IBILCE – Instituto de Biociências, Letras e Ciências Exatas

Campus de São José do Rio Preto

"CARACTERIZAÇÃO BIOQUÍMICA DE LIPASE EXTRAÍDA DE SEMENTES OLEAGINOSAS DE *Pachira aquatica*"

Patricia Peres Polizelli

Tese apresentada ao programa de pós-graduação em Biofísica Molecular do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista, como parte das exigências para a obtenção do título de Doutor em Biofísica Molecular.

São José do Rio Preto – SP

Agosto de 2008

Universidade Estadual Paulista

Instituto de Biociências, Letras e Ciências Exatas

"CARACTERIZAÇÃO BIOQUÍMICA DE LIPASE EXTRAÍDA DE SEMENTES OLEAGINOSAS DE *Pachira aquatica*"

Patricia Peres Polizelli

Prof. Dr. Gustavo Orlando Bonilla Rodriguez

Orientador

São José do Rio Preto – SP

2008

PATRICIA PERES POLIZELLI

CARACTERIZAÇÃO BIOQUÍMICA DE LIPASE EXTRAÍDA DE SEMENTES OLEAGINOSAS DE *Pachira aquatica*

COMISSÃO JULGADORA

TESE PARA OBTENÇÃO DO GRAU DE DOUTOR

Prof. Dr. Gustavo Orlando Bonilla Rodriguez

Profa. Dra. Maria de Lourdes Teixeira de Moraes Polizeli

Profa. Dra. Valéria Marta Gomes de Lima

Prof. Dr. Marcio José Tiera

Prof. Dr. Roberto da Silva

Profa. Dra. Fátima Ventura Pereira-Meirelles

Prof. Dr. Rubens Monti

Profa. Dra. Iêda Aparecida Pastre Fertonani

São José do Rio Preto, 22 de Agosto de 2008.

Dedico este trabalho,

À Deus, que foi meu suporte para chegar até aqui,

À toda minha família que me proporcionou a oportunidade de chegar até aqui e pela força e conforto,

Ao Marcos por tudo que ele foi, é, e sempre será na minha vida e,

À querida Izaura, que agora me olha do céu.

AGRADECIMENTOS

Agradeço inicialmente à Deus pela força espiritual durante toda minha vida.

Ao Prof. Dr. Gustavo Orlando Bonilla Rodriguez, que é um grande exemplo de profissional e foi mais que um orientador, foi sempre um amigo ao longo desta caminhada.

Ao Prof. Dr. Márcio José Tiera pelo apoio e sugestões feitas no decorrer do trabalho.

À todos os professores dos Departamentos de química e física, não menos importantes, minha gratidão pelo convívio e amizade.

Aos Profs. membros da banca do exame de qualificação, Prof. Dr. Roberto da Silva e Profa. Dra. Ieda Ap. Pastre Fertonani, pela colaboração crítica e atenciosa.

Aos Profs. membros da banca examinadora, meu muito obrigado.

Aos funcionários pela pronta ajuda e paciência: Ilva Cecílio Bernades, Rita de Cássia, Paulo Salinas, Antônio Barbosa, Eliani Nobuco I. Ohira, João Rodriguez dos Santos, Claudinei A. Nobile, Silvia Emiko Kasama, Rosana M. Gomes Ferro e Rosemar R. de Carvalho Brena.

Aos amigos, Rejane, Ana Lúcia, Letícia, Evandro, Leliane, Bárbara, Lílian, Fernanda, Laís, Angélica, Luciana Polotto, Maíra, Maraíssa, Daniela, Hamilton, Fábio, Patricia, Débora, Adriana, e a todos os não mencionados aqui, pela ajuda em incontáveis momentos, pela paciência e pela amizade.

Às estagiárias e amigas, Fernanda, Bárbara e Laís, pela colaboração com este trabalho e por todos os momentos que passamos juntas.

Aos departamentos de física e química e ao Instituo de Biociências, Letras e Ciências Exatas (UNESP) por propiciar minha formação cientifica e pelo apoio e disposição.

À todos os meus familiares, pelo carinho e estímulo que me tornaram capaz de suportar todos os desafios até a conclusão deste trabalho.

Ao Marcos, pelo incentivo, ajuda, encorajamento, paciência, amor e carinho.

À CAPES, pelo auxílio financeiro.

E, finalmente, a todos de maneira geral que contribuíram para que este trabalho se realizasse.

“Dizem que o que todos procuramos é um sentido para a vida. Não penso que seja assim. Penso que o que estamos procurando é uma experiência de estar vivos, de modo que nossas experiências de vida, no plano puramente físico, tenham ressonância no interior do nosso ser e da nossa realidade mais íntimos, de modo que realmente sintamos o enlevo de estar vivos.”

Joseph Campbell

Sumário

Lista de Figuras

Lista de Tabelas

Lista de abreviaturas

EDTA	Ácido etilenodiamino tetra-acético
DTT	Ditiotreitol
K_M	Constante de Michaelis
V_{max}	Velocidade máxima
p-NPP	*p*-nitrofenilpalmitato
p-NPA	*p*-nitrofenilacetato
DMSO	Dimetilsulfóxido
SDS	Dodecilsulfato de sódio
SDS-PAGE	Eletroforese em poliacrilamida desnaturante
SOS	Octilsulfato de sódio
CTAB	Cetiltrimetilamônio brometo
TTAB	Tetradeciltimetilamônio brometo
DTAB	Dodeciltrimetilamônio brometo
CMC	Concentração micelar critica
PEG	polietilenoglicol
$CaCl_2$	Cloreto de cálcio
v/v	Volume/volume

Resumo

Uma nova lipase extraída de sementes oleaginosas de *Pachira aquatica* foi purificada por SDS-PAGE obtendo-se uma enzima com massa molecular de aproximadamente 55 kDa. Esta exibiu atividade máxima a 40°C e pH 8,0 e foi estável na faixa de 4 a 50°C e a pH 9.0 mostrando estabilidade em pH alcalino. A atividade enzimática aumentou na presença de Ca^{++} e Mg^{++}, mas foi inibida por Hg^{++}, Mn^{++}, Zn^{++}, Al^{+++} e por vários agentes oxidantes e redutores, principalmente DTT e β-mercaptoetanol. Na presença de solventes orgânicos a atividade foi estimulada por metanol. Os valores de K_M e V_{max}, foram 1,65 mM e 37,3 $\mu mol.mL^{-1}.min^{-1}$, respectivamente usando *p*-nitrofenil acetato como substrato. A enzima mostrou preferência por ácidos graxos de cadeias longas, mas demonstrou atividade contra uma grande faixa de substratos. O extrato enzimático bruto foi testado para a hidrólise de óleo de soja e resíduos de frigorífico avícola. A condição ideal é 40°C e pH no valor de 7,0-9,0 para óleo de soja e o resíduo, respectivamente. A enzima foi estável na presença de várias formulações de detergentes comerciais, mas instável na presença de H_2O_2. A lipase não foi eficiente em reduzir o tamanho médio de partículas de gordura; altas doses foram requeridas para obter-se uma redução de 13 % após 72 horas de tratamento. A atividade enzimática foi aumentada na presença do surfactante catiônico brometo de cetiltrimetilamonio (CTAB) e sais biliares, onde surfactantes aniônicos e não-iônicos mostraram efeito inibitório. A incubação da enzima com soluções aquosas de PEG 12.000, 8.000 e 200, ativou a lipase e a máxima ativação (61%) ocorreu com PEG 12.000. Este efeito pode ser devido à exposição de alguns resíduos hidrofóbicos localizados na vizinhança do sitio ativo ou por agregação enzimática. No processo de imobilização da lipase em esferas de alginato de cálcio, estas foram preparadas pelo método de difusão usando cloreto de cálcio como agente geleificante. Foram avaliados: a quantidade de enzima encapsulada, a liberação, a

atividade e as características das esferas. Análises comparativas também foram realizadas com a enzima, nas formas livre e imobilizada, para a determinação da temperatura e pH ótimos e estabilidade de estocagem. A enzima imobilizada foi estável e reutilizada várias vezes sem perda da atividade lipolítica. O intervalo de temperatura ótima, tanto pra a enzima livre quanto imobilizada foi na faixa de 30 a 40°C. Ambas as formas da enzima mostraram maior atividade em pH 7,5-8,5. A estabilidade de estocagem foi maior para a enzima imobilizada que para a livre.

Abstract

A new lipase from seeds of *Pachira aquatica* was purified to homogeneity by SDS-PAGE obtaining an enzyme with a molecular weight of approximately 55 kDa. The lipase exhibited maximum activity at 40°C and pH 8.0, and was stable at the range from 4 to 50°C and pH 9.0 showed good stability in the alkaline pH. The enzyme activity increased in the presence of Ca^{++} and Mg^{++}, but was inhibited by Hg^{++}, Mn^{++}, Zn^{++}, Al^{+++} and various oxidizing and reducing agents, mainly DTT and β-mercaptoethanol. In the presence of organic solvents its activity was stimulated by methanol. The values of K_M and V_{max}, were 1.65 mM and 37.3 $\mu mol.mL^{-1}.min^{-1}$, respectively using *p*-nitrophenylacetate as substrate. The enzyme showed preference for esters of long chain fatty acids, but demonstrated significant activity against a wide range of substrates. The enzymatic extract was tested for the hydrolysis of soybean oil and wastewater from a poultry processing plant. It was determined that it should be carried out at a temperature of 40°C and an optimum pH 7.0-9.0 for soybean oil and poultry processing plant, respectively. The enzyme was stable in the presence of some commercial detergent formulations but unstable in the presence of H_2O_2. The lipase was not efficient in reducing average fat particle weight; high doses were required to obtain a reduction of 13% after 72 hours of treatment. The activity was enhanced by the cationic surfactant, CTAB and bile salts, whereas anionic and nonionic surfactants showed an inhibitory effect. Aqueous solutions of PEG, 12,000, 8,000 e 200, activated the lipase and maximum activation (61%) occurred in PEG 12,000. This effect on lipase that can be due to exposition of some hydrophobic residues located in the vicinity of the active site or enzymatic aggregation. In the immobilization of the lipase in beads of calcium alginate, these were prepared by diffusion methods using calcium chloride as gelling

agent. We evaluated enzyme loading, leaching, activity and characterized the beads. Comparative analyses on free and immobilized lipase for determination of optimum temperature, pH, and storage stability were carried out. The immobilized enzymes were stable and were reused several times without loss of enzyme activity. The optimum temperature interval for the free and immobilized enzyme was in the range from 30 to 40°C. Both forms of the enzyme showed higher activity at pH 7.5-8.5. Storage stability of immobilized lipase was higher than that of the free lipase.

1. Introdução

A aplicação biotecnológica de enzimas apresenta vantagens significativas sobre as reações químicas tradicionais. Embora, atualmente, a biotransformação não possa ainda competir com a produção química, já otimizada, de compostos industrialmente importantes, pode gradativamente, substituí-la devido à grande demanda de compostos naturais biodegradáveis, que não causam danos ao meio ambiente.

Dentre os processos biológicos utilizados nas biotransformações, as lipases se destacam por sua versatilidade em processos hidrolíticos e de síntese. São biocatalisadores de muita importância em diferentes áreas, podendo catalisar reações tanto em meio aquoso como em meio orgânico, com teor de água restrito. Sua aplicação é primariamente devido à sua capacidade de utilização de uma ampla gama de substratos, à sua estabilidade frente a temperatura, pH e solventes orgânicos, e à sua quimio-regio e enantiosseletividade. Entre as lipases, aquelas obtidas de vegetais são interessantes por sua facilidade de obtenção, abundância e pouca exploração.

As lipases têm sido utilizadas em uma variedade de segmentos biotecnológicos, como em indústrias de alimentos, de detergentes, agroquímica e oleoquímica. Mas, apesar de todas as vantagens, existem ainda problemas para serem resolvidos no desenvolvimento de processos de aplicações destas enzimas, principalmente do ponto de vista econômico, pois o custo de produção e purificação torna os processos enzimáticos mais caros.

Este trabalho contribui para o desenvolvimento do conhecimento na área da biotecnologia e a grande diversidade brasileira justifica a busca por novos produtores de enzimas que possam ser aplicados na produção de compostos através da biocatálise.

2. Revisão Bibliográfica

2.1Enzimas

A grande variedade de reações bioquímicas que compreendem a vida é mediada por uma série de catalisadores biológicos conhecidos como enzimas. As enzimas constituem a classe de moléculas protéicas mais numerosa e especializada; elas catalisam as milhares de reações químicas que, coletivamente constituem o metabolismo intermediário das células (LEHNINGER, 2004).

As enzimas são catalisadores dos sistemas biológicos altamente eficientes na catálise de diversas reações químicas, porque têm capacidade de se ligar especificamente a uma grande variedade de moléculas, sendo capazes de aumentar a velocidade em até 10^{14} vezes mais que uma reação não catalisada (CAMPBELL, 2001). Agem reduzindo a energia de ativação sem alterar a constante de equilíbrio.

Uma enzima geralmente catalisa uma reação química única ou um conjunto de reações estreitamente relacionadas, sendo que reações colaterais, com formação de subprodutos, raramente ocorrem, em contraste com as reações não catalisadas enzimaticamente. São moléculas extremamente versáteis; participam de diversas reações como hidrólises, transferência de grupos funcionais, oxidação-redução, desidratação e isomerização para mencionar somente as classes mais comuns de reações mediadas enzimaticamente (VOET e VOET, 2004).

A formação de um complexo enzima-substrato é a primeira etapa na catálise enzimática. Os substratos são ligados a uma região específica da enzima, chamada de sitio ativo, contendo os resíduos de aminoácidos que participam diretamente na formação e

quebra de ligações, bem como na ligação e posicionamento do substrato. As forças não covalentes através das quais substratos e outras moléculas ligam-se à enzima são idênticas, em caráter, às forças que ditam a conformação das proteínas: interações de van der Waals, eletrostáticas, hidrofóbicas e ligações de hidrogênio. Entre as teorias e racionalizações que têm sido desenvolvidas para entender a catálise enzimática, os modelos mais ilustrativos foram o mecanismo da chave e fechadura desenvolvido por Emil Fisher em 1894, e o mecanismo de encaixe induzido de Koshland Jr. desenvolvido no final da década de sessenta.

A regulação enzimática ocorre de duas maneiras: pelo controle da disponibilidade e/ou da atividade da enzima. No primeiro caso a quantidade de uma dada enzima na célula depende de ambos sua taxa de síntese e sua taxa de degradação. No segundo caso, a atividade catalítica da enzima pode ser diretamente regulada através de alterações conformacionais e estruturais. A atividade de uma enzima pode assim ser controlada pela variação da afinidade pelo substrato e ainda pode variar com a ligação de moléculas moduladoras (VOET e VOET, 2004).

As enzimas são classificadas e codificadas pelo NC-IUBMB (*Nomenclature Committee of the International Union of Biochemistry and Molecular Biology*) de acordo com a reação catalisada. A nomenclatura utiliza a abreviação E.C. (*Enzyme Commission*) seguida de até 4 dígitos referentes à classe e subclasses a que pertence a enzima.

2.2 Biotecnologia de proteínas

A biotecnologia representa a aplicação comercial de descobertas científicas no setor tecnológico (WALSH e HEADON, 1994). Muitos processos biotecnológicos foram desenvolvidos dependentes da conversão enzimática de substrato nos produtos desejados. Processos tais como a inclusão de enzimas proteolíticas em preparações de detergentes foram introduzidos no começo do século 20, embora não tenham sido aplicados em grande escala até os anos 60. De fato foi durante 1940 e 1960 que muitos processos industriais dependentes das biotransformações catalisadas por enzimas foram desenvolvidos.

Uma grande variedade de proteínas encontra aplicações industriais. Estas incluem enzimas, anticorpos, hormônios, fatores sanguíneos, fatores de crescimento e regulatórios e são obtidas de diferentes fontes incluindo microrganismos, plantas e animais (WALSH e HEADON, 1994).

A maioria das enzimas usadas pela indústria catalisa reações de hidrólise; estas incluem amilases, proteases e lipases. Tais enzimas são extensivamente usadas na produção de alimentos, bebidas e em vários setores. A aplicação de enzimas como biocatalisadores em processos industriais tem se dado em indústrias alimentícias, têxtil, de papel e celulose, detergentes, óleos e gorduras, na síntese de intermediários de fármacos, herbicidas e substâncias químicas finas (ROBERTSON e STEER, 2004; HASAN et al., 2006). Comparando-se catalisadores químicos e biológicos, a principal vantagem das enzimas é a capacidade que estas possuem de catalisar reações sob condições suaves de temperatura e pressão. Tais características têm como benefício a redução do consumo energético, dos riscos de desnaturação térmica de compostos termolábeis e menor formação de subprodutos (BRIGIDA, 2006).

Apesar das vantagens muitos processos ainda não são viáveis, devido aos custos de obtenção das enzimas e sensibilidade a altas temperaturas. Abordagens como a imobilização de enzimas e a obtenção de enzimas de microrganismos têm procurado ultrapassar esses obstáculos.

23 Lipases

As lipases são classificadas como hidrolases, enzimas solúveis em água que catalisam a hidrólise de ligações ésteres (figura 1) em moléculas de triacilgliceróis (SALAH et al., 2006). Entretanto, as lipases (E.C. 3.1.1.3) são capazes de catalisar não somente reações de hidrólise, mas também de síntese em meios com reduzida disponibilidade de água, como reações de esterificação, interesterificação, transesterificação, alcoólise e aminólise, e de operar sobre substratos não-naturais (BJORKLING et al., 1991; JAEGER e EGGERT, 2002; SHARMA et al., 2002; KAMBOUROVA et al., 2003; SAXENA et al., 2003). Essas reações catalisadas por lipases são processadas com alta régio, quimio e enatiosseletividade, tornando as lipases um grupo importante de biocatalisadores.

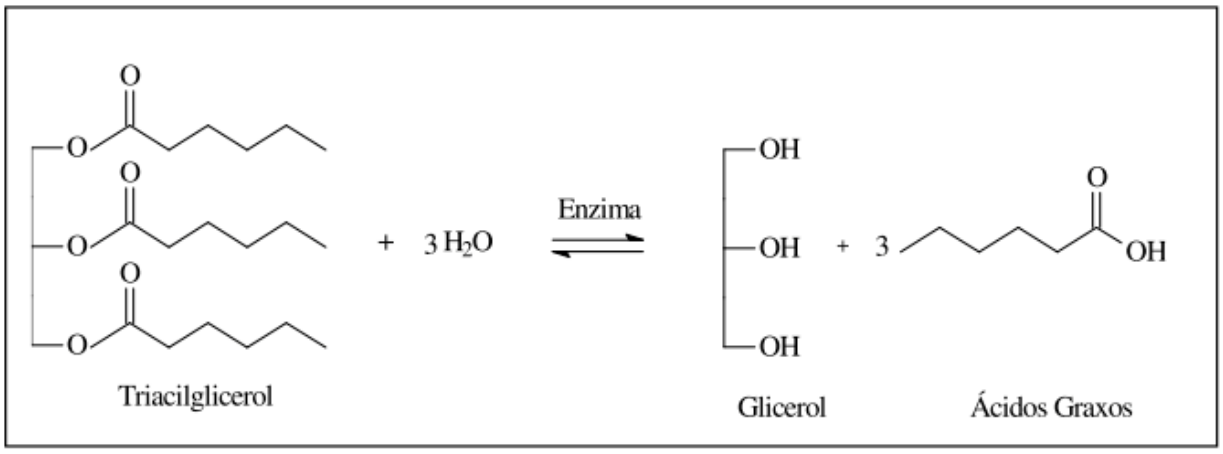


Figura 1. Reação de hidrólise catalisada por uma triacilglicerol lipase.

A diferenciação entre lipases (E.C. 3.1.1.3) e esterases (E.C. 3.1.1.1) foi estudada por vários autores mas ainda existem algumas controvérsias. Em 1958, Sarda e Desnuelle propuseram definir as lipases a partir da propriedade de ativação na presença de uma interface lipídeo/água. Assim, as lipases seriam ativadas na presença de ésteres emulsionados, enquanto as esterases não apresentariam esta propriedade, exercendo sua função hidrolítica sobre substratos solúveis em água.

A determinação da estrutura tridimensional de algumas lipases (BRADY et al., 1990; WINKLER et al., 1990; SCHRAG et al., 1991) propiciou a identificação de uma "tampa" hidrofóbica cobrindo o sítio ativo destas enzimas, que ao interagir com a interface lipídeo/água sofreria uma mudança conformacional, expondo o sítio ativo. Entretanto, observou-se que a presença da "tampa" não está necessariamente correlacionada com a ativação interfacial, pois, foram descritas lipases que apresentam "tampa", mas não sofrem ativação interfacial (JAEGER e REETZ, 1998). Além disso, as cutinases, lipases "verdadeiras", não apresentam a "tampa" e não precisam da interface para exercer sua

atividade catalítica (CYGLER e SCHRAG, 1997). Atualmente as lipases são definidas como carboxilesterases que hidrolisam acilgliceróis de cadeia longa, com mais de 10 átomos de carbono (FERRATO etal., 1997; VERGER, 1997). Contudo, a maioria das lipases pode hidrolisar os substratos de esterases, enquanto o inverso não é verdadeiro (JAEGER et al., 1999).

As lipases são encontradas em tecidos de vários animais e vegetais, e podem ser produzidas por fermentação usando várias espécies de microrganismos. Em eucariotos, as lipases estão envolvidas em vários estágios do metabolismo incluindo digestão de gordura, absorção, reconstituição e metabolismo de lipoproteínas. Em plantas, as lipases são encontradas em tecidos de reserva de energia (SHARMA, et al., 2001) e as sementes oleaginosas provavelmente usam esta enzima durante os primeiros estágios de germinação, iniciando a metabolização de triglicerídeos estocados através da hidrólise dos ácidos graxos. Os ácidos graxos liberados são levados às vias de produção de energia e assim, fornecem energia para o crescimento do embrião (STAUBMANN et al., 1999). Cabe aqui observar que os vegetais possuem a chamada Via do Glioxilato, a qual permite a obtenção de glicose a partir de ácidos graxos, permitindo a síntese posterior de celulose e outros carboidratos.

Em algumas sementes, as lipases estão presentes na forma dormente (NCUBE e READ, 1995), contudo, na maioria das sementes, as lipases são produzidas somente após a fase de embebição (BEISSON et al., 2001). A alta especificidade apresentada por algumas lipases encontradas em plantas é uma característica interessante que pode ser explorada para propostas biotecnológicas (LIN et al., 1986; HUANG et al., 1988 apud MOHAMED et al., 2000; STAUBMANN et al., 1999).

Muitos estudos são realizados para a compreensão da relação estrutura-função das lipases para fornecer suas propriedades e ampliar suas aplicações industriais. As estruturas tridimensionais de várias lipases mostram que apresentam um enovelamento comum do tipo α/β hidrolase (figura 2) (OLLIS et al., 1992 apud SALAH et al., 2006; NOBLE, et al., 1993; SCHRAG e CYGLER, 1997; UPPENBERG, et al., 1994; TYNDALL, et al., 2002). O núcleo da estrutura tridimensional é composto de uma folha β central consistindo de diferentes fitas β rodeado por 3porções de α-hélice.

Essas enzimas são classificadas como serino hidrolases possuindo um sitio catalítico composto pela tríade: Ser-His-Asp/Glu (BLOW et al., 1969; WRIGHT et al., 1969; BRADY et al., 1990; WINKLER et al., 1990; MARTINEZ et al., 1992; UPPENBERG et al., 1994; ROUSSELL et al., 2002) semelhante à encontrada em serino proteases. O nucleófilo catalítico (serina) está localizado especificamente no C-terminal da fita β5 em um pentapeptídeo altamente conservado GXSXG, que constitui o ângulo nucleofílico, que só é alcançado quando os resíduos localizados nas posições -2 e +2 em relação ao resíduo de serina tem substituições pequenas, o que explica a constante presença de glicina nestas posições (JAEGER e REETZ, 1998).

O sítio ativo fica localizado dentro de uma cavidade hidrofóbica que pode ser superficial ou profunda, de acordo com a homologia a que pertence a enzima. Nesta cavidade aloja-se o ácido graxo de modo a posicionar a ligação éster alinhada com o sítio ativo. Na maioria dos casos, o sitio ativo é coberto por uma "tampa" hidrofóbica composta de uma ou duas seqüências peptídicas em α-hélice, que previne o acesso do substrato. Após a interação da lipase com a interface lipídeo/água, o sitio ativo torna-se acessível e uma grande área hidrofóbica é exposta por rearranjos estruturais, os quais incluem o movimento

da “tampa” (BRADY et al., 1990; WINKLER, et al., 1990; BRZOZOWSKI et al., 1991; van TILBEURGH et al., 1993). Mas, como foi dito anteriormente nem todas as lipases apresentam “tampa”.

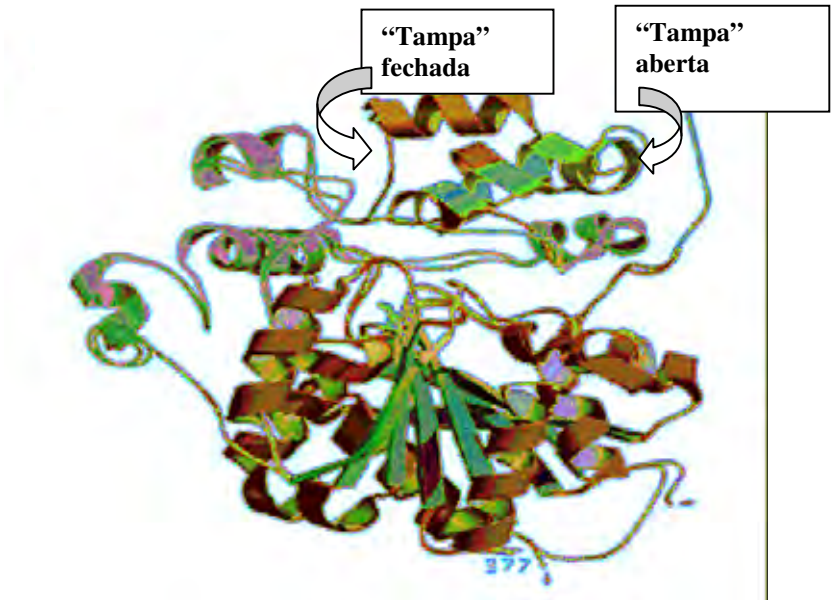


Figura 2. Estrutura esquemática de lipase gástrica de cachorro (ROUSSEL et al., 2002). As estruturas secundárias são: α hélices; folhas; *random coil*.

As lipases que possuem a “tampa” apresentam-se em duas conformações distintas: fechada, com a “tampa” cobrindo o sitio ativo, caracterizada por um sítio ativo desocupado preenchido por moléculas de solvente; e aberta com a “tampa” desobstruindo o sitio ativo e proporcionando uma grande superfície hidrofóbica de interação lipídeo/proteína. Ao menos algumas lipases podem estar em equilíbrio dinâmico entre a forma aberta e fechada, quando em solução, e a abertura da “tampa” pode ser facilitada por solventes orgânicos, provavelmente por diminuição da constante dielétrica do meio (BRZOZOWSKI et al., 2000).

Devido à semelhança estrutural entre o sítio ativo de lipases e proteases, o mecanismo catalítico para lipases segue o modelo proposto para a quimotripsina (figura 3).

A hidrólise ocorre em duas etapas. Inicialmente, a histidina aumenta a nucleofilicidade do grupo hidroxila da serina; ocorre, então, um ataque nucleofílico do oxigênio da hidroxila serínica ao carbono carbonílico da ligação da cadeia do substrato, formando um intermediário tetraédrico. O anel imidazólico da histidina fica protonado e carregado positivamente, sendo estabilizado pela carga negativa do resíduo ácido. O intermediário tetraédrico é estabilizado por duas pontes de hidrogênio formadas com ligações amida dos resíduos de aminoácidos. Um álcool é liberado, deixando um complexo acil-enzima.

Em um segundo ataque nucleofílico por um íon hidroxila e o ácido graxo é liberado e a enzima regenerada (JAEGER et al., 1994). Este mecanismo tem sido confirmado por estudos de ligação de inibidores a lipases e pela análise estrutural destas proteínas (JAEGER et al., 1999).

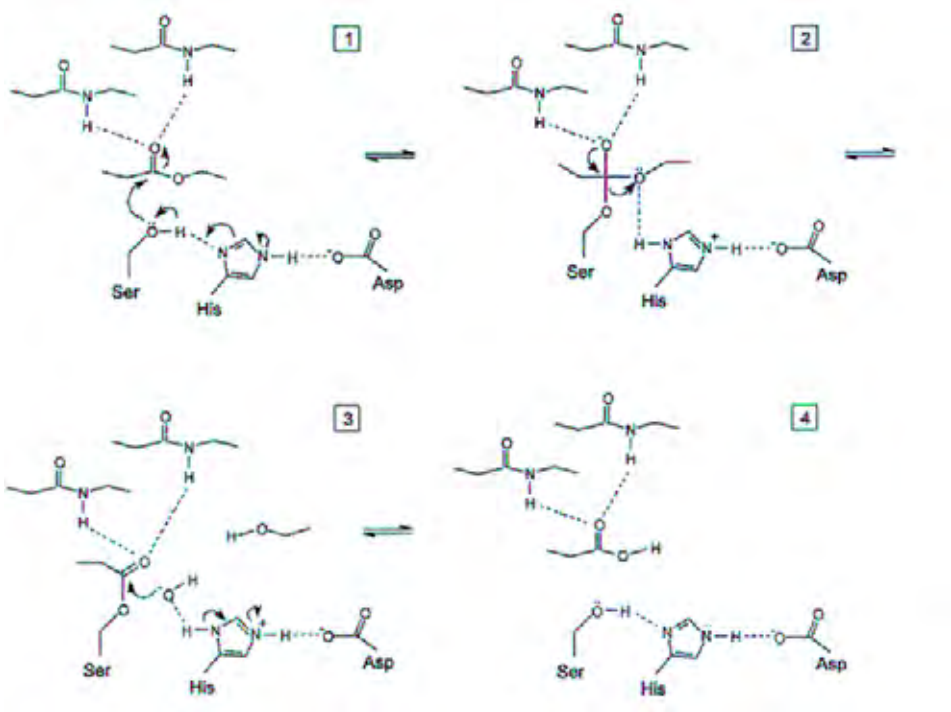


Figura 3. Mecanismo catalítico proposto para lipases. Adaptado de JAEGER et al., (1999).

2.4 Aplicação industrial de lipases

A aplicação de lipases é ampla podendo ser utilizada para a obtenção de diversos produtos. Diante da versatilidade das lipases frente às reações por elas catalisadas, a aplicação industrial destas enzimas engloba química orgânica, formulação de detergentes, industrias como oleoquímica, laticínios, agroquímica, manufatura de papel, nutrição, cosméticos,farmacêutica (RUBIN e DENNIS, 1997; SHARMA et al., 2001; SAXENA et al., 2003) e processamento de óleos e gorduras (MASSE et al., 2001; LEAL et al., 2002; MASSE et al., 2003; CAMMAROTA e FREIRE, 2006). Além destes, outros produtos de ampla aplicação como poliésteres e policarbonatos podem ser obtidos através de lipases. Uma linha crescente na aplicação destas enzimas é o estudo das reações de transesterificação para a obtenção de biodiesel, um produto promissor que tem recebido muita atenção nos últimos anos.

A aplicação de lipases advém, principalmente, da capacidade em catalisar reações com uma grande variedade de substratos não-naturais, permitindo o seu uso para a obtenção de diversos tipos de compostos. E ainda, podem ser usadas em solventes orgânicos em que a maioria dos compostos (substratos e produtos) é solúvel. A indústria farmacêutica tem demonstrado grande interesse nestas enzimas, visto que a atividade biológica de muitas drogas racêmicas, às vezes, reside em um único enantiômero (SUAN e SARDIMI, 2004, GOTOR-FERNANDEZ, et al.,2006). Atualmente, diversas enzimas são utilizadas em escala industrial em reações sintéticas e de isomerização, como para a produção de xaropes de milho com alto teor de frutose, pela ação da xilose isomerase, que catalisa a isomerização de D-glicose e D-frutose, e a preparação de penicilina semi-sintética catalisada pela penicilina amidase (SCHMID et al., 2001).

Dentre os processos bioquímicos reportados na literatura, as lipases representam cerca de 35% dentre as enzimas empregadas. No entanto, o uso dessas enzimas em escala industrial ainda é escasso, devido aos elevados custos de produção. Com o objetivo de intensificar a utilização de lipases em escala piloto e industrial, estudos de fontes vegetais, como sementes, látex, folhas e caules, têm crescido nos últimos anos. Dentre as lipases vegetais, as mais estudadas são as extraídas de cereais e óleos de sementes, localizadas em diferentes tecidos e normalmente ativadas durante a germinação (PASQUES e MACEDO, 2006).

2.5 Agentes tensoativos

A interação entre um surfactante carregado e uma lipase em solução aquosa é interessante para várias aplicações, tais como: liberação de drogas, cosméticos, inclusão em formulações de detergentes e para estudar as interações entre proteínas de membrana e lipídeos (SAVELLI et al., 2000).

Os agentes tensoativos ou surfactantes são moléculas compostas por uma porção hidrofílica, região da molécula que possui afinidade pela molécula de água e que é denominada ¨cabeça polar¨, e uma porção hidrofóbica, região da molécula que não possui afinidade pela molécula de água e que é denominada ¨cauda apolar¨ (figura 4).

Os tensoativos podem ser classificados em: iônicos, que possuem cabeça carregada positiva ou negativamente; não-iônicos, possuindo cabeça polar capaz de realizar ligações de hidrogênio com a água ou ainda zwiteriônicos possuindo cabeça caracterizada por um dipolo. As moléculas tensoativas em solução aquosa podem exibir diversos tipos de comportamento, dependendo da concentração e características da solução. Em soluções com concentração de tensoativo abaixo da concentração micelar crítica (CMC), específica

para cada tensoativo, as moléculas deste deslocam-se na interface ar-água (figura 4), projetando suas caudas apolares em direção à fase ar, de forma a minimizar o contato com a água (TANFORD, 1980).

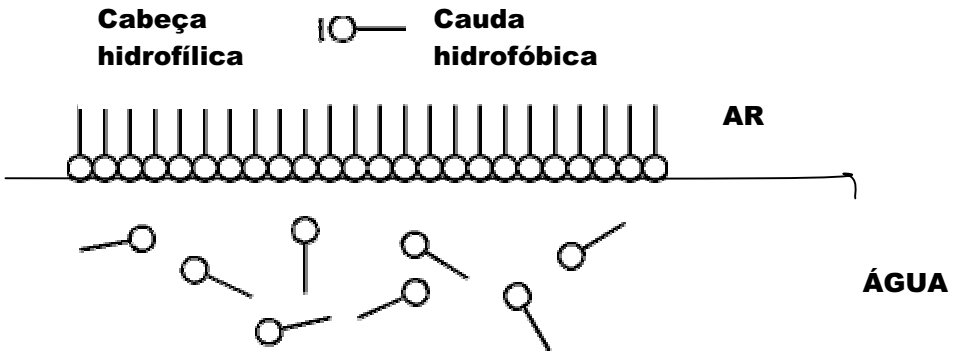


Figura 4. Estrutura do monômero de um tensoativo. Representação esquemática dos tensoativos na interface água/ar. Adaptado: **laser.physics.sunysb.edu**

Acima da CMC além da adsorção na interface as moléculas formam agregados, e estes em solução podem se agregar em uma variedade de estruturas: aumentando a concentração de surfactante na mistura surfactante/água, a estrutura formada obedece à seqüência: micela esférica, fase cúbica, micela cilíndrica, cúbica, lamela e as correspondentes estruturas reversas (JÖNSSON et al., 1998).

A formação de micelas envolve um balanço complexo de forças intermoleculares, que incluem interações de van der Waals, eletrostáticas, estéricas, hidrofóbicas e ligações de hidrogênio (TANFORD, 1980). As micelas são entidades dinâmicas formadas pela agregação não-covalente de monômeros de tensoativos e podem ser esféricas, cilíndricas ou planas (disco ou bicamada). A forma e o tamanho podem ser modificados trocando a estrutura química do tensoativo, bem como, variando sua concentração, temperatura, pH e força iônica (JÖNSSON et al., 1998).

Os tensoativos de uma forma geral são capazes de ligar-se à proteínas. As proteínas hidrofílicas são capazes de interagir cooperativamente com tensoativos iônicos, resultando em alterações conformacionais e até mesmo na desnaturação.

Os monômeros de um tensoativo iônico ligam-se primariamente por interações eletrostáticas aos resíduos de aminoácidos de carga oposta na superfície da proteína (ligação sitio-específica), que induz extensão da estrutura terciária permitindo maior interação das caudas apolares do tensoativo com o interior apolar da proteína (ligação cooperativa não-específica) (GODDARD e ANANTHAPADMANABHAN, 1993).

Na presença de tensoativos não-iônicos há evidências de que as proteínas interagem muito pouco, não sendo incorporadas nas micelas destes tensoativos. Entretanto, interações hidrofóbicas não específicas podem ocorrer .

A ligação de surfactantes a proteínas é influenciada por algumas variáveis tais como, a natureza e o comprimento da cadeia do surfactante, pH, força iônica, temperatura e a natureza da proteína (GODDARD e ANANTHAPADMANABHAN, 1993).

O polietilenoglicol (PEG) é um polímero sintético, solúvel em água, atóxico, muito utilizado em biotecnologia. É capaz de formar sistemas de duas fases com sal ou dextrana que pode ser utilizado para purificação e separação de proteínas. As forças motrizes para a separação parecem ser interações hidrofóbicas entre polímero-proteína (HUDDLESTON et al., 1994). A influência de moléculas de PEG na atividade catalítica de algumas enzimas foi verificada em alguns estudos reportados na literatura. Segundo Grimonprez e Johansson (1996) a atividade de fosfofrutoquinase e da gliceraldeído 1,3 bifostato desidrogenase é 1,3 e 1,55 vezes maior na presença de PEG. Esse efeito pode ser atribuído à hidrofobicidade das enzimas como citado por Lee e Lee (1981), Furness et al. (1998) e Jonsson et al. (2003), à atividade de água (NINNI et al., 1999; KULKARNI et al., 2000), à exclusão

estérica (ARAKAWA e THIMASHEFF, 1985) e estresse osmótico (PARSEGIAN et al., 1995).

Pancera (2002) observou que a atividade da glicose 6- fosfato desidrogenase aumentou em torno de 5 a 20% na presença de PEG 400 e 4000 independente da massa molecular e Grimonprez e Johansson (1996) observaram, na presença de PEG 3350, um aumento de 30 e 55% para fosfofrutoquinase e gliceraldeido 3- fosfato. Estudos da interação de PEG e lisozima mostraram que independentemente da massa molecular não ocorreu influência na atividade da enzima. A atividade de uma enzima está relacionada ao seu sitio catalítico e sua estrutura, assim, dependendo da estrutura e sitio de uma enzima pode ocorrer o aumento ou diminuição da atividade na presença de PEG. Dessa forma, predizer o efeito de PEG no comportamento das enzimas de forma generalizada é bastante complicado (PANCERA, 2006).

2.7 Imobilização de enzimas

A imobilização é um método de fixação ou encapsulação de uma enzima, que possibilita diversas aplicações deste catalisador com manutenção da atividade catalítica. O principal interesse em imobilizar uma enzima é obter um biocatalisador com atividade e estabilidade que não sejam afetadas durante um processo, em comparação à sua forma livre. Além disso, não deverão ocorrer alterações estruturais, nem modificações no sitio ativo (BRYJAK et al., 1997; MORENO et al., 1997).

Entre as vantagens deste processo estão o aumento da estabilidade térmica e operacional da maioria das enzimas (TELEFONCU et al., 1990; PANCREAC´H et al., 1997), a maior facilidade de recuperação e separação de produtos e catalisador, contínua reutilização (ZADA, 2006), utilização por períodos prolongados, catalisadores mais seletivos (FERNANDEZ-LORENTE et al., 2001 e 2003) e facilidade na interrupção da

reação. Em alguns casos é possível ainda obter-se produtos com seletividade alterada e hiperativados. A imobilização também auxilia na dispersão homogênea da enzima no meio, o que é essencial para a condução de reações enzimáticas. Contudo, algumas desvantagens são às vezes verificadas, como perda ou redução de atividade por unidade de volume, limitação difusional e custo de preparação (GOMES et al., 2006).

Os efeitos da imobilização sobre a atividade das enzimas resultam da interação de diferentes fatores, como: modificação da estrutura tridimensional da enzima, modificações do micro-ambiente, alterações nas interações eletrostáticas, fenômenos de difusão e transferência de massa no interior do complexo e impedimento estérico (di CARLI, 2006). A imobilização pode ocorrer através da adsorção ou ligação da enzima em um material insolúvel, pelo uso de um reagente multifuncional através de ligações cruzadas e por confinamento em matrizes formadas por géis poliméricos ou encapsulação (MURRAY et al., 1997).

Na escolha de um suporte algumas características devem ser observadas, como: permeabilidade, resistência a bactérias, morfologia, resistência mecânica, insolubilidade, composição e área superficial. Os suportes podem ainda ser classificados de acordo com a morfologia: em porosos, não-porosos ou estrutura de gel. Os porosos apresentam grande área superficial interna e protegida da turbulência; os não-porosos demonstram baixa área superficial interna, mas não apresentam resistência à transferência de massa; quanto aos géis, estes são utilizados nos casos em que a grade formada seja de malha suficiente para reter a proteína sem implicar em restrição difusional para o substrato (BRIGIDA, 2006)

A morfologia do poro é de fundamental importância, pois o poro não deve liberar a enzima, mas assegurar livre acesso do substrato e produtos de reação. Todavia, quanto maior o poro menor a área interfacial.

A imobilização via confinamento ou microencapsulação consiste em confinar a enzima em um polímero insolúvel (envolvimento em fibra ou gel) ou em uma microcápsula (microencapsulação). Dessa forma, moléculas grandes, tais como as enzimas, não são capazes de se difundir, enquanto que pequenas moléculas, como substratos e produtos, são. A vantagem da utilização desta técnica é que a enzima não interage quimicamente com o polímero, evitando assim, a desnaturação. Contudo, a velocidade de difusão dos substratos e produtos é um fator limitante (DELLA-VECHIA et al., 2004). O mais popular método de aprisionamento é o que utiliza matrizes de gel, sendo que os géis de cálcio são os mais utilizados devido a sua resistência ao ataque por microrganismos ou enzimas. Um aspecto negativo deste método é a limitação difusional dos substratos e produtos na matriz (BATISTA, 2005).

Os hidrogéis, constituídos de polímeros naturais (polissacarídeos) apresentam uma série de vantagens para a imobilização: atoxicidade, biocompatibilidade, baixo custo e são encontrados em abundância na natureza. As partículas de hidrogéis podem ser preparadas usando vários tipos de polímeros: gelatina, alginatos, carragenatos, pectina, agar. O alginato é um polímero linear constituído de resíduos de α-L-gulurônico (G) e β-D-manurônico (M) (figura 5), presentes em proporções e seqüência variável. São encontrados em algas marrons, onde agem como componente estrutural na parede celular e nos espaços intracelulares, promovendo rigidez e ao mesmo tempo flexibilidade. Podem ser encontrados também em algumas espécies de microrganismos, atuando como polissacarídeo capsular (NUSSINOVITCH, 1997 apud OLIVEIRA, 2004).

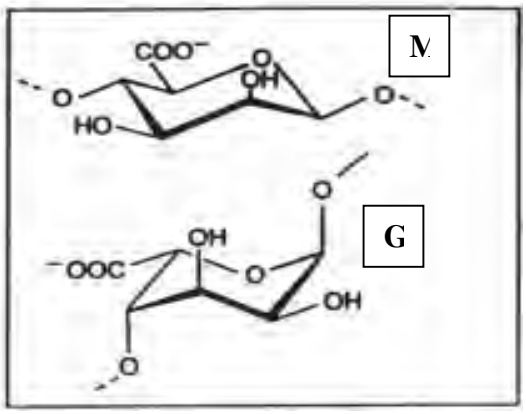


Figura 5. Resíduos de açúcares presentes na composição do alginato, (**M)** representa um resíduo (1-4)-β-D- Manurônico e (**G**) um resíduo (1-4)α-L-gulurônico. (Adaptado de NUSSINOVITCH, 1997 citado por OLIVEIRA, 2004).

Os resíduos de M e G podem estar dispostos continuamente, formando blocos homopoliméricos ou alternadamente formando blocos heteropoliméricos. As geometrias assumidas por blocos M, G ou alternados depende das formas particulares dos monômeros e de seu modo de ligação no polímero (NUSSINOVITCH, 1997 apud OLIVEIRA, 2004).

A figura 6 mostra as configurações dos resíduos G e M presentes no alginato.

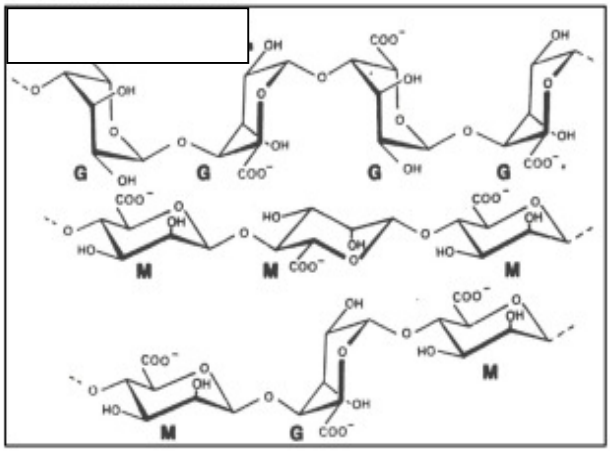


Figura 6. Representação da estrutura molecular do alginato. (Adaptado de NUSSINOVITCH, 1997 apud OLIVEIRA, 2004).

Segundo Onsonyen (1997) apud Oliveira (2004), as regiões do bloco G geram um polímero denso e rígido, o bloco M um polímero flexível, e as regiões de bloco GM alternadas formam polímeros com dureza intermediária. A ligação G-G em seqüência

forma uma estrutura de “zig zag”, com cavidades que são importantes na ligação de íons e na formação do gel (SMIDSROD e DRAGET, 1997 apud OLIVEIRA, 2004).

O alginato forma géis em temperatura ambiente, estáveis ao calor, através de reação com cátions divalentes. Apresenta alta seletividade em relação a esses íons, como metais terrosos (Ba>Sr>Ca>Mg), o que indica que as interações entre alginatos e esses íons não são puramente eletrostáticas, mas também através de complexação nos blocos G (SMIDSROD e DRAGET, 1997 apud OLIVEIRA, 2004). A geleificação ocorre por meio da ligação iônica de íons cálcio com dois grupos carboxila presentes em resíduos G adjacentes. Os blocos M também formam ligações intermoleculares, embora sejam menos efetivos que blocos G, portanto a formação e a força do gel estão diretamente ligados com a quantidade de resíduos G e comprimento médio destes blocos (ONSOYEN, 1997 apud OLIVEIRA, 2004).

A formação do gel de alginato de cálcio é considerada como um processo de troca iônica, onde o sódio do alginato é trocado com o cálcio presente no meio geleificante. Na literatura, o uso de partículas preparadas com alginato na imobilização de enzimas é amplo, bem como, na encapsulação de drogas farmacêuticas ou microrganismos e em preparações alimentícias (OLIVEIRA, 2004).

2.7 Pachira aquatica

A *Pachira aquatica,* conhecida popularmente como munguba ou castanheira d’água, é uma árvore pertencente à família das Bombacaceae, nativa das áreas do sudeste

do México, Guiana e nordeste do Brasil (figura 7). Estudos desenvolvidos sobre a composição das sementes demonstram que a *Pachira aquatica* tem um elevado teor de óleo. Destes (44,1%) é de acido palmítico, o seu principal componente (LORENZI, 1992; OLIVEIRA et al., 2000).

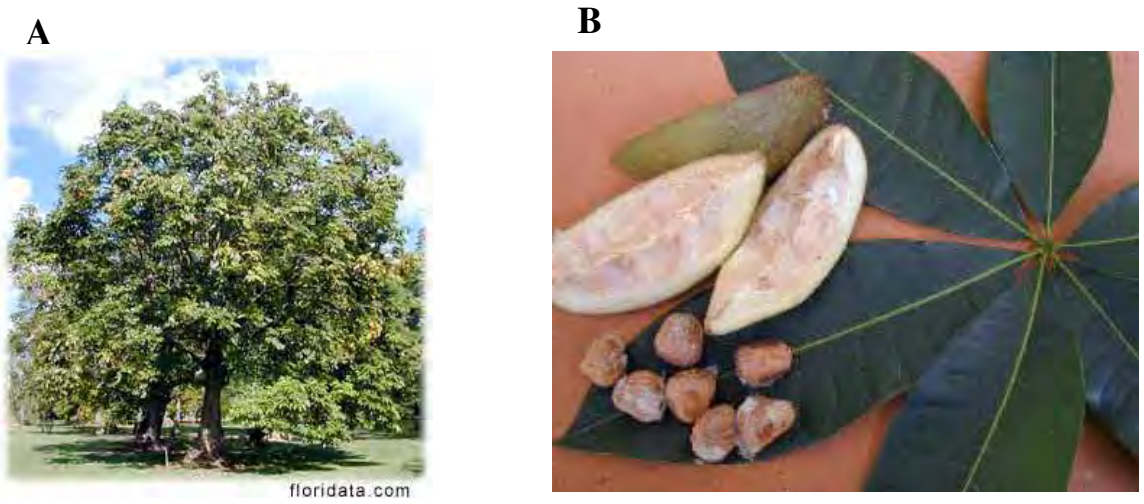


Figura 7 A. Representação de um espécime de *Pachira aquatica*. **B.** Representação das folhas, frutos e sementes de *Pachira aquatica.*

A munguba apresentou repelência sobre a broca-do-café e o extrato das sementes também apresentou diferença significativa no crescimento micelial e esporulação de Fusarium sp. (http://www.bdtd.unir.br/tde_busca/arquivo.php?codArquivo=30). Dos Santos e colaboradores (2007) utilizaram essa planta para a produção de biodiesel e obtiveram a identificação dos ésteres metílicos que o compõem.

3. Objetivos

As plantas representam uma fonte tradicional de uma gama de moléculas ativas biologicamente e um grande número de proteínas importantes industrialmente, como a papaína, é obtido de plantas. Diante desses fatores, este trabalho trata do isolamento, purificação e caracterização bioquímica da lipase de *Pachira aquatica*. Contribui, desta forma para o desenvolvimento do conhecimento em área de importância significativa dentro da biotecnologia: a tecnologia enzimática. Os estudos adicionais relativos à imobilização da lipase visam a diminuição dos custos-benéficos para uma possível aplicação da enzima e a grande biodiversidade brasileira justifica a busca por novas fontes de enzimas que possam ser aplicadas na biocatálise.

Assim, o presente trabalho teve por objetivo geral o estudo da enzima lipase extraída de sementes oleaginosas de *Pachira aquatica,* e adicionalmente avaliar seu potencial biotecnológico.

Para atingir os objetivos acima, foi necessário padronizar e efetuar:

1) A extração e purificação da enzima;

2) A determinação do peso molecular;

3) A análise da estabilidade da enzima, com relação às alterações de pH, temperatura, e a presença de agentes químicos;

4) Analisar os efeitos de pH, temperatura, presença de íons metálicos, sais e agentes químicos, e solventes orgânicos com relação à atividade da enzima;

5) Determinar o grau de especificidade frente a diferentes substratos sintéticos e naturais;

6) Determinar os parâmetros cinéticos de hidrólise;

7) Testar a eficiência da lipase no pré-tratamento de partículas de gorduras e resíduos provenientes de frigorífico avícola;

8) Analisar o efeito de agentes tensoativos, PEG e uréia na atividade lipolítica da enzima.

9) Desenvolvimento e otimização de protocolo de imobilização em gel de alginato de cálcio.

Referências

ARAKAWA, T.; TIMASHEFF, S. N. Mechanism of poly(ethylene glycol) interactions with proteins. **Biochemistry,** 24(24), 6756-6762, 1985.

BATISTA, M.A. **Estudo da imobilização de células de *Saccharomyces cerevisiae* em gel de alginato de cálcio no processo de fermentação alcoólica**, Uberlândia, 2005, 105f. Dissertação (Mestrado em Engenharia Química) - Universidade Federal de Uberlândia.

BEISSON, F.; FERTE, N.; BRULEY, S.; VOULTOURY, R.; VERGER, R.; ARONDEL, V. Oil-bodies as substrates for lipolytic enzymes. **Biochim. Biophys. Acta**, 1531(1-2), 47-58, 2001.

BJORKLING, F.; GODTFREDSEN, S. E.; KIRK, O. The future impact of industrial lipases. **Trends Biotechnol.**, 7, 330-335, 1991.

BLOW, D. M.; BIRKTOFT, J. J.; HARTLEY, B. S. Role of buried acid group in the mechanism of action of chymotrypsin. **Nature**, 221, 337-340,1969.

BRADY, L.A.; BRZOZOWSKI, M.; DEREWENDA, Z. S.; DODSON, E.; DODSON, G. G.; TOLLEY, S.; TURKENBURG, J. P.; CHRISTIANSEN HUGE-JENSEN, L. B.;

NORSKOV, L.; THIM MENGE, L. U. A serine protease triad forms the catalytic center of a triacylglycerol lipase. **Nature**, 343, 767-770, 1990.

BRÍGIDA, A. I. S. **Estudo da imobilização de lipase tipo B de *Candida antarctica* utilizando fibra de casca de coco verde como suporte**, Fortaleza, 2006, 205f. Dissertação (Mestrado em Engenharia Química) - Universidade Federal do Ceará.

BRYJAK, J.; BACHMANN, K.; PAWLÓW, B.; MALISZEWSKA, I.; TROCHIMCZUK, A.; KOLARZ, B.N. Immobilization of lipase on various acrylic copolymers. **Chem. Eng. J.**, 65, 249-256, 1997.

BRZOZOWSKI, A. M. ; DEREWENDA, U. A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. **Nature**, 351, 491-494, 1991.

BRZOZOWSKI, A.M.; SAVAGE, H.; VERMA, C.S.; TURKENBURG, J.P.; LAWSON, D.M.; SVENDSEN, A.; PATKAR, S. Structural origins of the interfacial activation in *Thermomyces (Humicola) lanuginosa* lipase. **Biochemistry**, 39, 15071-15082, 2000.

CAMMAROTA, M. C.; FREIRE, D. M. G. A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content. **Bioresour. Technol**., 97, 2195-2210, 2006.

CAMPBELL, M. K. **Bioquímica**. 3. ed. Porto Alegre: Artmed, 2001.

CYGLER, M.; SCHRAG, J. D. Structure as basis for understanding interfacial properties of lipases. **Methods Enzymol.,** 284(A), 3-27, 1997.

DALLA-VECCHIA, R,; NASCIMENTO, M.G.; SOLDI, V. Aplicações sintéticas de lipases imobilizadas em polímeros. **Química Nova**, 27 (4), 623-630, 2004.

DE CARLI, I. M. **Síntese de ésteres derivados de carboidratos com propriedades surfactantes utilizando lipases imobilizadas em suporte sólido**. Blumenau, 2006, 70f. Dissertação (Mestrado em Química) – Centro de Ciências Naturais, Universidade Regional e Blumenau.

dos SANTOS, R. B.; LACERDA Jr., V.; de CASTRO, E. V.R.; de SOUZA, T. S.; GARCIA, P. R. Identificação dos ésteres que compõem o biodiesel da munguba (*Pachira aquatica* AUBL*)*. **30ª Reunião Anual da Sociedade Brasileira de Química,** 2007.

FABER, K. **Biotransformations in organic chemistry**, 3rd. ed. Berlin: Springer Verlag, 1997.

FERNÁNDEZ-LORENTE, G.; FERNÁNDEZ-LORENTE, R.; PALOMO, J.M.; MATEO, C.; BASTIDA, A.; COCA, J.; HARAMBOURE, T.; HERNÁNDEZ-JUSTIZ, O.; TERRENI, M.; GUISÁN, J.M. Biocatalist engineering exerts a dramatic effect on

selectivity of hydrolysis catalyzed by immobilized lipases in aqueous medium**. J. Mol. Catal. B: Enzimatic,** 11, 649-656, 2001.

FERNÁNDEZ-LORENTE, G.; PALOMO, J. M.; COCCA, J.; MATEO, C.; MORO, P.; TERRINI, M.; FERNÁNDEZ-LAFUENTE, R.; GUISÁN, J.M. Regio-selective deprotection of peracetylated sugars via lipase hydrolysis. **Tetrahedron**, 59,5705-5711, 2003.

FERRATO, F.; CARRIERE, F.; SARDA, L.; VERGER, R. A critical reevaluation of the phenomenon of interfacial activation. **Methods Enzimol.,** 284, 327-347, 1997.

FURNESS, E.L.; ROSS, A.; DAVIS, T.P.; KING, G.C. A hydrophobic interaction site for lysozyme binding to polyethylene glycol and model contact lens polymers. **Biomaterials,** 19 (15), 1361-1369, 1998.

GODDARD, E. D.; ANANTHAPADMANABHAN, K. P. editors. **Interaction of surfactants with polymers and proteins**, Boca Raton: CRC Press, 1993.

GOMES, F. M.; PAULA, A. V.; SILVA, G.S.; CASTRO, H.F. Determinação das propriedades catalíticas em meio aquoso e orgânico da lipase de *Candida rugosa* imobilizada em celullignina quimicamente modificada por carbonildiimidazol. **Química Nova**, 29, 710-718, 2006.

GOTOR-FERNANDEZ, V.; BRIEVA, R.; GOTOR, V. Lipases: Useful biocatalysts for the preparation of pharmaceuticals. **J. Mol. Catal. B: Enzym.,** 40, 111-120, 2006.

GRIMONPREZ, B.; JOHANSSON, G. Liquid-liquid partitioning of same enzymes, especially phosphofructokinase, from *Saccharomyces cerevisiae* at sub-zero temperature. **Chromatog. B- Biomedical Applications,** 680 (1-2), 55-63, 1996.

HASAN, F.; SHAN, A. A.; HAMEED, A. A industrial applications of microbial lipases. **Enz. Microbial Technol.,** 39, 235-251, 2006.

http://www.bdtd.unir.br/tde_busca/arquivo.php?codArquivo=30. Acessado em 30/04/2008.

HUANG, A. H. C.; LIN, Y. H.; WANG, S. Characteristics and biosynthesis of seed lipases in maize and other plant species**. J. Am. Oil Chem. Soc**., 65, 897-899, 1988.

HUDDLESTON, J. G.; WANG, R.; FLANAGAN, J.A.; O´BRIEN, S.; LYDDIATT, A. Variation of protein partition coefficients with volume ratio in poly (ethylene glycol) –salt aqueous 2-phase systems. **J. Chomatogr. A,** 688 (1), 3-11, 1994.

JAEGER, K.E.; RANSAK, S.; KOCK, H.B., FERRATO, F.; DIJKSTRA, B. W. Bacterial lipases. **FEMS Microbiol. Rev.,** 15,29-63, 1994.

JAEGER, K.E.; REETZ, M. T. Microbial lipases form versatile tools for biotechnology. **Trends Biotechnol**., 16, 396-403, 1998.

JAEGER, K.E.; DIJKSTRA, B.W.; REETZ, M.T. Bacterial biocatalyst: molecular biology, three dimensional structures and biotechnological applications of lipases. **Annu. Rev. Microbiol.,** 53, 315-351, 1999.

JAEGER, K.E.; EGGERT, T. Lipases for biotechnology. **Current Opin. Biotechnol.,** 13, 390-397, 2002.

JÖNSSON, B.; LINDMAN, B.; HOLMBERG, K.; KRONBERG, B**. Surfactants and Polymers in Aqueous Solution**, John Wiley & Sons: Chichester, 1998.

JONSSON, M.; SKEPO, M.; TJERNELD, F.; LINSE, P. Effect of spatially distributed hydrophobic surface residues on protein-polymer association. **J. Phys. Chem. B,** 107(23), 5511-5518, 2003.

KAMBOUROVA, M.; KIRILOVA, N.; MANDEVA, R.; DEREKOVA, A. Purification and properties of Thermostable lipase from a thermophilic *Bacillus stearothermophilus* MC 7. **J. Mol. Catalysis B: Enzymatic**, 22, 307-313, 2003.

KULKARNI, A. M.; CHATTERJEE, A.P.; SCHWEIZER, K.S.; ZUKOSKI, C.F. Effects of polyethylene glycol on protein interactions. **J. Chem Phys.,** 113 (21), 9863-9873, 2000.

LEAL, M. C. M. R.; CAMMAROTA, M. C.; FREIRE, D. M. G.; SANT'ANNA JR., G. L. Hydrolytic enzymes as coadjuvants in the anaerobic treatment of dairy wastewaters. **Braz. J. of Chem. Eng**., 19 (2), 175-180, 2002.

LEE, J.C.; LEE, L. L. I. Preferential solvent interaction between protein and polyethylene glycols. **J. Biological Chem.,** 256 (2), 625-631, 1981.

LEHNINGER, A. L.; COX, M. M. **Principles of Biochemistry,** 4. ed. Editora: W. H. FREEMAN, 2004.

LIN, Y. H.; HUANG , A. H.; YU, C. Substrate specificities of lipases from corn and other seeds. **Arc. Biochem. Biophys.,** 244, 346-356, 1986.

LORENZI, H. **Árvores Brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil**, Editora Plantarum LTD., São Paulo, 1992.

MARTINEZ, C.; DE GEUS, P.; LAUWEREYS, M.; MATTHYSSENS, G.; CAMBILLAU, C. Fusarium-solani cutinase is a lipolytic enzyme with a catalytic serine accessible to solvent. **Nature**, 356, 615-618, 1992.

MASSE, L.; KENNEDY, K. J.; CHOU, S. Testing of alkaline and enzymatic hydrolysis pretreatments for fat particles in slaughterhouse wastewater. **Bioresour. Technol**., 77, 145-155, 2001.

MASSE, L.; MASSE, D. I.; KENNEDY, K. J. Effect of hydrolysis pretreatment on fat degradation during anaerobic digestion of slaughterhouse wastewater. **Process Biochem**., 38, 1365-1372, 2003.

MOHAMED, M. A.; MOHAMED, T. M.; MOHAMED, S. A.; FAHMY, A. S. Distribution of lipases in the Gramineae. Partial purification and characterization of esterase from *Avenua fatua*. **Bioresource Technol.,** 73, 227-234, 2000.

MORENO, J. M.; ARROYO, M.; HERNÁIZ, J.M.; SINISTERRA, J.V. Covalent immobilization of pure isoenzymes from lipase of *Candida rugosa*. **Enz. Microbial Technol.**, 21, 552-558, 1997.

MURRAY, M.; ROONEY, D.; VAN NEIKERK, M.; MONTENEGRO, A.; WEATHERLEY, LR. Immobilization of lipase onto lipophilic polymer particles and applications to oil hydrolysis. **Process biochem.**, 22,479-786, 1997.

NCUBE, I.; READ, J. S. Evaluation of *Veronia galamensis* lipase (acetone powder) for use in biotechnology. **Ind. Crops. Products***,* 3, 285-292, 1995.

NINNI, L.; CAMARGO, M.S.; MEIRELLES, A.J.A. Water activity in poly (ethylene glycol) aqueous solutions. **Thermochimica Acta,** 328 (1-2), 169-176, 1999.

NOBLE, M. E. M.; CLEASBY, A.; JOHNSON, L.N.; EGMOND, M. R.; FRENKEN, L. G. J. The crystal structure of triacylglycerol lipase from *Pseudomonas glumae* reveals a partially redundant catalytic aspartate. **FEBS Lett.,** 331,123-128, 1993.

NUSSINOVITCH, A. **Hydrocolloid applications – Gum technology in the food and other industries.** Cap. 2 Alginates, 19-39, 1997.

OLIVEIRA, A. P. C. **Produção e caracterização de partículas de hidrogéis para aplicações em cosméticos**,Campinas, 2004, 178f. Dissertação (Mestrado em Engenharia Química) –Faculdade de Engenharia Química, Universidade de Campinas.

OLIVEIRA, J.T.A.; VASCONCELOS, I.M.; BEZERRA, L.C.N.M.; SILVEIRA, S.B; MONTEIRO, A.C.O.; MOREIRA, R.A. Composition and nutritional properties of seeds from *Pachira aquatica* Aubl, *Sterculia striata* St Hil et Naud and *Terminalia catappa* Linn. **Food Chem.,** 70, 185-191, 2000.

OLLIS, D.L.; CHEAH, E.; CYGLER, M. The hydrolase fold**. Protein Eng,** 5, 197-211, 1992.

ONSONYEN, E., In: IMENSON, A. **Thickening and gelling agents for food.** 2 ed. Cap. 2: Alginates, 1997.

PANCERA, S. M. **Estudo da interação entre polímeros e proteínas.** São Paulo, 2006, 133f. Tese (doutorado em Química) – Instituto de Química, Universidade de São Paulo.

PANCERA, S. M.; PETRI, D. F. S.; da SILVA, L. H. M.; LOH, W.; ITRI, R.; PESSOA Jr. A. The effect of poly(ethylene glycol) on the activity and structure of glucose-6 phosphate dehydrogenase in solution. **Coll. Surfaces B: Interfaces,** 26, 291-300, 2002.

PANCREAC´H, G.; LEULLIER, M.; BARATTI, J.C. Properties of free and immobilized lipase from *Pseudomonas cepacia*. **Biotechnol. Bioeng.**, 57, 181-189, 1997.

PARSEGIAN, V. A.; RAND, R.P.; RAU, D.C. **Methods in enzymology,** 259, 43-94, 1995.

PASQUES, F.W.; MACEDO, G.A. Lipases de látex vegetais: propriedades e aplicações industriais. **Química nova,** 29, 1, 2006.

ROBERTSON, D.E.; STEER, B. A. Recent progress in biocatalyst discovery and optimization. **Current Opin. Chem. Biol.**, 8, 141-149, 2004.

ROUSSEL, A.; MILED, N.; BERTI-DUPUIS, L.; RIVIÉRE, M.; SPINELLI, S.; BERNA, P.; GRUBER, V.; VERGER, R.; CAMBILLAU, C. Crystal structure of the open form of dog gastric lipase in complex with a phosphonate inhibitor. **J. Biol. Chem.,** 277(3), 2266-2274, 2002.

RUBIN, B.; DENNIS, E. A. Editors**. Lipases. Part A: biotechnology,** In: Methods Enzymology, New York: Academic Press,1-284. 1997.

SALAH, R. B.; MOSBAH, H.; FENDRI, A.; GARGOURI, A.; GARGOURI, Y.; MEJDOUB, H. Biochemical and molecular characterization of a lipase produced by *Rhizopus oryzae.* **FEMS Microbiol lett.,** 260, 241-248, 2006.

SARDA, L.; DESNUELLE, P. Action de la pancreátique sur les esteres en emulsion. **Biochem. Biophys. Acta**, 30, 513-521,1958.

SAVELLI, G.; SPRETI, N.; di PROFIO, P. Enzyme activity and stability control by amphiphilic self-organizing systems in aqueous solutions. **Curr. Opin. in Collid & Interface Sci.,** 5, 111-117, 2000.

SAXENA, R. K.; DAVIDSON, W. S.; SHEORAN, A.; GIRI, B. Purification and characterization of an alkaline thermostable lipase from *Aspergillus carneus.* **Process Biochem**., 39, 239-247, 2003.

SCHMID, A.; DORDICK, J.S.; HAUER, B.; KIENER, A.; WUBBOLTS, M.; WITHOLT, B. Industrial biocatalysis today and tomorrow. **Nature insight**, 409, 258-268, 2001.

SCHRAG, J.D.; CYGLER, M. Lipases and alpha/beta hydrolase fold. **Methods Enzymol,** 284,85-107, 1997.

SCHRAG, J.D.; LI, Y.; WU, S.; CYGLER, M. Ser-His-Glu triad form the catalytic site of the lipase from *Geotrichum candidum.* **Nature**, 351, 761-764, 1991.

SHARMA, R.; CHISTI, Y.; BANERJEE, U. C. Production, purification, characterization, and applications of lipases. **Biotechnol. Advance**, 19, 627-662, 2001.

SHARMA, R.; SONI, S. K.; VOHRA, R. M.; GUPTA, L. K.; GUPTA, J. K. Purification and characterization of a thermostable alkaline lipase from a new thermophilic *Bacillus* sp.RSJ-1. **Process Biochem**, 37, 1075-1084, 2002.

SMIDSROD, O.; DRAGET, K. I. In: BERGHSTAHL, E. D. **Food colloids- protein, lipids and polysaccharides.** Alginate Gelation Technologies, 1997.

STAUBMANN, R.; NCUBE, I.; GÜBITZ, G. M.; STEINER ,W.; READ, J.S. Esterase and lipase activity in *Jatropha curcas* L. seeds. **J. of Biotechnol.,** 75, 117-126,1999.

SUAN, C.; SARDIMI, M.R. immobilised lipase-catalysed resolution of (r,s) – 1-phenylethanol in recirculated packed bed reactor. **J. Mol. Catal. B: Enzym.,** 28,111-119, 2004.

TANFORD, C. **The hydrophobic effect: formation of micelles and biological membranes.** 2. ed. New York: Wiley, 1980.

TELEFONCU, A.; DINCKYA, E.; VORLOP, K.D.,Preparation and characterization of pancreatic lipase immobilized in Eudragit-matrix. **Applied Biochem. Biotechnol**, 26, 311-317, 1990.

TYNDALL, J. D. A.; SINCHAIKUL, S.; FOTHERGILL-GILMORE, L. A.; TAYLOR, P.; WALKINSHAW, M. D. Crystal structure of a thermostable lipase from *Bacillus stearothermophilus* P1. **J. Mol. Biol.,** 323, 859-869, 2002.

UPPENBERG, J.; HANSEN, M.T.; PATKAR, S.; JONES, T.A. The sequence, crystal structure determination and refinement of two crystal forms of lipase B from *Candida Antarctica.* **Structure,** 2 (4), 293-308, 1994.

van TILBEURGH, H.; EGLOFF, M. P.; MARTINEZ, G.; RUGANI, N.; VERGER, R.; CAMBILLAU, C. Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography. **Nature,** 362, 814-820, 1993.

VERGER, R. Interfacial activation of lipases: facts and artifacts. **Trends Biotechnol.,** 15, 32-38, 1997.

VOET, D.; VOET, J., **Biochemistry,** 3nd ed. Editora Wiley & Sons, 2004.

WALSH, G. E HEADON, D. **Protein Biotechnology**, Editora John Wiley & Sons, 1994.

WINKLER, F. K.; D´ARCY, A.; HUNZIKER, W. Structure of human pancreatic lipase. **Nature**, 343, 771-774, 1990.

WRIGHT, C. S.; ALDEN, R. A.; KRAUT, J. Structure of subtilisin BPN´at 2. 5 Å resolution. **Nature**, 221, 235-242, 1969.

ZADA, A.; HAREL, M. Enzimatic transesterification of racemic lavanduol: preparation of the two enantiomeric alcohls and the two enantiomers of lavanduol senecionate. **Tetrahedron: Asymetry**, 15,2339-2343, 2006.

Capítulo 1

A new lipase isolated from oleaginous seeds from *Pachira aquatica* (Bombacaceae)

Patricia Peres Polizelli, Fernanda Dell Antonio Facchini, Hamilton Cabral e Gustavo Orlando Bonilla Rodriguez

Artigo publicado na revista: Applied Biochemistry and Biotecnology (ISSN: 0273-2289, Impact factor 1.102

Resumo

Uma nova lipase extraída de sementes oleaginosas de *Pachira aquatica* foi purificada por SDS-PAGE obtendo-se uma enzima com massa molecular de aproximadamente 55 kDa. A lipase exibiu atividade máxima a 40°C e pH 8,0, com um tempo de incubação de 90 minutos. Com respeito à estabilidade a temperatura, na faixa de 4 a 50°C a enzima permaneceu com aproximadamente 47% de sua atividade original após 3 horas de exposição. A atividade enzimática aumentou na presença de Ca^{++} e Mg^{++}, mas foi inibida por Hg^{++}, Mn^{++}, Zn^{++}, Al^{+++} e vários agentes oxidantes e redutores. A enzima foi estável na presença de solventes orgânicos e sua atividade foi estimulada por metanol (50% v/v). Os valores de K_M e V_{max}, foram 1,65 mM e 37,3 $\mu mol.mL^{-1}.min^{-1}$, respectivamente usando *p*-nitrofenil acetato como substrato. A enzima mostrou preferência por ácidos graxos de cadeias longas, mas demonstrou atividade significativa contra uma grande faixa de substratos.

Palavras-chave: Enzima, lipase, sementes, *Pachira aquatica,* hidrólise.

Appl Biochem Biotechnol
DOI 10.1007/s12010-008-8145-z

A New Lipase Isolated from Oleaginous Seeds from *Pachira aquatica* (Bombacaceae)

Patricia Peres Polizelli · Fernanda Dell Antonio Facchini · Hamilton Cabral · Gustavo Orlando Bonilla-Rodriguez

Received: 31 August 2007 / Revised: 28 December 2007 / Accepted: 2 January 2008

Abstract A new lipase from seeds of *Pachira aquatica* was purified to homogeneity by SDS-PAGE obtaining an enzyme with a molecular weight of approximately 55 kDa. The purified lipase exhibited maximum activity at 40°C and pH 8.0, for an incubation time of 90 min. Concerning temperature stability, at the range from 4 to 50°C, it retained approximately 47% of its original activity for 3 h. The enzyme activity increased in the presence of Ca^{++} and Mg^{++}, but was inhibited by Hg^{++}, Mn^{++}, Zn^{++}, Al^{+++} and various oxidizing and reducing agents. The lipase was highly stable in the presence of organic solvents, and its activity was stimulated by methanol. The values of K_m and V_{max} were 1.65 mM and 37.3 μmol mL^{-1} min^{-1}, respectively, using *p*-nitrophenylacetate as substrate. The enzyme showed preference for esters of long-chain fatty acids, but demonstrated significant activity against a wide range of substrates.

Keywords Enzyme · Lipase · Seeds · *Pachira aquatica* · Hydrolysis

Introduction

Lipases (E.C.3.1.1.3) are enzymes that are able to catalyze fat hydrolysis at an oil–water interface. This reaction is reversible, and the enzyme also catalyzes the synthesis of esters and transesterification in microaqueous conditions. Lipases are common among living beings [1].

Lipases that are present in oilseeds help to hydrolyze the ester bonds of storage triacylglycerols, and it has been reported that with a few exceptions, lipase activity is absent in ungerminated (or dormant) seeds and increases rapidly in post-germination [2]. In those cases where the enzyme is active in the dormant seeds, as in castor bean seed [2], peanut [3],

P. P. Polizelli
Department of Physics, State University of São Paulo, Rua Cristovão Colombo, 2265, São José do Rio Preto, SP CEP 15054-000, Brazil

F. D. A. Facchini · H. Cabral · G. O. Bonilla-Rodriguez (✉)
Department of Chemistry and Environmental Sciences, IBILCE-UNESP, State University of São Paulo, Rua Cristovão Colombo, 2265, São José do Rio Preto, SP CEP 15054-000, Brazil
e-mail: bonilla@ibilce.unesp.br

and Barbados nut [4, 5], it is apparently inactive in vivo but highly active in vitro. In other words, as long as the seed is in the intact state, the enzyme will remain inactive, but any slight change in the seed or storage conditions will probably initiate activity [6].

Oilseed lipases have great potential for commercial exploitation as industrial enzymes, especially those oilseeds that are presently considered underutilized [6]. They have attracted much interest because of their many potential applications in synthetic reactions. The applications of lipases in industry include the synthesis of food ingredients, oil chemistry, food, organic synthesis, paper manufacturing, biosurfactants, cosmetics, pharmaceuticals, biodiesel and the use as additives in detergents [7–9].

However, our knowledge in the area of plant lipases is still very limited, especially when compared with information on mammalian and bacterial lipases, and although lipases used in industry have been obtained from microbes, the unique substrate specificity of plant lipases, not found in microbes or mammalian systems, may be of special value in industrial utilization [8,10].

Pachira aquatica is a tree belonging to the Bombacaceae family, found in a large area, from Southern Mexico to Northeastern Brazil. Its seeds are sometimes consumed raw or as roasted beans having a chestnut flavor, being considered a delicacy. Furthermore, its young leaves and flowers are cooked and used as vegetables [11].

A preliminary characterization showed the presence of a lipase, and accordingly, this study intended to carry on its purification and characterization.

Materials and Methods

Materials

The seeds were collected from trees of our Campus, at São José do Rio Preto, State of São Paulo. They were washed, peeled, and extracted by homogenization in a food processor with a solution containing: 3 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium metabissulfite, and 50 mM Tris–HCl buffer (pH 8.0) at 25°C. A 0.3 ratio seed *w*/*v* extraction buffer was used, and the enzyme was recovered in the supernatant. The samples were clarified and concentrated by centrifugation at 9,000×*g* by 40 min at 4°C using a *Jouan* CR3i refrigerated centrifuge and stored until use in liquid nitrogen.

Purification

The purification procedure involved a zymogram method based on the protocol described by Fuciños et al. [12], with modifications. The SDS-PAGE was performed using 13% polyacrilamide slab gels with 0.1% sodium dodecylsulfate (SDS). For esterase detection, the gel was submerged in a solution containing α- and β-naphtyl acetate in the presence of Fast Blue [13]. The band was cut off, and the enzyme was allowed to diffuse in Tris–HCl buffer for 24 h. Purity of the enzyme was established as described by Laemmli [14]. For estimation of the relative molecular weight, we carried out 13% SDS-PAGE using molecular weight standards: aprotinin (6.5 kDa), alfa-lactalbumin (14.2 kDa), soybean trypsin inhibitor (20 kDa), trypsinogen (24 kDa), carbonic anhydrase (29 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), ovalbumin (45 kDa), and albumin (66 kDa) purchased from Sigma. For protein detection, the gel was stained with Coomassie Brilliant Blue.

Assay of Lipase Activity

Lipolytic activity was estimated by a spectrophotometric assay method [15] using *p*-nitrophenyl palmitate (*p*-NPP) as substrate for this standard assay. The assay mixture contained 2.5 mM of the substrate, 2% of Triton X-100 as emulsifier, 50 mM Tris–HCl buffer pH 8.0 and 5 mg/ml of the pure enzyme. The assay mixture was incubated for 90 min at 37°C, and the reaction proceeded almost linearly in this time. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute at 37°C and pH 8.0.

As *p*-NPP is not stable at high pH values, assays of the pH effect were carried out by alkali titration using soybean oil as substrate as described by Saxena et al. [16] with some modifications. The assay mixture consisted of 50% soybean oil, 31.3% 30 mM Tris–HCl buffer pH 8.0, 10.15% Triton X-100, 0.002% of 10 mM $CaCl_2$, and 9.6% of the enzyme crude solution. Incubation was also performed for 90 min at 37°C. This assay was used also to determine the hydrolysis of natural oils.

The reaction was ended by adding of a mixture of acetone:ethanol (1:1), and the amount of released fatty acids was titrated with 50 mM KOH in the presence of phenolphthalein as indicator. One unit of enzyme activity was defined as the amount able to release 1 μmol of free fatty acids per minute and specific activities as micromole of product per minute per milligram protein, under the assay conditions. Protein concentration of the samples was estimated according to the dye binding assay method using bovine serum albumin as a standard [17].

Effect of Temperature

The effect of temperature on lipase activity was studied by carrying out the assays at different temperatures in the range of 25–60°C at pH 8.0 using 50 mM Tris–HCl buffer. Lipase thermostability was tested by preincubating the enzyme at varying temperatures ranging from 4 to 60°C for different time intervals, from 0 to 240 min.

pH Effect

The pH activity profile was studied by the alkali titration assay procedure described above in a pH range from 3.0 to 10.0 using different buffers at 50 mM concentration: sodium citrate (pH 3 to 5), Ada (pH 6 and 6.5), sodium phosphate (pH 7 and 7.5), Tris–HCl (pH 8 and 9), and glycine (pH 10).

Effect of Oxidizing, Reducing, and Chelating Agents on Lipase Activity

The effect of the chemicals was studied using a final concentration of 10 mM at 37°C and pH 8.0.

Effect of Solvents

The effect of several solvents was analyzed with 50% of solvent using the standard assay.

Kinetic Study

The effect of substrate concentration (*p*-nitrophenyl acetate) on the reaction rate was assayed at 37°C pH 7.5 (due instability of substrate) with 50 mM Hepes buffer measuring

the absorbance at 410 nm using a Varian Cary 100 Spectrophotometer. The limiting rate V_{max} and the Michaelis concentration, K_m, were calculated by nonlinear regression using GnuPlot 4.0 [18].

Substrate Specificity

Substrate specificity of the enzyme was determined at 37°C using 50 mM Hepes buffer pH 7.5 (due instability any substrates) and the assay method described above. Several nitrophenylesters were analyzed: *p*-nitrophenyl acetate (2:0), butyrate (4:0), caprate (10:0), myristate (14:0), palmitate (16:0), and stearate (18:0), as well as *O*-nitrophenyl palmitate. The specificity was also tested using soybean, olive, corn, sunflower, cotton, and rapeseed oils by titration with KOH as described previously.

Effect of Different Ions and Salt Concentration

The assays were performed using standard method in the presence of different ions: $CaCl_2$, $CoCl_2$, KCl, $HgCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $AlCl_3$, and $NaCl_2$. We also tested the effect of salt concentration for LiCl, NaCl, and KCl.

Results and Discussion

The results obtained in the present study show lipase activity in ungerminated (dormant) seeds of the *P. aquatica*.

Purification

The lipase was purified 9.6-fold (Table 1). The purity of the lipase was checked by SDS-PAGE, and the enzyme had a molecular weight of approximately 55 kDa stained with Coomassie blue, but silver staining also showed the same results. (Fig. 1).

Effect of Temperature and pH

The activity reached a maximum at 40°C when tested with p-NPP and soybean oil (Fig. 2a) for a 90-min incubation period. The thermal stability was studied from 4 to 60°C (Fig. 2b). The data indicates that enzyme had appreciable stability during prolonged incubation (3 h) from 4 to 50°C. The enzyme exhibited maximum stability at 4°C and decreased at other temperatures.

The pH optimum (Fig. 3) was found at pH .0. This finding agrees with results reported for other plant lipases that display pH optima at alkaline values [3, 8, 19, 20] and is also consistent with the pH optima reported for lipases from other sources [1, 16, 21, 22].

Table 1 Purification of the lipase from *P. aquatica*.

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2.5	168.5	2.625	64.2	100	1.0
electrophoresis	0.5	11.6	0.019	614.3	7	9.6

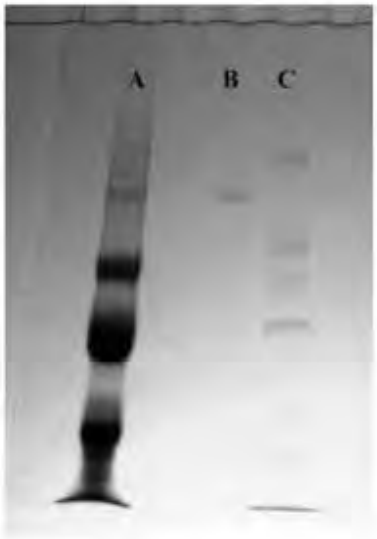


Fig. 1 SDS-PAGE electrophoresis pattern of the purified lipase from *P. aquatica* stained with Coomassie Brilliant Blue R-250. *Lane A*: crude sample, *lane B*: purified lipase, *lane C*: molecular mass markers

Effect of Oxidizing, Reducing, and Chelating Agents on Lipase Activity

The activity of the lipase from *P. aquatica* decreased in the presence of all the tested chemicals (Table 2). Only with oxidized glutathione, we observed the lowest inhibition; the enzyme retained 98% of the relative activity. Almost 30% of the activity is lost in the presence of 10 mM EDTA, and the effect was more prominent in the presence of reduced glutathione, DTT, and β-mercaptoethanol. This inhibition suggests that the lipase requires one or more intact disulfide bonds for maintaining its native conformation, as postulated for other lipases [23].

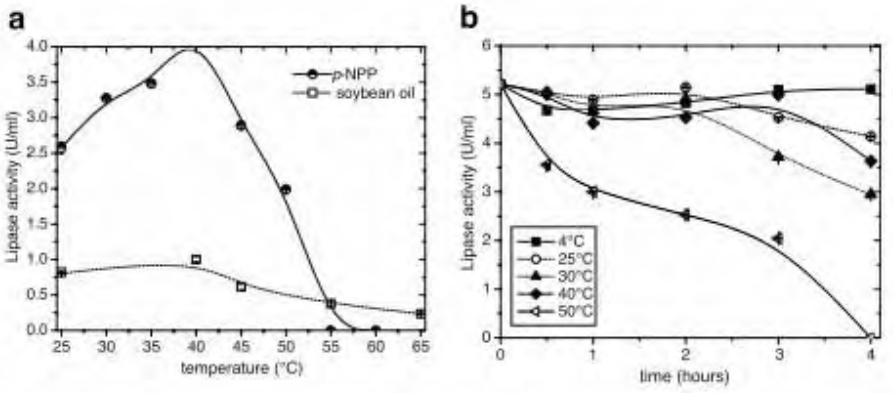


Fig. 2 **a** Effect of temperature on the activity of the lipase from *P. aquatica*. Activity was measured with *p*-NPP (*empty squares*) and soybean oil (*filled circles*) at pH 8.0. **b** Temperature stability measured using *p*-NPP as substrate. The experiments were performed in triplicate

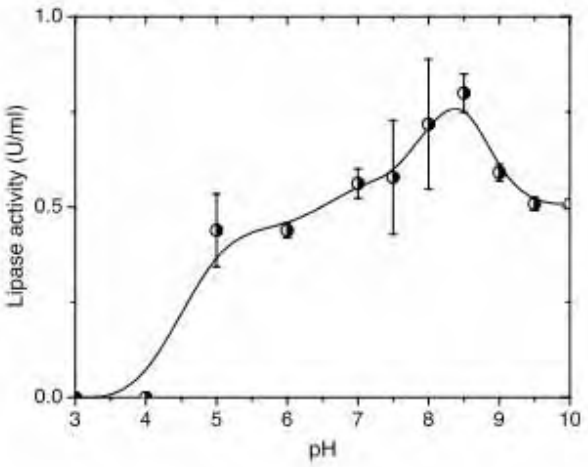


Fig. 3 Effect of pH on lipase activity, measured at 37°C. The experiments were performed in triplicate

EDTA inhibition of the enzyme activity could be attributed to its chelating ability. It will naturally perform what is known as the "chelation process" of the system and thereby disrupt the formation of the enzyme–substrate complex [6].

Effect of Solvents

Lipases are known for their ability to work in aqueous and in organic solvents. Table 3 shows the relative lipolytic activity towards *p*-nitrophenyl palmitate in the presence of 50% (*v*/*v*) organic solvents. The activity in the presence of methanol and glycerol was higher. Heller and Mozes [24] also observed increase of activity in the presence of methanol to phospholipase from peanut. The activities were lowered by the addition of ethanol and butanol, in agreement with findings reported by Kambourova et al. [22] and Takeda et al. [25] for lipases from *Bacillus stearothermophilus* and *Burkholderia cepacia*, respectively. The results from the present study indicate that *P. aquatica* lipase could be suitable for organic synthesis, as an important criterion in the selection of biocatalyst is its ability to be adequately stable under process conditions [26].

Table 2 Effects of chemical agents (10 mM) on lipase activity.

Agents	Relative activity (%)
Control	100.0
Ammonium persulfate	32.4
Potassium iodide	53.6
Glutathione (reduced)	65.7
Glutathione (oxidized)	98.
Ascorbic acid	44.0
DTT	1.0
β-mercaptoethanol	1.3
Sodium citrate	64.6
EDTA	70.9

The experiments were performed in triplicate.

Table 3 Relative activity of the purified lipase in organic solvents (50% v/v).

Solvents	Relative activity (%)
Control	100.0
Methanol	130.0
Ethanol	82.5
Glycerol	116.2
DMSO	93.6
Hexane	99.0
Butanol	82.0
Acetone	86.6

The experiments were performed in triplicate.

Kinetic Studies

The values of K_m and V_{max} of the purified lipase from *P. aquatica*, using *p*-NPP were 1.65± 0.026 mM and 37.3±0.024 μmol/mg min, respectively. The K_m value is higher than that found by Mohamed et al. [8] for the esterase III from *Avenua fatua* (0.38 mM) and higher than that described by Staubmann et al. [19] for esterases from *Jatropha curcas* (0.02 and 0.07 mM). The value of V_{max} is higher than the values reported for the lipase from *M. cephalus* (20 μmol/mgmin) and for esterases from *J. curcas* (0.26 and 0.24 μmol/mg min) [19, 27].

Substrate Specificity

The enzyme was able to hydrolyze both soluble and insoluble emulsified substrates. The hydrolysis rate for nitrophenylesters (Fig. 4a) were as follows: myristate > caprate > palmitate > stearate > *o*-palmitate > butyrate > acetate. The enzyme preferentially hydrolyzed long-chain fatty acid esters and showed the highest activity for myristate esters as found by Takeda et al. [25] for cholesterol esterase of the bacterium *B. cepacia*. The enzyme showed activity in different oils, but exhibited the highest one for olive oil (Fig. 4b)

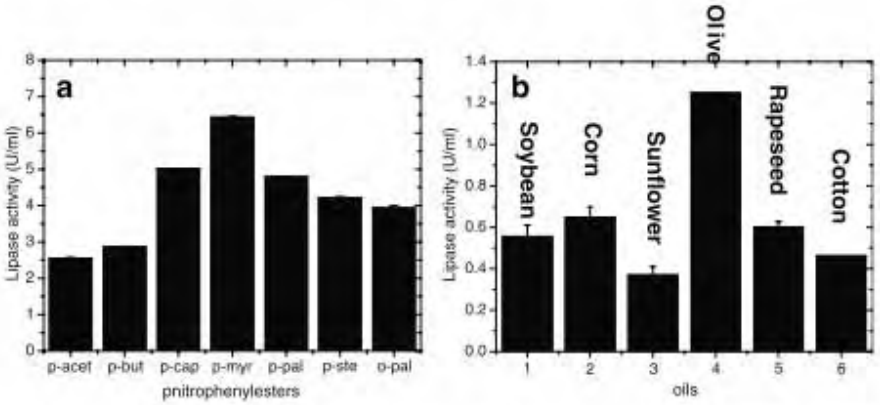


Fig. 4 Lipase activity on different substrates (**a**) using nitrophenylesters, measuring at 37°C and pH 7.5. **b** enzyme activity on different oils at 37°C and pH 8.0. The experiments were performed in triplicate

Table 4 Effects of various cations at 10 mM on the lipolytic activity.

Cation	Relative activity (%)
None	100.0
Ca	222.0
Mg	208.0
Li	92.0
Co	68.0
Na	85.0
K	77.0
Zn	38.0
Al	15.0
Mn	13.5
Hg	9.4

The experiments were performed in triplicate.

followed by corn and rapeseed oil. This enzyme is a lipase that displays some esterase activity as well, as noted by Gilbert et al. [28] and Kambourova et al. [22] for bacterial lipases from *Pseudomonas aeruginosa* and *Bacillus stearothermophilus*, respectively.

This comparison is, however, tentative, due to different physical state of the substrates and interfacial parameter which have been shown to influence the rate of lipase hydrolysis [27].

Effect of Different Cations and Salt Concentration

Table 4 shows the effect of different ions on lipase activity. Mg^{++} and Ca^{++} ions doubled the lipolytic activity, whereas Hg^{++}, Mn^{++}, Zn^{++}, and Al^{+++} caused significant inhibition. This inhibition was also demonstrated by esterase from *Jatropha curcas* [19]. Similarly, Aisaka and Terada [29], Sanz and Olias [30], and Saxena et al. [15] have reported for other lipases from Rhizopus *japonicus*, lupin seed, and *Aspergillus carneus*, respectively, activation effects for Ca^{++} and Mg^{++}. Some enzymes were activated only by Ca^{++}, such as the lipase from *A. fatua* esterases [8], *J. curcas* L [4], castor bean acid lipase [31], and

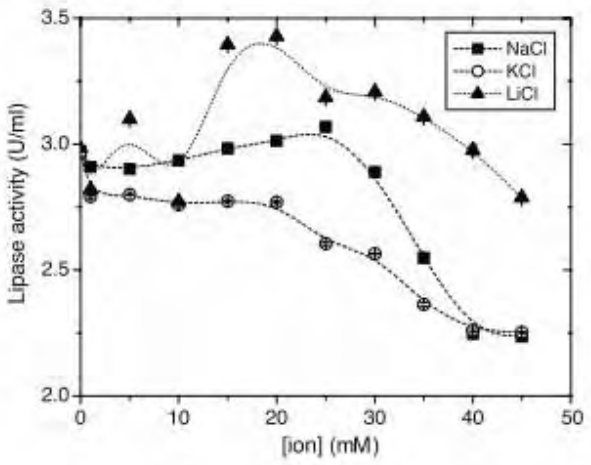


Fig. 5 Effect of three cations: Na, K and Li on lipase activity, determined at 37°C and pH 8.0. The experiments were performed in triplicate

also *Pentaclethra macrophylla* lipase [6]. In general, lipases are strongly inhibited by Hg^{2+} (a thiol group inhibitor). This is likely due to the proximity of the SH group to the catalytic and interfacial binding site but spatially remote from catalytic site, this may have induced the marked loss of activity [27, 32, 33]. The catalytic triad of lipases has been recognized to consist of Ser, His and Glu or Asp [34, 35], thus the bulky Hg^{2+} group might cause steric interference to the approach of the substrate to the active site.

Figure 5 shows the effect of cation concentration in the lipolytic activity. In agreement with our data, Yu et al. [36] observed that Li^{+} stimulated a lipase from *Candida rugosa* more than Na^{+} and K^{+}. It is not clear why Li, Na, and K have different effects on lipase activity. One reason suggested by Yu et al. [36] is that lithium is chemically more efficient to facilitate the contact between the substrates and the lipase.

Ions at lower concentrations have a stimulant effect, probably because the salt exerts a screening effect and disperse the enzyme molecules. When salt concentration increases, the activity would decrease because it could cover the lipase surface and prevent substrate binding. Rather, they provide a driving force for substrate binding and catalysis by lowering energy barriers in the ground and/or transition states and enhance enzyme activity through conformational transitions triggered upon binding to a site where the ion makes no direct contact with substrate [37].

The lipase from *P. aquatica* presented in this study has some interesting characteristics such as wide substrate specificity and could be used for different purposes in the oil industry.

References

1. Salah, R. B., Mosbah, H., Fendri, A., Gargouri, A., Gargouri, Y., & Mejdoub, H. (2006). *FEMS Microbiology Letters, 260*, 241–248.
2. Huang, A. H. C. (1984). In B. Borgstrom, & H. L. Brockman (Eds.) *Lipase: Plant lipases* (pp. 419–422). Amsterdam: Elsevier Publishers.
3. Sanders, T. H., & Pattee, H. E. (1975). *Lipids, 10*(1), 50–54.
4. Abigor, R. D., Uadia, P. O., Foglia, T. A., Haas, M. J., Scott, K., & Savary, B. J. (2002). *Journal of the American Oil Chemists Society, 79*(11), 1123–1126.
5. Haas, M. J., Cichowicz, D. J., & Bailey, D. G. (1992). *Lipids, 27*, 571–576.
6. Enujiugha, V. N., Thani, F. A., Sann, T. M., & Abigor, R. D. (2004). *Food Chemistry, 88*, 405–410.
7. Rubin, B., & Dennis, E. A. (1997). *In methods enzymology: Lipases*. New York: Academic Press.
8. Mohamed, M. A., Mohamed, T. M., Mohamed, S. A., & Fahmy, A. S. (2000). *Bioresource Technology, 73*, 227–234.
9. Sharma, R., Soni, S. K., Vohra, R. M., Gupta, L. K., & Gupta, J. K. (2002). *Process Biochemistry, 37*, 1075–1084.
10. Huang, A. H. C., Lin, Y. H., & Wang, S. (1988). *Journal of the American Oil Chemists Society, 65*, 897–899.
11. Lorenzi, H. (1992). *Árvores Brasileiras: Manual de identificação e cultivo de plantas arbóreas nativas do Brasil*. São Paulo: Editora Plantarum LTD.
12. Fuciños, P., Abadín, C. M., Sanromán, A., Longo, M. A., Pastrana, L., & Rúa, M. L. (2005). *Journal of Biotechnology, 117*, 233–241.
13. Beguin, P. (1983). *Analytical Biochemistry, 131*, 333–336.
14. Laemmli, U. K. (1970). *Nature, 227*, 680–685.
15. Chang, C. R., Chou, S. J., & Shaw, J. F. (1994). *Biotechnology and Applied Biochemistry, 19*, 93–97.
16. Saxena, R. K., Davidson, W. S., Sheoran, A., & Giri, B. (2003). *Process Biochemistry, 39*, 239–247.
17. Bradford, M. (1976). *Analytical Biochemistry, 72*, 248–254.
18. Williams, T., & Kelley, C. (2004). GnuPlot for Windows 4.0.
19. Staubmann, R., Ncube, I., Gübitz, G. M., Steiner, W., & Read, J. S. (1999). *Journal of Biotechnology, 75*, 117–126.
20. Bhardwaj, K., Raju, A., & Rajasekharan, R. (2001). *Plant Physiology, 127*, 1728–1738.

21. Ghanem, E. H., Al-Sayed, H. A., & Saleh, K. M. (2000). *World Journal of Microbiology and Biotechnology, 16*, 459–464.
22. Kambourova, M., Kirilova, N., Mandeva, R., & Derekova, A. (2003). *Journal of Molecular Catalysis B: Enzymatic*, *22*, 307–313.
23. Nawani, N., Khurana, J., & Kaur, J. (2006). *Molecular and Cellular Biochemistry*, *209*, 17–22.
24. Heller, M., & Mozes, N. M. (1981). *Methods Enzymology, 35*, 226–232.
25. Takeda, Y., Aono, R., & Doukyu, N. (2006). *Extremophiles*, *10*, 269–277.
26. Faber, K., & Franssen, M. C. R. (1993). *Trends in Biotechnology, 11*, 461–470.
27. Aryee, A., Simpson, B. K., & Villalonga, R. (2007). *Enzyme and Microbial Technology, 40*, 394–402.
28. Gilbert, E. J., Cornish, A., & Jones, C. W. (1991). *Journal of General Microbiology, 137*, 2223.
29. Aisaka, K., & Terada, O. (1981). *Journal of Biochemistry*, *89*, 817–822.
30. Sanz, L. C., & Olias, J. M. (1990). *Food Chemistry*, *37*, 221–228.
31. Eastmond, P. J. (2004). *The Journal of Biological Chemistry*, *279*(44), 45540–45545.
32. Raso, B. A., & Hultin, H. (1988). *Comparative Biochemistry and Physiology*, *89*(B), 671–677.
33. Verger, R., Srada, L., & Desnulle, P. (1971). *Biochimica et Biophysica Acta*, *242*, 580–592.
34. Guidoni, A. A., Bendouka, F., De Caro, J. D., & Rovery, M. (1981). *Biochimica Et Biophysica Acta*, *660*, 148–150.
35. Jin, W., Broedl, U. C., Monajemi, H., Gllick, J. M., & Rader, D. J. (2002). *Genomics*, *80*, 268–273.
36. Yu, H. W., Chen, H., Yang, Y. Y., & Ching, C. B. (2005). *Journal of Molecular Catalysis B: Enzymatic*, *35*, 28–32.
37. Di Cera, E. (2006). *The Journal of Biological Chemistry*, *281*(3), 1305–1308.

Capítulo 2

Test of enzymatic hydrolysis for the treatment of soybean oil and fat particles in wastewater

Patricia Peres Polizelli, Fernanda Dell Antonio Facchini e Gustavo Orlando Bonilla Rodriguez

Artigo submetido à revista: Bioresource technology (ISSN: 0960-8524, Elsevier, Impact factor 1,863)

Resumo

Um extrato enzimático bruto de sementes de *Pachira aquatica* foi obtido por homogenização e foi testado para a hidrólise de óleo de soja e resíduos de frigorífico avícola. Diferentes condições de hidrólise foram testadas, e foi determinado que a condição ideal é a 40°C de temperatura, com um tempo de hidrólise de 90 e 60 minutos e pH ótimo no valor de 7,0-9,0 para óleo de soja e o resíduo, respectivamente. A enzima foi estável a pH 9,0 e mostrou boa estabilidade em pH alcalino. A enzima foi estável na presença de várias formulações de detergentes comerciais, mas instável na presença de H_2O_2. A lipase não foi eficiente em reduzir o tamanho médio de partículas de gordura; altas doses foram requeridas para obter-se uma redução de 13 % após 72 horas de tratamento.

Palavras-chave: Triacilglicerol, pré-tratamento hidrolítico, lipase, *Pachira aquatica*

Test of enzymatic hydrolysis for the treatment of soybean oil and fat particles in wastewater

P. P. Polizelli [a], F. D. A. Facchini[b], G. O. Bonilla-Rodriguez[c,*]

[a]*Department of physics and b Chemistry and Environmental Sciences, IBILCE-UNESP, State University of São Paulo, Rua Cristovão Colombo 2265, São José do Rio Preto, SP, CEP 15054-000, Brazil.*

Corresponding author. Tel.: Phone: 55-17- 3221-2361 FAX: 55-17- 2356

E-mail address: bonilla@ibilce.unesp.br

Abstract

An enzymatic extract from seeds of *Pachira aquatica* was obtained by homogenization and it was tested for the hydrolysis of rancified soybean oil and wastewater from a poultry processing plant. Different hydrolysis conditions were tested, and it was determined that it should be carried out at a temperature of 40°C, a hydrolysis time of 90 and 60 minutes and an optimum pH 7.0-9.0 to rancified soybean oil and poultry processing plant. The enzyme was stable at pH 9.0 and showed good stability in the alkaline pH. The enzyme was stable in the presence of some commercial detergent formulations but unstable in the presence of H_2O_2. The lipase was not efficient in reducing average fat particle size; high doses were required to obtain a reduction of 13% after 72 hours of treatment.

Keywords: Fat; Triacylgrycerols, Hydrolysis pretreatment; Lipase; *Pachira aquatica*

1. Introduction

Lipases (glycerol ester hydrolases, E.C. 3.1.1.3) are hydrolases, which catalyze the hydrolysis of carboxyl ester bonds present in acylglycerols with the subsequent release of fatty acids and glycerol. They are particularly important due to the fact that they specifically hydrolyze oils and greases, which are of great interest for different industrial applications (Leal et al., 2002).

Lipids are an important component of domestic wastes, what create severe environmental pollution. It can form oily films on water surfaces preventing the diffusion of oxygen from air into water, leading to the death of many forms of aquatic life (Dharmsthiti and Kuhasuntisuk, 1998).

A previous research suggested that the application of a pretreatment to partially hydrolyze fat particles could accelerate the anaerobic treatment of slaughterhouse wastewater (Masse et al., 2001) and there are several research studies available on the treatment of oily wastes using lipase (Zinebi et al., 1994; Scioli and Vollaro, 1997; Cammarota et al., 2001; Masse et al., 2001; Mongkolthanaruk and Dharmsthiti, 2002; Masse et al., 2003; Jeganathan et al., 2006).

Major constituents of animal fat are triglycerides consisting of straight chains of saturated fatty acids (Wakelin and Forster, 1997). The component fatty acids of edible fats vary considerably depending on the source of wastewater. They may be free, dispersed or emulsified.

For wastewater treatment several processes, aerobic and anaerobic, are used; however, it is necessary to reduce the concentration of fats, oils and proteins or to eliminate these materials altogether, in order to enable the biological treatment to proceed without any inhibition of the biological reduction of organic matter in wastewater (Cammarota and Freire, 2006). High fat concentration can be cause sludge flotation and/or the development of sludges with different physical characteristics and/or poor activity as reported by Perle et al. (1995). Rinzema et al (1993) reported sludge flotation and a total sludge washout in a UASB reactor with a lipid loading rate exceeding 2-3 g COD/l.d. Vidal et al. (2000) account also problems of sludge flotation during anaerobic biodegradation.

Sayed et al. (1988) investigated the effect of high concentrations of suspended solids, mostly lipids, on the anaerobic treatment of slaughthouse wastewater.Their results suggested that the process-controlling factor was the liquefaction of colloidals adsorbed on the bacteria and the hydrolysis of coarse suspended solids entrapped within the biomass bed.

The application of a pretreatment process to hydrolyze and dissolve fats may improve the biological degradation of fatty wastewaters, accelerating the process and therefore reducing time by decreasing the fat adsorption to the surface of the anaerobic sludge and not limiting transport of the soluble substrate to the biomass and consequently avoid conversion rate in substrate decrease (Sayed et al., 1988; Rinzema et al., 1993; Perle et al., 1995). The utilization of a hybrid technology-enzymatic treatment associated with anaerobic biologic treatment- enables a reduction in hydraulic retention time and, consequently, in reactor volume, since it promotes hydrolysis of fats which cause problems of clogging of the sludge bed in anaerobic reactors of the UASB type (Chernicharo, 1997).

In effluent treatments, the use of low-cost enzymatic preparations is vital, since the use of high-cost commercial enzymatic preparations would make the pretreatment procedure not feasible (Cammarota and Freire, 2006).

Pachira aquatica is a tree belonging to the Bombacaceae family, found in a large area, from Southern Mexico to Northeastern Brazil. Its seeds are sometimes consumed raw or as roasted beans which have a chestnut flavor, being considered a delicacy. Furthermore, its young leaves and flowers are cooked and used as vegetables (Lorenzi, 1992).

In this study the lipase from *P. aquatica* seeds was characterized to determine its potential for use in wastewater treatment as an enzymatic pretreatment.

2. Methods

2.1 Materials

The *p*-nitrophenylpalmitate (*p*-NPP), and other products of analytical quality were purchased from Sigma Chemical Co, St Louis, MO, USA, and various oil substrates were obtained from a local market.

The seeds were collected from trees at our Campus, at São José do Rio Preto, State of São Paulo, Brazil. They were washed, peeled and extracted by homogenization in a food processor with a solution containing: 3mM DTT (dithiotreitol), 1 mM EDTA, 10mM sodium metabissulfite and 50mM Tris-HCl buffer (pH 8.0) at 25°C. The samples were clarified by centrifugation at 9,000g for 40min at 4°C using a Jouan CR3i refrigerated centrifuge and the supernatant was concentrated and stored in aliquots in liquid nitrogen until use.

2.2 Lipase assay

The activity of lipase was measured as previously described (Saxena et al., 2003). In this assay, by a titration method, the free fatty acids concentration was determined by a standard curve done with linoleic acid. The solution contained 50% rancified soybean oil, 31.3% 30 mM Tris-HCl buffer (pH 8.0), 10.15% Triton X-100, 0.002% of 10 mM $CaCl_2$ and 9.6% of the enzyme crude solution. The reaction was ended by adding acetone:ethanol (1:1), and the amount of released fatty acids was titrated with 50 mM KOH in the presence of phenolphthalein as indicator. Rancified soybean oil was used to use to advantage a industrial product.

The residual activity was measured by the cleavage of *p*-NPP and was analyzed at 37°C and pH 8.0 according to Chang et al. (1994). The pH residual activity profile was studied in a pH range from 4.0 to 10.0 using different 100mM buffers: Sodium Citrate (pH 4 to 5), Ada (pH 6 and 6.5), Sodim Phosphate (pH 7 and 7.5), Tris-HCl (pH 8 and 9) and Glycine (pH 10). Aliquots of the enzyme were left in the presence of each buffer for 240 minutes,

and subsequently incubated for 90 minutes at 37°C and analyzed spectrophotometrically at 410 nm.

Protein content was measured according to Bradford (1976). Briefly, a sample volume of 50 µl was added to 2.5 ml of the reagent in a disposable cuvette and thoroughly mixed before measuring the absorbance at 595 nm using BSA (bovine serum albumine) as standard protein.

2.3 Effect of detergent formulations on the lipolytic activity

In order to determine if the enzyme is suitable for use in cleaning formulations, its compatibility was studied in the presence of hydrogen peroxide (0-20%) and commercial detergents by directly incorporating them into the assay mixture, using soybean oil as substrate.

2.4 Stability of the enzyme preparation in the presence of commercial detergents

The stability of lipase was tested in the presence of several commercial detergents (3.3, 6.6 and 10 mg/mL) available in Brazil: Ariel®, Surf®, Tixan®, Ace® and Omo® under normal assay conditions when added directly to the assay mixture; the stability was determined at 37°C. The tests were performed with soybean oil as substrate.

2.5 Wastewater hydrolysis

The wastewater from a poultry processing plant was treated with enzyme crude solution. Wastewater hydrolysis was carried out with several enzyme concentrations (3-20 mg/mL) and incubation times (0-200 min) at 37°C and pH 8.0. Different conditions of temperature (25-55°C) and pH (4-10) were also examined.

2.6 Particles degradation

Pieces of beef fat were cut as uniform small particles of about 5 x 5 x 5 mm and stored at 4°C. An experiment included a mixture of one pieces and crude enzyme solution (5, 10, 15 and 20 mg/mL) in 100mM Tris-HCl buffer pH 8.0 and always incorporated a control. The mixtures were added to beakers and were gently mixed (30 RPM) in a shaker MA 832 *Marconi* with temperature and speed control, at 40°C in varying incubation times (from 0 to 72 hours).

Beef fat was choice because demonstrated better results than pork fat as found by Masse et al. (2001) possibly due to different triglyceride compositions. Grinstone et al (1986) reported that in beef, 18.4% of the triglycerides are composed of saturated fatty acids, while 45.1% have only one unsaturated fatty acid; pork fat has 6.6% and 29.7% of its triglycerides with zero and one unsaturated fatty acid, respectively. In other study (Polizelli et al. 2008) we verify that lipase from *P. aquatica* shown better enzymatic activity with polyunsaturated oleic (18:1) that linoleic (18:2) and linolenic (18:3) acids.

3. Results and Discussion

3.1 Lipase characterization

The tests were performed with rancified soybean oil analyzing the effects of pH, temperature and incubation time in the enzymatic activity.

Lipolytic activity was assayed at various pH values using the titration method with rancified soybean oil as substrate, displaying maximum activity around 7.0 to 9.0 (figure 1). Concerning the effect of pH on the stability, residual activity showed that the lipase was more stable at pH 9.0. At acids pH (that are preferable to work subsequent biological treatment), activity remained 45% after 3h (figure 2), that is more long than period required for optimal hydrolytic treatment.

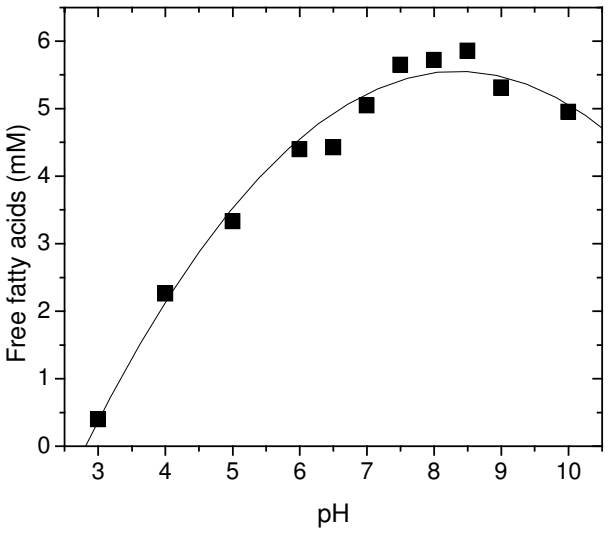


Figure 1. Effect of pH on activity of *P. aquatica* lipase. Activity was measured with oxidized soybean oil as substrate at 37°C.

The lipase showed maximum activity at 40°C for an incubation time of 90 minutes (data not shown) and the hydrolysis increased with time up to 90 min.; after that there was a decrease in the hydrolysis, probably due to accumulating free fatty acids that could destabilize the interface or decrease the pH.

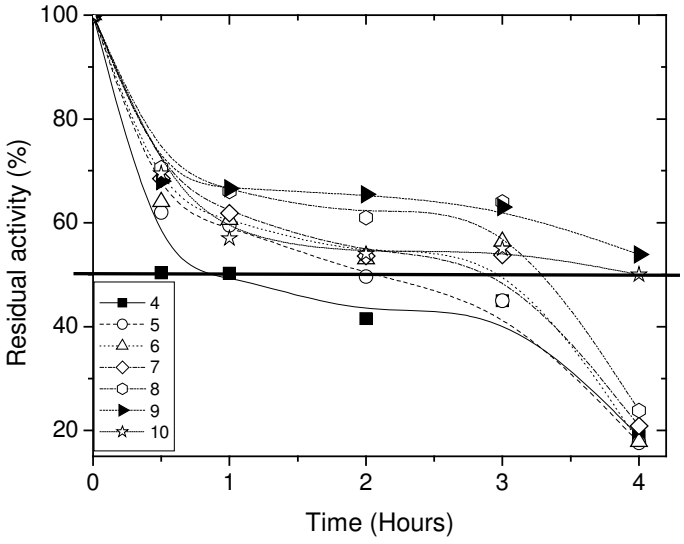


Figure 2. Effect of pH on stability of the lipase. Activity was determined by a colorimetric method with *p*-NPP at pH 8.0 and 37°C.

3.2 Effect of detergents on the lipolytic activity

The information on the stability of enzymes against tensoactives and oxidants is an important property to be analyzed, because to determine the suitability of enzyme for use in detergents, should also be stable in the presence of bleaching agents and commercial detergents. The activity in the presence of hydrogen peroxide showed that the enzyme was stable at very low concentration of H_2O_2, it retained 30% of the activity in the presence of 1% of H_2O_2 (table 1). On the other side, the lipase from *Pachira aquatica* in the presence of commercial detergents (table 2) as the enzyme retained approximately 80% of its activity in the presence of 10 mg/ml Surf®, Ace® and Omo® 60 and 54% of Tixan® and Ariel®, respectively.

Table 1. Effects of the percentage of H_2O_2 on lipase activity. The assay was realized with soybean oil as substrate in 100mM Tris-HCl pH 8.0 buffer by 60 min of time incubating at 37 °C.

H_2O_2 (%)	Relative activity (%)
0	100
0.05	103
0.25	102
0.3	103
0.35	102
0.4	62.2
0.45	56.9
0.5	45
1	30
5	23
10	0
20	0

Table 2. Relative activity of the lipase in the presence of commercial detergents. The assay was performed with soybean oil as substrate in 100mM Tris-HCl pH 8.0 buffer by 60 min of time incubating at 37 °C.

Detergent	Relative activity (%)		
	3.3 mg/ml	6.6 mg/ml	10 mg/ml
Ariel	80.35	60.7	54
Tixan	100	60.5	60
Surf	100	100	80
Ace	100	100	80
Omo	60	80.35	80.35

3.3 Stability of the enzyme preparation in the presence of commercial detergents

The lipase was found to be stable; it showed ~ 40% of activity in the presence of Tixan® detergent after 4 hours of exposition and 20% activity in Surf® and Omo® (figure 3). This detergent compatibility is interesting for a potential application in detergent industry.

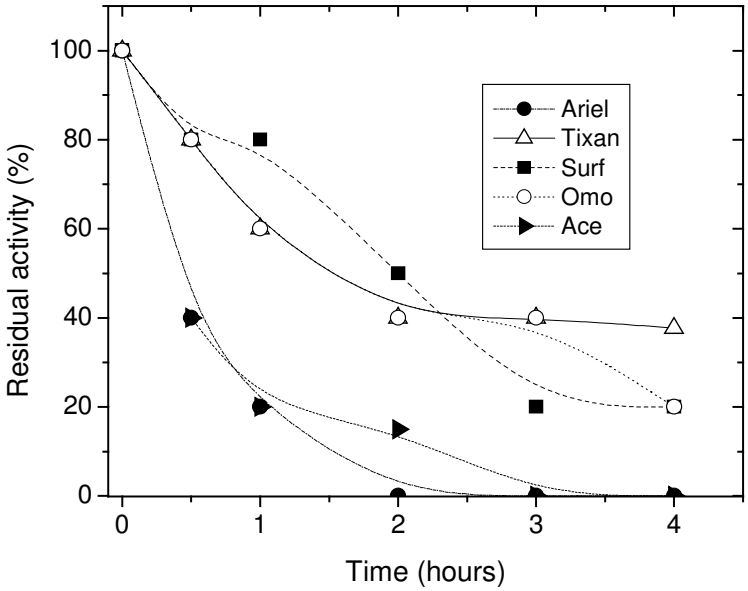


Figure 3. Compatibility of the lipase from *Pachira aquatica* with different commercial detergents (10 mg/ml). Activity was determined by titration method with soybean oil at pH 8.0 and 37°C.

3.4 Wastewater hydrolysis

A poultry processing plant usually contains large amounts of wastewater, both in the process itself and in the washing of equipment and facilities, characterized by high organic rates and suspended solids concentrations. However, the characteristics of the wastewater vary from plant to plant, depending on the industrial process and the water consumed per

slaughtered bird (Del Nery et al., 2001). The optimum conditions of pH, temperature, incubation time and enzyme concentration were investigated.

Since the lipolytic activity was maximal at pH 8.0 (figure 4) and 40°C, these results demonstrated that the enzyme is active in the substrates found in wastes and its characteristics are not changed by the substrates, since they were similar to those found for soybean oil and p-NPP. The concentration of free fatty acids in the wastewater before lipolytic treatment was 0.07 mM and the maximal activity was reached with 60 minutes of incubation shown 0.35 mM of free fatty acids.

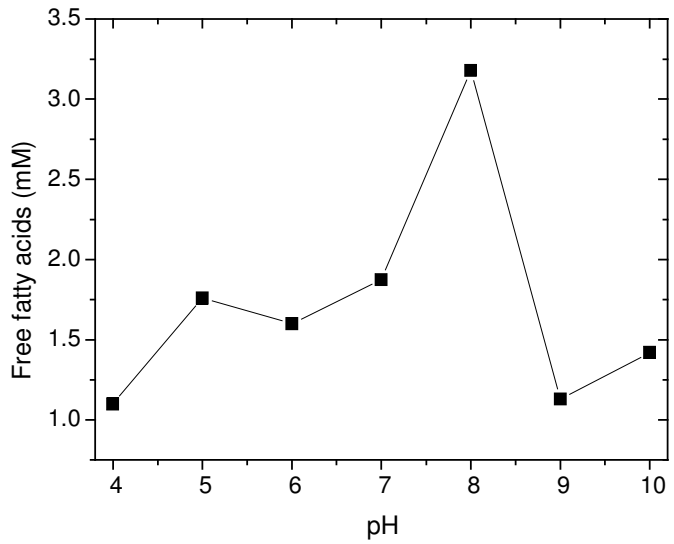


Figure 4. Effect of pH on activity of *P. aquatica* lipase. Activity was measured by alkaline titration with wastewater at 37°C.

3.5 Particles degradation

The effect of the enzyme in the fat particles degradation was tested with different crude enzyme concentrations and incubation time (figures 5 and 6). The average particle size data obtained during these experiments was analyzed statistically by the Student´s *t* test (Zar, 1999), using the BioEstat 4.0 program (Ayres et al., 2005) and $p < 0.05$. After 72 hours of treatment with the lipase occurred a decrease in the fat particle size in solution to about 13% of the original. Higher doses of enzyme were needed to obtain a maximal particle size reduction, but the found values did not show significant differences. Masse et al. (2001) also reported no significant particle size reduction after 4h of pretreatment with the plant lipase *EcoSystem Plus*.

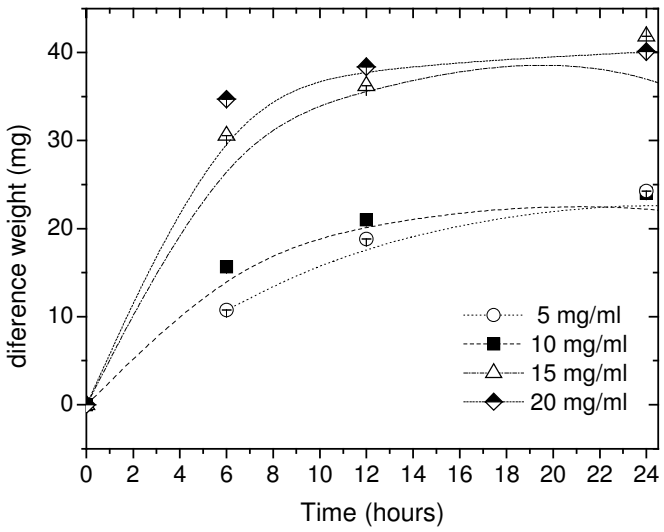


Figure 5. Reduction in the difference weight of beef fat particles after until 24 hours of pretreatment with different amount of lipase from *P. aquatica* at 40°C and pH 8.0.

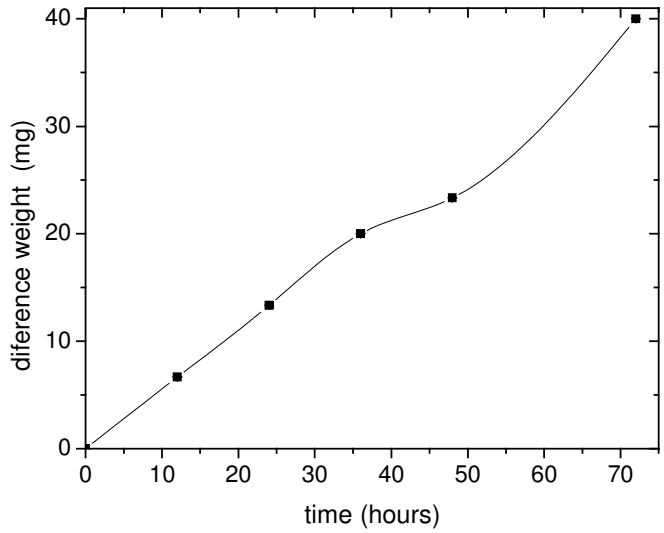


Figure 6. Reduction in the difference weight of beef fat particles after until 72 hours of pretreatment with lipase 10 mg/ml from *P. aquatica* at 40°C and pH 8.0.

4. Conclusion

The present work found the optimal hydrolysis conditions, in terms of the enzyme concentration, incubation time and optimum pH and temperature, as well as its stability against detergents and H_2O_2. This analysis shows the potential of this enzyme for applications in pre-treatments in waste of oils and greases.

These results suggest that crude lipase of *P. aquatica* can be used for oil and fats wastewater treatment for hydrolysis of used soybean oil that is the major waste of frying. It is also stable to detergents powder formulations.

References

Ayres, M.; Ayres Jr, M.; Ayres, D.I.; Santos, A.S., 2005. BioEstat.4.0: Aplicações estatísticas nas áreas das ciências biológicas e médicas. Sociedade Civil Mamirauá, MCt e Imprensa Oficial do Estado do Pará, Belém, Brazil.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72, 248-54.

Cammarota, M. C., Freire, D.M.G., 2006. A review on hydrolytic enzymes in the treatment of wastewater with high oil and grease content. Bioresour. Technol. 97, 2195-2210.

Cammarota, M.C., Teixeira, G.A., Freire, D.M.G., 2001. Enzymatic pre-hydrolysis and anaerobic degradation of wastewaterrs with high high fat contents. Biotech. Lett. 23, 1591-1595.

Chang, C. R., Chou, S.J., Shaw, J.F., 1994. Multiple forms and functions of *Candida rugosa* lipase. Biotechnol. Appl. Biochem. 19, 93-97.

Chernicharo, C.A.L., 1997. Reatores Anaeróbicos, DESA/UFMG.

Del Nery, V., Damianovic, M.H.Z., Barros, F.G., 2001. The use of a upflow anaerobic sludge blanket reactor in of poultry slaughterhouse wastewater. Water Sci Tecnol 44(4): 83-8.

Dharmsthiti, S.,Kuhasuntisuk, B., 1998. Lipase from *Pseudomonas aeruginosa* LP602: biochemical properties and application for wastewater treatment. Journal of industrial Microbiology & Biotechnology 21, 75-80.

Jeganathan, J., Nakhla, G., Bassi, A., 2006. Hydrolytic pretreatment of oily wastewater by immobilized lipase. Journal of Hazardous Materials 37 (1), 121-128.

Hydrolytic enzymes as coadjuvants in the anaerobic treatment of dairy wastewaters. Brazilian Journal of Chemical Engineering 19 (2), 175-180.

Leal, M.C.M.R. , Cammarota, M.C., Freire, D.M.G., Sant'Anna Jr., G.L., 2002.

Lorenzi, H., 1992. Árvores Brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil, 2nd ed. Editora Plantarum LTD., Nova Odessa.

Masse, L., Kennedy, K.J., Chou, S., 2001.Testing of alkaline and enzymatic hydrolysis pretreatments for fat particles in slaughterhouse wastewater. Bioresour. Technol. 77, 145-155.

Masse, L., Masse, D.I., Kennedy, K.J., 2003. Effect of hydrolysis pretreatment on fat degradation during anaerobic digestion of slaughterhouse wastewater. Process Biochem. 38, 1365-1372.

Mongkolthanaruk, W. and Dharmsthiti, S., 2002. Biodegradation of lipid-rich wastewater by a mixed bacterial consortium. Int. Biodeterior. Biodegrad. 50, 101-105.

Perle, M., Kimchie, Sh., Shelef, G., 1995. Some biochemical aspects of the anaerobic degradation of dairy wastewater. Water Research 29(6), 1549-1554.

Rinzema, A., Alphanaar, A., Lettinga, G., 1993. Anaerobic digestion of long-chain fatty acids in UASB and expanded granular sludge bed reactor. Process Biochemistry 28, 527-537.

Saxena, R.K., Davidson, W. S., Sheoran A., Giri, B., 2003. Purification and characterization of alkaline thermostable lipase from *Aspergillus carneus.* Proc. Biochem. 39, 239-247.

Sayed, S., Zanden, J., Wijiffels, R., Lettinga, G., 1988. Anaerobic degradation of the various fractions of slaughterhouse wastewater. Biological Wastes 23(2), 117-142.

Scioli, C. and Vollaro, L., 1997. The use of *Yarrowia lipolytica* to reduce pollution in olive mill wastewater. Water Res. 31 (10), 2520-2524.

Vidal, G., Carvalho, A., Méndez, R., Lema, J.M., 2000. Influence of the content in fats and proteins on the anaerobic biodegradatibility of dairy wastewaters. Bioresource Technol 74, 231-239.

Wakelin, N. G., Forster, C.F., 1997. An investigation into microbial removal of fats, oils and greases. Bioresour. Technol. 59, 37-43.

Zar, J.H., 1999. Biostatistical Analysis, 4th ed. Prentice Hall, New Jersey.

Zinebi, S., Henriette, C., Petitdemange, E., Joret, J.C, 1994. Identification and characterization of bacterial activities involved in wastewater treatment by aerobic fixed-bed reactor. Wat. Res. 28, 2575-2582.

Capítulo 3

Effect of surfactants and polyethylene glycol on the activity and stability of a lipase from oilseeds of *Pachira aquatica*

Patricia Peres Polizelli, Márcio José Tiera e Gustavo Orlando Bonilla Rodriguez

Artigo submetido à revista: Journal of the American Oil Chemists´ Society (ISSN: 0003-021X, Elsevier)

Resumo

As lipases de sementes oleaginosas apresentam grande potencial para exploração comercial como enzimas industriais. Lipases são usadas misturadas com surfactantes em produtos de limpeza e outras formulações, e assim, ambos componentes devem ser compatíveis um com o outro. Este trabalho apresenta o resultado do efeito de surfactantes aniônicos, catiônicos e não-iônicos, polietilenoglicol e uréia na atividade e estabilidade da lipase. A enzima purificada foi analisada por ensaios espectrofotométricos realizados usando *p*-nitrofenil acetato (*p*-NPA) como substrato a pH 7,5 e 25°C. A atividade enzimática foi aumentada na presença do surfactante catiônico brometo de cetiltrimetilamonio (CTAB). Sais biliares aumentam a atividade da lipase na faixa de concentração estudada, onde surfactantes aniônicos e não-iônicos mostram efeito inibitório. A incubação da enzima com soluções aquosas de PEG, 12.000, 8.000 e 200, ativou a lipase e a máxima ativação (161%) ocorreu em 2 mM de PEG 12.000. Este efeito na lipase pode ser devido à exposição de alguns resíduos hidrofóbicos localizados na vizinhança do sitio ativo ou por agregação enzimática.

Palavras-chave: sementes oleagionosas, lipase, surfactantes, polietilenoglicol, sais biliares, uréia.

Biotechnology and Biocatalysis

Effect of surfactants and polyethylene glycol on the activity and stability of a lipase from oilseeds of *Pachira aquatica*

Patricia Peres Polizelli[a], Márcio José Tiera[b], Gustavo Orlando Bonilla-Rodriguez[b*]

[a] *Department of Physics and* [b] *Department of Chemistry and Environmental Sciences, IBILCE-UNESP, Rua Cristovão Colombo 2265, São José do Rio Preto, SP 15054-000, Brazil.*

[*]Corresponding author. Tel.: +5517 3221 2361 Fax: +5517 3221 2356

E-mail address: bonilla@ibilce.unesp.br (G.O. Bonilla-Rodriguez)

Abstract

Lipases from oilseeds have a great potential for commercial exploration as industrial enzymes. Lipases are used mixed with surfactants in cleaning and other formulated products, and accordingly, both components must be compatible with each other. This work presents the results of the effects of anionic, cationic and nonionic surfactants, polyethylene glycol and urea on the activity and stability of a lipase extracted of oilseeds from *Pachira aquatica*. The enzyme was purified and the spectrophotometric assays were done using *p*-nitrophenyl acetate (*p*-NPA) as substrate pH 7.5 and 25°C. The activity was significantly enhanced by the cationic surfactant CTAB. Bile salts increased the lipase activity in the tested concentration range, whereas anionic and nonionic surfactants showed an inhibitory effect. Aqueous solutions of PEG activated the lipase and maximum activation (161%) occurred in PEG 12,000.This effect on lipase that can be due to exposition of some hydrophobic residues located in the vicinity of the active site or aggregation.

Key words: oilseeds, lipase, surfactants, polyethylene glycol, bile salts, urea.

Introduction

Enzymes are highly selective catalysts, able to operate under mild reaction conditions, properties that make them potentially attractive for industrial purposes. The biological function of lipases is mediated by an interaction between lipid and water interfaces that affects their activity [1]. Surfactant-enzyme interactions in aqueous solutions have been extensively studied for technical applications, such as drug delivery, cosmetics and detergency, and for studying interactions between membrane proteins and lipids [2], and then both components must be compatible with each other.

Lipases improve the washing capacity of the protease-containing detergents and improve the removal of fatty food stains and sebum from fabrics, which are difficult to remove under normal washing conditions [3]Another important factor is that using hydrolytic enzymes (lipases, proteases and amylases) in detergent formulations allows laundering at lower temperatures, and thus reduces energy expenditure [4]. Accordingly, lipases must be characterized in terms of their stability towards surfactants[5-7].

Polyethylene glycol (PEG) is a synthetic polymer having low toxicity, soluble in aqueous solution, largely used in industrial biotechnological process [8].

Urea is a well-known protein denaturating agent [9] that can affect the enzyme structure by direct interaction with the macromolecule or by an indirect action through effects on the structure and properties of the surrounding solvent or by a combination of both of these mechanisms [10].

In this work we compared the effect of urea, PEG and investigated the influence of surfactant charge and chain length on the lipase activity extracted from *Pachira aquatica.*

Experimental Procedures

Materials

Pachira aquatica also known in Brazil as Munguba or Castanheira d´água, is a tree belonging to the Bombacaceae family, found in South America, and studies [11] developed on the composition of the seeds demonstrate that *Pachira aquatica* has a high oil content (44.1%) and could be a candidate for biodiesel production, so we were motivated to investigate the presence of a lipase that could be isolated from that source.

The seeds from *Pachira aquatica* were collected from trees of our Campus, at São José do Rio Preto, State of São Paulo. They were washed, peeled and extracted by homogenization in a food processor with a solution containing: 3mM DTT, 1 mM EDTA, 10mM sodium metabissulfite and 50mM Tris-HCl buffer (pH 8.0) at 25°C. The samples were clarified by centrifugation at 9,000xg by 40min at 4°C using a Jouan CR3i refrigerated centrifuge and the supernatant was concentrated and stored until use in liquid nitrogen. The purification was performed as described [12]. The lipase was purified 9.6 fold with specific activity of approximately 641 U/mL, showing one band in electrophoresis. The enzyme is a lipase as demonstrated in assays using soybean oil as substrate (U >1). This lipase has a molecular weight of ~55 kDa and maximum activity at 40°C and pH 8.0. The surfactants sodium dodecyl sulfate (SDS), sodium octyl sulfate (SOS), dodecyltrimethylammonium bromide (DoTAB), tetradecyltrimethylammonium bromide (TTAB) and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma-Aldrich Co., (USA) and recrystallized from ethanol and ethyl acetate. The reagents, Triton X- 100, Tween 80 (Vetec, Brazil) and polyethylene glycols (Fluka, Switzerland) were used as provided.

Methods

Protein concentration

Protein concentration was determined according to the method of Bradford [13], using bovine serum albumin (BSA) as standard.

Lipase assay

We used a modified version of the procedure proposed by Chang et al. [14], based on the hydrolysis of p-NPA. The substrate was dissolved in 6% dimethyl sulfoxide (DMSO) and added to 30 mM Hepes buffer pH 7.5 and 0.264U/mL of the enzyme. We had to choose this substrate because it is soluble in aqueous solutions, allowing to investigate the effect of added tensoactives. The assay mixture was incubated for 5 minutes at 25°C and lipase activity was measured at 410 nm using a double-beam spectrophotometer Cintra 10e (GBC Scientific Equipment (USA) LLC), allowing to discount the spontaneous hydrolysis of *p*-NPA. Each assay was done in triplicate.

Effects of surfactants

The effects of surfactants on lipase activity were studied by carrying out the assays at different surfactant concentrations (0 to 50 mM). We used SDS, SOS, tauricholic acid, sodium glycholate, CTAB, DTAB, TTAB, Triton X-100 and Tween 80.

Lipase stability was also analyzed by preincubating the enzyme with 10 mM of each surfactant, in the presence or not of 10 mM calcium chloride. Aliquots were withdrawn at intervals and used for the assay, from 0 to 180 min.

Effects of Polyethylene glycol

The effects of polyethylene glycol of various molecular weights (0.2, 8 and 12 kDa) were studied at a range concentration from 0 to 25 mM, 25°C and pH 7.5. The enzyme stability was measured by preincubating the enzyme with a 10 mM concentration of the different MW PEGs and the test was performed at 25°C and pH 7.5. Aliquots were withdrawn at intervals and used for the assay.

Effects of urea

The effects of urea (0 to 50 mM) was determined at 25°C and 30 mM Hepes buffer pH 7.5 by the assay methods described above. Lipase stability in the presence of 10 mM urea was analyzed by preincubating the enzyme, and aliquots were withdrawn at different intervals for assays, from 0 to 240 min.

Results and discussion

Effects of surfactants

As shown in Figure 1 A, SOS and SDS showed inhibitory effects in the whole concentration range. Taurocholic acid did not show a clear trend. Lipolytic activity increased initially and afterwards remained lower but very close to the 100% reference value. Glycholate induced an interesting response: increased lipase activity up to 10 mM (108% relative activity), but above 20 mM there was a decrease, obtaining similar values to those observed for SOS. The results observed for glycholate are similar to those reported [15] for a lipase from *Aspergillus carneus*. SDS induced the strongest inhibition on lipase

activity; at 50 mM, reduced approximately 40% the relative activity, probably due to a low cmc (critical micelle concentration) value or by influence its chain length.

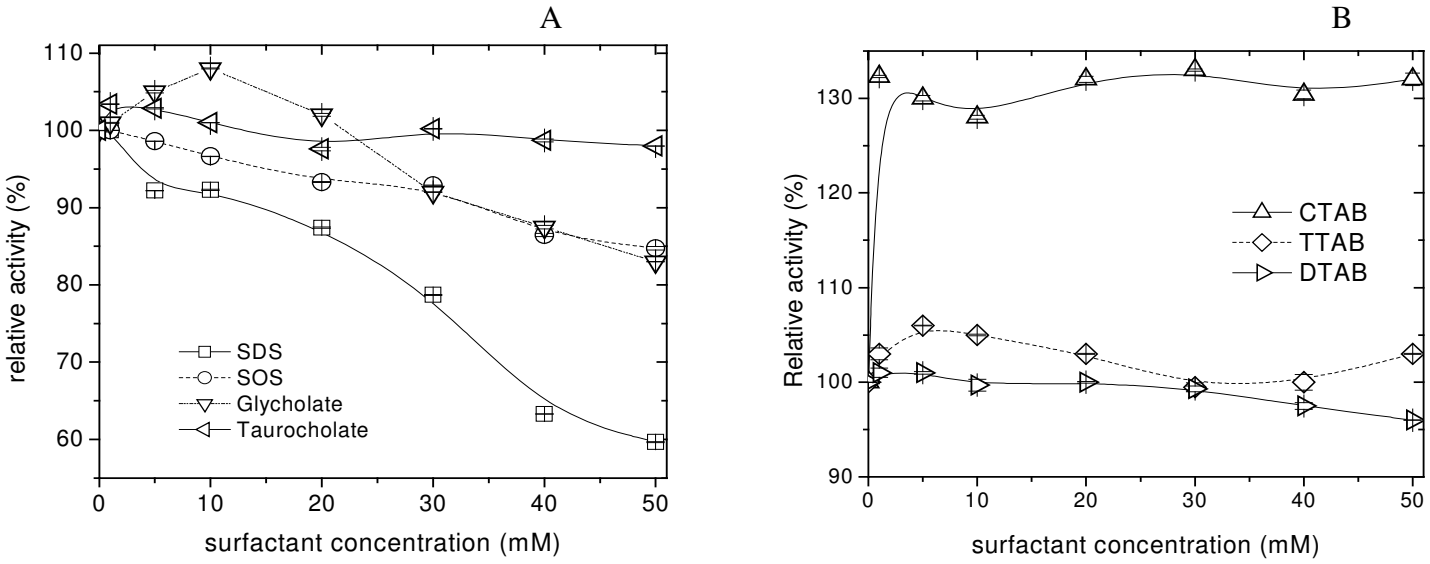


Figure 1. Effect of anionic (A) and cationic (B) surfactants concentration on the activity of the lipase from *Pachira aquatica* using *p*-NPA as substrate at 25°C and pH 7.5 for 5 min. Each point is the average of three replicates ± S.D.

Among the tested cationic surfactants (Fig. 1 B), CTAB showed clear stimulatory effects, inducing a 32% activity increase in lipase activity. The effect seems to be proportional to the chain length (CTAB>TTAB>DTAB). This behavior can be also due to micelle formation, because CTAB cmc is low, facilitating the interaction substrate-enzyme. The lipase activity was also slightly increased (6%) by TTAB. On the other side, DTAB did not induce a clear response up to 20 mM, but caused a very weak inhibition that reached 4% at 50 mM.

In the presence of nonionic surfactant (Fig. 2) the lipase activity was inhibited for all the tested concentrations. However, above 20 mM, tween 80 inhibiton was more pronounced

than that in the presence of Triton X 100. A similar inhibition produced by triton X-100 was found also [16] for chicken pancreatic lipase.

A stimulating effect of surfactants on enzymatic hydrolysis has been reported several times. Explanations for the surfactant effect include an increase of enzyme stability and increasing accessibility of the substrate [17].

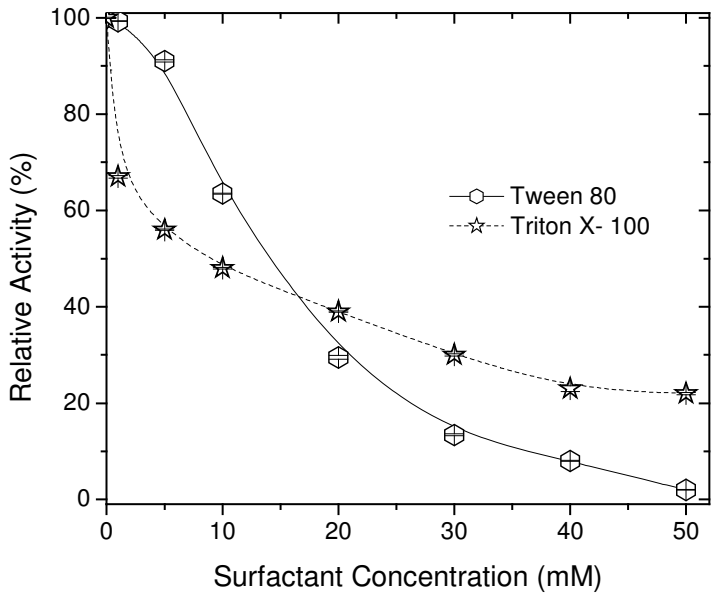


Figure 2. Effect of nonionic surfactants concentration on lipase activity using *p*-NPA as substrate at 25°C and pH 7.5. Each point is the average of three replicates ± S.D.

An interaction surfactant-protein involves some kind of protein denaturation.

However, the reduced activity can include other explanations beyond conformational changes of the protein. Gargouri et al. [18] related the inhibition of pancreatic lipase activity by tensoactives to a desorption of the enzyme from its substrate occurring after a change in the interfacial quality.

In order to explain the correlation between the surfactant concentration and the decrease of enzymatic activity it can be conjectured that the active site is occupied by surfactant molecules when it reaches a certain concentration, or alternatively by interaction of the substrate molecules with surfactants.

Figure 3 shows that the only increase of activity was obtained for CTAB at 10 minutes of exposure, but a reduction of activity was evident after 20 minutes. Up to 1 hour, inhibition was larger in the presence of Triton, followed by SDS and CTAB. After that time, the effect of Triton was lower than for SDS and CTAB.

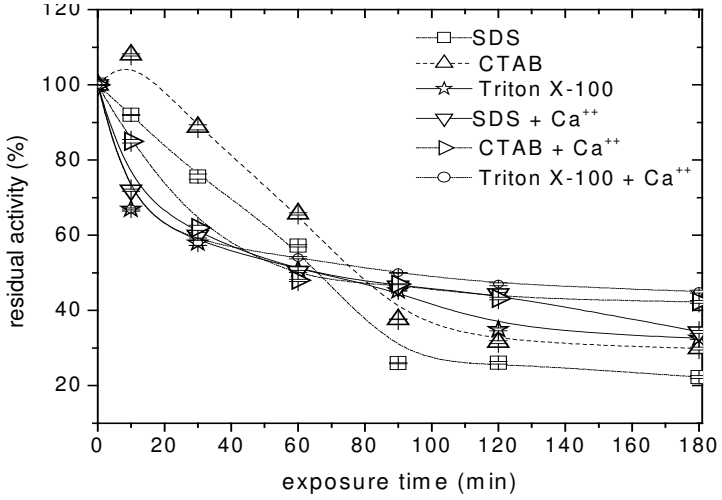


Figure 3. Lipase stability in the presence of surfactants in the absence and in the presence of 10 mM $CaCl_2$.

Assay performed using *p*-NPA as substrate at 25°C and pH 7.5. Each point is the average of three replicates ± S.D.

Lipase stability also was tested in the presence of 10 mM $CaCl_2$ (Fig. 3.3). The enzyme showed greater stability to SDS and CTAB than in the absence of calcium, but the data obtained in the presence of triton X-100 did not show significant differences. Ions Ca^{++} are known to provide a stabilizing effect on the three-dimensional structure of any lipase but surfactant aggregates and interactions Ca^{++}-micelles compete favorably with enzymes for bound calcium ions [19]. Since triton X 100 has a low value for cmc, this could explain the absence of significative differences of stability.

Effect of Polyethylene glycol

Incubation of lipase with various concentrations of PEG 0.2, 8 and 12 kDa increased the hydrolytic activity in aqueous solution (Fig. 4) as described by Grimonprez and Johansson [20] for phosphofructokinase and glyceraldehydes-3-phosphate dehydrogenase, Otero et al. [21] for a family of *Candida rugosa* isolipases and by Börjesson et al. [22] for lignase from *Trichoderma reesei*. The increase in activity was proportional to PEG molecular mass as described by Pancera, 2006 [23] and Otero, 2005 [21], and no inhibition was observed within the tested range, although the stimulation effect tends to decrease with time after an initial burst. This behavior can be due to an enhance in hidrophobicity promoted by increase in the chain length as suggested by Forciniti et al. [24] and then facilities the coupling of substrate in the active site or still by reduced water activity and favoring the exposure of hydrophobic residues in the opening lid, or even enzyme aggregation. A comparative analysis of crystallographic models of isolipases from *Candida rugosa*

suggests a role for PEG in the enzyme activation and further stabilization of the actived form via dimerization in aqueous media [21].

The lipase showed stability in the presence of several PEGs maintaining its activity significantly higher than the control after 4 hours (Fig. 5).

It is noteworthy that differently from the data shown previously (Fig. 4) the lipase stability in the presence of PEG was larger for PEG 200 and 8,000 than for 12,000 (Fig. 5). The results agree with those reported that indicate that when the molecular mass of PEG is larger than 1,000 g.mol^{-1} the chains are excluded of bigger aqueous compartments around the protein; Larger chains can dehydrate the protein [25].

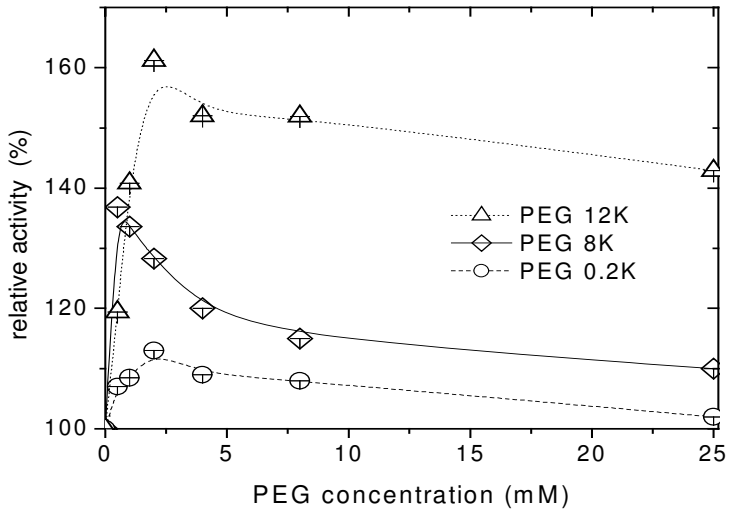
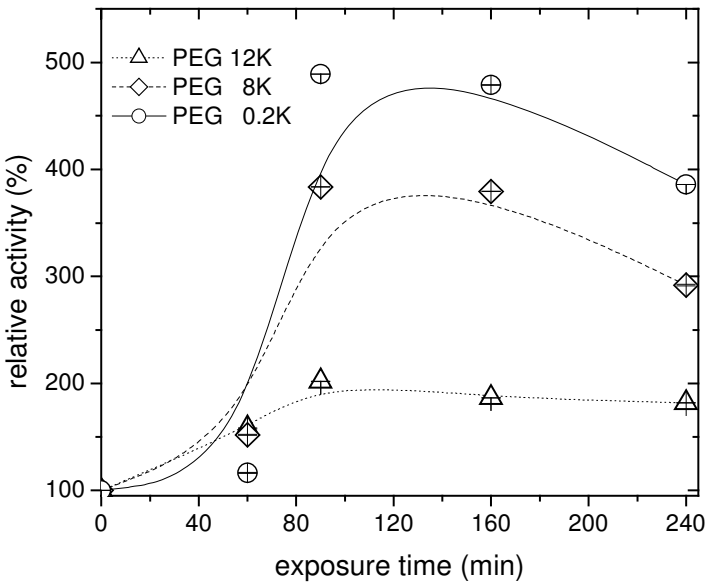


Figure 4. Lipase activity in the presence of PEG. The assays were done using *p*-NPA as substrate at 25°C and pH 7.5. Each point is the average of three replicates ± S.D.

Figure 5. Lipase stability in the presence of 10 mM PEG. The assays were done using *p*-NPA as substrate at 25°C and pH 7.5. Each point is the average of three replicates ± S.D.

Effect of urea

Up to 10 mM we can observe an increase and afterwards an inhibition of lipase activity (Fig. 6). We attribute this effect to a



conformational change (unfolding) caused by urea. In the presence of 10 mM urea (Fig. 6)

the enzyme was inhibited, been nearly 40% of initial after 30 minutes of exposure to urea and remained so thereafter. At 240 min. of exposure the relative activity was 58%.

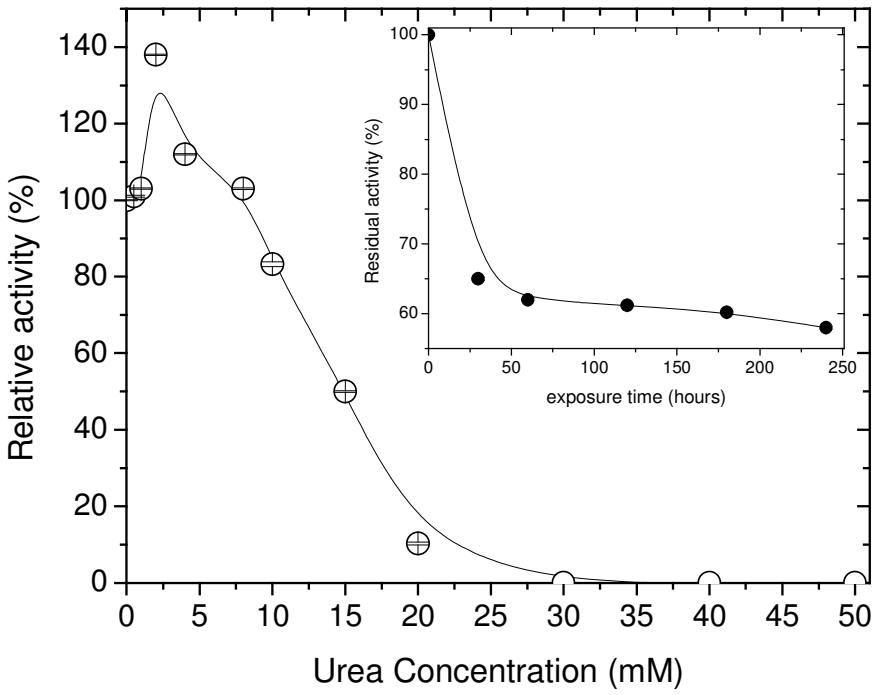


Figure 6. Effect of urea concentration on lipase activity. Inset: Lipase stability in the presence of 10 mM urea. The assays were done using *p*-NPA as substrate at 25°C and pH 7.5. Each point is the average of three replicates ± S.D.

Acknowledgements

This work received financial support from Brazilian Agencies CAPES (PPP), FAPESP and CNPq (GOBR).

References

[1] Miyake Y, Ohkubo M, Teramoto M (1991) Lipase-catalyzed hydrolysis of 2-naphtyl

esters in biphasic system. Biotechnol. Bioeng. 38 (1): 30-36.

[2] Savelli G, Spreti N, Di Profio P (2000) Enzyme activity and stability control by amphiphilic self-organizing systems in aqueous solutions. Curr Opin. in Colloid Interface Sci. 5: 111-117.

[3] Antrim R L, Buchert J, Burrows H, Herbots I, Kottwitz B, Lenting HBM, Niku-Paavola M-L, Reilly P J, Suurnäkki A, Viikari L (2004) Industrial Enzymes: Enzymes in Nonfood Applications in: Aehle W (ed) Enzimes in Industry (2nd Edition), Wiley-VCH Verlag GmbH & Co. KGaA pp 155-244.

[4] Davranov K (1994) Microbial lipases in biotechnology. Appl. Biochem. Microbiol. 30: 427-32.

[5] Stamatis H, Xenakis A, Kolisis F N (1999) Bioorganic reactions in microemulsions the case of lipases: Biotechnol. Adv.17(4-5): 293-318;

[6] Holmberg K (1994) Organic and Bioorganic Reactions in Microemulsions: Adv. Colloid Interface Sci. (51): 137-174.

[7] Holmberg K (2003) Organic reactions in microemulsions: Curr. Opin. Colloid Interface Sci. 8: 187-196.

[8] Johansson G (1992) Poly (ethylene glycol) chemistry: Biotechnical and biomedical Applications, Plenum Press, New York.

[9] Brandts Jf, Hunt L (1967) Thermodynamics of protein denaturation .3. Denaturation of ribonuclease in water and in aqueous urea and aqueous ethanol mixtures. J. Am. Chem. Soc. 89 (19): 4826-4838.

[10] Dias LG, Florenzano FH, Baptista MS, Souza SMB, Alvarez EB, Chaimovich H, Cuccovia IM, Amaral CLC, Brasil CR, Romsted LS, Politi MJ (2002) Effect of urea on

biomimetic systems: Neither water 3-D strucuture rupture nor direct mechanism, simply a more ¨polar water¨. Langmuir 18 (2): 319-324.

[11-] Oliveira J T A, Vasconcelos I M, et al. (2000) Composition and nutritional properties of seeds from Pachira aquatica Aubl, Sterculia striata St Hil et Naud and Terminalia catappa Linn. Food Chem. 70(2): 185-191.

[12] Polizelli PP, Facchini FDA, Cabral H, Bonilla-Rodriguez GO (2008) A new lipase isolated from oleaginous seeds from *Pachira aquatica* (Bombacaceae). Appl. Biochem. Biotechnol. Available on line.

[13] Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem.72: 248-254.

[14]Chang C R, Chou SJ, Shaw JF (1994) Multiple forms and functions of *Candida rugosa* lipase. Appl. Biochem. Biotechnol. 19: 93-97.

[15] Saxena RK, Davidson WS, Sheoran A, Giri B (2003) Purification and characterization of an alkaline thermostable lipase from *Aspergillus carneus*. Process Biochem. 39: 239-247.

[16] Fendri A, Frikha F, Mosbah H, Miled N, Zouari N, Bacha A B, Sayari A, Mejdoub H, Gargouri Y (2006) Biochemical characterization, cloning, and molecular modelling of chicken pancreatic lipase. Arch.Biochem.Biophys. 451: 149-159.

[17]Kristensen JB, Börjesson J, Bruun MH, Tjerneld F, J rgensen H (2007) Use of surface active additives in enzymatic hydrolysis of wheat straw lignocellulose. Enzyme Microb. Technol. 40: 888-895.

[18] Gargouri Y, Julien R, Bois AG, Verger R, Sarda L (1983) Studies on the detergent inhibition of pancreatic lipase activity. J. Lipid Res. 24: 1336-1342.

[19] Stoner MR, Dale DA, Gualfetti PJ, Becker T, Randolph TW (2005) Ca^{2+}-surfactant interactions affect enzyme stability in detergent solutions. Biotechnol. Prog. 21: 1716-1723.

[20] Grimonprez B, Johansson G (1996) Liquid-liquid partitioning of some enzymes, especially phosphofructokinase, from Saccharomyces cerevisiae at sub-zero temperature. J. Chromatogr. B- Biomedical applications, 680 (1-2) 55-63.

[21] Otero C, Fernández-Pérez M, Hermoso JA, Ripio MM (2005) Activation in the family of *Candida rugosa* isolipases by polyethylene glycol. J. Mol. Catal. B: Enzym. 32: 225-229.

[22] Börjesson J, Peterson R, Tjerneld F (2007) Enhanced enzymatic conversion of softwood lignocellulose poly(ethylene glycol) addition. Enzyme Microb. Technol. 40:754-762.

[23] Pancera SM, da Silva LHM, Loh W, Itri R, Pessoa A, Petri DFS (2002) The effect of poly(ethylene glycol) on the activity and structure of glucose-6-phosphate dehydrogenase in solution. Colloids Surf. B-Biointerfaces 26 (4): 291-300.

[24] Forciniti D, Hall CK, Kula MR (1991) Protein Partitioning At The Isoelectric Point - Influence Of Polymer Molecular-Weight And Concentration And Protein Size. Biotechnol. Bioeng. 38 (9): 986-994.

[25] Rand RP, Fuller NL, Butko P, Francis G, Nicholls P (1993) Measured Change in Protein Solvation with Substrate-binding and Turnover. Biochemistry 32 (23): 5925-5929.

Capítulo 4

Immobilization of a plant lipase in calcium alginate beads

Patricia Peres Polizelli, Bárbara Martinelli Bonine, e Gustavo Orlando Bonilla Rodriguez

Artigo submetido à revista: Process Biochemistry (ISSN: 0168-1656, Elsevier, Impact factor 2,687)

Resumo

No estudo realizado para a imobilização da lipase em esferas de alginato de cálcio, estas foram preparadas pelo método de difusão usando cloreto de cálcio como agente gelificante. Foram avaliados: a quantidade de enzima encapsulada, a liberação, a atividade e as características das esferas. Análises comparativas também foram realizadas com a enzima, nas formas livre e imobilizada, para a determinação da temperatura e pH ótimos e estabilidade de estocagem. A enzima imobilizada foi estável e reutilizada várias vezes sem perda da atividade lipolítica. O intervalo de temperatura ótima, tanto para a enzima livre quanto imobilizada foi na faixa de 30 a 40°C. Ambas formas da enzima mostram maior atividade em pH 7,5-8,5. A estabilidade de estocagem e termoestabilidade foram maiores para a enzima imobilizada que para a livre.

Palavras-chave: Lipase, enzima imobilizada, alginate, reutilização enzimática, estabilidade, *Pachira aquatica.*

Immobilization of a plant lipase in calcium alginate beads

Patricia Peres Polizelli[1], Bárbara M. Bonine[2], Gustavo O. Bonilla-Rodriguez[2*]

[1]Department of Physics and [2]Department of Chemistry and Environmental Sciences, IBILCE-UNESP, São José do Rio Preto SP, Brazil

[*]Corresponding author. Tel.: +5517 3221 2361 Fax: +5517 3221 2356

E-mail address: bonilla@ibilce.unesp.br (G.O. Bonilla-Rodriguez)

Abstract

This study reports the immobilization of a lipase from oleaginous seed of *Pachira aquatica*, using beads of calcium alginate. Alginate beads were prepared by ionic gelation using calcium chloride as the cross-linking agent in the gelling solution. We evaluated enzyme loading, leaching, activity and characterized the beads. Comparative analyses on free and immobilized lipase for determination of optimum temperature, pH, and storage stability were carried out. The immobilized enzymes were stable and were reused several times without loss of enzyme activity. The optimum temperature interval for the free and immobilized enzyme was in the range from 30 to 40°C. Both forms of the enzyme showed higher activity at pH 7.5-8.5. Storage stability of the immobilized lipase was higher than that of the free lipase.

Key words: Lipase, immobilized enzyme, alginate, enzyme reusability, enzyme stability, *Pachira aquatica.*

Introduction

Enzymes have a wide variety of biotechnological, biomedical and pharmaceutical applications. They are used as catalysts for chemical or biochemical reactions, and purified enzymes can be rather costly to discard them after use [1].

Enzyme immobilization has proved to be a useful technique for improving enzyme activity in several uses. Immobilized enzymes typically exhibit greater stability and enzyme activity over a broader range of pH and temperature [2,3] and possess other advantages such as recovery yield and reusability, possibility of continuous process, rapid termination of reactions, controlled product formation, easy enzyme removal from the reaction mixture, and adaptability to various engineering designs [4].

Entrapment, one of the immobilization techniques, can be defined as a physical restriction of the enzyme within a confined space or network. Gelation of polyanionic or polycationic polymers by the addition of multivalent counter-ions is a simple and common method of enzyme entrapment. Alginates are one of the most used polymers due to their mild gelling properties and non-toxicity [5] it is a water-soluble anionic linear polysaccharide composed of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid in different proportions an sequential arrangements, which can be precipitated by the addition of Ca^{2+} ions [6,7], giving microspheres with good strength and flexibility [8].

A number of enzymes have been formulated in microcapsules involving various polysaccharides. Some examples include invertase [9], lactase [10], lipase [1,11-13] and horsedish peroxidase [14].

Among hydrolases, lipases occupy a prominent position and have a wide spectrum of biotechnological applications. They are able to catalyze fat hydrolysis at an oil-water

interface, this reaction is reversible and these enzymes also catalyze the synthesis of esters and transesterification in microaqueous conditions. Lipases found in oilseeds help to hydrolyze the ester bonds of storage triacylglycerols and have great potential for commercial exploitation as industrial enzymes [15]. The applications of lipases in industry include the synthesis of food ingredients, oilchemistry, food, organic synthesis, paper manufacturing, biosurfactants, cosmetics, pharmaceuticals and the use as additives in cleaning products [16].

This study had the goal of immobilizing a lipase extracted from oleaginous seeds of *Pachira aquatica* by entrapment in alginate beads, describing some parameters of the immobilized lipase and comparing them to the free enzyme.

Materials and methods

Materials

A lipase from seeds of trees of *Pachira aquatica* collected at our Campus (State University of São Paulo) , as described elsewhere [17].

Entrapment of the lipase in calcium alginate beads

Beads were prepared using four different polymer concentrations (2, 2.5, 3, 3.5%). Alginate, $CaCl_2$ and lipase solutions were prepared in pH 8.0, 0.03M Tris buffer. A 2 ml aliquot of the alginate dissolved in buffer solution was mixed with 0.5 ml of lipase and this mixture was added dropwise into 5 ml of $CaCl_2$ (0.025-0.2 M) solution with a hypodermic syringe. Washing was completed twice as before with ultrapure water and the beads were used as such for further studies. A similar method was followed for preparation of control

alginate beads.

The amounts of protein in the enzyme solution and in the washing solutions were determined as described by Bradford [18], with bovine serum albumin as a standard.

The amount of the bound enzyme was calculated according to de Queiroz et al. [8] with modifications:

$$q = \frac{(Ci - Cf)}{W} V$$

where q is the amount of the encapsulated enzyme in alginate beads (U/g); Ci and Cf are the initial and final enzymatic activity (U/mL) of the medium, respectively; V is the volume of the medium (mL); W is the microspheres' mass in grams.

The curing time for alginate beads (1, 2, 3, 4, 8 and 24 hours) and the effect of beads' size were tested by the enzyme assay described below. The needles used to drop a mixture of lipase and alginate a $CaCl_2$ solution had several diameters: 0.3, 0.55 and 0.7 mm.

Leaching

The leaching or release of lipase from each batch of beads was studied at a constant temperature of 4°C. A 10 mL aliquot of a 0.03M, pH 8.0, Tris buffer was used as the release medium. Samples were taken at specific time intervals for protein determination as described [18]. Samples taken from buffer exposed to control beads and subject to a similar process were used.

Lipase assay

The substrate for the catalytic tests was *p*- nitrophenylpalmitate (*p*-NPP). The assay was realized spectrophotometrically at 410 nm. This substrate was dissolved in 1% isopropanol,

2% Triton X-100 as emulsifier, 50 mM Tris buffer pH 8.0, to which 0.7 g of microspheres were added. One unit of enzyme was defined as the amount of enzyme that released 1µmol de *p*-nitrophenol per minute at 37°C and pH 8.0.

A negative control, without the enzyme, was also studied and the reaction mixture in each case was incubated for exactly 60 min. The reaction was stopped by removing the immobilized enzyme with a sieve.

Beads' characterization

The beads were characterized in terms of their number, volume and morphology (using about 20 units) was performed for each entrapment test. The number of beads obtained from each test was counted, and the average data obtained during these experiments was analyzed statistically by the Student's *t* test [19], using the BioEstat 4.0 program [20] and $p< 0.05$.

The beads' surface morphology was studied using a magnifying glass Olympus SZ2-ILST.

Measurement of pH optimum

The effect of pH on the activity of the free and immobilized enzymes were determined in the pH range of pH 4.0-10.0 with 0.03M buffers (sodium acetate for pH 4 and 5.0; sodium citrate for 6.0; hepes for 7.0; Tris for 8.0, glycine for 9.0 and 10.0) measuring the activity of enzyme. The enzyme assays were carried out by the titration protocol described below.

For the experiments of optimum pH was used titration with soybean oil. The solution contained 50% soybean oil, 31.3% 30 mM Tris buffer (pH 8.0), 10.15% Triton X-

100, 0.002% of 10 mM $CaCl_2$, and 9.6% of the enzyme. The reaction was ended by adding acetone:ethanol (1:1), and the amount of released fatty acids was titrated with 50 mM KOH in the presence of phenolphthalein as indicator. One unit of enzyme activity (U) was defined as the amount of enzyme able to release 1 μmol of free fatty acids per minute and the specific activity was calculated as U per milligram of protein, under the assay conditions. The experiments were carried out in duplicate.

The effect of pH on enzyme binding also was investigated. In a typical experiment beads were prepared using the different pH values cited above.

Effectof temperature optima and stability of free and immobilized lipase

The temperature optima of free and immobilized lipase were determined by carrying out the enzyme assay at various temperatures ranging from 30 to 60°C at pH 8.0. The thermal stability of free and immobilized lipase was ascertained by measuring the activity of the residual enzyme exposed to various temperatures (30-60°C) in 0.03M Tris pH 8.0 for until 24 hours. Activities of the samples were performed at optimum conditions.

The storage stability of free and immobilized lipase

The activity of free and immobilized lipase after storage in 0.03M Tris buffer pH 8.0 was measured with the experimental conditions given above.

Repeated use of lipase immobilized in the alginate beads

In order to test the stability of the lipase entrapped in the Ca-alginate beads were used

several times for the hydrolysis reaction. Each run lasted 60 min.; after that period the beads were separated and washed with ultrapure water. The reaction medium was then replaced with a fresh one. The activity of freshly prepared beads in the first experiment was defined as 100%.

Results and Discussion

Entrapment of lipase in calcium alginate beads

Because cross-linking between alginate and Ca^{2+} ions leads to gelation, alginate and $CaCl_2$ concentration are major parameters for enzyme gel entrapment [5]. Alginate concentration was increased from 2 to 3.5% (w/v), maintaining the $CaCl_2$ concentration (50 mM) and the ratio by weight (E/A) of enzyme to alginate 5:2. The best condition was 3% alginate and 0.5 mM $CaCl_2$.

The best curing time was 30 min, larger than that found by Betigeri and Neau [1] for *Candida rugosa* lipase, that was 20 min.

As with most heterogeneous catalysts, immobilized enzymes generally experience mass transfer limitations. The size of beads in which lipase is entrapped may be one of the most important parameters of lipase immobilization. It is expected that enzymes in smaller beads will show higher catalytic activity due to reduced substrate transfer resistance. To overcome the diffusion resistance, the size of the bead was reduced from 0.7 to 0.3 mm and verified a 67% increase in the enzyme activity (Fig. 1). Other works have also reported that the activity of immobilized enzymes decreases with increasing bead size due to mass transfer resistance [5,21,22].

–

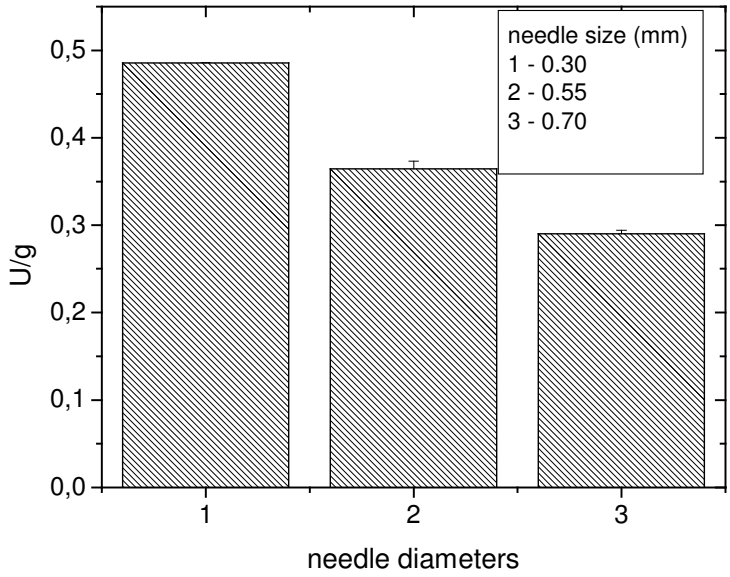


Figure 1. Dependence of the activity of the immobilized lipase on the diameter of the needle used to obtain the microspheres.

Different specific activity (4.5, 9, 15.75 and 22.50 U/mL) were tested in order to determine which is more suitable for immobilization, analyzing by equation 1. The highest tested (22.50 U/mL) showed more immobilized enzyme benn the specific activity in the washing water + $CaCl_2$ smaller in 9 U/mL. Accordingly, this was the best concentration for immobilization.

Leaching

We estimated the enzyme leaching from the beads over a period of time to judge the potential reuse of the beads in a long term biotechnological process or chemical reaction. The rate of release was found to increase with an increase in the alginate concentration, although there is no difference between 2 and 2.5%.

Beads' characterization

The results of the characterization of beads are present in Tables 1 and 2. The number of beads formed at each alginate and $CaCl_2$ concentration were not statistically different from each other as reported by Betigeri and Neau [1] for a lipase from *Candida rugosa* immobilized in alginate beads. The dependence of beads morphology on alginate and $CaCl_2$ concentration was examined also. The surface of beads was observed using a magnifying glass are shown in fig. 2 (A and B). It was found that the beads morphology was not different on alginate and $CaCl_2$ concentration, with the only exception of 25 mM of $CaCl_2$ (Fig. 2B), when the beads displayed malformations.

Table 1. Mean diameter ±S.D. in mm and number of calcium alginate microspheres obtained in different alginate concentrations.

Alginate content (% (w/v))	Average diameter (mm)	Number of beads
2	2.65 ± 0.032	214.5 ± 6.4
2.5	2.83 ± 0.112	173.5 ± 10.6
3	2.99 ± 0.000	159.5 ± 2.1
3.5	2.73 ± 0.204	157.5 ± 9.2

The Calcium concentration was 0.05M.

Table 2. Mean diameter ±S.D. in mm and number of calcium alginate microspheres obtained in different Ca^{2+} concentrations.

Calcium concentration (M)	Average diameter (mm)	Number of beads
0.025	3.03 ± 0.549	92 ± 0.0
0.05	2.68 ± 0.011	178.5 ± 3.5
0.1	2.55 ± 0.00	189 ± 7.1
0.2	2.52 ± 0.099	190 ± 5.6

The alginate content was 3%.

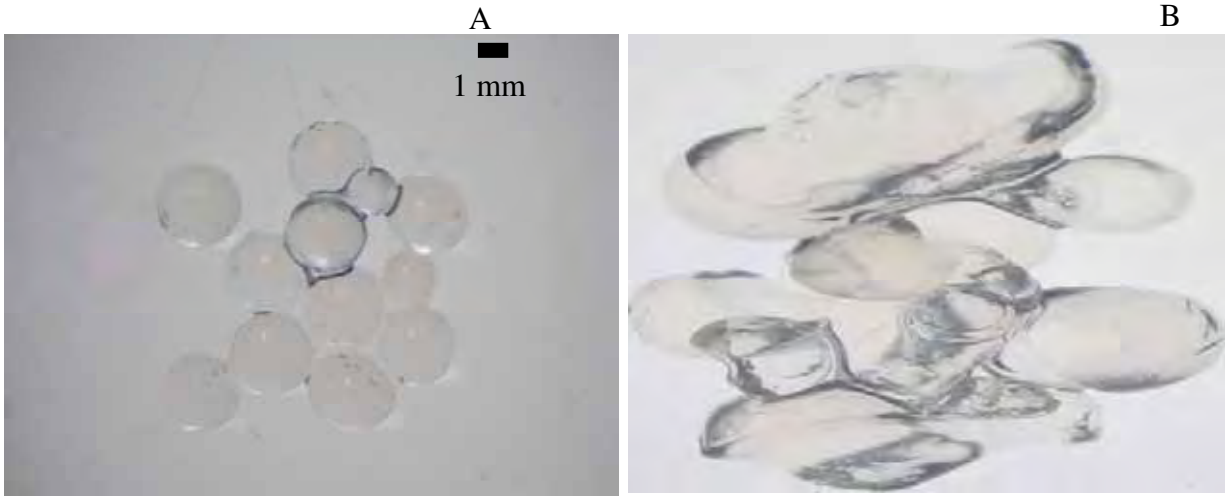


Figure 2. Micrograph of the alginate beads. $CaCl_2$ concentration (A) 0.025 and (B) 0.05M.

Measurement of pH optima

The pH activity profiles of free and immobilized lipase were compared. The results are given in Figure 3. The optimum pH values for free and immobilized enzyme remain uncharged. The optimum pH range was obtained as 7.5-9.0 for free and immobilized lipase. Mondal et al. [13] and Altun and Cetinus [23] reported unaltered optimum pH values with immobilization process.

The effect of pH on enzyme binding not demonstrated different performance, with exception at pH 4.0 in that the lipase show zero activity.

Measurement of temperature optima and stability of free and immobilized lipase

Utilization of non- thermophilic enzymes in industrial processes often faces the problem of thermal inactivation of the enzyme. At high temperature, enzyme undergoes partial unfolding by heat-induced destruction of non-covalent interactions [24]. The optimum temperature for free and immobilized (fig. 4) lipase was found to be about 40 °C for 90

minutes of incubation, and the immobilized enzyme displays higher stability at 60ºC, keeping 40% of the maximum activity.

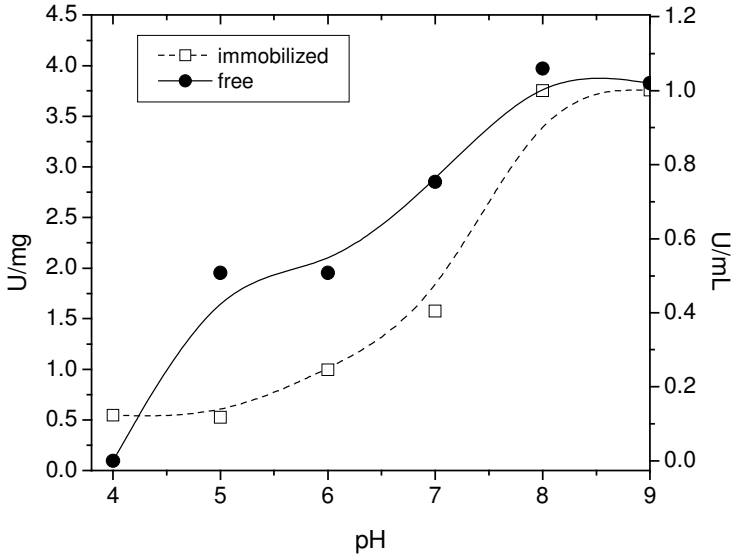


Figure 3. Effect of pH on the activities of the free and immobilized lipase at 37°C.

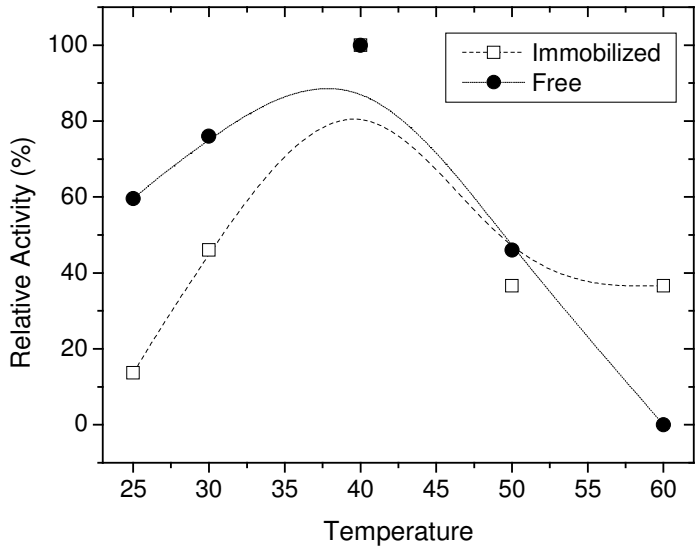


Figure 4. Effect of temperature on the activities of the free and immobilized lipase at 37°C.

Thermal stability was investigated by incubating free and immobilized lipase on alginate beads at temperatures ranging from 30-60 °C for until 4 h. The immobilization increased the thermal stability of the enzyme, as evident from Figure 5 (A to D). While at 50 and 60 °C, the free enzyme lost completely its activity at the end of 4 hours, the immobilized lipase preserved 50% and 22% activity. As demonstrated by Altun and Cetinus [23] the thermal stability may be increased if the enzymes are immobilized.

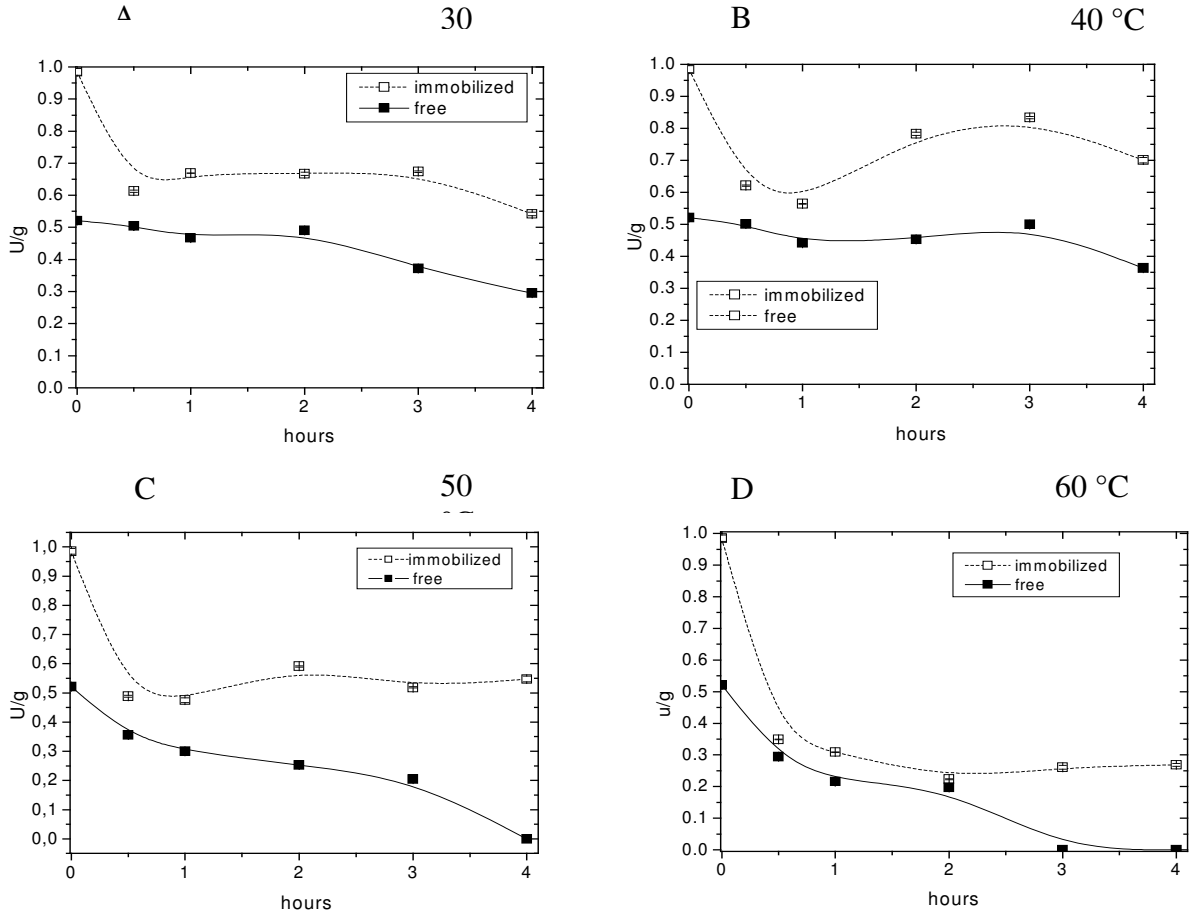


Figure 5. Thermal stability of free and immobilized lipase. (A) 30°C, (B) 40°C, (C) 50°C e (D) 60°C. The experiments was realized with *p*-NPP as substrate and pH 8.0.

The storage stability of free and immobilized lipase

The storage stability of free and immobilized lipase were analyzed. The stability of an

immobilized enzyme without appreciable loss of enzyme activity is important for the economic viability. The immobilized lipase exhibited good stability with no significant decrease in activity up to 6 months kept at 4°C, in opposition to 8 hours for the free enzyme. The time dependent natural loss can be prevented to a significant degree by immobilization and this is one of the most important aims of the enzyme engineering, enhance the conformacional stability of enzymes.

Repeated use of lipase immobilized in the alginate beads

When comparing the performance of immobilized biocatalysts, aiming preparative or industrial use, characterization of their operational stabilities is very important. The operational stability was evaluated in repeated batch process. The repeatability test conducted at optimum conditions of beads and the results (Fig. 6) showed that the immobilized lipase could be used until 6 cycles with little loss of activity; after that, activity decreased drastically. This data are in agreement with the literature [13,21, 25].

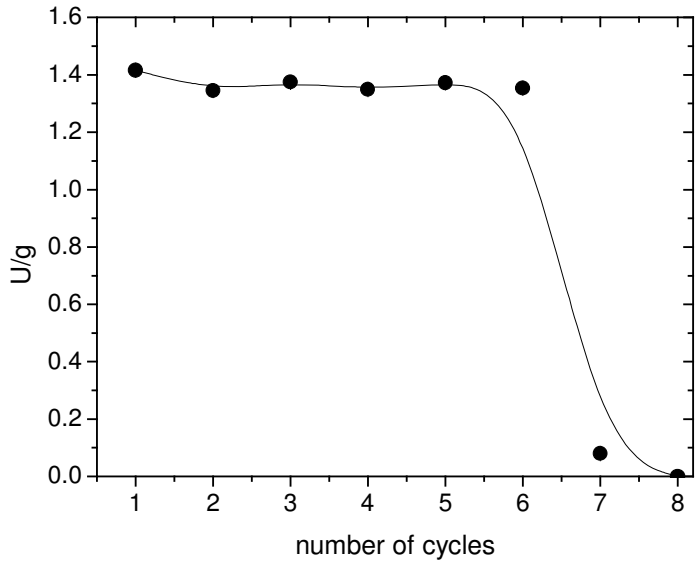


Figure 6. Reusability of the immobilized lipase at 37°C.

Acknowledgements

Thanks are due Prof. Francisco Langeani Neto from the Department of Zoology and Botany of our Institute, for assistance concerning the photographs. The authors thank the financial support provided by CAPES (PPP), CNPq and FAPESP 05/02418-6 (GOBR).

References

[1] Betigeri SS, Neau SH. Immobilization of lipase using hydrophilic polymers in the form of hydrogel beads. Biomaterials 2002; 23: 3627-3636.

[2] Telefoncu A, Dinckya E, Vorlop KD. Preparation and characterization of pancreatic lipase immobilized in Eudragit-matrix. Applied Biochemistry and Biotechnology 1990; 26: 311-317.

[3] Pancreac´h G, Leullier M, Baratti JC. Properties of free and immobilized lipase from *Pseudomonas cepacia*. Biotechnology Bioengeenering 1997; 57: 181-189.

[4] Cao L, Langen LV, Sheldon RA. Immobilized enzymes: carrier-bound or carrier-free? Current Opinion in Biotechnology 2003; 14: 387-394.

[5] Won K, Kim S, Kim K, Park HW, Moon S. Optimization of lipase entrapment in Ca-alginate gel beads. Process Biochemistry 2005; 40: 2149-2154.

[6] Ertesvag H, Valla S. Biosynthesis and applications of alginates. Polymer Degradation and Stability 1998; 59:85-91.

[7] SmidsrØd O, Skajak-Braek G. Alginate as immobilization matrix for cells. Trends Biotechnology 1990; 8: 71-78.

[8] De Queiroz AAA, Passo ED, Alves SB, Silva GS. Alginate-poly(vinyl alcohol) core-shell microspheres for lipase immobilization. Journal of Applied Polymer Science 2006;

102:1553-1560.

[9] Arruda LM. Vitolo M. Characterization of invertase entrapped into calcium alginate beads. Applied Biochemistry and Biotechnology 1999; 81: 23-33.

[10] Dashevsky A. Protein loss by the microencapsulation of an enzyme (lactase) in alginate beads. International Journal of Pharmaceutics 1998; 161:1-5.

[11] Hertzberg S, Kvittingen L, Anthonsen T, Skjak-Braek G. Alginate as immobilization matrix and stabilizing agent in a two-phase liquid system: application in lipase catalyzed reactions. Enzyme Microbial Technology 1992; 14: 42-47.

[12] Mohapatra SC, Hsu JT. Optimizing lipase activity, enantioselectivity, and stability with medium engineering ad immobilization for beta-blocker synthesis. Biotechnology Bioengineering 1999; 64: 213-220.

[13] Mondal K, Mehta P, Mehta BR, Varandani D, Gupta MN. A bioconjugate of *Pseudomonas cepacia* lipase with alginate with enhanced catalytic efficiency. Biochimica et Biophisica Acta 2006; 1764: 1080-1086.

[14] Patil RT, Speake TJ. Water-based microsphere delivery systems for proteins. Journal Pharmaceutical Science 2000; 89: 9-15.

[15] Enujiugha VN, Thani FA, Sanni TM, Abigor RD. Lipase activity in dormant seeds of the African oil bean (*Pentaclethra macrophylla* Benth). Food Chemistry 2004; 88: 405-410.

[16] Rubin B, Dennis EA, editors. Lipases.Methods Enzymology: Part A biotechnology. New York: Academic Press; 1997.

[17] Polizelli PP, Facchini FDA, Cabral H, Bonilla-Rodriguez GO. A New Lipase Isolated from Oleaginous Seeds from *Pachira aquatica* (Bombacaceae) Applied Biochemistry and Biotechnology 2008; available online.

[18] Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochemistry 1976; 72: 248-254.

[19] Zar JH. Biostatistical Analysis, 4th ed. New Jersey: Prentice Hall; 1999.

[20] Ayres M, Ayres Jr M, Ayres DI, Santos AS. BioEstat.4.0: Aplicações estatísticas nas áreas das ciências biológicas e médicas. Belém: Sociedade Civil Mamirauá, MCt e Imprensa Oficial do Estado do Pará; 2005.

[21] Knezevic Z, Bobic S, Milutinovic A, Obradovic B, Mojovic L, Bugarski B. Alginate-immobilized lipase by electrostatic extrusion for the purpose of palm oil hydrolysis in lecithin/isooctane system. Process Biochemistry 2002; 38:313-318.

[22] Fadnavis NW, Sheelu G, Kumar BM, Bhalerao MU, Deshpande AA. Gelation blends with alginate: gels for lipase immobilization and purification. Biotechnology Progress 2003; 19: 557-564.

[23] Altun G D, Cetinus SA. Immobilization of pepsin on chitosan beads. Food Chemistry 2007; 100: 964-971.

[24] Nirprit SD, Jagdeep K. Immobilization, stability and esterification studies of a lipase from a *Bacillus* sp. Biotechnology and Applied Biochemistry 2002; 36:7-12.

[25] Jeganthan J, Bassi A, Nakla G. Pre-treatment of high oil and grease pet food industrial wastewaters using immobilized lipase hydrolysation. Journal of Hazardous Materials B 2006; 137: 121-128.

4. Conclusão

A enzima estudada foi extraída e purificada, apresentando aproximadamente 55 KDa de massa molecular. A atividade máxima foi alcançada a 40°C e pH 8,5 e, foi estável na faixa de temperaturas de 4 a 50°C e em valores de pH entre 6 e 10. A atividade lipolítica foi diminuída na presença de Hg^{++}, Mn^{++}, Zn^{++} and Al^{+++}, mas aumentada na presença de Mg^{++} e Ca^{++}. Ainda, foi capaz de hidrolisar substratos solúveis e insolúveis em solução aquosa.

Na presença de resíduos de frigorífico avícola a enzima demonstrou atividade lipolítica e manteve as características encontradas com outros substratos naturais e sintéticos. Quanto à degradação de pedaços de gorduras animal a enzima não mostrou-se eficaz.

Nos estudos com tensoativos, foi notado efeito estimulativo na atividade lipolítica quando na presença de CTAB, TTAB e sais de bile. Efeitos inibitórios para surfactantes aniônicos e não-iônicos. PEG demonstrou um efeito estimulativo na atividade da enzima, que pode ser devido à exposição de alguns resíduos hidrofóbicos localizados na vizinhança do sitio ativo, ou por dimerização.

Os estudos de imobilização enzimática, em alginato de cálcio, mostraram a obtenção de micro-esferas com enzimas estáveis, o que comprova o potencial deste polímero para imobilização. E a imobilização promoveu a obtenção de enzimas termicamente mais estáveis.

Conclusão

As análises mostraram o potencial desta enzima para aplicação em pré-tratamentos de resíduos de óleos e gorduras, principalmente para a hidrólise de óleos provenientes de frituras, podendo ser aproveitada na indústria de produtos de limpeza.

O processo de imobilização proporcionou a obtenção de esferas com características de suma importância para viabilizar futuras aplicações.