fuentes de carbono, oligoelementos y vitaminas.

En efecto, el ejemplo 2 de la solicitud de patente pendiente ilustra el proceso de ejecución de la invención con un medio de cultivo sintético.

Sobre la base de las enseñanzas de D1, una persona versada en la materia no se vería motivada a utilizar una fuente de carbono y nitrógeno distintas de las de origen animal o de mezclas no caracterizadas de los materiales proteicos de origen animal o de mezclas no caracterizadas de proteínas de origen animal origen o de productos de la digestión enzimática, tales como bactopectona o bactotripton, sin comprometer el rendimiento de la producción de la lipasa. D1 no enseña o sugiere que el uso de bactotripton es particularmente ventajoso para aumentar la actividad de la lipasa.

Además, D2 alejaría a una persona versada en la materia de emplear fuentes de nitrógeno distintos de los de origen animal o de mezclas no caracterizadas que consiste en proteínas de origen animal o de los productos de la digestión enzimática.

Así entonces, ninguno de los documentos citados enseña o sugiere el uso de un medio de producción de la enzima (es decir, medio de cultivo de fermentación) desprovisto de nitrógeno orgánico.

Por lo tanto, es evidente que una persona experta en la técnica no se vería motivada a emplear las enseñanzas de los documentos D1 y D2 con el fin de obtener el objeto reivindicado en las reivindicaciones 1 a 8 de la presente solicitud.

Además, la preparación de lipasas de la reivindicación 9 difiere de la de D1 y de D2, ya que está desprovista de impurezas y tiene una mayor actividad de la lipasa y concentración de lipasa.

Asimismo, es posible extrapolar la actividad lipolítica en aceite de oliva de la actividad lipolítica medido en trioctanoin como sustrato (basado en la especificación de la solicitud internacional publicada (página 10, líneas 18-20) y en Aloulou et al. Biochimica et Biophysica Acta 1771 (2007) 228-237, 1 U = 1,4 U trioctanoin en aceite de oliva); 20000 U en trioctanoin corresponde por tanto a 14 5000 U en aceite de oliva.

De lo anterior, se concluye que la presente solicitud es inventiva respecto a los documentos más cercanos del estado de la técnica, ya que todas las características esenciales técnicas de la reivindicación 9 no se enseñan o sugieren en los documentos D1 y D2 por si solos o combinados.

Por otra parte, la siguiente tabla compara las propiedades de las cepas de la técnica anterior y de la cepa según la reivindicación 11:

Strain	citation	copy number of expression cassette of the LIP2 gene	Genetic stability	Lipase yield	Lipolytic activity	
					Substrate	Activity
Wild type (Po1d)	D1	1	Yes	-	olive oil	50-60 U/ml
MS4 (Po1d-22-1-6-4)	D1	10	No	0,5g/l	olive oil	12 000 U/ml
YL-LIP2-6C I-3542	Pending application	6	Yes (industrial scale)	1 to 3 g/l	trioctanoin	20 000 U/ml
					olive oil	14 500 U/ml

Como se explicó anteriormente, la actividad enzimática medida usando trioctanoin como sustrato con la preparación de lipasa obtenida con la cepa I-3542 ha sido extrapolada a la actividad con aceite de oliva teniendo en cuenta que: 1U en triactanoin U = 1,4 en aceite de oliva (basado en la especificación de la solicitud internacional publicada una (página 10, líneas 18-20) y en Aloulou et al. Biochimica et BiophysicaActa 1771 (2007) 228-237).

La estabilidad es un requisito esencial para el desarrollo de un proceso industrial; una cepa se considera que es genéticamente estable si su número de copia del casete de expresión LIP2 permanece constante durante toda la etapa de fermentación.

Como se muestra en la tabla anterior y como se demuestra en los ejemplos de la presente solicitud, la reivindicación 11 se refiere a una cepa específica de Yarrowia lipolytica que muestra:

- Una velocidad inesperada de la producción de lipasa que es mucho más alta que las tasas de producción obtenidas con cepas de D1 (MS4) a pesar de que esta cepa contiene menos copia del casete de expresión LIP2;
- Una actividad enzimática mejorada en comparación con cepas ya conocidas del estado de la técnica; y
- Excelente estabilidad genética que permite su utilización a escala industrial.

Dado que los documentos D1 y D2 no enseñan o sugieren la cepa específica Yarrowia lipolytica de la reivindicación 11 de la presente solicitud o las ventajas en relación con la tasa de producción de lipasa, la mejora de la actividad enzimática de la lipasa y la

estabilidad genética, al igual que la presente solicitud, es claro que la presente solicitud es inventiva frente a las enseñanzas de dichos documentos.

Así entonces, teniendo en cuenta que las enseñanzas de las anterioridades D1 y D2 citadas por su Despacho no llevan a un técnico versado en la materia a obtener la materia de invención acá reivindicada toda vez que no enseñan ni sugieren como un todo las características técnicas esenciales de la misma, es claro que a partir de dichos documentos no era posible para un técnico medio obtener de manera obvia y/o evidente la presente invención. Lo anterior, si se tiene en cuenta que para el estudio de nivel inventivo el Examinador no puede adoptar la posición de un inventor y que además no puede partir de la solución dada sino simplemente de lo que expresamente revelen el o los documentos más cercanos del estado del arte, que en el presente caso son D1 y D2.

Dado que la presente solicitud cumple con las disposiciones de los artículos 28, 30, 16 y 18 de la Decisión 486, respetuosamente solicito a ese Despacho que se proceda con la concesión de la presente solicitud.

Anexos

Nuevo capítulo reivindicatorio conformado por 10 reivindicaciones.

Aloulou et al. Biochimica et Biophysica Acta 1771 (2007) 228-237.

Señor Director,



Margarita Castellanos Abondano

C.C. No. **39.779.654** de Usaquén

T.P.A. No. **70.819** del Consejo Superior de la Judicatura

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REIVINDICACIONES

1. Un método de producción de la lipasa LIP2 recombinante, que comprende:

a) una etapa en la cual se procede a poner en cultivo las células *Yarrowia lipolytica* transformadas por un vector seleccionado del grupo que consiste de JMP6 y JMP10 expresando la lipasa ácido-resistente de levadura LIP2, y

b) una etapa en la cual se recoge, en el sobrenadante de cultivo, la lipasa recombinante producida por las células anteriores, con las etapas de

b1) separación de lipasa del sobrenadante de cultivo, en donde dicha separación es efectuada por una técnica elegida entre la filtración tangencial en fibra honda, la filtración frontal y la centrifugación continua o discontinua, y la purificación es efectuada por una técnica elegida entre la filtración, la precipitación fraccionada, la cromatografía de intercambio iónico, la cromatografía de interacción hidrofóbica y la cromatografía por gel-filtración.

b2) la purificación de lipasa obtenida en b1, en donde el método es caracterizado porque la etapa a) para el cultivo es llevada a cabo en un medio de cultivo libre de productos de origen animal o de mezclas no caracterizadas constituidas de materias proteicas de origen animal o de sus productos de digestión enzimática,

en donde dicho medio de cultivo comprende:

- como fuente de nitrógeno, nitrógeno inorgánico,
- una fuente de carbono seleccionada entre las fuentes de carbono de origen glucídico, los polialcoholes tales como glicerol, y las fuentes de carbono de origen lipídico tales como ácidos grasos y acilgliceroles; y
- sales minerales, oligoelementos y vitaminas.

2. El método de producción de lipasa recombinante de acuerdo con la reivindicación 1, caracterizado porque dicha fuente de nitrógeno es sulfato de amonio.

3. Método de producción de acuerdo con la reivindicación 1 o 2, caracterizada porque la etapa a) comprende:

a1) el pre-cultivo de células transformadas de *Yarrowia lipolytica* en un medio que contiene una fuente de carbono de origen glucídico;

a2) una etapa de fermentación que comprende una fase de crecimiento celular en un medio que contiene una fuente de carbono de origen glucídico y una fase de producción de lipasa en un medio que contiene como una sola fuente carbono en ácido graso entre los triglicéridos de cadena corta, media o larga.

4. Método de producción de acuerdo con la reivindicación 3, caracterizado porque la fermentación es ventajosamente llevada a cabo con un pO_2 constante comprendido entre 15% y 25% y un pH de preferencia inferior a 6.5.

5. Método de producción de acuerdo con la reivindicación 3 o la reivindicación 4, caracterizado porque la etapa a1) de pre-cultivo es realizada hasta obtenerse un valor de DO_{600nm} entre 3 y 10 por 1 ml, y porque en la etapa a2) de fermentación, la fase anterior de producción de lipasa es iniciada cuando DO_{600nm} del cultivo alcanza un valor entre 60 y 80 por 1 ml.

6. Método de producción de acuerdo con cualquiera de las reivindicaciones 1 a 5, caracterizado porque la etapa b) en la cual se recoge la lipasa es realizada cuando DO_{600nm} alcanza un valor entre 300 y 350 por 1 ml.

7. Método de producción de acuerdo con cualquiera de las reivindicaciones 1 a 6, caracterizado porque la etapa a) consiste de poner en el cultivo del clon denominado YL-LIP2-6C, registrado ante la Colección Nacional de Cultivos de Microorganismos (CNCM, por sus siglas en Francés), 28 rue de DocteurRoux, 75724 Paris Cedex 15, bajo el número I-3542, el

15 de diciembre de 2005.

8. Preparación de lipasa LIP2 recombinante ácido-resistente de levadura, caracterizada porque presenta una actividad catalítica de pH6 superior a 20000 unidades por ml de sobrenadante de cultivo, una unidad que corresponde a la cantidad de enzima capaz de catalizar la liberación de 1 μ mol de ácido graso por minuto puesto que el sustrato utilizado es la trioctanoína, y/o que la concentración de la lipasa en la preparación es superior a 1 g de lipasa por litro.

9. La célula de *Yarrowia lipolytica* transformada por un vector que comprende un casete de expresión de una lipasa extracelular ácido-resistente de levadura, caracterizada porque se trata de un clon denominado YL-LIP2-6C, registrado ante la Colección Nacional de Cultivos de Microorganismos (C.N.C.M.) bajo el número I-3542, el 15 de diciembre de 2005.

10. Medicamento, caracterizado porque comprende una preparación de lipasa de acuerdo con la reivindicación 8.



Purification and biochemical characterization of the LIP2 lipase from *Yarrowia lipolytica*

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Abstract

The LIP2 lipase from the yeast *Yarrowia lipolytica* (YLLIP2) was obtained from two genetically modified strains with multi-copies of the *lip2* gene and further purified using gel filtration and cation exchange chromatography. Four YLLIP2 isoforms were identified and subjected to N-terminal amino-acid sequencing and mass spectrometry analysis. These isoforms differed in their glycosylation patterns and their molecular masses ranged from 36,874 to 38,481 Da, whereas the polypeptide mass was 33,385 Da. YLLIP2 substrate specificity was investigated using short (tributyrin), medium (trioctanoin) and long (olive oil) chain triglyceride substrates at various pH and bile salt concentrations, and compared with those of human gastric and pancreatic lipases. YLLIP2 was not inhibited by bile salts at micellar concentrations with any of the substrates tested, and maximum specific activities were found to be $10,760 \pm 115$ U/mg on tributyrin, $16,920 \pm 480$ U/mg on trioctanoin and $12,260 \pm 700$ U/mg on olive oil at pH 6.0. YLLIP2 was found to be fairly stable and still active on long chain triglycerides (1590 ± 430 U/mg) at pH 4.0, in the presence of bile salts. It is therefore a good candidate for use in enzyme replacement therapy as a means of treating pancreatic exocrine insufficiency. © 2006 Elsevier B.V. All rights reserved.

Keywords: Microbial lipase; Lipolysis; pH-dependency; Bile salts; Yeast; Triglycerides

1. Introduction

The yeast *Yarrowia lipolytica*, formerly known as *Candida*, *Endomycopsis* or *Saccharomycopsis lipolytica*, is one of the most extensively studied non-conventional yeasts. This yeast, the only species recognized so far in the *Yarrowia* genus, is quite different from the other frequently studied yeasts such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* in terms of its phylogenetic evolution, physiology, genetics, and molecular biology. *Y. lipolytica* is non-pathogenic to humans and has been approved for several Generally Recognized As Safe (GRAS) industrial processes. *Y. lipolytica* is an obligate aerobic dimorphic ascomycete that naturally secretes large amounts of various metabolites (such as organic acids and extracellular proteins) [1]. Since an increasing number of studies have clearly shown that *Y.*

lipolytica occurs naturally in various kinds of food, information about this yeast is of great importance to the agro-food industry [2]. Strains of this species have mostly been isolated from lipid-rich food such as cheese and olive oil, as well as from sewage, and this yeast grows readily on hydrophobic substrates such as alkanes, fatty acids and oils [2–4]. Its substrate preferences and physiological properties have been attributed to the fact that this yeast efficiently produces and secretes proteolytic and lipolytic enzymes [1]. Several processes have been developed involving the use of *Y. lipolytica* in bioconversion reactions with hydrophobic substrates [2], some of which have been patented in the fields of bioremediation, fine chemistry and the food industry. For instance, *Y. lipolytica* can be used for alkane and fatty-acid bioconversion, the production of aromas, single-cell protein (SCP), single-cell oil (SCO) and citric acid, as well as for steroid biotransformation.

Since *Y. lipolytica* can use fat and oils as carbon sources, increasing attention has been paid to the lipases produced by this yeast. Lipases (EC 3.1.1.3) catalyze the hydrolysis of long-chain

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triacylglycerols and play an important physiological role in several metabolic processes (fat digestion in the gastro-intestinal tract, lipolysis in the adipose tissue, lipolysis of lipoproteins) in the conversion of oils and fats into free fatty acids and partial acylglycerols. Considerable efforts have been made to elucidate their role in dietary fat digestion [5–7] and to describe in detail the molecular and kinetic events occurring during the catalytic process at lipid/water interfaces [8]. Lipases with new properties (substrate specificity, stereoselectivity, thermostability) are of particular interest, and these can be obtained either by isolating them from various natural sources, by using protein engineering methods, or by performing directed evolution [9]. Increasing attention has been paid to lipases of microbial origin, due to their wide range of biochemical properties and because they are relatively easy to produce in bioreactors and to recover from the culture medium [10]. The catalytic properties of microbial lipases have made it possible to develop many biotechnological applications in the food, cosmetic, detergent, and pharmaceutical industries [11–14].

In *Y. lipolytica*, the *lip2* gene which codes for a 301-amino acid extracellular lipase YLLIP2 has been cloned and amplified and it was established that most of the extracellular lipase activity results from *lip2* expression [15,16]. The authors of recent studies reported that methyl oleate modulates YLLIP2 expression in *Y. lipolytica* [2]. Although YLLIP2 seems involved in the use of triglycerides as a carbon source for the yeast, the finding that a *lip2* knock-out strain is still able to use this source suggested the presence of other lipases in *Y. lipolytica*. The cloning and functional analysis of the *lip7* and *lip8* genes encoding two predicted lipases were recently performed [2]. Unlike YLLIP2, which is secreted into the culture medium, it was observed that YLLIP7 and YLLIP8 are mainly associated with the cell wall.

Fungal lipases mainly belong to two families: (i) lipases which are homologous to acetylcholine esterase (i.e. *Geotrichum candidum* and *Candida rugosa* lipases) and (ii) triacylglycerol lipases including *Rhizopus oryzae*, *Rhizomucor miehei*, *Candida antarctica* and *Thermomyces lanuginosus* [17]. Amino acid sequence comparisons have shown that YLLIP2 belongs to the second group (Fig. 1). The highest amino acid sequence similarity was 91.6% with *Candida deformans* lipase LIP1. Great similarities were also found to exist with the lipases from *Thermomyces lanuginosus* (30.3%), *Candida ernobii* (27.7%) and *Rhizomucor miehei* (25.1%).

Unlike these extensively characterized fungal lipases, no purified YLLIP2 has been biochemically characterized so far, to our knowledge. In the present study, we therefore purified this enzyme for the first time, and determined its lipase activity on triglycerides with various chain lengths. The effects of pH and bile salts were tested and compared with those observed with human pancreatic and gastric lipases.

2. Materials and methods

2.1. YLLIP2 production

Two genetically modified *Y. lipolytica* strains overproducing YLLIP2 were used in this study [15]. These strains had a mean number of 6 and 20 integrated copies of the *lip2* gene under the control of the strong inducible *POX2* promoter.

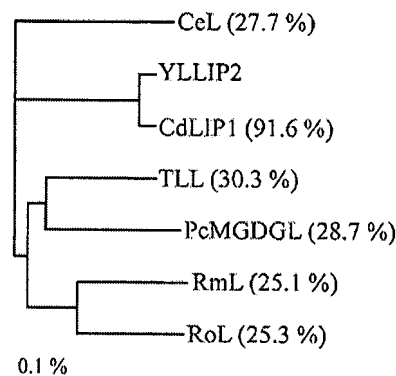


Fig. 1. Dendrogram of fungal lipase sequence alignment showing the homologies between YLLIP2 and other lipases. YLLIP2 (*Yarrowia lipolytica* lipase, access no CAB91111), TLL (*Thermomyces lanuginosus* lipase, AAC08588), RmL (*Rhizomucor miehei* lipase, CAA00250), CdLIP1 (*Candida deformans* lipase LIP1, CAD21428), PcMGDGL (*Penicillium camemberti* mono- and diacylglycerol lipase, BAA14345), CeL (*Candida ernobii* lipase, CAC15040) and RoL (*Rhizopus oryzae* lipase, AAF32408). Accession numbers are those of the NCBI protein data base. Sequence alignment was performed using the ClustalW program [42]. Identity percentages versus YLLIP2 are in brackets.

The 20-copy strain produces more active lipase than the 6-copy strain under lipase-inducing conditions (see below). The media and techniques used here to grow and handle *Y. lipolytica* have been described elsewhere [18]. The production of YLLIP2 was performed using fed-batch cultures under controlled conditions at 28 °C in a 6 L bioreactor (BIOSTAT®A-DCU, Sartorius BBI systems) with a working volume of 4.5 L. Dissolved oxygen was continuously monitored with an oxygen probe (Ingold, Urdorf, Switzerland). The stirrer was used at a speed of 1000 rpm to ensure that the dissolved oxygen reached a saturation level of at least 30%. Air flow was set at 2 L/min and the pH was automatically held at 5.6±0.1 by adding 28% NH₄OH in water. In order to prevent excess foam formation, a level sensor triggering the addition of an antifoam compound (J673, Struktol, Germany) was placed 10 cm from the top of the vessel. Initially, the bioreactor was filled with 3 L of YPD culture medium and then inoculated at 12.5% (v/v) with *Y. lipolytica* liquid pre-culture, having an optical density (600 nm) of 1, in order to start the cell growth directly in the exponential growth phase, as well as to establish reproducible cell culture conditions. During the first 25-h fermentation phase, the biomass level was increased using the glucose (10 g/L) present in the YPD culture medium. Then, the lipase expression was induced by feeding the bioreactor with culture medium containing 1% (v/v) of oleic acid as the only source of carbon during the following 35 h. Biomass and lipase activity were monitored as a function of time until the lipase production level in the culture broth reached a maximum value, which was generally around 3 g lipase/L of culture supernatant after 60 h of fermentation. The lipase mass concentration was estimated from the lipase activity measurement (32,280 U/mL using tributyrin as substrate) and the specific activity of the lipase (10,760±115 U/mg) determined in the present study. The culture (4.5 L) was then stopped to avoid any proteolysis and the cells were pelleted by centrifugation at 3000×g for 20 min at 4 °C. Benzamidin was then added to the supernatant at a final concentration of 2 mM in order to limit the proteolysis of YLLIP2. The culture supernatant (2.5 L) was immediately filtered by tangential flow ultrafiltration using hollow cartridges (Millipore) with a fiber diameter of 0.1 µm. The filtrate was then lyophilised and stored at –20 °C.

2.2. YLLIP2 purification

All subsequent purifications steps were performed at 4 °C. One hundred mg of the resulting powder containing 120,640 lipase U (tributyrin as substrate) were suspended in a minimum volume (~10 mL) of 10 mM Tris buffer, pH 7.5, containing 150 mM NaCl and 2% (v/v) protease inhibitors (Complete®, Roche), and filtered. Gel filtration chromatography was performed after loading the previous solutions on a Superdex 200 HR 26/60 column (Pharmacia), using

10 mM Tris/HCl pH 7.5, 150 mM NaCl buffer. The flow rate was adjusted to 0.5 mL/min and the pressure kept below 0.5 MPa using an ÄKTA explorer chromatographic device (Amersham Biosciences). The protein elution profile was recorded spectrophotometrically at 280 nm. Fractions with lipase activity eluted from the gel filtration column were pooled and concentrated by ultrafiltration on a YM 10 Amicon ultrafiltration membrane and dialysed against 10 mM MES, pH 7.0. A second anion-exchange chromatography step was performed on a Mono Q 5/50 GL column (Tricorn, Amersham Biosciences). A linear NaCl concentration gradient ranging from 0 to 80 mM NaCl in 10 mM MES pH 7.0 was applied for 2 h. The flow rate was adjusted to 1 mL/min and the pressure was kept between 3.0 and 3.5 MPa using the ÄKTA explorer device. Fractions showing lipase activity were analysed separately before they were either pooled or not.

2.3. Analysis of YLLIP2

YLLIP2 present in the culture medium and in purification fractions was analysed electrophoretically on 12% polyacrylamide gels in the presence of SDS as described by Laemmli [19]. The amino acid composition was analysed as previously described [20]. To check that the cleavage of the YLLIP2 signal peptide had occurred appropriately, and to ensure that no proteolytic degradation had taken place, the purified protein was subjected to N-terminal sequence analysis using an Applied Biosystem Model 473A gas-phase sequencer. MALDI-TOF mass spectrometry was performed using Voyager DE-RP equipment (Perseptive Biosystems Inc.).

2.4. Lipase activity measurements using a pH-stat

Lipase activity was assayed potentiometrically by automatically titrating the free fatty acids released from mechanically stirred triacylglycerol emulsions, using 0.1 N NaOH and a pH-stat device (TTT 80 Radiometer, Copenhagen). Each assay with short-chain triglycerides (e.g. tributyrin (Fluka)) or medium-chain triglycerides (e.g. trioctanoin (Sigma)) was performed in a thermostated (37 °C) vessel containing 0.5 mL triglyceride and 14.5 mL of a 150 mM NaCl, 5 mM CaCl₂ solution and various concentrations of sodium taurodeoxycholate (NaTDC) ranging from 0 to 8 mM. The triglycerides were added directly to the pH-stat vessel containing the assay solution and were emulsified by mechanical stirring. Corrections were made to take into account the partial ionization of butyric and octanoic acids occurring at pH levels below 6.0. Long-chain triglycerides (e.g. olive oil) had first to be pre-emulsified with gum arabic (GA) by mixing 5 mL of olive oil with 45 mL of a 10% (w/v) GA solution in 150 mM NaCl, 5 mM CaCl₂ prepared as previously described [21]. Five mL of this olive oil-GA emulsion were then mixed in the pH-stat vessel with 10 mL of a solution containing 150 mM NaCl, 5 mM CaCl₂ and various NaTDC concentrations. The final amounts of long-chain triglyceride and GA in the pH-stat vessel were 0.5 mL and 3% (w/v), respectively. The activity on short and medium chain TG was determined by directly titrating FFA, whereas the activity on long chain TG was determined by performing back titration. After incubating the enzyme with the substrate for variable periods of time at a given pH, the pH end-point value was increased to pH 9.0 in order to ensure full ionization and titration of FFA. A blank assay without any lipase was performed at each initial pH of incubation.

Specific activities are expressed here in international units (U) per milligram of protein, quantified by means of amino acid composition analysis. One U corresponds to 1 μmol of fatty acid released per minute.

2.5. Study of YLLIP2 stability as a function of pH and temperature

To study the effects of the pH on the enzyme stability, buffered 1-mL samples containing 100 μg YLLIP2 were incubated at various pH values ranging from 1 to 8 for 1 h at 37 °C. To keep the sample pH constant, the following 50 mM buffers systems were used: HCl/KCl, pH 1.0–2.0; Na₂HPO₄/Citrate, pH 3.0–5.0 and KH₂PO₄/Na₂HPO₄, pH 6.0–8.0. During the incubation period, the residual YLLIP2 activity was measured at *t*=0, 1, 15, 30, 45 and 60 min in order to determine the changes with the time in the residual YLLIP2 activity. Each measurement was performed twice.

To determine whether the loss of activity occurring at low pH values was reversible, we incubated samples of purified enzyme at pH 3.0 for 1 h in a

thermostated vessel (37 °C) under stirring. After complete inactivation of the lipase, the pH was increased to 6.0, a pH level at which YLLIP2 is known to remain stable for more than 1 h. During this period, we measured the residual enzymatic activity at regular intervals ranging from 1 to 120 min, to determine whether any enzyme reactivation had occurred. Similar experiments were performed to establish whether the loss of YLLIP2 activity occurring at high pH values (8.5) was reversible when the pH was decreased to 6 after 2 h of incubation at pH 8.5.

The thermal stability was studied by incubating YLLIP2 at various temperatures and measuring the residual activity with time under standard titrimetric assay conditions.

2.6. Time course of long chain triglyceride hydrolysis by YLLIP2 and analysis of the products formed

The lipolysis of pure triolein with YLLIP2 was monitored with a pH-stat (718 STAT Titrimetric, Metrohm, France) under the following conditions: 20 mL of a solution containing 150 mM NaCl, 5 mM CaCl₂, 4 mM NaTDC and 0.7 mL of triolein were mixed and vigorously stirred in a pH-stat vessel at a temperature of 37 °C. YLLIP2 (5–100 μg) then was added to the mixture, which was maintained at constant pH (6.0) by titrating the fatty acids released with 0.1 M NaOH. Samples (1 mL) of the reaction mixture were collected at various times for 22 h and lipid extraction was immediately performed in order to determine the time course of the release of partially acylated glycerols and free fatty acid during hydrolysis. Each 1-mL sample was mixed vigorously with 200 μL of 0.1 N HCl and 5 mL of a hexane:n-butanol mixture (3:2 v/v) in a 15-mL glass tube with a screw cap. After phase separation, the upper organic phase was collected using a Pasteur pipette and transferred to a 15-mL test tube. The remaining water phase was subjected to a second extraction step with 5 mL of a hexane:isopropanol mixture (3:2 v/v). After phase separation, the upper organic phase was collected, mixed with the previous one in the 15-mL test tube, and dried over anhydrous magnesium sulfate (MgSO₄). Once MgSO₄ had precipitated, 1–2 mL of the clear organic phase was transferred to a 2-mL vial with a screw cap and the vial was kept at –20 °C until lipid analysis was performed. Analysis of lipolysis product by thin layer chromatography coupled to flame ionisation detection (TLC-FID) was performed with a Iatroscan MK5 (Iatron laboratories, Japan) as described by Carriere et al. [22].

The TLC-FID mass detection data were converted into moles using the molecular masses of triolein (885.43 Da), diolcin (586.97 Da), monoolein (356.54 Da) and oleic acid (282.46 Da).

The rate of hydrolysis at various times *t* was defined as the percentage of the acyl chain released from TG (the complete hydrolysis of 1 molecule of triglyceride releases 3 molecules of fatty acid) and calculated using the following equation:

$$\text{Hydrolysis}(\%) = 100 \cdot \text{FFA} / 3 \cdot \text{TG}_0 = 100 \cdot \text{FFA} / (3 \cdot \text{TG} + 2 \cdot \text{DG} + \text{MG} + \text{FFA})$$

where TG₀ is the number of triglyceride moles initially present, and FFA, TG, DG and MG are the number of moles of free fatty acid, triglycerides, diglycerides and monoglycerides present at time *t* in the incubation mixture.

The lipase stereoselectivity was investigated by analyzing the diglyceride enantiomers formed during the lipolysis of triolein as described previously [23]. The diglycerides were derivatized with optically active R-(+)-1-phenylethylisocyanate to obtain diastereo isomeric carbamates, which were then separated by high-performance liquid chromatography (HPLC). The enantiomeric excess (ee) of 1,2-diolein versus 2,3-diolein was then calculated to determine the lipase stereopreference [23,24]. Before derivatization, it was checked that no acyl migration had occurred from the secondary to primary ester position in the diglycerides formed.

3. Results

3.1. Purification of YLLIP2

The YLLIP2 lipase was purified from the culture medium of *Y. lipolytica* mutants with increased lipase secretion capacities.

When a genetically modified *Y. lipolytica* strain with 6 copies of the *lip2* gene was used to overproduce YLLIP2 (at a level of around 2 g of lipase/L of culture medium), only one lipase activity peak was observed during the gel filtration chromatography (peak I, Fig. 2A). This peak was eluted with the dead volume of the Superdex 200 column. The corresponding fractions contained high molecular mass lipase aggregates with tightly bound lipids, as shown by thin-layer chromatography on silica gel (data not shown). Most of the lipids bound to the lipase were free fatty acids, probably the residual oleic acid used for inducing lipase secretion. The complete separation of the bound lipids from this so-called fast lipase required drastic conditions (treatment with organic solvent), leading to the extensive loss of enzymatic activity (data not shown). When using a 20-copy strain (producing around 3 g of lipase/L of culture medium), a “fast lipase” (peak I, Fig. 2B) was also observed upon performing gel filtration, but a second peak of lipase activity was observed at 1.8-fold the dead volume of the column (peak II, Fig. 2B). The molecular mass of the lipase present in the corresponding fraction was estimated to be around 38–40 kDa. At this purification stage, SDS-PAGE analysis showed the existence of one major protein band corresponding to a molecular mass of around 38 kDa in fractions corresponding to both peak I and peak II (Fig. 3).

These results confirmed that the apparent high molecular mass of the lipase present in peak I was due to the partial aggregation of YLLIP2 with lipids present in the culture medium. The larger the amounts of YLLIP2 produced, the more the proportion of lipid-free lipase increased. It is worth noting that the lipase activity corresponding to the “fast lipase” peak was similar with both lipase productions, with an active

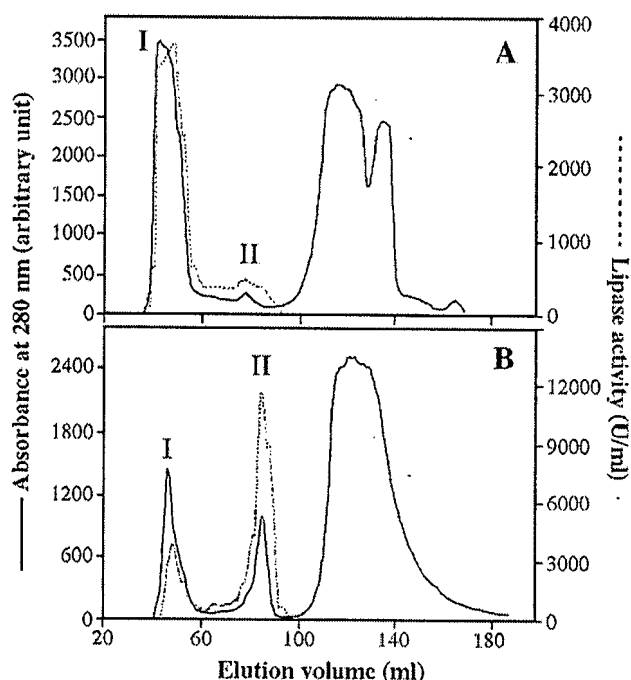


Fig. 2. Superdex 200 gel filtration of YLLIP2 produced by 6-copy (A) and 20-copy (B) *Y. lipolytica* strains. Protein elution was monitored at 280 nm. Lipolytic activity was measured using a trioctanoin substrate at pH 5.5.

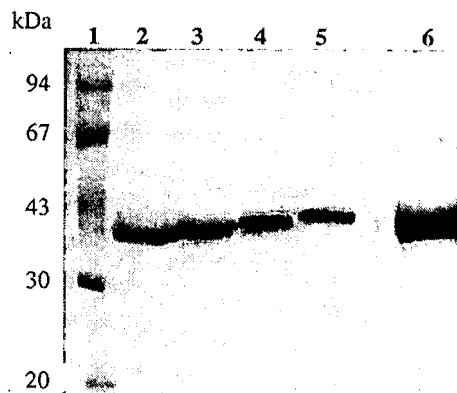


Fig. 3. SDS-PAGE (12% acrylamide) of YLLIP2 at various purification stages. Lane 1, molecular mass markers; lanes 2, 3, 4 and 5, YLLIP2_A, YLLIP2_B, YLLIP2_C and YLLIP2_D isoforms purified by Mono Q chromatography, respectively; lane 6, pooled fractions of the gel filtration peak II.

lipase concentration reaching 3000–4000 U/mL in the fractions collected. It thus seems likely that lipid-free lipase is released once the lipids have been saturated with lipase molecules.

It was checked that “fast lipase” and lipid-free lipase showed similar specific activities on long-, medium- and short-chain triglycerides (data not shown). The gel filtration fractions corresponding to the lipid-free lipase (peak II) produced by the 20-copy strain were further used to complete the YLLIP2 purification procedure. It was observed that the single peak II of the active lipase obtained by gel filtration chromatography did not correspond to a homogenous protein. Unlike the pattern obtained by SDS-PAGE analysis, the isoelectrofocusing pattern of YLLIP2 purified by gel filtration showed several bands with isoelectric points ranging from 5.0 to 5.4. In order to purify some of the YLLIP2 molecular species detected by isoelectrofocusing analysis, gel filtration fractions (peak II) were pooled, dialysed and loaded onto a Mono Q 5/50 GL anion exchange column. Four enzymatically active proteins were separated, as shown in Fig. 4. No lipolytic activity was detected in the fractions passing through the column. These YLLIP2 isoforms were found to be homogenous, as the corresponding fractions taken from the top of each peak gave a single SDS-PAGE band (Fig. 3) and also a single peak when re-chromatographed using the same chromatographic system. These four isoforms were found, however, to differ in their apparent molecular masses as determined by SDS-PAGE, and these differences were confirmed by MALDI-TOF mass spectrometry, which gave molecular masses of $36,850 \pm 34$ Da, $37,644 \pm 31$ Da, $38,168 \pm 34$ Da, $38,573 \pm 129$ Da for purified isoforms denoted YLLIP2_A, YLLIP2_B, YLLIP2_C and YLLIP2_D, respectively. These masses were all found to be higher than that of the YLLIP2 polypeptide deduced from cDNA (33,385 Da), which suggests that all four YLLIP2 were glycosylated, as previously shown with total YLLIP2 present in culture medium and treated with endoglycosidases [16] (NB: we later produced an YLLIP2 mutant without glycosylation sites and the theoretical molecular mass of the mature protein was confirmed; unpublished data). Furthermore, the

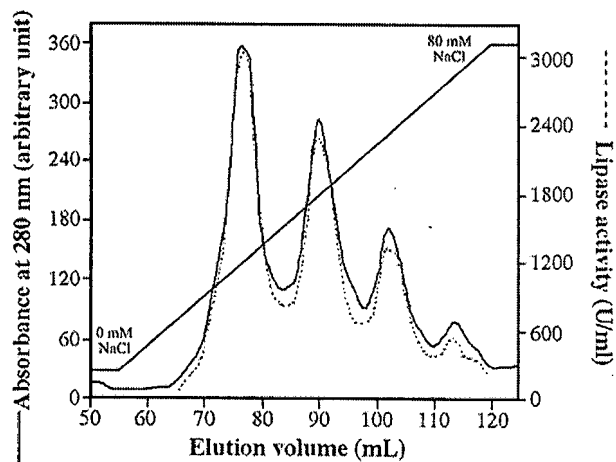


Fig. 4. Separation of YLLIP2 isoforms by anion-exchange chromatography on Mono Q 5/50 GL. The column was loaded with fraction II from the Superdex 200 gel filtration of YLLIP2 produced by the 20-copy *Y. lipolytica* strain. The column was equilibrated with 10 mM MES-NaOH buffer (pH 7.0). Protein was eluted using a linear NaCl concentration gradient ranging from 0 to 80 mM.

four isoforms were found to have the same amino acid composition and N-terminal sequence (VYTSTETSHI), which corresponds to the N-terminal sequence of the mature YLLIP2 already determined by Pignède et al. from SDS-PAGE separation, western blotting and Edman degradation of the isolated lipase band [16]. Hence, *Y. lipolytica* secretes four major YLLIP2 isoforms, which differ in their glycosylation pattern and have glycan moieties of 3,465 Da, 4,259 Da, 4,783 Da and 5,188 Da, corresponding to isoforms YLLIP2_A, YLLIP2_B, YLLIP2_C and YLLIP2_D, respectively. It was observed that all these isoforms showed the same specific activity on various substrates, and the enzyme activity therefore does not seem to be affected by the various glycosylation patterns. As shown in Table 1, which summarizes the data on the purification of YLLIP2 molecular species, YLLIP2_A was found to be the major isoform. The initial proportions of YLLIP2 isoforms in the culture medium

Table 1
Flowsheet of the procedure used to purify YLLIP2 from 20-copy *Y. lipolytica* strain

Steps	Total units (U)	Proteins (mg)	Specific activity (U/mg of protein)	Activity recovery (%)	Purification factor
Culture filtrate (10 mL)	120640	15,00	8043	100	1
S-200 (fraction II)	82940	6,02	13782	69	1,71
Mono Q					
Isoform A	36759	2,33	15769	30,47	1,96
Isoform B	24695	2,17	11405	20,47	1,41
Isoform C	9731	0,79	12384	8,07	1,54
Isoform D	5161	0,46	11284	4,28	1,4
Mono Q Total	76346	5,74		63,28	

Protein amounts were determined by performing quantitative amino-acid analysis. One international unit (U) is defined as the release of 1 μ mol of fatty acid per minute, using a trioctanoin substrate.

are difficult to assess, however, since part of YLLIP2 interacts with lipids at this stage and the corresponding active fractions, in which some isoforms might have been concentrated, were discarded prior to the chromatography on Mono Q.

3.2. Study of YLLIP2 stability

The purified YLLIP2 was incubated at 37 °C and at various pH values and its stability was found to be maximum at pH 5.0 and 6.0 (Fig. 5): 90 and 98% of the initial activity was still present after 1 h of incubation, respectively. YLLIP2 was also found to be fairly stable at pH 7.0 and 4.0: 88% and 74% of the initial activity was still present after 1 h of incubation, respectively. YLLIP2 activity decreased rapidly at pH 3.0 and pH 8.0, giving half inactivation times of 13 ± 1 and 35 ± 1 min, respectively.

To determine whether the pH-inactivation of YLLIP2 was reversible, samples of purified YLLIP2 were completely inactivated after incubating them at either pH 3.0 or pH 8.5 for 2 h. The pH value was then changed to pH 6.0 and activity measurements were performed at regular intervals for 2 h. In both cases, no reactivation of YLLIP2 activity was found to occur, which indicated that the inactivation of YLLIP2 was irreversible.

YLLIP2 inactivation kinetics were measured at different temperatures. While the half-life was more than 4 h at 40 °C, it was found to be only approximately 9 min at 50 °C.

3.3. Effects of bile salts on YLLIP2 activity

When the activity of purified YLLIP2 was assayed using an emulsion of pure trioctanoylglycerol, no activity was detected (Fig. 6A). Some lipase activity was immediately detected, however, when YLLIP2 was injected after adding NaTDC (Fig. 6A). No activity was detected when the enzyme was injected before adding NaTDC, which suggested that YLLIP2 was irreversibly denaturated at the pure oil–water interface. YLLIP2 therefore seems to be sensitive to interfacial denaturation in the absence of any amphiphile decreasing the interfacial tension, as

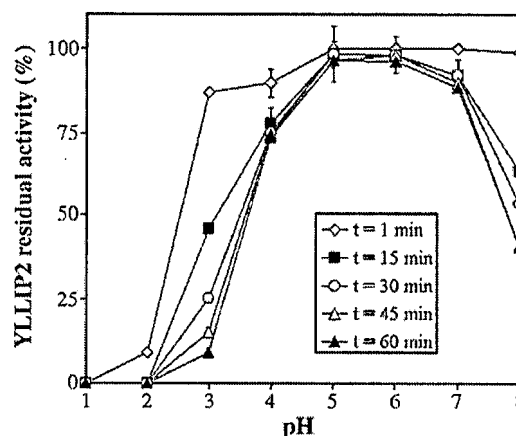


Fig. 5. Residual activity of purified YLLIP2 as a function of pH and time. Residual YLLIP2 activity is expressed as a percentage of the initial activity. Values are means $\pm$ SD ($n=3$).

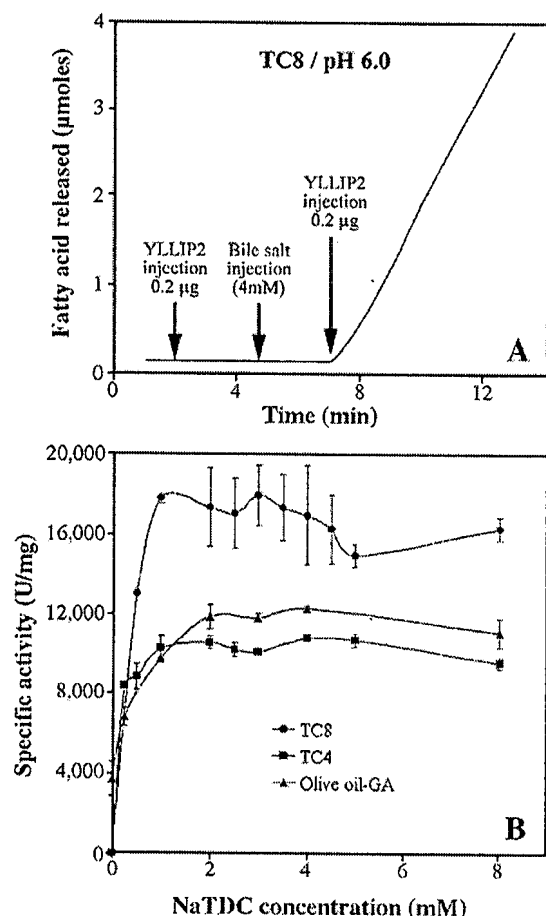


Fig. 6. Effects of bile salts on the lipase activity of YLLIP2. (A) Kinetics of the hydrolysis of trioctanoin emulsion by YLLIP2. Arrows indicate successive injections of YLLIP2 (0.2 μg), bile salt (NaTDC) at a final concentration of 4 mM and YLLIP2 again. (B) Effects of increasing concentrations of NaTDC on the rates of hydrolysis of tributyrin (■), trioctanoin (●) and olive oil-GA (▲) emulsions by YLLIP2. Experiments were performed at pH 6.0.

previously reported to occur in the case of gastric lipase [25,26] and other lipases.

The effects of the bile salt concentration on the lipase activity were then tested at pH 6.0, using short-, medium- and long-chain triglycerides. As previously mentioned with trioctanoin, NaTDC was also required to be able to measure the lipase activity on tributyrin. A fairly high level of activity (3700 ± 800 U/mg) on olive oil emulsified with gum arabic was detected without any prior addition of NaTDC (Fig. 6B), probably because gum arabic already decreased the interfacial tension [21]. With all the substrates tested, YLLIP2 specific activity was found to increase with the bile salt concentrations, and maximum activities were reached at concentrations above 2 mM NaTDC, i.e. above the critical micellar concentration (CMC) of NaTDC.

3.4. Optimization of assay conditions

NaTDC was used at a concentration of 4 mM in all the assays. When tributyrin and trioctanoin were used as substrates, a lag time of about 1 min was consistently observed before the

establishment of the steady-state velocity. Adding various amounts of bovine serum albumin (0–40 μM) did not increase the YLLIP2 specific activity or abolish the lag times (data not shown). No lag phase was observed with olive oil-GA emulsions. A solution containing 150 mM NaCl, 5 mM CaCl₂ and 4 mM NaTDC was selected for the emulsification of triglyceride substrate and the standard pH-stat assay of YLLIP2. Efficient and stable emulsification of short and medium triglycerides was obtained by mechanical stirring, whereas gum arabic had to be added to be able to measure YLLIP2 activity on long-chain triglycerides. When long chain triglycerides were used, the fatty acids released were estimated by back-titration at pH 9.0 after a 3-min incubation period at the pH of the assay. We checked experimentally that the release of fatty acid was linear with time up to 15 min of incubation.

3.5. Effects of pH on YLLIP2 activity with various triglyceride substrates

The effects of pH on YLLIP2 specific activity were tested using emulsions of short-chain (tributyrin), medium-chain (trioctanoin) and long-chain (olive oil-GA) triglycerides as substrates (Fig. 7). In the presence of 4 mM NaTDC, the maximum specific activities were observed at pH 6.0 with all the substrates tested. The highest activity was measured with trioctanoin ($16,920 \pm 480$ U/mg), followed by olive oil ($12,260 \pm 700$ U/mg) and tributyrin ($10,760 \pm 115$ U/mg). In the absence of NaTDC, the maximum activity of YLLIP2 on olive oil-GA emulsion shifted from pH 6.0 to 8.0 (Fig. 7). YLLIP2 specific activity on long-chain triglyceride emulsion (olive oil-GA) was still high (1590 ± 430 U/mg) at pH of 4.0.

3.6. Time course of triolein hydrolysis by YLLIP2 and specificity

Fig. 8A shows the time course of the hydrolysis of triolein by YLLIP2 and the release of partial glycerides (DG, MG glycerol

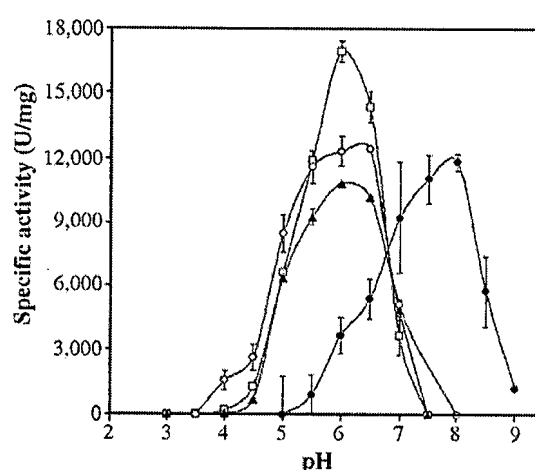


Fig. 7. Influence of pH on YLLIP2 specific activity on tributyrin (▲), trioctanoin (□) and olive oil-GA, in presence (○) or in absence (●) of 4 mM NaTDC, measured at 37 °C. All measurements with tributyrin and trioctanoin were performed in the presence of 4 mM NaTDC.

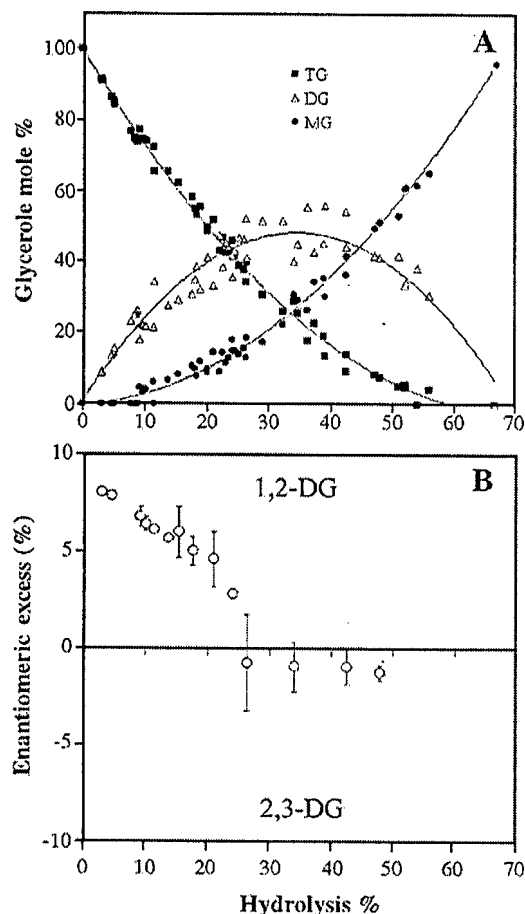


Fig. 8. Hydrolysis of triolein emulsion by YLLIP2 at pH 7.0 and 37 °C in the presence of 4 mM NaTDC. (A) Glyceride species (TG, triglyceride; DG, 1,2 (2,3)-diglycerides; MG, monoglycerides) are expressed as glycerol mole percentages and plotted as a function of the percentage rate of hydrolysis. (B) Enantiomeric excess (*ee*) of 1,2- vs. 2,3-diolein expressed as triolein hydrolysis %. $ee = ([R] - [S]) / ([R] + [S]) \times 100$. [R] and [S] are concentrations of the respective enantiomers 1,2- and 2,3-diglycerides.

mol%) as a function of the rate of hydrolysis. It is worth noting the transient accumulation of diglycerides, which reached a maximum value at around 33% hydrolysis, while the MG increased continuously up to 66% hydrolysis, by which stage TG and DG had almost disappeared. The rate of DG production is therefore faster than the rate of hydrolysis into MG. The variation profiles are typical of a 1,3-regioselective lipase generating 2-monoglyceride, but not hydrolysing the ester bond at the *sn*-2 position, since MG accumulate in the reaction mixture.

In the course of the same experiments, the stereoselectivity of YLLIP2 toward the chemically alike, but sterically non-equivalent ester groups at the *sn*-1 and *sn*-3 positions of the prochiral triolein substrate was tested by measuring the enantiomeric excess of 1,2-diolein versus 2,3-diolein (Fig. 8B). A clear enantiomeric excess (*ee*) of 1,2-diolein was observed at low rates of hydrolysis (8% *ee* of 1,2-diolein at 5% hydrolysis), indicating that YLLIP2 shows a slight stereopreference for the hydrolysis of the ester bond at the *sn*-3 position of triolein. The apparent stereopreference of YLLIP2

changes however with the hydrolysis rate and the enantiomeric excess of diglycerides is slightly reversed at lipolysis rates higher than 25%. This change is probably due to the subsequent reaction step of diglyceride hydrolysis into monoglycerides by YLLIP2, in which a stereospecific hydrolysis of 1,2-diolein might occur, as previously observed in the cases of dog gastric and pancreatic lipases [27].

3.7. YLLIP2 activity on long chain triglycerides emulsified with phospholipids and search for any phospholipase activity

In order to assay the effects of phospholipids on the hydrolysis of long chain triglycerides by YLLIP2, we used Intralipide™, which is a fine, well-calibrated emulsion of purified soybean oil stabilized by egg yolk lecithin. Five mL of 30% Intralipide™ (Amersham Pharmacia) were mixed in the pH-stat vessel with 10 mL of a solution containing 150 mM NaCl, 5 mM CaCl₂ and 4 mM NaTDC concentrations, and the activity of YLLIP2 was measured by back-titration. The specific activity of YLLIP2 was found to be 2,200 ± 40 U/mg at pH 6.0, i.e. 6-fold lower than the maximum activity measured using olive oil at the same pH value. Since egg lecithin is present in Intralipide™, we checked whether the lipolytic activity displayed by YLLIP2 could result from phospholipid hydrolysis. YLLIP2 did not show any phospholipase activity when pure egg lecithin was used as substrate (data not shown). Although YLLIP2 activity on Intralipide™ is markedly reduced versus that measured with olive oil, it remains however rather high when compared to those of other known lipases. For the sake of comparison, human gastric lipase displays a specific activity of 600 U/mg at pH 5.4 [26] and human pancreatic lipase does not act on pure Intralipide™ although it is the main lipase involved in the digestion of triglycerides [28]. It is therefore difficult to predict the *in vivo* effects of phospholipids from the *in vitro* measurement of lipase activity on Intralipide™.

4. Discussion

The results presented in this paper clearly indicate that YLLIP2 is able to catalyze the hydrolysis of both long-, medium- and short-chain triglycerides at high rates. The maximum specific activity of YLLIP2 measured on long chain triglycerides, namely 12,260 ± 700 U/mg at pH 6.0, is much higher than the values measured previously with human gastric (600 U/mg at pH 5.4 [26]) and pancreatic (3000 U/mg at pH 7.5 [21]) lipases. Unlike human pancreatic and gastric lipases [26], which hydrolyze tributyrin more rapidly than long-chain triglycerides, YLLIP2 hydrolyzes long-chain triglycerides faster than tributyrin and therefore shows a similar chain length specificity to that of dog gastric lipase (DGL) [25]. It is worth noting that at pH 4.0, the ratio between the YLLIP2 activities on long-chain and short-chain triglycerides is 23. Although YLLIP2 shows its maximum activity at pH 6.0, its activity on long chain TG at pH 4.0 (1590 ± 430 U/mg) is higher than the maximum activity of DGL (1000 U/mg) expressed at pH 4.0, and thus greater than the highest rate of hydrolysis of long-chain triglyceride previously recorded with a lipase [25]. Although

olive oil emulsified in the presence of gum arabic is not really a dietary emulsion, these data indicate that YLLIP2 is potentially active on dietary triglycerides under acidic pH conditions such as those present in the stomach, as well as the small intestine of patients with exocrine pancreatic insufficiency.

The pH profiles of YLLIP2 acting on triglycerides with various chain lengths are very difficult to interpret because the pH can affect both the lipase adsorption at the oil–water interface and the catalytic step [29]. The dramatic downward shift of the optimum activity on long-chain triglycerides towards acidic pH values in the presence of bile salt is extremely puzzling. Since the pH dependence of interfacial enzyme adsorption can make a major contribution to the overall enzymatic process and activity [29,30], the shift in the optimum activity of YLLIP2 occurring at acidic pH values in the presence of NaTDC might reflect the effect of bile salts on the enzyme adsorption rates.

The K_d value of the lipase–interface interaction can be decreased by NaTDC at high pH values, as already observed with DGL [29]. Under these conditions, the pH corresponding to the optimum enzyme turnover is only apparent.

Whatever the substrate used here, YLLIP2 activity increased with increasing bile salt concentration. This behaviour contrasts with the strong inhibitory effects of bile salts observed with many lipases, including pancreatic lipase in the absence of colipase. As previously established in the case of HPL, this inhibition results from the lack of enzyme adsorption occurring at the interface [31]. Both gastric lipase (alone) and pancreatic lipase associated with colipase can counteract the inhibitory effects of bile salts and bind at the oil–water interface in the presence of these competing amphiphiles. It can therefore be concluded that YLLIP2, like gastric lipase, is able to adsorb to triglyceride interfaces in the presence of bile salts, without any requirement for a specific cofactor.

In the absence of bile salts, a lack of activity is observed on both tributyrin and trioctanoin, probably due to the denaturation of YLLIP2 occurring at the high interfacial energy triglyceride–water interface [26]. It has been established that lipases can be rapidly denatured by the unfolding process due to the physical and chemical properties of the interfaces [32–34]. The interfacial denaturation of YLLIP2 (Fig. 6A) can be prevented by adding bile salts, which lower the interfacial energy and prevent the irreversible inactivation of YLLIP2. Likewise, YLLIP2 is not inactivated at the surface of olive oil droplets emulsified with gum arabic, because GA is tensioactive and therefore decreases the oil–water interfacial energy [21]. Since amphiphilic dietary proteins can also prevent lipase denaturation at the oil–water interface, one can assume that YLLIP2 will not be inactivated in the presence of dietary fat in the stomach, although bile salts are not present in this upper part of the GI tract.

YLLIP2 has been assumed to be an acid stable enzyme [15]. However, no systematic studies on its stability at various pH values have been published so far. Here we established for the first time that YLLIP2 is stable under acid conditions (pH 4.0 to 7.0), especially between pH 5.0 and 6.0, but not as stable as preduodenal lipases [35].

The triolein hydrolysis profile was found to be typical of a 1,3-regioselective lipase generating 2-monoglycerides. YLLIP2 therefore shows a similar regioselectivity to that of fungal lipases belonging to the same lipase family [23] as well as those of pancreatic lipases [27,36]. Diglycerides occur only transiently, whereas monoglycerides accumulate, unless the non-enzymatic isomerisation of 1,2(2,3)-DG occurs and renders them subject to lipase hydrolysis (data not shown), as previously observed with pancreatic lipase [36]. YLLIP2 is thus strictly specific of primary ester bond hydrolysis in glycerides. In addition, YLLIP2 was found to display a slight stereopreference for the ester group at position *sn*-3 of triglycerides.

In conclusion, YLLIP2 possesses several biochemical properties that are similar to those of mammalian digestive lipases: (i) a high level of activity on long-chain TG reaching an optimum at pH 6.0, (ii) the absence of inhibition by bile salts, (iii) the same pattern of regioselectivity as human pancreatic lipase, generating 2-MG and FFA, the lipolysis products absorbed at the intestinal level, and iv) a fairly high level of stability at low pH values at 37 °C. YLLIP2 is therefore tailored to act optimally under the physiological conditions pertaining in the gastro-intestinal tract. Its sensitivity to digestive proteases still remains to be tested, however. The activity of YLLIP2 at pH 4.0 is very high in comparison with other characterized lipases, and YLLIP2 might be an ideal candidate for enzyme replacement therapy for pancreatic exocrine insufficiency. Mammalian-like digestive lipases obtained from either bacterial [37] or fungal [38] sources or by over-expressing cloned mammalian gastric lipase genes [22,39] are urgently required at present for the treatment of fat malabsorption in patients with pancreatic exocrine lipase insufficiency [40], such as those with cystic fibrosis, chronic pancreatitis, and prematurely born infants. According to Raimondo et al. [41], to alleviate steatorrhea in patients with human pancreatic insufficiency, an ideal lipase should (1) maintain adequate levels of lipolytic activity at acidic and relatively neutral pH levels and in the presence of normal and low physiological concentrations of intra-intestinal bile acids, (2) resist proteolytic digestion, and (3) actively hydrolyse dietary TG. However, most of the lipases tested to date, including microbial lipases, are either inactivated by gastric acidity or physiological concentrations of bile salts in the lumen of the small intestine [38] or degraded by digestive tract proteases. Among the lipases currently under investigation apart from pancreatic lipase, only gastric lipase has been reported so far to be active and stable at very low pH levels and in the presence of bile salts [28]. Due to its unique biochemical properties, YLLIP2 has great potential for use as a therapeutic tool on patients with pancreatic exocrine insufficiency. It is however less stable than gastric lipase at low pH values such as those found in the stomach in the absence of a meal [28] and an enteric coating might be required for this enzyme.

Due to its extremely high lipase activity and the high production yield (g/L) obtained with genetically-modified strains of *Y. lipolytica*, YLLIP2 is also a good candidate for biotechnological applications on the industrial scale (e.g. in the

production of food ingredients, it could be used to catalyze triglyceride transesterification reactions [11]). It will be most interesting to follow future developments in clinical research as well as the biotechnological applications of this unique microbial enzyme.

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