UNIVERSIDADE ESTADUAL PAULISTA
IBILCE - Câmpus São José do Rio Preto

Letícia Maria Zanphorlin

Caracterização Bioquímica de uma Serino-Protease produzida pelo fungo termofílico *Myceliophthora* sp

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Biofísica Molecular, junto ao Departamento de Física, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientadora: Prof[a]. Dr[a]. Eleni Gomes

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São José do Rio Preto, 23 de fevereiro de 2010

Zanphorlin, Letícia Maria.
Caracterização bioquímica de uma serino-protease produzida pelo fungo termofílico Myceliophthora sp. / Letícia Maria Zanphorlin. - São José do Rio Preto : [s.n.], 2010.

81 f. : il. ; 30 cm.

Orientador: Eleni Gomes
Co-orientador: Gustavo Orlando Bonilla-Rodriguez
Dissertação (mestrado) - Universidade Estadual Paulista,

Instituto de Biociências, Letras e Ciências Exatas

1. Enzimas proteolíticas. 2. Enzimas por fungos. 3. Proteases. 4. Fungos terrmofílicos. 3. Susbstratos fluorogênicos. I. Gomes, Eleni. II. , Rodriguez, Gustavo Orlando Bonilla. III. Universidade Estadual Paulista, Instituto de Biociências, Letras e Ciências Exatas. IV. Título.

CDU - 663

Dedico esse trabalho as quatro pessoas mais importantes da minha vida: meus pais queridos, Sirineu e Maria José, meu irmão amado Lucas e meu marido Mário.

Agradecimentos

Em primeiro lugar, a Deus, pela vida, por ter me colocado junto a pessoas tão maravilhosas e amadas que é minha família.

Aos meus orientadores, Prof. Dr. Gustavo Bonilla e Profª. Drª. Eleni Gomes, pela oportunidade. Muito obrigada pela amizade, pela parceria, pelos preciosos conhecimentos. Serei eternamente grata.

Aos meus pais queridos, Branco e Nina. O que falar de vocês? Vocês são minha fortaleza, obrigada por tudo, pela paciência, pelo carinho, pelo amor incondicional e o mais importante, por sempre me apoiar. Eu amo vocês com toda força do mundo.

Ao meu irmão Lucas, por fazer da minha vida mais alegre.

Ao meu marido Mário, grande amante da Ciência. Obrigada por me guiar nessa caminhada e pelo constante companheirismo. Eu te amo!!

A toda minha família: meus tios, primos e avós.

As minhas eternas amigas de infância: Dandara, Aline, Fer Mastrocola, Paulinha. Muitas saudades!!

Aos meus eternos amigos de graduação: Lívia, Michele, Leandro, Samantha, Soraia, Ane e Ivana.

Aos meus amigos do laboratório de Bioquímica: Ana Lúcia, Angélica, Rejane, Patrícia, Bárbara, Bruna, Lílian, Laís, Tássia. Obrigada pela maravilhosa convivência. Sentirei muuuuuitas saudades das nossas risadas.

Aos alunos do laboratório de Microbiologia, UNESP – Rio Preto.

Aos Professores que aceitaram o convite de participar da minha banca de qualificação: Prof. Hamilton e Prof. Roberto da Silva.

Ao Prof. Dr. Luiz Juliano Neto e todo o pessoal do laboratório de Biofísica da UNIFESP – São Paulo que abriram as portas e me receberam com muito carinho.

A todos os professores do Departamento de Física. Tenho por vocês muito respeito e gratidão.

Ao Prof. Dr. Valmir Fadel, por me orientar no estágio docência.

Aos funcionários do Departamento de Física: Ilva, Paulinho e Barbosa. E aos funcionários do Departamento de Química: Rita e Eliane.

E por fim, a CAPES, pela bolsa concedida.

O meu MUITO OBRIGADO!!

Letícia Maria Zanphorlin

"Sempre que pensamos em mudar queremos tudo o mais rápido possível. Não tenha pressa, pois as pequenas mudanças são as que mais importam. Por isso, não tenha medo de mudar lentamente, tenha medo de ficar parado."

Provérbio chinês.

APRESENTAÇÃO

Essa dissertação, dividida em três capítulos, descreve desde a identificação de um fungo termofílico do gênero *Myceliophthora* produtor de enzimas proteolíticas até a caracterização detalhada da enzima majoritária pura.

No primeiro capítulo é apresentada uma revisão bibliográfica que descreve o contexto da pesquisa realizada, assim como sua a relevância e importância em processos industriais.

O segundo capítulo é dedicado aos dados de identificação do fungo do gênero *Myceliophthora* bem como a produção e caracterização inicial do extrato enzimático bruto obtido por fermentação sólida (FES) e submersa (FSM). Estes resultados culminaram em um artigo intitulado "*Production, partial characterization and immobilization in alginate beads of an alkaline protease from a new thermophilic fungus Myceliophthora sp*" que foi aceito para publicação no *The Journal of Microbiology*.

E no terceiro capítulo são apresentados os dados de purificação e caracterização detalhada da protease pura buscando revelar sua especificidade quanto aos subsítios de reconhecimento do substrato. Tais resultados estão expressos na forma de artigo cujo título é *"Functional properties and subsite specificity mapping of a new alkaline serine-protease from the thermophilic fungus Myceliophthora sp"* e que está em fase final de preparação.

Além dos resultados obtidos com a protease do fungo termofílico *Myceliophthora* sp foram realizados estudos de cinética enzimática com uma metalo-protease produzida pelo fungo termofílico *Thermoascus aurantiacus* em colaboração com o grupo do Prof. Dr. Roberto da Silva que contribuíram para o artigo "*Biochemical and Functional Characterization of a Metalloprotease from the Thermophilic Fungus Thermoascus aurantiacus"* publicado no *Journal of Agricultural and Food Chemistry*.

RESUMO

Fungos termofílicos têm despertado grande interesse acadêmico e industrial por produzirem uma variedade de enzimas termoestáveis com potenciais aplicações em processos biotecnológicos como biocatálise nas indústrias de couro, farmacêutica, têxtil e alimentícia, e na preparação de produtos de limpeza e cosméticos. Particularmente, as proteases, além de participarem de inúmeros processos fisiológicos vitais como vias metabólicas, hemostasia e sinalização celular, também representam hoje cerca de 60% do mercado mundial de enzimas. Neste trabalho, descrevemos a produção, purificação e caracterização bioquímica de uma serino-protease produzida por um fungo termofílico do gênero *Myceliophthora*. As taxas de atividade proteolítica foram avaliadas através de fermentação em meio sólido (FES) e submerso (FSM) e observou-se um rendimento na atividade proteolítica 4,5 vezes maior para o meio sólido. A enzima bruta obtida por ambos os procedimentos (FES e FSM) exibiu a mesma temperatura ótima de 50 ºC, porém em relação ao pH ótimo houve um deslocamento de 7 (FSM) para 9 (FES) sugerindo que o perfil enzimático do fungo difere de acordo com suas condições de fermentação. Baseado nesses resultados prosseguiu-se os estudos com o extrato bruto obtido por FES. A imobilização da enzima bruta em esferas de alginato de cálcio resultou no aumento da temperatura ótima e na estabilidade térmica quando comparado com a enzima livre. O extrato bruto obtido por FES foi, então, fracionado por métodos cromatográficos como exclusão molecular e troca-iônica que resultaram na protease pura com peso molecular de 28,2 kDa determinado por espectrometria de massa. A protease pura demonstrou pH ótimo de 9,0 e temperatura ótima de 45 °C que corroboram a caracterização prévia do extrato bruto. Testes de inibição com a protease purificada resultaram na inibição completa por PMSF, um inibidor canônico de serino-proteases. A atividade proteolítica foi testada na presença de NaCl, agentes redutores, detergentes, diferentes íons e solventes orgânicos, e somente o íon Mg^{2+} e o detergente tween 20 resultaram no aumento da atividade proteolítica. Por outro lado, acetona, butanol e SDS aboliram a ação enzimática provavelmente devido à desnaturação protéica. Agentes redutores e alterações da força iônica (0-0.5M NaCl) não apresentaram efeitos sobre a atividade proteolítica. Testes com íons divalentes sugerem um papel específico do Mg^{2+} para sua atividade. A seqüência N-terminal da protease apresentou similaridade com outras proteases de fungos. A deconvulação dos dados de dicroísmo circular indicou uma predominância de estrutura α-hélice. Estudos de especificidade de subsítio utilizando peptídeos de fluorescência apagada (FRET) da família Abz-KLRSSKQ-EDDnp mostraram que o substrato sintético Abz-KLISSKQ-EDDnp é o melhor dentre aqueles testados (k_{cat}/K_m = 1,275.30 mM^{-1} s^{-1}). Em geral, a enzima apresentou preferência por aminoácidos hidrofóbicos nas posições P_1, P_2, P_3 e P_1', P_2', P_3' que corrobora com outros dados experimentais obtidos neste trabalho como o efeito inerte de agentes redutores e alterações da força-iônica.

ABSTRACT

Thermophilic fungi have attracted great academic and industrial interest because they produce a variety of thermostable enzymes with potential applications in biotechnological processes such as biocatalysis in the industries of leather, pharmaceutical, textile and food, and the preparation of detergents and cosmetics. In particular, proteases not only participate in many vital physiological processes such as metabolic pathways, cell signaling and homeostasis, but also currently represent about 60% of the world market of enzymes. In this work, we describe the production, purification and biochemical characterization of a serine protease produced by a thermophilic fungus of the genus *Myceliophthora*. The levels of proteolytic activity were evaluated either by solid fermentation (SSF) and submerged (SmF). The crude enzyme obtained by both procedures (SSF and SmF) exhibited similar optimum temperature of around 50 ºC, but in relation to the optimum pH was shifted of 7 (SmF) to 9 (SSF), suggesting that the enzymatic profile of the fungus differs from according to its fermentation conditions. Based on these results, the studies were followed with crude extract obtained by SSF. The immobilized enzyme on beads of calcium alginate resulted in increased optimum temperature and thermal stability when compared to the free enzyme. The crude extract obtained by SSF was then fractionated by chromatographic methods including molecular exclusion and ion-exchange that resulted in the pure protease with molecular weight of 28.2 kDa as determined by mass spectrometry. The pure protease showed optimum pH of 9.0 and optimum temperature of 45 °C that corroborate to the preliminary characterization of the crude extract. Inhibition tests resulted in complete inhibition by PMSF, a canonical inhibitor of serine proteases. The proteolytic activity was also tested in the presence of NaCl, reducing agents, detergents, various ions and solvents, and only the Mg^{2+} ion and Tween 20 resulted in increased proteolytic activity. On the other hand, acetone, buthanol and SDS abolished the enzymatic activity probably due to protein denaturation. Reducing agents as well as changes in ionic strength (0-0.5M NaCl) showed no effects on the proteolytic activity. Experiments with divalent ions suggests a specific role of Mg^{2+} for its activity. The N-terminal sequence of the protease showed similarity with other fungal serine proteases. CD data deconvolution indicated a predominance of α-helix structure. Studies of subsite specificity using quenched fluorescent peptides derived from Abz-KLRSSKQ-EDDnp were conducted and showed that the synthetic substrate Abz-KLISSKQ-EDDnp is the best among those tested (k_{cat}/K_m = 1,275.30 mM^{-1} s^{-1}). In general, the enzyme showed preference for hydrophobic residues at positions P_1, P_2, P_3 e P_1', P_2', P_3'.

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ABREVIAÇÕES

AMINOÁCIDOS	3 LETRAS	1 LETRA
ALANINA	Ala	A
ASPARAGINA	Asn	N
ÁCIDO ASPÁRTICO	Asp	D
ARGININA	Arg	R
CISTEÍNA	Cys	C
FENILALANINA	Phe	F
GLICINA	Gly	G
GLUTAMINA	Gln	Q
ÁCIDO GLUTÂMICO	Glu	E
HISTIDINA	His	H
ISOLEUCINA	Ile	I
LEUCINA	Leu	L
LISINA	Lys	K
METIONINA	Met	M
PROLINA	Pro	P
SERINA	Ser	S
TIROSINA	Tyr	Y
TREONINA	Thr	T
TRIPTOFANO	Trp	W
VALINA	Val	V

Outras Abreviações

AA	Aminoácido
λ_{em}	Comprimento de onda de emissão
λ_{ex}	Comprimento de onda de excitação
BSA	Albumina do Soro Bovino
CAPS	(3-[Ciclohexilamino]-1-ácido propanosulfonico)
DMSO	Dimetilsulfóxido
DTT	Ditiotreitol
EDTA	Ácido etilenodiaminotetracético
E-64	N-[N-(L-3-transcarboxiirano-2-carbonil)-L-Leucil]-agmatina
HEPES	4- (2-hydroxyethyl) – 1 ácido piperazina etano sulfônico
MES	ácido 2-(N-morfolino) etano sulfônico
pI	Ponto Isoelétrico
PMSF	Fluoreto de fenil-metano-sufonil
Sn	Nomenclatura para subsítios do sítio ativo de acordo com Schechter e Berger
Pn	Nomenclatura para os resíduos do substrato de acordo com Schechter e Berger
TAPS	N-tris[hidroximetil]metil-3-acido aminopropanosulfonico
Tris	Tri(hidroximetil)aminometano

SUMÁRIO

FUNDAMENTAÇÃO DO PROJETO

A aplicação biotecnológica de enzimas apresenta vantagens significativas sobre as reações químicas tradicionais e atualmente, milhares de preparações enzimáticas, incluindo misturas de biocatalisadores, estão disponíveis no mercado.

Dentre as enzimas mais utilizadas, as proteases se destacam devido à sua ampla versatilidade catalítica que se reflete nas suas inúmeras aplicações biotecnológicas, principalmente nas indústrias farmacêutica, têxtil, alimentícia e na preparação de produtos de limpeza e cosméticos. Por outro lado, as proteases são também de grande importância fisiológica devido às suas diferentes funções bioquímicas na célula que incluem sinalização celular, regulação do sistema sangüíneo e participação de inúmeras vias metabólicas. Em particular, as proteases são alvos da indústria farmacêutica para o desenvolvimento de inibidores para doenças como hipertensão, trombose, AIDS e câncer.

Devido à sua extraordinária importância clínica e industrial, as proteases têm sido foco de muitos grupos de pesquisa e muito se espera avançar quanto a seus aspectos bioquímicos e estruturais para impulsionar suas aplicações biotecnológicas. Assim, neste trabalho visamos contribuir no conhecimento de enzimas proteolíticas produzidas por fungos termofílicos que possuem interesse industrial para produção de compostos através da biocatálise.

OBJETIVOS

Um projeto anterior de iniciação cientifica permitiu o isolamento de um fungo termofílico produtor de proteases, o que nos motivou a investigar as características funcionais e estruturais da enzima. Diante disso, os objetivos gerais deste trabalho foram: em primeiro lugar, estabelecermos o protocolo de purificação da enzima para depois efetuarmos a caracterização da enzima pura em termos de efeito do pH, temperatura, ativadores, inibidores, e determinação da massa molecular. Além disso, realizamos um estudo mais profundo envolvendo a determinação dos parâmetros cinéticos de hidrólise de substratos sintéticos fluorogênicos (FRET) e pontos de clivagem.

CAPÍTULO 1

"Revisão bibliográfica"

1.1 ENZIMAS: ASPECTOS GERAIS

De todas as funções das proteínas, provavelmente a mais importante é a catálise; na sua ausência, a maioria das reações nos sistemas biológicos seria por demais lenta para fornecer produtos na quantidade adequada para um organismo metabolizante. A aceleração das reações químicas só é possível graças à participação das enzimas. Com exceção de um pequeno grupo de moléculas de RNA com propriedades catalíticas (ribozimas), todas as enzimas são proteínas. As enzimas são capazes de aumentar 10^{14} vezes mais a velocidade de uma reação quando comparada com uma reação não catalisada, pois diminuem a energia de ativação da reação química e não alteram o equilíbrio da reação (CAMPBELL, 2000).

1.1.1 Nomenclatura

A nomenclatura das enzimas foi definida por uma comissão especializada, a *Enzyme Commission* (EC), que pertence à União Internacional de Bioquímica e Biologia Molecular (IUBMB). Segundo esta comissão, as enzimas são classificadas de acordo com sua ação catalítica (VOET & VOET, 2004). Assim, foram divididas dentro de seis classes: Óxidorredutases, Transferases, Hidrolases, Liases, Isomerases e Ligases (Tabela 1).

Tabela 1: Classificação das enzimas de acordo com o tipo de reação e atuação:

CLASSE DA ENZIMA	TIPO DE REAÇÃO CATALISADA
1- Óxidorredutases	Reação de Oxido-Redução ou transferência de elétrons
2- Transferases	Transferência de grupos funcionais
3- Hidrolases	Reações de Hidrólise
4- Liases	Quebra de ligações covalentes para formar duplas ligações, ou adição a duplas ligações
5- Isomerases	Transferência de grupos dentro da mesma molécula para formar isômeros
6- Ligases	Formação de ligações acoplada à hidrólise de ATP

1.2 PROTEASES

Dentro da classe das Hidrolases, encontra-se a sub-classe das enzimas proteolíticas ou proteases, enzimas que hidrolisam ligações peptídicas de proteínas e peptídeos.

Estas enzimas constituem uma grande família, dividida em endopeptidases ou proteinases e exopeptidases, de acordo com a posição da ligação peptídica a ser clivada na cadeia polipeptídica (Figura 1). As endopeptidases atuam preferencialmente nas regiões internas da cadeia polipeptídica, entre as regiões N e C terminal, enquanto que as exopeptidases atuam somente nas extremidades das cadeias polipeptídicas na região N ou C terminal.

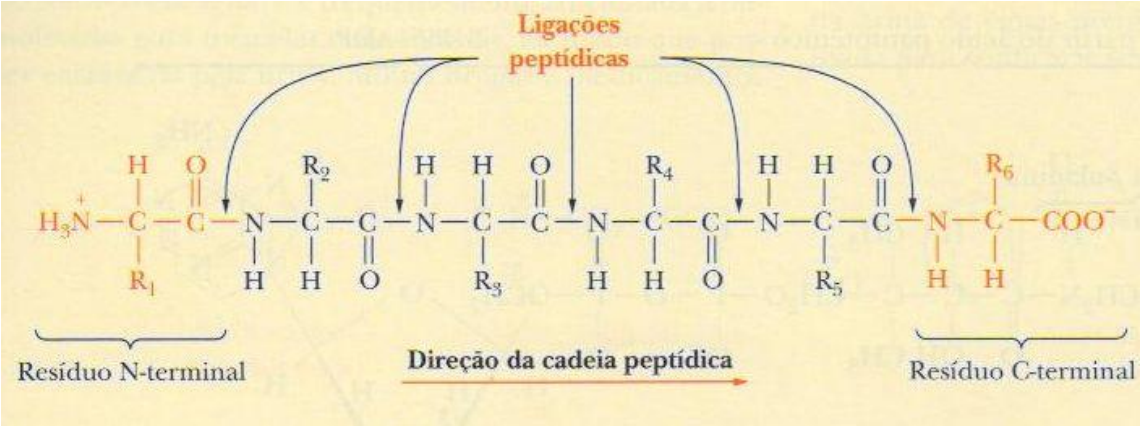

Figura 1: Um oligopeptídeo mostrando as ligações peptídicas, eventuais locais de clivagem por proteases (CAMPBELL, 2000).

As endopeptidases podem ser ainda subdivididas em sub-sub-classes, de acordo com o grupo reativo no sítio ativo envolvido com a catálise, em serino- (EC 3.4.21), cisteíno- (EC 3.4.22), aspártico- (EC 3.4.23) e metaloproteinases (EC 3.4.24); estas últimas usam um íon metálico no seu mecanismo catalítico. Existem, ainda, as treonina-proteinases, descritas somente em proteossomas (http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/4/25/). A tripsina (Figura 2) e a pepsina são exemplos de proteases que atuam na degradação de proteínas ingeridas, e a trombina que tem função reguladora na coagulação sanguínea (NEURATH et al., 1967; ADAMS & BIRD, 2009).

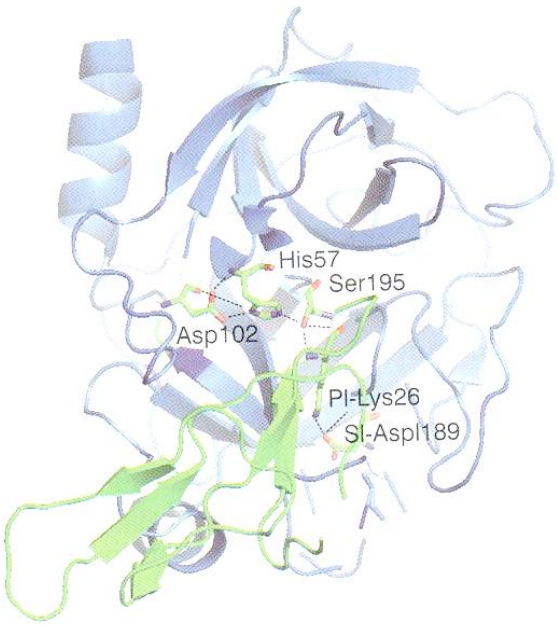

Figura 2: Estrutura tridimensional do complexo formado pela tripsina (azul) e o inibidor (verde) de serino-proteases (BARBOSA et al., 2007)

1.2.1 Especificidade

O sítio ativo da enzima desempenha a dupla função de ligação de um substrato e catalisar uma reação e, portanto, determina a especificidade da enzima. Assim, é possível obter informações sobre o sítio ativo pela cinética de reação da enzima com diferentes substratos e inibidores (POLGÁR, 2005; BERGER et al., 1970). A terminologia para descrever a especificidade das proteases depende do modo em que é considerado o sítio catalítico, podendo ser flanqueado por um ou ambos lados dos subsítios específicos, cada um capaz de acomodar uma única cadeia lateral do resíduo de aminoácido (BARRET et al., 1998). Seguindo a padronização proposta por Schechter e Berger (1967), os subsítios são numerados S1-Sn em direção ao N-terminal do substrato, e S1`- Sn` em direção ao C-terminal, enquanto os resíduos do substrato acomodados são numerados de P1 - Pn, e P1`- Pn`, respectivamente (Figura 3).

A estrutura do sítio ativo da protease determina, portanto, quais resíduos do substrato podem se ligar aos subsítios específicos da protease, determinando assim a especificidade da enzima (TURK, 2006).

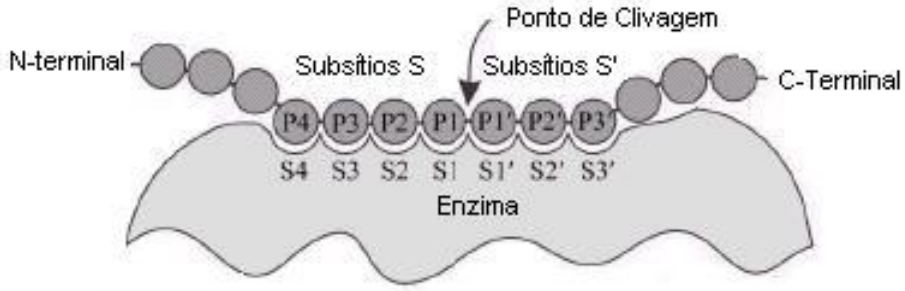

Figura 3: Representação esquemática do substrato (proteína) ligado à protease. A superfície da protease que é capaz de acomodar uma única cadeia lateral de resíduo do substrato é chamada de subsítio (TURK, 2006).

1.2.1.1 Substratos Peptídicos Fluorogênicos

Substratos peptídicos contendo uma seqüência de aminoácidos entre um grupo fluorescente e um supressor de fluorescência são compostos indicados para o estudo da especificidade das proteases. Com a fragmentação da molécula, desaparece a supressão intramolecular e há um aumento na emissão da fluorescência, a qual pode ser mensurada em um espectrofluorímetro (Figura 4).

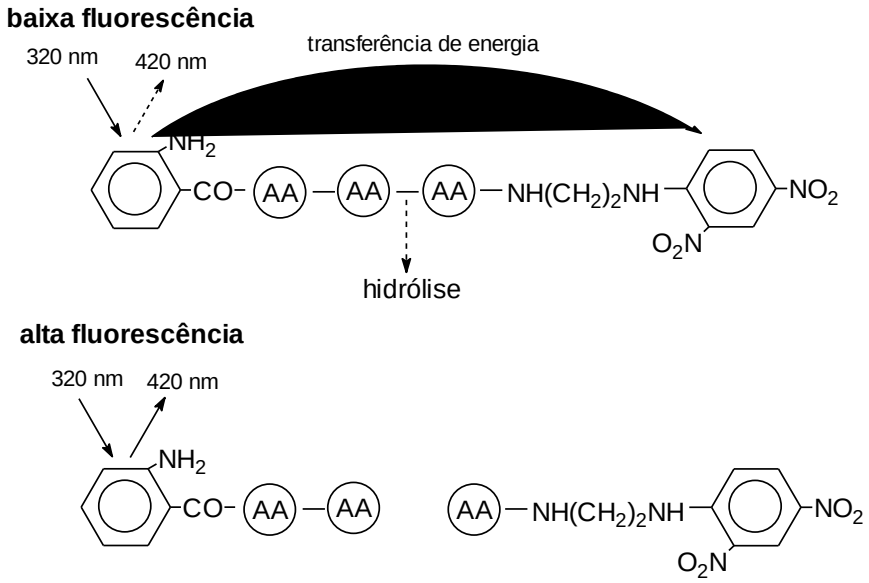

Figura 4: Representação do mecanismo de supressão intramolecular da fluorescência do Abz devido à transferência de energia.

Essas observações permitiram um avanço significativo no desenvolvimento de substratos artificiais para as proteases. Nos métodos cromogênicos, a ligação amida entre o peptídeo e os grupos cromogênicos constitui o único sítio detectável de clivagem do substrato pelas peptidases e a grande maioria dos cromóforos utilizados apresenta propriedades diferentes dos resíduos de aminoácidos. A necessidade de se colocar esses grupos diretamente nos pontos de clivagem catalítica, além da ligação química diferir das ligações peptídicas normais, constitui a grande desvantagem destes substratos. Sob o ponto de vista da interação enzima-substrato é evidente que, para esses substratos sintéticos, a interação fica limitada ao lado carboxílico da ligação susceptível, isto é, P1, P2, P3, etc. Porém tratando-se de um substrato fluorogênico com supressão intramolecular, o grupo supressor e o grupo fluorescente estão situados em regiões opostas do peptídeo, isto permite aumentar a especificidade dos substratos pela enzima, pois agora é permitido incluir os resíduos P1', P2', etc. Desta forma, evita-se a maior parte dos inconvenientes citados anteriormente para os substratos com o grupo cromóforo ou fluoróforo colocado diretamente no sítio de hidrólise (DIAMOND, 2007; KORKMAZ et al., 2008).

1.2.2 Aplicações das proteases

As proteases representam uma classe de enzimas com importantes papéis em processos fisiológicos. Além disto, elas possuem diversas aplicações comerciais, sendo responsáveis por 60% do comércio internacional de enzimas (Figura 5) (JOHNVESLY et al., 2001).

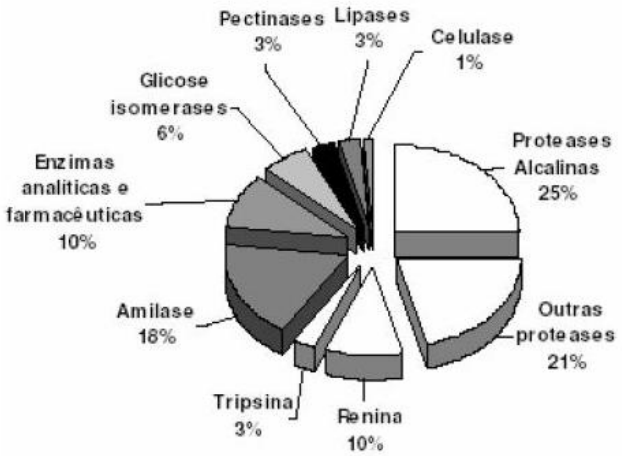

Figura 5: Enzimas no mercado internacional (RAO et al., 1998).

Na indústria de alimentos, preparados de enzimas proteolíticas podem ser aplicados nos processos de fermentação (cervejaria) (SCHMIDELL, 2001) e produção de alimentos orientais e queijos (POOLMAN et al., 1995), na produção de gelatina hidrolisada e de leite de soja (WORKMAN et al., 1986), no processamento de carnes vermelhas (DONAGHY et al., 1993), na produção de pães e biscoitos (LYONS, 1998).

Nos processos de higienização, proteases são muito empregadas em formulações de soluções para limpeza, pois possibilitam a remoção de manchas e resíduos protéicos, provenientes, por exemplo, de carne, sangue, leite e ovo e por serem aceitas ecologicamente (KUMAR et al., 1999).

Na área de saúde, as proteases são utilizadas na eliminação de vermes, na cicatrização de feridas e queimaduras, para remoção de tecidos necrosados e para lise de coágulos sanguíneos (LANDAU & EGOROV, 1996). Além disso, são agentes antiinflamatórios e tem sido estudada sua eficiência na inibição do desenvolvimento de células cancerígenas (TURK, 2006). Atualmente, alguns medicamentos comercializados são inibidores de proteases. Como exemplo tem-se o Captopril (Capoten®), utilizado no tratamento de hipertensão já que age inibindo a enzima conversora de angiotensiva (CONSTANTINESCU et al., 1998). E ainda, o Indinavir (Crixivan®), inibidor da aspártico-protease do HIV (MISHRA et al., 2010).

Portanto, estudos envolvendo proteases, seus substratos e inibidores são de grande relevância para a biofísica, biotecnologia e medicina.

1.2.3 Proteases microbianas

Proteases são encontradas em vários microrganismos, como vírus, bactérias, protozoários, leveduras e fungos. A impossibilidade das proteases de plantas e animais atenderem à demanda mundial de enzimas tem levado a um interesse cada vez maior pelas proteases de origem microbiana. Os microrganismos representam uma excelente fonte de proteases devido a sua grande diversidade bioquímica e facilidade de manipulação genética (PATEL et al., 2005). Proteínas são degradadas por microrganismos, que utilizam os produtos da degradação como nutrientes para o seu crescimento (VAN DEN HOMBERGH et al., 1997). Numerosas proteases são produzidas por microrganismos distintos dependendo da espécie, ou mesmo por diferentes cepas de uma mesma espécie. Proteases diferentes também podem ser produzidas pela mesma cepa, variando as condições de cultura.

As proteases microbianas contam com aproximadamente 40% do total das enzimas comercializadas (RAO et al., 1998). São divididas em três grupos, de acordo com o pH no qual exibem maior atividade: proteases ácidas, neutras e alcalinas. As proteases ácidas possuem atividade na faixa de pH 2,0 a 5,0, perdendo rapidamente a atividade em valores mais elevados. Elas são ativas na presença de agentes sulfidrílicos, agentes quelantes, metais pesados e do agente organofosfórico DFP (Diisopropil-fluorfosfato). As proteases neutras possuem atividade entre pH 7,0 a 8,0, são metaloenzimas e geralmente são inibidas por agentes quelantes como o EDTA. O terceiro grupo de proteases são as alcalinas, tendo pH de atividade entre 9,0 e 11,0. Elas possuem serina no seu sítio ativo, sendo assim chamadas de serino-proteases e são inibidas por agentes organofosfóricos como DFP e não são inibidas por agentes quelantes (GUERRA, 1991).

1.2.4 Microrganismos termofílicos

Nos últimos anos têm aumentado significativamente as pesquisas sobre produção de enzimas por microrganismos termofílicos, assim como a diversidade de substratos alternativos já que a temperatura tem grande influência no funcionamento de moléculas e estruturas biológicas (MAHESHWARI et al., 2000; MARTINS et al., 2002). De fato, a maioria dos organismos atualmente conhecidos pode crescer somente dentro de uma faixa estreita de temperatura. Entretanto, a existência de ambientes geotermicamente estáveis tem permitido a seleção ou a persistência, de microrganismos que não apenas resistem, mas também requerem altas temperaturas para sobreviver. Estes organismos são chamados de termófilos ou termofílicos.

Microrganismos que vivem em condições extremas são, usualmente, uma rica fonte para obtenção de bioprodutos com propriedades diferenciadas, em particular, enzimas. Devido às suas propriedades únicas, esses bioprodutos podem ser empregados em condições drásticas, que com frequência ocorrem na prática industrial. Em geral, há uma correlação alta entre a termofilia do organismo e a termoestabilidade de suas proteínas (intra e extracelulares). Enzimas de termófilos são usualmente moléculas mais termoestáveis e não perdem a sua conformação original e a sua atividade em elevadas temperaturas, o que as torna atraentes para serem incorporadas em processos industriais, havendo, portanto um grande interesse comercial na busca desses catalisadores biológicos (BRUINS et al., 2001).

1.2.5 Fungos e Fermentação

Os fungos constituem um grupo microbiano extremamente diverso, com uma ampla variedade morfológica, metabólica e de habitat. Uma das mais surpreendentes propriedades desses microrganismos é sua habilidade em adaptar-se a ambientes extremos, nos quais fatores como pH, temperatura, pressão e concentração de sal ultrapassam os valores considerados como padrão para a maioria dos organismos (LASA et al., 1993). São obtidos através de processos fermentativos e apresentam vantagens já que não é necessário o uso de métodos caros de filtração, pois seu micélio pode ser facilmente removido, por filtração a vácuo ou centrifugação, obtendo-se um extrato livre de micróbios (ANDRADE et al., 2002). Além disso, o uso de fungos, como produtores de enzimas, é mais seguro do que bactérias já que normalmente são considerados como GRAS (*Generally Regarded As Safe*) (GERMANO et al., 2003).

A produção de enzimas por processos fermentativos é um vasto campo da biotecnologia no qual ainda tem muito para se conhecer e explorar. Estes processos viabilizam economicamente a produção de enzimas em quantidades industriais. Sistemas de fermentação em estado sólido (FES) e líquida ou submersa (FSM) têm sido utilizados para produção de proteases (SANDHYA et al., 2005). No caso da fermentação submersa, o material fermentado é centrifugado ou filtrado para a recuperação das enzimas nos sobrenadantes das culturas. No caso da fermentação sólida, os biocatalisadores são extraídos da massa do substrato fermentado. Sendo que, a FES apresenta algumas vantagens em relação à FSM como a utilização de resíduos agroindustriais de custos baixos, utilização de pouca quantidade de água, e ainda a produção enzimática pode ser maior.

1.3 IMOBILIZAÇÃO DE ENZIMAS

A aplicação de biocatalisadores na indústria é objeto de muitas investigações, devido à alta atividade catalítica em comparação com os catalisadores convencionais, e ao fato de serem biodegradáveis (DALLA-VECCHIA et al., 2004). O desenvolvimento de tecnologias que evitem a inativação de enzimas é extremamente importante para aplicação dessas nas indústrias. No entanto, aplicações de enzimas na indústria são viáveis somente se as enzimas estiverem estabilizadas em relação à temperatura, extremos de pH, e na presença de sais, alcalóides e surfactantes (IYER et al., 2008). Uma alternativa viável que permitiria o emprego das enzimas na maior parte das reações de biocátalise presentes nas indústrias seria o uso do processo de imobilização de enzimas. As principais vantagens que ratificam o emprego dos métodos de imobilização de biocatalisadores em indústrias seriam: garantir maior estabilidade, assegurar a reutilização do biocatalisador e facilidade de separação do meio de reação, o que acarreta economia significativa no custo global do processo (VILLENEUVE et al., 2000). Contudo, a velocidade de difusão dos substratos é um fator limitante e geralmente são necessárias altas concentrações de substratos a fim de limitar esta influência (GOMES et al., 2006). A imobilização de enzimas pode ser realizada por meio de diferentes métodos: adsorção ou ligação da enzima em um material insolúvel, pelo uso de um reagente multifuncional através de ligações cruzadas, por confinamento em matrizes formadas por géis poliméricos ou encapsulação através de uma membrana polimérica (DALLA-VECCHIA et al., 2004).

Na escolha de um suporte algumas características devem ser observadas, como: permeabilidade, resistência à bactérias, morfologia, resistência mecânica, insolubilidade, composição e área superficial (BRIGIDA, 2006). Para fins de imobilização, alginatos são um dos polímeros mais utilizados devido às suas propriedades geleificantes e não-toxicidade (WON et al., 2005); ele é um polissacarídeo linear constituído de resíduos de α-L-gulurônico (G) e ß-D-manurônico (M), presentes em proporções e seqüência variável (Figura 6) (SANKALIA et al., 2005). Assim, pode ser precipitado pela adição de íons Ca^{2+} (ERTESVAG et al., 1998) permitindo a obtenção de microesferas com boa resistência e flexibilidade (DE QUEIROZ et al., 2006). 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 (OLIVEIRA, 2004). Na literatura, o uso de partículas preparadas com alginato na imobilização de enzimas é amplo, bem como, na encapsulação de

drogas farmacêuticas ou microganismos e em preparações alimentícias (KAWAGUTI & SATO, 2008).

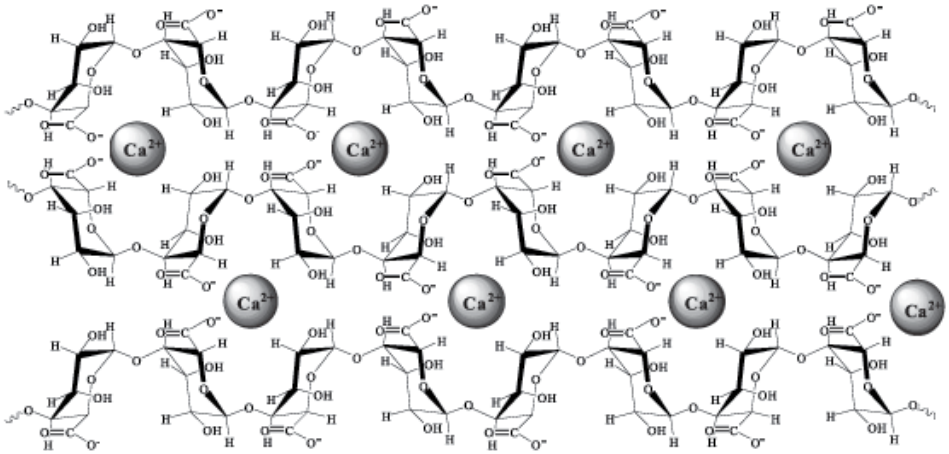

Figura 6: Formação do gel de alginato de cálcio (KAWAGUTI & SATO, 2008).

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CAPÍTULO 2

"Production, partial characterization and immobilization in alginate beads of an alkaline protease from a new thermophilic fungus Myceliophthora sp"

Production, partial characterization and immobilization in alginate beads of an alkaline protease from a new thermophilic fungus *Myceliophthora* sp.

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Running title: alkaline protease from *Myceliophthora* sp.

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Este artigo foi aceito para publicação no *The Journal of Microbiology* (ISSN: 1225-8873, IF: 1.5) em 29/12/2009.

ABSTRACT

Thermophilic fungi produce thermostable enzymes which have a number of applications, mainly in biotechnological processes. In this work, we describe the characterization of a protease produced in solid-state (SSF) and submerged (SmF) fermentations by a newly isolated thermophilic fungus identified as a putative new species in the genus *Myceliophthora*. Enzyme-production rate was evaluated for both fermentation processes, and in SSF, using a medium composed of a mixture of wheat bran and casein, the proteolytic output was 4.5-fold larger than that obtained in SmF. Additionally, the peak of proteolytic activity was obtained after 3 days for SSF whereas for SmF it was after 4 days. The crude enzyme obtained by both SSF and SmF displayed similar optimum temperature at 50 ºC, but the optimum pH shifted from 7 (SmF) to 9 (SSF). The alkaline protease produced through solid-state fermentation (SSF), was immobilized on beads of calcium alginate, allowing comparative analyses of free and immobilized proteases to be carried out. It was observed that both optimum temperature and thermal stability of the immobilized enzyme were higher than for the free enzyme. Moreover, the immobilized enzyme showed considerable stability for up to 7 reuses.

***Keywords**:* alkaline protease, immobilized enzyme, *Myceliophthora* sp., solid state fermentation, thermophilic fungus.

2.1 INTRODUCTION

Enzymes have a wide range of biotechnological, biomedical, and pharmaceutical applications. Proteases constitute the most prominent group of commercially available enzymes (Joo *et al.*, 2002); they account for about 65 % of the worldwide sale of industrial enzymes in the world market (Johnvesly and Naik, 2001). Microorganisms are a valuable source of proteases mainly due to their short generation time, the ease of bulk production and genetic manipulation (Patel *et al.*, 2005). Microbial proteases account for approximately 40 % of the total worldwide enzyme sales (Rao *et al.*, 1998), and among them alkaline proteases have ample biotechnological potential for industrial sectors like laundry detergents, leather processing, brewing, food and pharmaceutical industries (Kembhavi *et al.*, 1993).

Thermophilic fungi are known to produce thermostable enzymes. The use of these enzymes may exhibit several advantages due to the high processing temperatures that can be applied, which are related to increased reaction rates, improved solubility of reagents, and a reduction in mesophilic contamination. Besides thermal stability, these enzymes also exhibit higher stability towards other protein denaturating conditions when compared to similar mesophilic enzymes (Gusek and Kinsella, 1988).

The available literature shows that a large amount of work has been devoted to the immobilization of enzymes in polymeric carriers (Kennedy *et al.*, 1990). The main advantage of using immobilized enzymes as biocatalysts is that it is possible to reuse them since they can be easily recovered, thereby making the process economically feasible (da Silva *et al.*, 2009). Therefore, for industrial applications, the immobilization of enzymes can provide several advantages and may contribute to an increase in their stability, making feasible their widespread use in industry.

For immobilization purposes, alginates are one of the most used polymers due to their mild gelling properties and non-toxicity (Won *et al.*, 2005). 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 in sequential arrangements, which can be precipitated by the addition of Ca^{2+} ions (Ertesvag and Valla, 1998; Smidsrod and Skajak-Braek, 1990) allowing the formation of microspheres with considerable strength and flexibility (De Queiroz *et al.*, 2006).

In this work we describe the isolation of a thermophilic fungus and protease production through solid-state and submerged fermentations, and present a comparative study

on the properties of the protease produced in both fermentation systems. Additionally, we show data on the immobilization of the enzyme from SSF on beads of calcium alginate and also compare the properties of the free and immobilized proteases.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Casein, glycine, agar and $CaCl_2$ were obtained from Vetec (Duque de Caxias, Brazil). Yeast extract was obtained from Oxoid (Cambridge, England). NaCl, Na_2CO_3, $MnCl_2.4H_2O$, $FeSO_4.7H_2O$ and sodium phosphate were obtained from Synth (Diadema, Brazil). K_2SO_4 and $MgCl_2$ were obtained from Nuclear (Sao Paulo, Brazil). Glycerol, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, EDTA, H_3BO_3, $(NH_4)_2HPO_4$, $CoCl_2.5H_2O$ and $(NH_4)Mo_7O_{24}.4H_2O$ were obtained from Merck (Darmstadt, Germany). $(NH_4)_2SO_4$, trichloroacetic acid, Hepes, Taps, Caps and BSA were obtained from Sigma (St. Louis, USA). $MgSO_4$ $.7H_2O$ and NH_4NO_3 was obtained from Dinamica (Diadema, Brasil). Sodium citrate dehydrate and sodium acetate were obtained from Mallinckrodt Baker (Phillipsburg, USA). Sodium alginate was obtained from Fluka (Deisenhofen, Germany).

2.2.2 Isolation and Identification

Several thermophilic fungal strains were isolated from box fat, poultry, poultry litter and a composting plant. Approximately, 1 g or 2 mL of collected material was transferred directly to flask containing the following nutrient medium adapted from Jensen *et al.* (2002) and Sandhya *et al.* (2005): 0.5 % (v/v) glycerol, 2 % casein, 0.3 % yeast extract, 0.5 % NaCl, 0.15 % Na_2CO_3, 0.1 % K_2SO_4, 0.02 % $MgCl_2$, 0.3 % $(NH_4)_2HPO_4$ in the proportion (w/v) and a 0.5 % (v/v) solution of trace elements (2.2 % $ZnSO_4.7H_2O$; 1.1 % H_3BO_3; 0.5 % $MnCl_2.4H_2O$; 0,5 % $FeSO_4.7H_2O$; 0,16 % $CoCl_2.5H_2O$; 0.16 % $CuSO_4.5H_2O$; 0.11 % $(NH_4)Mo_7O_{24}.4H_2O$; 5 % EDTA in the proportion (w/v)). After 24, 48 or 72 hours of incubation at 45 ^oC, the samples were transferred by means of grooves, to Petri dishes. After the emergence of fungi colonies, they were separated based on the appearance of the mycelium, color of spores and other characteristics of the obverse and reverse of the colonies. These colonies were reinoculated by streaking to obtain the pure cultures.

The fungal isolate that showed the highest protease activity was identified by conventional and molecular approaches. Cultures grown in 2 % (w/v) malt agar for seven days at 45 °C (Van Oorschot, 1980) were observed under a stereomicroscope (Zeiss, Stemi SV6) in order to study the colony morphology. In addition, details on the micromorphology were observed under light microscope (Zeiss, Axioskop 40) using squashed lumps stained with cotton blue. Molecular identification was carried out by sequencing the ITS1-5.8S-ITS2 rDNA region coupled with phylogenetic analyses as described in Sette *et al.* (2006).

2.2.3 Enzyme production in solid-state (SSF) and submerged (SmF) fermentations

Erlenmeyer flasks (250 mL) containing media composed of 4.75 g of wheat bran and 0.25 g of casein and hydrated with 7 mL of distilled water and 3 mL of nutrient solution 0.1 % (w/v) $(NH_4)_2SO_4$, $MgSO_4$ $.7H_2O$ and NH_4NO_3 were inoculated with 2 mL of a spore suspension and cultivated at 45 °C for 168 hours, taking samples every 24 h. The fermented material was mixed with 30 mL of distilled water per 5 grams of fermented material, stirred for 30 min, filtered and centrifuged at 10,000 x g, at 6 °C. The supernatant was used as a crude enzyme solution for proteolytic assays.

The submerged fermentation (SmF) was carried out in 125 mL Erlenmeyer flasks containing 25 mL of medium composed by 2 % (w/v) casein, 0.1 % (w/v) $(NH_4)_2SO_4$, $MgSO_4$ $.7H_2O$ and NH_4NO_3. This medium was inoculated with 2 mL of a spore suspension and incubated at 45 °C under agitation of 100 rpm, for 168 hours. After each 24 hours period of fermentation, the material in a flask was vacuum filtered using Whatman paper N°1, centrifuged at 10,000 x g for 40 minutes at 4 ^oC and the supernatant was used as crude enzyme solution for testing proteolytic activity. The fermentation experiments were performed in triplicate and the results are reported as mean averages.

2.2.4 Protease assay

The proteolytic activity was assayed as described by Sarath *et al.* (1996) with modifications. The reaction mixture was made up of 0.2 mL (or 0.5 g immobilized) crude enzyme and 0.8 mL of 1 % (w/v) casein dissolved in glycine buffer (50 mM, pH 9.0). The reaction was carried out at 50 °C and stopped after 30 min by adding 0.5 mL of 15 % trichloroacetic acid (TCA). Test tubes were centrifuged at 15,000 xg for 30 min and the absorbance of the supernatant was measured at 280 nm using a Cary 100 (Varian)

spectrophotometer. A control was prepared by adding TCA before the enzyme solution. One unit of enzyme activity (U mL^{-1}) was arbitrarily defined as the amount of enzyme required to cause an absorbance increase of 0.01 at 280 nm under the assay conditions (Merheb *et al.*, 2007). The experiments were performed in triplicate.

2.2.5 Entrapment of the protease in calcium alginate beads

The procedure for enzyme immobilization in alginate was adapted from Betigeri and Neau (2002). Predetermined quantities of sodium alginate (2, 2.5, 3, 3.5 % w/v) were prepared in 4 mL of 50 mM glycine buffer pH 9.0. To this solution 1 mL of crude protease was added and thoroughly mixed. The mixture was added drop wise to 5 mL $CaCl_2$ solutions (0.04, 0.06, 0.08, 0.1 M). The cure time was fixed in 20 min, and thereafter, the formed microcapsules were washed with the buffer twice, to remove non-encapsulated enzyme. A similar method was followed for preparation of control alginate beads in the absence of the enzyme. The ideal concentrations of alginate and calcium were established guided by the highest activity of the immobilized enzyme. The amount of immobilized enzyme was estimated by subtracting the value of specific activity determined in the solution after immobilization from the value of specific activity (8 U mg^{-1}) used for immobilization (Worsfold, 1995). The total protein concentration was determined using the method of Bradford (1976) and bovine serum albumin as a standard.

2.2.6 Effect of pH and temperature on free and immobilized enzyme activity

The effect of temperature and pH on the activity of free and immobilized protease was investigated. The enzyme was incubated at 50 ^{o}C in 0.05 M buffer solutions containing 1 % (w/v) casein: sodium citrate dehydrate (pH 5), sodium acetate (pH 5.5 and pH 6), sodium phosphate (pH 6.5 and pH 7), Hepes (pH 7.5), TAPS (pH 8 and pH 8.5), Glycine (pH 9 and pH 9.5), CAPS (pH 10, pH 10.5 and pH 11). Optimum temperature was determined by incubating the reaction mixture at temperatures ranging from 0 to 75 ^{o}C at the optimum pH.

2.2.7 Effect of temperature on free and immobilized enzyme stability

The thermal stability was investigated by measuring the enzyme activity after keeping the enzyme solution for 24 h in the absence of substrate, at temperatures between 30 and 70 °C. Remaining protease activity was determined at optimum pH and temperature.

2.2.8 Reusability of protease immobilized in alginate beads

The initial activity of the immobilized enzyme was measured and the conjugate was then subjected to cycles of repeated use. The results of pH, temperature and reusability stability of free and immobilized protease are presented in a normalized form, with the highest value of each set being assigned the value of 100 % activity.

2.3 RESULTS AND DISCUSSION

2.3.1 Isolation and Identification

We isolated thirty thermophilic fungi with the ability to grow at 45 °C on medium containing wheat bran and casein as carbon sources and produced proteases in both SSF and SmF (data not shown). The fungus strain F.2.1.4 presented the highest protease activity and was subjected to taxonomic characterization.

Analyses of macro and micro-morphological characteristics accommodated the isolate F.2.1.4 in the genus *Myceliophthora* due to the presence of spreading colonies with dense aerial mycelium and blastic conidia often borne in ampulliform swellings (Van Oorschot, 1980). Particularly, this isolate produced pulverulent pale mycelium, obovoid conida measuring 5.65-7.91 x 3.39-4.52 µm as well as finely ornamented conidia walls. Based on such characteristics the isolate F.2.1.4 is similar to *Myceliophthora thermophila*, however, some slight morphological differences were observed, for instance, *M. thermophila* has pale brown, woolly colonies and a variable range of conidia sizes 4.5-11 x 3.0-4.5 µm (Van Oorschot, 1977).

Data derived from BLAST (ITS-rDNA region) showed that the isolate F.2.1.4 had high sequence similarities (99-96%) with three unidentified *Myceliophthora* spp. isolated from geothermal soils in China; and 95-94% of sequence similarities with different strains of the Ascomycota genus *Corynascus* (one of the *Myceliophthora* teleomorphic states), including three new species: *C. verrucosus* sp. nov., *C. similis* sp. nov., *C. sepedonium, C. sexualis* sp. nov. (Stchigel *et al.*, 2000). The phylogenetic tree showed that the isolate F.2.1.4 clustered with the three *Myceliophthora* strains from China (Figure 1).

Molecular and phylogenetic results corroborate the data from morphological analyses and considering the combined methods used for fungus identification, the thermophilic isolate F.2.1.4 was identified as *Myceliophthora* sp. Species in this genus are commonly found in soil, molding open-stack alfalfa ensilage, fir and spruce pulpwood chips and wheat straw compost (Van Oorschot, 1980). Moreover, results derived from conventional, molecular and phylogenetic analyses suggest that isolate F.2.1.4 likely represent a new species in the genus *Myceliophthora*. Additional taxonomic analyses will be performed in order to confirm the new species hypothesis.

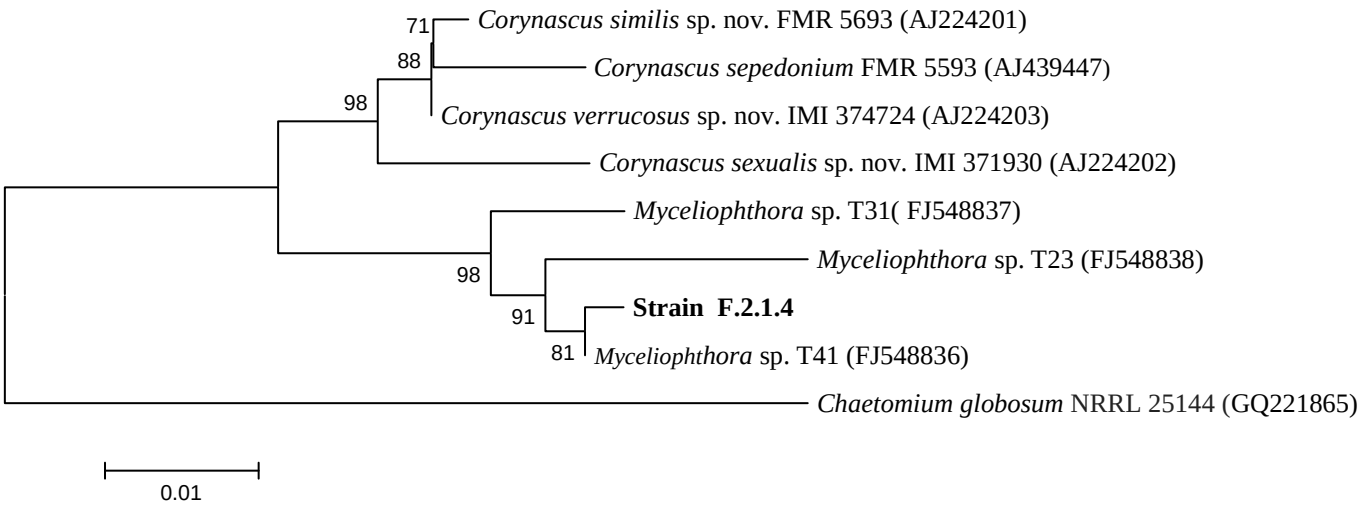

Figure 1: Phylogenetic tree based on ITS-rDNA analyses showing closest relatives of thermophilic fungus strain F.2.1.4 (Kimura two-parameter model; Neighbor-Joining algorithm). Bootstrap values (1,000 replicate runs) greater than 70% are listed.

2.3.2 Enzyme production in solid-state (SSF) and in submerged (SmF) fermentations

Figure 2 shows the profile of protease production by *Myceliophthora* sp. in SSF and SmF. The maximum enzyme production was obtained in the third day of fermentation in SSF (1.78 U mL^{-1}) and in the fourth day in SmF (0.38 U mL^{-1}). The higher protease production achieved in SSF corroborate data reported by several investigators and reinforce the hypothesis that production of extracellular enzymes is higher in SSF since the conditions of this fermentation system are similar to natural fungal growth media (Aguilar *et al.*, 2004; Silva *et al.*, 2007; Morita *et al.*, 1999; Ramesh and Lonsane, 1991). The data also showed that wheat bran that contains approximately 18 % protein, 5 % fat and 62 % carbohydrate was an effective medium for protease production because it supplies adequate nutrients for microorganisms (Ellaiah *et al.*, 2004; Beg *et al.*, 2000). Protease production in state solid and submerged fermentation by thermophilic fungi have been reported by Germano *et al.* (2003), Sandhya *et al.* (2005), Macchione *et al.* (2008), O'Donoghue *et al.* (2008) and Merheb *et al.* (2007). Also Badhan *et al.* (2007) reports the production of protease by *Myceliophthora* sp. IMI 387099.

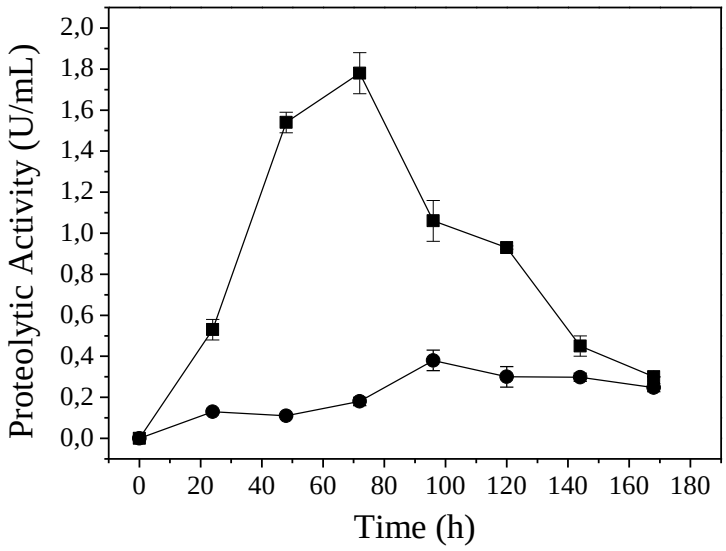

Figure 2: Production of protease by *Myceliophthora* sp. in SSF (■) and SmF (●) fermentations. Each symbol represents the average ± S.D.

2.3.3 Characterization of the free crude enzyme

Figure 3a shows that the optimum pH value for the free protease produced by SSF was 9. However, the protease produced in submerged fermentation exhibited the maximum activity at pH 7 with accentuate decreasing of the activity in alkaline pH, suggesting a different protease expression in both fermentation systems. Extracellular alkaline proteases from thermophilic and thermotolerant fungi have been described from *Thermomyces lanuginosus* (Li *et al.*, 1997), *Aspergillus clavatus* (Tremacoldi and Carmona, 2005) and *Fusarium culmorum* (Pekkarinen *et al.*, 2002).

Alkaline proteases have many applications in the industry such as laundry detergents, leather processing, brewing, food and pharmaceutical industries therefore the alkalophilic properties exhibited by proteases from *Myceliophthora* sp. in SSF are an important feature to be explored.

The maximum activity for both crude enzymes was at 50 ºC, but the crude enzyme from SSF maintained 95 % of the maximum activity at 60 ºC while for the protease from SmF the activity was 79 % (Figure 3b). This property is consistent with the data reported for extracellular enzymes from thermophiilic fungi. Similar results were reported for proteases from *Thermoascus aurantiacus var levisporus* (55 ^{o}C) (Marcy *et al.*, 1984). Proteases from

thermophilic *Scytalidium* (Hasbay and Ögel, 2002) and thermotolerant *Aspergillus fumigatus* (Santos *et al.*, 1996) showed optimal activities at 45 ^{o}C.

Since proteolytic activity was higher in the crude enzyme solution from SSF and additionally, it showed alkaline optimum pH and activity at higher temperature range (50-65 ºC), it was used for the immobilization procedure.

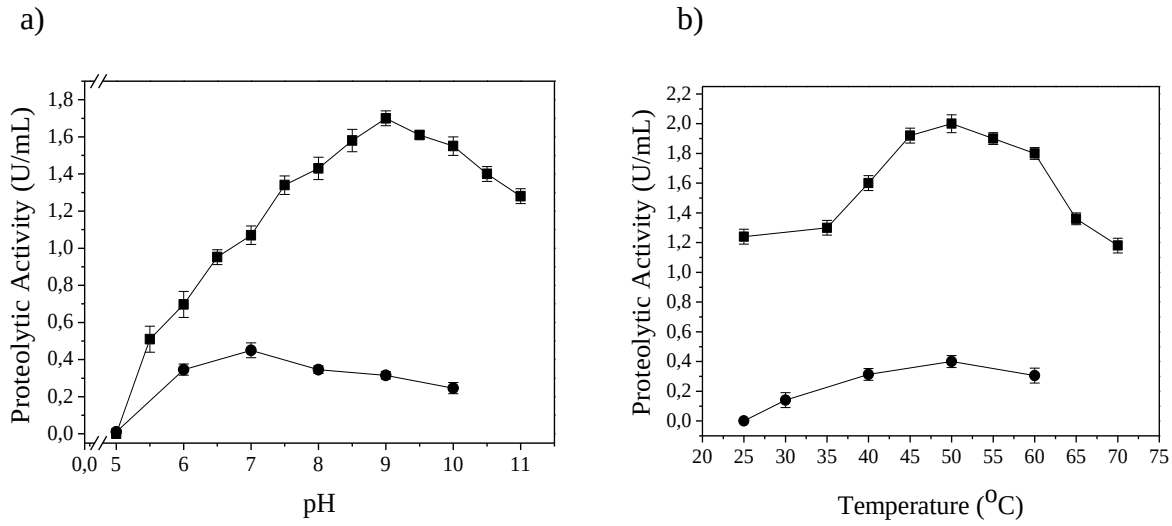

Figure 3: Effect of pH (a) and temperature (b) on free crude proteolytic activity produced by *Myceliophthora* sp. in SSF (■) and SmF (●). For the pH effect the samples were incubated at 50 ^{o}C using different buffers, and for the temperature analysis the samples were incubated at the optimum pH. Each symbol represents the average ± S.D.

2.3.4 Entrapment of the protease in calcium alginate beads

We tested several concentrations of sodium alginate (2, 2.5, 3, 3.5 % (w/v)) and $CaCl_2$ (0.04, 0.06, 0.08, 0.1 M). The best condition for immobilization of the protease was 2.5 % alginate and 0.08 mM $CaCl_2$ (data not shown), obtaining 2 mm (average diameter) beads.

The immobilized enzyme was reevaluated concerning its physical-chemical properties (Figure 4). The profile of activity of the immobilized protease was very similar to the free enzyme in terms of its optimum pH and temperature (Figure 4a and b), but the thermal stability was strongly increased by the entrapment procedure. The immobilized enzyme maintained around 50 % of the initial activity at 50 to 65 ºC for five hours (Figure 4c), but the free enzyme was stable at 50 ºC for five hours but it was inactivated at temperatures above 55 ºC (Figure 4d). This result suggested that the conformational flexibility of the enzyme was affected by immobilization causing an increase in enzyme rigidity which was reflected by

increase in stability towards thermal denaturation, as proposed by Abdel-Naby (1993) and Worsfold (1995) for other proteases.

The immobilization of proteases in alginate beads was reported by Ko *et al.* (2008) and Ahmed *et al.* (2008), and the last author reported the increased thermal stability of an immobilized enzyme; at 65 ºC the free enzyme was inactivated, while the immobilized enzyme maintained around 80 % of the initial activity.

Concerning pH stability, the free protease isolated from *Myceliophthora* sp. exhibited 95% of activity after 24 h at pH values from 8 to 11 at room temperature (data not shown).

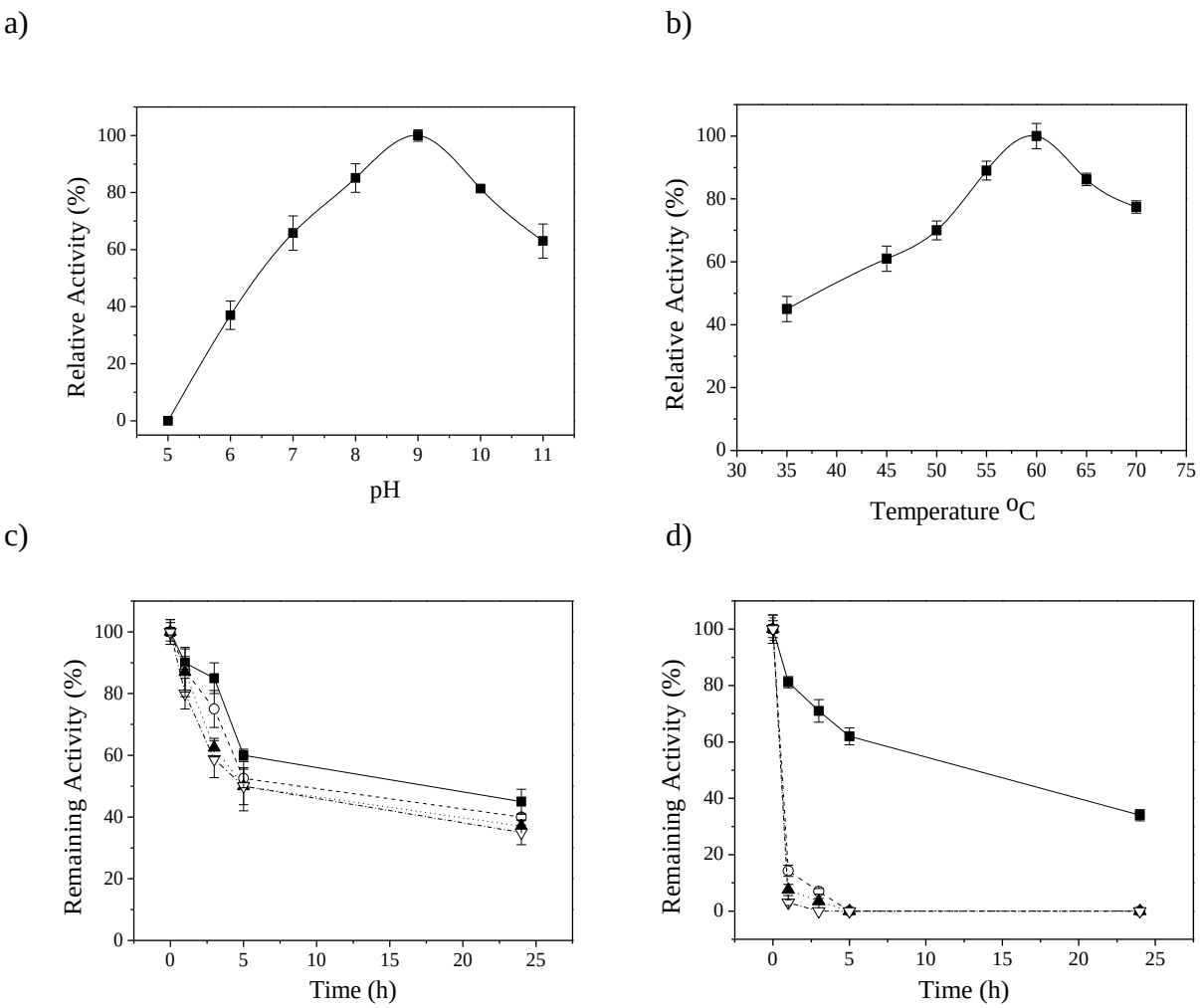

Figure 4: Properties of the immobilized protease produced by SSF. (a) Effect of pH on immobilized enzyme activity; (b) Effect of temperature on immobilized enzyme activity; (c) effect of temperature on stability of immobilized enzyme when in the absence of substrate;(d) effect of temperature on stability of free enzyme when in the absence of substrate. ■ 50 ºC, ○55 ºC, ▲60 ºC, ∇65 ºC. The results were expressed as perceptual relative activity (the ratio of activity of the sample to that of the maximum x 100). Each symbol represents the average ± S.D.

2.3.5 Reusability of protease immobilized in the alginate beads

When comparing the performance of immobilized biocatalysts intended for industrial use, knowledge of their operational stability is very important. The stability of an immobilized enzyme without appreciable loss of enzyme activity is important mainly for the economic viability. Figure 5 shows the effect of repeated use on the activity of the immobilized protease; it decreased gradually during 7 cycles of reuse (its activity at the 7th cycle being greater than 50% of the original activity) and more sharply after that.

In conclusion, the thermophilic fungus *Myceliophthora* sp. was able to produce alkaline and thermostable proteases in SSF using wheat bran as culture medium. The protease immobilization in alginate not only maintained some properties, but significantly improved its thermal stability and permitted its reuse for seven cycles.

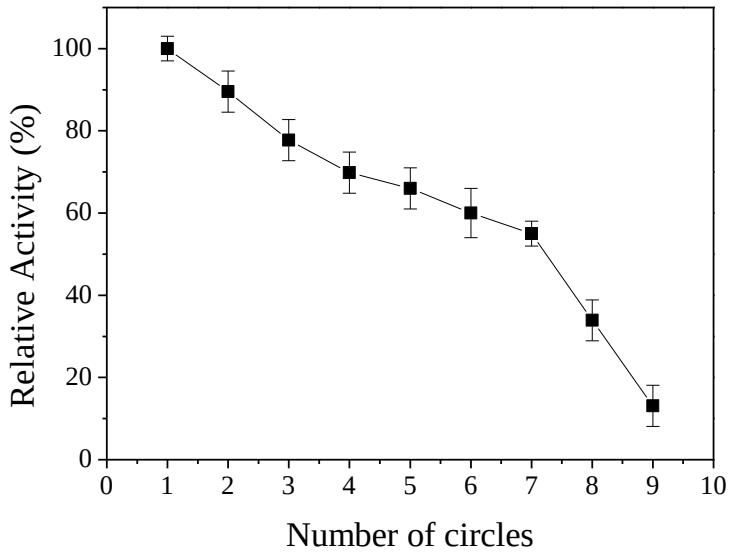

Figure 5: Reusability of the immobilized protease at 50 °C. The results were expressed as percentual relative activity (the ratio of activity of the sample to that of the maximum x 100). Each symbol represents the average ± S.D.

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ACKNOWLEDGEMENTS

This work was supported by CAPES (LMZ), FAPESP and CNPq (GOBR and EG). The authors thank Austin Vogt for a helpful English review of this manuscript.

CAPÍTULO 3

"Functional properties and subsite specificity mapping of a new alkaline serine-protease from the thermophilic fungus Myceliophthora sp."

Functional properties and subsite specificity mapping of a new alkaline serine-protease from the thermophilic fungus *Myceliophthora* sp.

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Running title: Characterization of an alkaline serine-protease from *Myceliophthora* sp.

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ABSTRACT

A novel alkaline serine protease was purified from a thermophilic fungus belonging to genus *Myceliophthora*. The enzyme has a molecular weight of 28.2 kDa as determined by MALDI-TOF mass spectrometry and showed optimum pH and temperature at 9.0 and 40-45 °C, respectively. The proteolytic activity was completely inhibited by PMSF, but benzamidine did not affect. Several ions have been tested and only Mg^{2+} ions resulted in increased catalytic activity indicating a specific role of Mg^{2+} for its functioning. The addition of reducing agents did not affect the catalytic activity suggesting that the enzyme is not dependent on thiol groups. In other hand, the presence of acetone, buthanol and SDS the enzyme was inhibited probably due to denaturing effects of these compounds. Subsite specificity mapping using quenched fluorescent peptides showed that the Abz-KLISSKQ-EDDnp peptide is the best substrate among those tested for the purified protease (k_{cat} /K_m = 1,275.30 mM^{-1} s^{-1}). Additionally, the specificity data indicated that subsites P_1, P_2, P_3 and P_1', P_2', P_3', in general, have preference for hydrophobic residues.

Keywords: *Myceliophtora* sp, serine-protease, quenched fluorescent peptides, subsite mapping.

3.1 INTRODUCTION

Proteases are the most important industrial enzymes accounting for approximately 60 % of the total industrial enzyme market (RAO et al., 1998). The relevance of this large family of enzymes, which exhibits a wide structural and functional diversity, is reflected in their number of industrial applications such as food and drug processing, detergent enhancing, and textile treatment (GUPTA et al., 2002). Therefore, the industrial demand of proteolytic enzymes, with appropriate specificity and stability to pH, temperature and chemical agents, continues to stimulate the search for new sources.

Many microorganisms secrete proteases to the external environment in order to degrade proteins; their products of hydrolysis are used as carbon and nitrogen sources for cell multiplication (VAN DEN HOMBERGH et al., 1997). Proteases featuring high activity levels and stability at a wide range of pH are of great interest for bioengineering and biotechnological applications. A number of studies have described the production and isolation of alkaline proteases by bacteria and fungi (JELLOULI et al., 2009; WANG et al., 2009). The alkaline proteases are widely used as detergent enhancers owing to their stability at alkaline conditions since the pH of cleansing products is generally between 9.0 and 12.0. Moreover, the alkaline proteases found in cleansing solutions allow reducing toxic chemicals such as solvents and caustic substances, decreasing their environmental impact (CASTRO et al., 2004).

Currently, the majority of proteases used in detergents belong to the serine protease family. The serine proteases can be classified into the (chymo)trypsin or subtilisin subfamilies depending on its structure and function, and both subfamilies share a common catalytic triad (Ser195, His57, Asp189; numbering based on the trypsinogen sequence, reference) encompassing a reactive serine supported by a hydrogen bond network (RAWLINGS et al., 1993; POLGÁR, 2005). Thus, their specificity is strictly related to the physical-chemical properties and spatial arrangements of the residues forming the subsites around the active-site pocket and kinetic studies using different substrates and inhibitors can be valuable to understand its biological roles and potential industrial applications (BERGER et al., 1970).

Recently, we have isolated a thermofilic fungus *Myceliophhtora* sp. strain, which produces an extracellular proteolytic enzyme (ZANPHORLIN et al., 2010). In the present work, we report the purification, biochemical characterization and subsite specificity mapping using FRET peptides derived from the sequence Abz-KLRSSKQ-EDDnp (Abz = o-aminobenzoic acid; EDDnp = N-[2,4 dinitrophenyl]ethylenediamine; Abz/EDDnp =

donor/acceptor fluorescent pair) of the major extracellular protease produced by *Myceliophthora* sp.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Protease production in solid-state fermentation (SSF)

Erlenmeyer flasks (250 mL) containing media composed by 4.75 g of wheat bran and 0.25 g of casein and hydrated with 7 mL of distilled water and 3 mL of nutrient solution 0.1 % (w/v) $(NH_4)_2SO_4$, $MgSO_4$.$7H_2O$ and NH_4NO_3 were inoculated with 2 mL of a spore suspension and cultivated at 45 °C for 72 hours. The fermented material was mixed with 30 mL of distilled water per 5 g of fermented material, stirred for 30 min, filtered and centrifuged at 10,000 x g, at 6 °C. The supernatant was used as a crude enzyme solution.

3.2.2 Protease activity

Proteolytic activity was assayed as described by Sarath et al. (1996) with modifications. The reaction mixture was made up of 0.2 mL crude enzyme to and 0.8 mL of 1 % (w/v) casein dissolved in glycine buffer (50 mM, pH 9.0). The reaction was carried out at 50 °C and stopped after 30 min with 0.5 mL of 15 % trichloroacetic acid (TCA). Test tubes were centrifuged at 15,000 x g/30 min and the absorbance of the supernatant was measured at 280 nm using a Cary 100 (Varian) spectrophotometer. A control was prepared by adding TCA before the enzyme solution. One unit of enzyme activity (U mL^{-1}) was arbitrarily defined as the amount of enzyme required to cause an absorbance increase of 0.01 per minute at 280 nm under the assay conditions (MERHEB et al., 2007).

3.2.3 Purification Protocol

The crude enzyme was concentrated using a precipitation procedure at 4 °C with ethanol (proportion 1:2). The crude precipitate was collected by centrifugation at 9,000 x *g* for 30 min at 4 °C and then dissolved in 20 mM Tris pH 8.0 buffer with 0.2 M NaCl. The concentrated enzyme was then further purified by gel filtration on a Sephacryl S-100 column (1.6 x 75 cm) equilibrated with 20 mM Tris pH 8.0 containing 0.2 M NaCl. Fractions of 3 mL each were collected at a flow rate of 0.15 mL/min and analyzed for protease activity and protein concentration. Some fractions exhibiting protease activities were pooled and applied

to a Source 15-Q column (1 x 6 cm) that was previously equilibrated with 20 mM Tris, pH 8.5 buffer. Proteins not bound were washed of the column with the equilibration buffer until the absorbance at 280 nm reached the baseline. Bound proteins were eluted with a linear gradient of sodium chloride in the range of 0-2.0 M in the equilibration buffer. Fractions of 1 mL each were collected at a flow rate of 1 mL/min and analyzed for protease activity and protein concentration.

3.2.4 Electrophoresis

Polyacrylamide gel electrophoresis under denaturing conditions was carried out to determine the purity and to estimate the molecular weight of the purified enzyme. Protein bands were visualized after staining with silver nitrate (LAEMMLI, 1970). For the methods described below we used the purified protease.

3.2.5 Mass spectrometry analysis

Protease molecular masses determination was carried out on a TOF Spec E mass spectrometer (Micromass, Manchester, UK) operating in linear mode using the matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) method, using α-cyano-4-hydroxycinnamic acid as the matrix.

3.2.6 Determination of protein concentration

Protein concentration was determined by the Bradford method (BRADFORD, 1976), using bovine serum albumin as a standard.

3.2.7 Peptide Synthesis

All the FRET peptides used to characterize the protease were synthesized by solid-phase synthesis and purified as earlier described (HIRATA et al., 1994; KORKMAZ et al., 2008). The molecular mass and purity of the peptides were confirmed by amino acid analysis and by MALDI-TOF using a Microflex-LT mass spectrometer (Bruker — Daltonics, Billerica, MA, USA). Stock solutions of peptides were prepared in DMSO, and their concentrations were measured through of spectrophotometer Cary 100 (Varian) using a molar extinction coefficient of 17.300 M^{-1} cm^{-1} at 365 nm.

3.2.8 Kinetic Measurements

The FRET peptides were assayed in a Shimadzu RF-5301 spectrofluorimeter at 45 °C in 50 mM Glicine buffer, pH 9.0. The enzyme was pre-incubated in the assay buffer for 3 min before the addition of substrate. Fluorescence changes were monitored continuously at λ_{ex} 320 nm and λ_{em} 420 nm. The enzyme concentrations for initial rate determinations were chosen at a level intended to hydrolyze less than 5 % of the amount of added substrate over the time course of data collection. The slope of the generated fluorescence signal was converted to micromoles of substrate hydrolyzed per min based on a calibration curve obtained from the complete hydrolysis of each peptide. The kinetic parameters K_m and k_{cat} were calculated by nonlinear regression using the GraFit® software (Erithacus Software, Horley, Surrey, U.K.). Errors were less than 5 % for each of the obtained kinetic parameters.

3.2.9 Effects of pH and temperature on enzyme activity

Optimal pH was determined by performing standard activity assays in a pH range from 3-10.5 at 45 °C using suitable buffers: sodium citrate dehydrate, sodium acetate, sodium phosphate, Hepes, Taps, Glycine and Caps. In order to determine optimal temperature, the enzymatic assay was carried out at different temperatures (25-60 °C), at pH 9.0. The reactions were performed under pseudo first-order conditions ($[S] << K_m$) of hydrolysis of substrate Abz-KLRFSKQ-EDDnp. The data was fitted with the GraFit® software to the appropriate equation (SARKANY et al., 2001).

3.2.10 Effect of inhibitors on enzyme activity

The effects of inhibitors on protease activity were studied using PMSF, benzamidine, iodoacetic acid, EDTA and E-64. The purified enzyme was pre-incubated with inhibitors for 3 min in pH 9.0, at 45 °C, and then the remaining enzyme activity was estimated using Abz-KLRFSKQ-EDDnp as a substrate. The reactions were performed under pseudo first-order conditions of hydrolysis of substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as 100 %.

3.2.11 Salt Influence on enzyme activity

The influence of NaCl on the proteolytic activity was investigated over a salt range of 0-0.5 M. The protease was pre-incubated with NaCl for 3 min in pH 9.0, at 45 °C, and then the remaining enzyme activity was estimated using Abz-KLRFSKQ-EDDnp as a substrate. The reactions were performed under pseudo first-order conditions of hydrolysis of substrate. The activity of the enzyme assayed in the absence of salt was taken as 100 %.

3.2.12 Effects of surfactants and oxidizing agents on enzyme activity

The effect of some surfactants (Triton X-100, Tween 20, Tween 80, and SDS) and oxidizing agents (DTT and β-mercaptoethanol) was studied by pre-incubating the protease for 3 min, pH 9.0, at 45 °C. The activity of the enzyme without any additive was taken as 100 %. The reactions were performed under pseudo first-order conditions of hydrolysis of substrate.

3.2.13 Effect of divalent ions on enzyme activity

The purified protease was subjected to addition of different metal ions including Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Hg^{2+}, Ni^{2+} at 5 mM final concentration. The reactions were performed under pseudo first-order conditions of hydrolysis of substrate. The activity of the enzyme assayed in the absence of divalent ions was taken as 100 %.

3.2.14 Effect of organic solvents on enzyme activity

The effect of organic solvents like methanol, ethanol, acetone, buthanol and isopropanol (20 % v/v) on purified protease activity was studied. The reactions were performed under pseudo first-order conditions of hydrolysis of substrate. The activity of the enzyme assayed in the absence of organic solvents was taken as 100 %.

3.2.15 N-terminal sequence

The N-terminal sequence was determined using the Protein Sequencer PPSQ-33A (Shimadzu Corporation, Kyoto, Japan). The PPSQ-33A system sequentially cleaves the N-terminal amino acids of proteins and peptides using Edman degradation. The PTH-amino acid obtained with Edman degradation was separated using high-performance liquid

chromatography, and it was identified and quantified by analyzing a previously quantified standard and comparing retention time/s and UV absorption, respectively.

3.2.16 Circular dichroism measurements

CD spectra were recorded with a Jasco-710 spectropolarimeter. The absorbance spectra of pure protease (5 µM) in deionized water were collected in near-UV range (195-260 nm) using a 1 cm path length. Ellipticity is reported as molar ellipticity, [θ] ($deg.cm^2.dmol^{-1}$). The spectrometer conditions were typically 10 mdeg sensibility; 0.2 nm resolution; 1 s response time; 20 nm min^{-1} scan rate, and 21 accumulations at 25 °C. The control baseline was obtained with deionized water and was subtracted of the spectrum protease.

3.2.17 Determination of the substrate cleavage sites

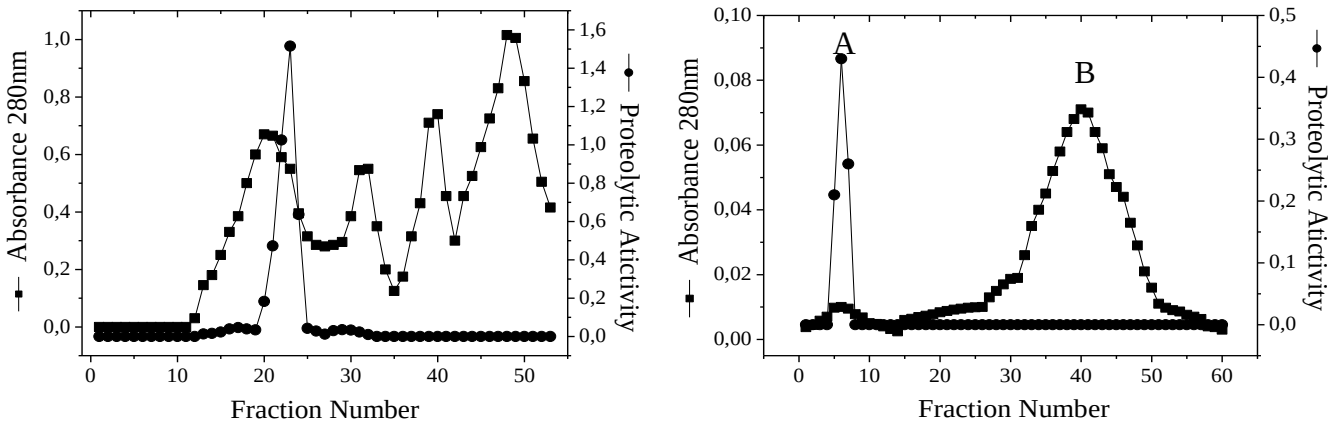
The scissile bond of hydrolyzed peptides was identified by isolation of the fragments using analytical HPLC followed by determination of their molecular mass by LC/MS using an LCMS-2010 equipped with an ESI-probe (Shimadzu, Japan).

3.3 RESULTS

3.3.1 Protease purification

The protease was purified by three chromatographic steps which are summarized in Table 1. After ethanol precipitation, a Sephacryl 100 column (Amersham Biosciences) was used to purify the enzyme and four peaks were eluted. However, only the first peak presented proteolytic activity on casein. The fractions containing activity were then pooled and applied on an anion-exchange column (Source 15Q, Amersham Biosciences). The anion-exchange chromatography displayed two peaks, the first (A) eluted in the absence of NaCl, washing not bound proteins and the second (B) eluted with a NaCl gradient of 0-2 M to remove the bound proteins. The proteolytic activity was observed in peak A, indicating that the enzyme did not bind to the Source 15-Q and therefore must have a pI higher than 8.5 or perhaps it is a glycosylated protein. The enzyme was purified 128.3 fold with a final yield of 51.5 %. The purified protease was homogenous on SDS-PAGE (Figure 1), and its molecular weight was determined to be 28.2 kDa by MALDI-TOF mass spectrometry (Figure 2). The low molecular weight exhibited by the purified protease is in agreement with other works that describe fungal proteases featuring molecular weights generally lower than 50 kDa (YANG et al., 2007; BARATA et al., 2002).

(a) **(b)**



(c)

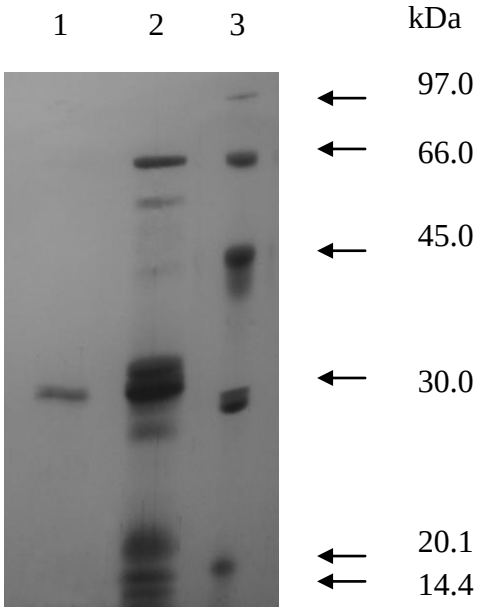


Figure 1: (a) Protease gel filtration chromatography on Sephacryl S100 HR. Conditions: 20 mM Tris buffer, 0.2 M NaCl, pH 8.0. (b) Protease ion exchange chromatography on Source 15Q. Peak A: the protease elutes in the absence of the saline gradient. Peak B: elutes during the linear gradient from 0 to 2 M of NaCl. Conditions: 20 mM Tris buffer, pH 8.5. (c) SDS-PAGE 12 %. Lane 1, pure protease; lane 2, crude extract and lane 3, molecular weight marker: α-lactalbumin (14.4 kDa), Trypsin inhibitor (20.1 kDa), Carbonic anhydrase (30 kDa), Ovalbumin (45 kDa), Albumin (66 KDa), Phosphorylase b (97 kDa).

Table 1: Parameters of purification of protease from fungus thermophilic *Myceliophthora* sp.

Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Purification factor (fold)	Recovery (%)
Crude Extract	2.49	19.80	7.95	1	100
Precipitation	1.25	15.75	12.6	1.58	79.54
Gel filtration	0,03	11,50	383.3	48.21	58.08
Ion exchange	0.01	10.2	1020	128.30	51.51

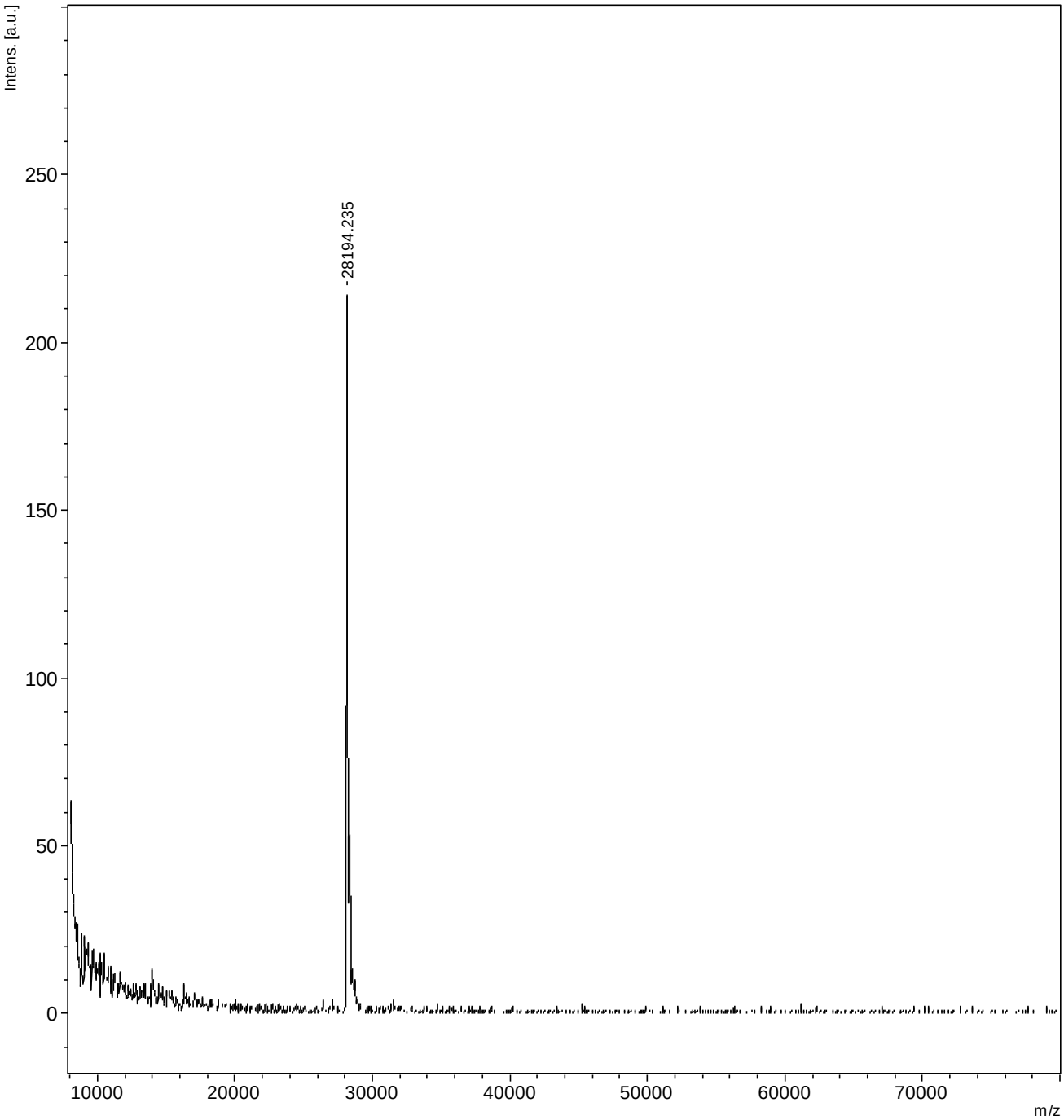


Figure 2: Mass spectrometry MALDI-TOF. The purified protease has molecular weight of 28.2 kDa.

3.3.2 Effect of pH and temperature on enzyme activity

The effect of pH on the hydrolysis of Abz-KLRFSKQ-EDDnp by the purified protease is shown by figure 3a. The protease under study is more active at pH in the range 7.0-10.5 with higher activity in pH 9.0 suggesting that it is an alkaline protease. For values below pH 7.0, the enzyme presented insignificant activity (data not shown). Purified alkaline proteases from fungi have been described from *Clonostachys rosea* (pH 9) (LI et al., 2006) and *Lecanicillium psalliotae* (pH 10) (YANG et al., 2005). The temperature dependence was determined from 25 to 60 °C (Figure 3b). Optimal hydrolysis occurred at 40-45 °C. Beyond that, the enzyme retained about 90 % of its catalytic activity at 55 ºC and 45 % at 60 °C. Proteases from fungi show similar results for optimum temperature such as 45 °C for *Colletotrichum gloeosporioides* (DUNAEVSKY et al., 2007), 50 °C for *Trichoderma reesei QM9414* (DIENES et al., 2007) and for *Fusarium culmorum* (PEKKARINEN et al., 2002).

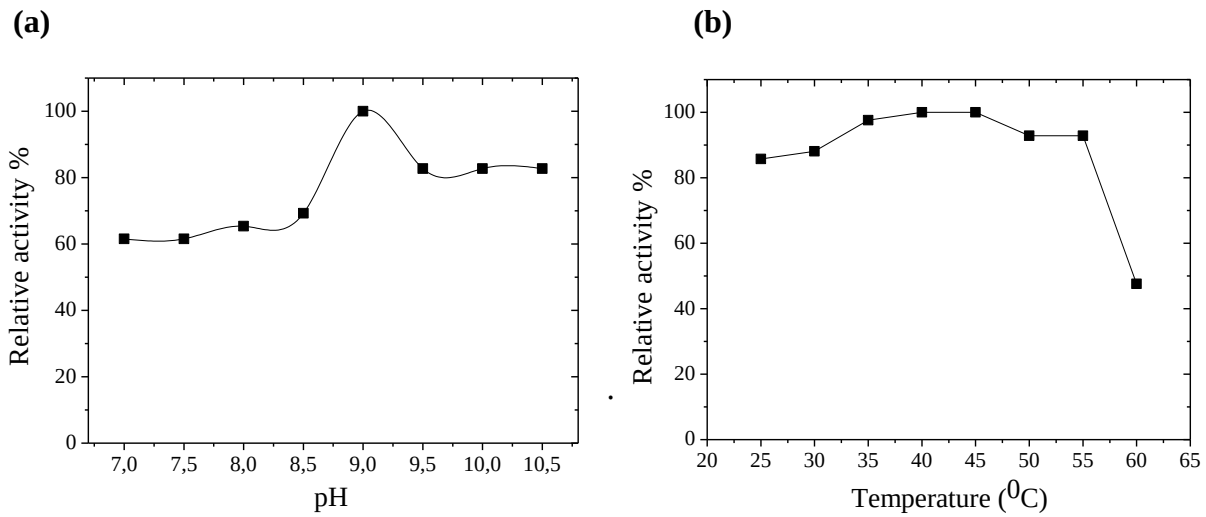


Figure 3: The influence of (a) pH, (b) temperature on hydrolysis of Abz-KLRFSKQ-EDDnp by the purified protease.

3.3.3 Effect of inhibitors on purified enzyme activity

The effect of some protease inhibitors was investigated on the hydrolysis of Abz-KLRFSKQ-EDDnp (Table 2). The assay occurred in pH 9.0 and at 45 °C. Proteolytic activity was strongly inhibited by 5 mM PMSF (100 % inhibition), suggesting that this enzyme would be a serine-protease; however it was not inhibited by benzamidine. Other protease inhibitors like EDTA, benzamidine, iodoacetic acid and E-64 had none or weak effect on the activity.

Khan et al. (2003) also reported serine protease from thermophilic fungi *Paecilomyces lilacinus,* and Charles et al. (2008) and Hajji et al. (2007) reported serine protease by thermotholerants fungi *Aspergillus nidulans* HA-10 and *Aspergillus clavatus* ES1.

Table 2: Effect of different types of inhibitors (5mM) on the hydrolysis of Abz-KLRFSKQ-EDDnp by serine-protease from *Myceliophthora* sp.

Inhibitors	Relative Activity %
Control	100
EDTA	95
PMSF	0
Benzamidine	100
E-64	80
Iodine-acetic acid	100

3.3.4 Salt Influence on enzyme activity

The figure 4 shows the effect of NaCl concentration on the catalytic activity of the serine protease from *Myceliophthora* sp. The enzyme was activated about 8 % in the presence of up to 250 mM NaCl, and with 500 mM NaCl, the protease retained approximately 97 % of its activity in the hydrolysis of Abz-KLRSFKQ-EDDnp. This result suggest that alterations of ionic strength did not significantly affect the function of this serine protease from *Myceliophthora* sp. Wang et al. (2005) has also reported that variations of NaCl had little or no effect on the activity of the protease from *Aspergillus fumigatus*.

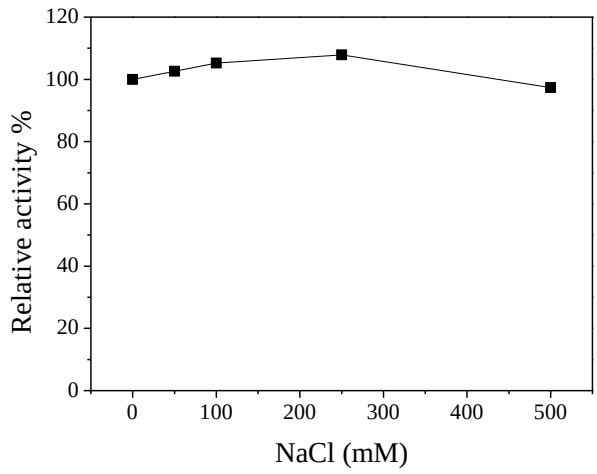


Figure 4: The influence of ionic strength on hydrolysis of Abz-KLRFSKQ-EDDnp by the purified protease.

3.3.5 Effect of surfactants and oxidizing agents on protease activity

The effect of some surfactants and reducing agents on protease activity is shown in table 3. The enzyme presented a smaller loss of activity in the presence of the non-ionic surfactant Triton X-100 and it was activated in the presence of Tween 20. However, in the presence of other non-ionic surfactant with higher chain Tween 80 displayed only 20 % of protease activity. Also, the protease was completely inactivated by the anionic surfactant SDS (0.1 %).

The protease alkaline from *Myrothecium verrucaria* has its activity little affected by the presence of 2.5 % Triton 100 (13 %) (MOREIRA-GASPARIN et al., 2009). The increase of activity by Tween-20, was also reported for the serine protease from *Clonostachys rosea* (LI et al., 2006), which exhibited 16 % of activation by Tween-20. Also, the serine protease from *Aspergillus clavatus* ES1 (HAJJI et al., 2007) presented about 33 % it activity in the presence 0.5 % SDS. On the other side, thiol reducing agents cleave disulfide bonds, important for stabilizing the tertiary structure of protein (VIEILLE et al., 2001). DTT and β-mercaptoethanol did not affect significantly the protease activity suggesting that the serine protease from *MyceliophtHora* sp. is not dependent of thiol groups. Also, the trypsin like protease from *Cordyceps militaris* in the presence 1 mM DTT did not suffer significant loss in its activity (HATTORI et al., 2005). In contrast, the serine protease from *Cordyceps sinensis* had it activity increased in the presence 2 mM DTT and β-mercaptoethanol, 29.8 % and 14.5 %, respectively (LI et al., 2007).

Table 3: Effect of reducing agents and surfactants on the hydrolysis of Abz-KLRFSKQ-EDDnp by serine protease from *Myceliophthora* sp.

-	**Concentration (mM or v/v)**	**Relative Activity %**
Control	-	100.0
ß-Mercaptoethanol	2mM	91.5
DTT	2mM	82.9
Tween 20	1%	117.2
Triton X-100	1%	88.6
Tween 80	1%	20.0
SDS	0.1%	0.0

3.3.6 Effect of divalent ions on enzyme activity

Table 4 shows the effect of some divalent ions. The addition of Mg^{2+} ions increased the enzyme activity by about 22 % of the control. Ca^{2+}, Ba^{2+} and Mn^{2+} showed little influence on the enzyme activity. Zn^{2+} exerted more influence decreasing activity by 22 %, whereas Hg^{2+} and Ni^{2+} greatly affected the enzyme activity with more than 60 % inhibition. Also, with addition Cu^{2+}, the purified protease was strongly affected with about 92 % inhibition. A serine-protease from *Aspergillus clavatus* ES1 was actived in the presence of 5 mM Mg^{2+} (HAJJI et al., 2007), and serine proteases from *Scedosporium apiospermum* (LARCHER et al., 1996) and *Cordyceps sinensis* (LI et al., 2007) were also inhibited in the presence of 5 mM Cu^{2+}.

Table 4: Effect of several divalent ions (5 mM) on the hydrolysis of Abz-KLRFSKQ-EDDnp by serine protease from *Myceliophthora* sp.

Compound	Relative Activity %
Control	100.0
Mg^{2+}	122.2
Ca^{2+}	97.2
Ba^{2+}	94.4
Mn^{2+}	91.7
Zn^{2+}	77.8
Ni^{2+}	41.7
Hg^{2+}	33.3
Cu^{2+}	8.3

3.3.7 Effect of organic solvents on enzyme activity

In the presence of acetone and buthanol, the enzyme was inhibited completely. Ethanol and methanol caused inhibition of more than 50 % in the enzyme: 47.2 % and 38.9 % respectively. Also, addition of isopropanol did not affect drastically the activity enzyme (Table 5). Differently, serine protease from *Aspergillus fumigatus* (DUNAEVSKY et al., 2007) presented 78 % of its activity in the presence of 50 % ethanol.

Table 5: Effect of organics solvents (20 %) on the hydrolysis of Abz-KLRFSKQ-EDDnp by serine protease from *Myceliophthora* sp.

-	**Relative Activity %**
Control	100.0
Acetone	0.0
Buthanol	0.0
Ethanol	47.2
Methanol	38.9
Isopropanol	91.7

3.3.8 N-terminal sequence

The first six N-terminal residues of the purified protease was determined by chemical sequencing and compared to other proteases through the gene bank. Multiple sequence alignment indicated that the N-terminal of the serine protease from *Myceliophthora* sp share significant similarity with other fungal proteases. Moreover, the serine protease from *Myceliophthora* sp displayed high identity to a protease from *Methylocella silvestris* BL2 (unpublished results) and to a serine protease from *Aedes aegypti* (NENE et al., 2007).

Table 6: Amino terminal sequence of a serine-protease from *Myceliophthora* sp as compared to other proteases.

Protein	Sequence	NCBI Blast
Serine-protease *Myceliophthora* sp	**G V V G V C**	**-**
Serine-protease *Metarhizium flavoviride var. minus*	S **V V G V** Q	gb|ACT66133.1|
Serine-protease *Metarhizium álbum*	S **V V G V** Q	gb|ACT66132.1|
Serine-protease *Metarhizium anisopliae*	S **V V G V** Q	gb|ACT66131.1|
Serine-protease *Metarhizium majus*	S **V V G V** Q	gb|ACT66127.1|
Protease *Coccidioides immitis RS*	L **V V G V** I	gb|EAS33583.1|
Trypsin-like protease *Septobasidium carestianum*	I **V V G V** S	gb|AAR91723.1|
Protease *Candida glabrata*	S **V V G V** R	emb|CAG61409.1|
Serine-protease *Monacrosporium haptotylum*	**G V V G** R R	gb|ABV46590.1|
Protease *Verticillium dahliae*	**G V V G** A S	gb|AAR10769.1|
Protease *Methylocella silvestris BL2*	**G V V G V C**	gb|ACK49719.1|
Serine-protease *Aedes aegypti*	**G V** I **G V C**	gb|EAT46743.1|

3.3.9 Circular dichroism measurement

The CD spectrum of the serine protease from *Myceliophthora* sp showed a maximum relative peak at 208 nm, which is characteristic of α-helical structures (Figura 5) (KELLY et al., 2005). Data deconvolution was carried out using different algorithms (K2D, Selcon 2 and Selcon 3) as implemented in the package DICROPROT and all of them indicated a high content of α-helical structure (Table 7).

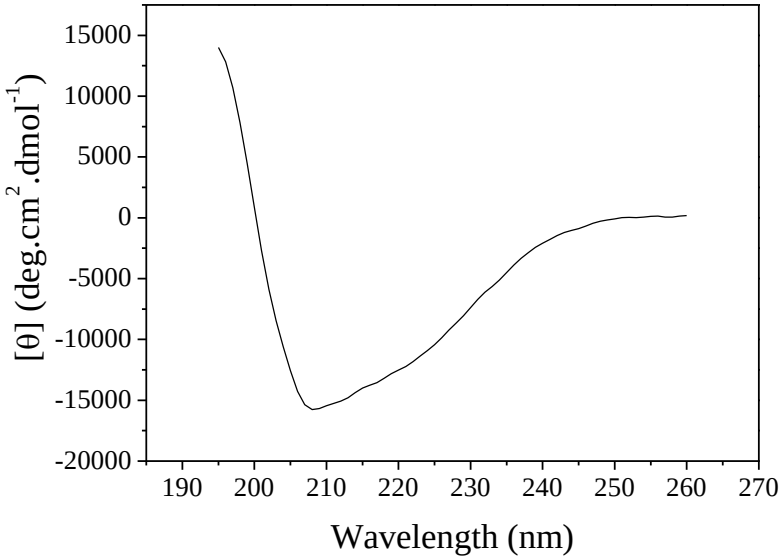


Figura 5: CD spectra for the serine-protease from *Myceliophthora* sp.

Table 7: Secondary structure estimation for serine-protease from *Myceliophthora* sp based on CD spectra data.

Method	α-helix (%)	ß-strands (%)	Unordered (%)
K2D	0.41	0.16	0.43
Selcon2	0.39	0.16	0.45
Selcon3	0.40	0.14	0.46

3.3.10 Determination of the substrate cleavage sites

FRET Peptides derived from Abz-KLRSSKQ-EDDnp - The FRET peptide series Abz-**X**LRSSKQ-EDDnp, Abz-K**X**RSSKQEDDnp, Abz-KL**X**SSKQ-EDDnp, Abz-KLR**X**SKQ-EDDnp, Abz-KLRS**X**KQ-EDDnp and Abz-KLRSS**X**Q-EDDnp were taken as reference to explore the specificity of the subsites S_3, S_2, S_1, and S_1', S_2', S_3', respectively. Assays were performed in 50 mM Glycine, pH 9.0 at 45 °C. The kinetic parameters for their hydrolysis by the protease are shown in tables 8 to 13. The parameters of hydrolysis for FRET peptides of six series showed a clear preference for hydrophobic residues with exception of Glu in P_3, His in P_2' and Arg in P_3'. Moreover, the positions P_1 and P_2 are remarkably interesting due to the higher catalytic efficiency when compared to values obtained from variations at positions P_1',

P_2', P_3' and P_3. Moreover, the variation of serine at position P_1' resulted in significant decrease of k_{cat}/K_m values and these values were increased again when serine is fixed at P_1' and P_2' is varied.

Analyzing the protease behavior in relation to specificity of cleavage, table 8 shows globally a preferential single cleavage occurred at the side C of P_1 position. The only exception was Pro in P_1 position, that showed a displaced cleavage for the C side of P_3' position. However, it is important to point out that for most substrates, the enzyme showed a second point of cleavage (5 – 10 %) at the side C of P_2' position. For P_1, the protease displayed a greater preference for the hydrophobic residues Ile, Met, Trp, followed of Ala and Phe.

For P_2 (Table 9), the preference was for the hydrophobic residues Phe and Pro. For P_3 (Table 10), the preference was for the acid residue Glu followed of hydrophobic residues Ile and Phe. For P_1' (Table 11), the protease displayed lower values of hydrolysis and the preference was for the hydrophobic residues Phe and Ile. For P_2' (Table 12), the preference was again for the hydrophobic residue Phe followed of basic residue His. In P_3' (Table 13), for the few peptides tested, the values of hydrolysis were lowers and showed preference for the basic residue Arg.

Thus, the specificity of the alkaline-serine protease from *Myceliophthora* sp. differs from the alkaline-serine protease from *Trichoderma reesei* QM9414, classified as being a trypsin-like enzyme (DIENES et al., 2007).

Table 8: Kinetic parameters for the hydrolysis of the FRET peptide series Abz-KLXSSKQ-EDDnp by serine-protease from *Myceliophthora* sp. for the characterization of its S_1 subsite specificity**.**

Substrate	k_{cat} $(s)^{-1}$	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Abz-KL**I**↓SSKQ-EDDnp	0.12	0.09	1275.30
Abz-KL**M**↓SSKQ-EDDnp	0.78	1.15	676.40
Abz-KL**W**↓SSKQ-EDDnp	0.82	1.28	639.00
Abz-KL**A**↓SSKQ-EDDnp	0.97	3.77	259.15
Abz-KL**F**↓SSKQ-EDDnp	2.16	9.37	230.12
Abz-KL**T**↓SSKQ-EDDnp	1.31	7.58	172.73
Abz-KL**K**↓SSKQ-EDDnp	1.44	8.88	162.23
Abz-KL**R**↓SSKQ-EDDnp	0.61	4.60	132.60
Abz-KL**P**SSK↓Q-EDDnp	0.01	0.08	121.00
Abz-KL**N**↓SSKQ-EDDnp	0.41	3.88	105.00
Abz-KL**S**↓SSKQ-EDDnp	0.99	10.63	92.86
Abz-KL**D**↓SSKQ-EDDnp	0.13	1.41	92.20
Abz-KL**E**↓SSKQ-EDDnp	0.14	1.90	73.68
Abz-KL**L**↓SSKQ-EDDnp	0.38	6.80	55.46
Abz-KL**Q**↓SSKQ-EDDnp	0.16	3.10	53.00
Abz-KL**H**↓SSKQ-EDDnp	0.17	4.82	35.35
Abz-KL**V**↓SSKQ-EDDnp	0.07	4.81	13.72
Abz-KL**C**↓SSKQ-EDDnp	0.04	5.25	7.45
Abz-KL**G**SSKQ-EDDnp		No hydrolysis	

Table 9: Kinetic parameters for the hydrolysis of the FRET peptide series Abz-KXRSSKQ-EDDnp by serine-protease from *Myceliophthora* sp. for the characterization of its S_2 subsite specificity.

Substrate	k_{cat} $(s)^{-1}$	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Abz-K**F**R↓SSKQ-EDDnp	0.04	0.06	682.90
Abz-K**P**R↓SSKQ-EDDnp	0.02	0.07	299.10
Abz-K**V**R↓SSKQ-EDDnp	0.44	5.08	85.62
Abz-K**A**R↓SSKQ-EDDnp	0.25	6.8	37.00
Abz-K**I**R↓SSKQ-EDDnp	0.37	10.70	34.70
Abz-K**Y**R↓SSKQ-EDDnp	0.05	1.96	24.73
Abz-K**T**R↓SSKQ-EDDnp	0.17	7.19	23.92
Abz-K**Q**R↓SSKQ-EDDnp	0.10	5.50	17.64
Abz-K**G**R↓SSKQ-EDDnp	0.03	2.30	11.50
Abz-K**E**R↓SSKQ-EDDnp	0.07	8.46	11.50
Abz-K**N**R↓SSKQ-EDDnp	0.02	2.42	7.32
Abz-K**H**RSSKQ-EDDnp		No hydrolysis	

Table 10: Kinetic parameters for the hydrolysis of the FRET peptide series Abz-XLRSSKQ-EDDnp by serine-protease from *Myceliophthora* sp. for the characterization of its S_3 subsite specificity.

Substrate	k_{cat} $(s)^{-1}$	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Abz-**E**LR↓SSKQ-EDDnp	0.71	6.16	114.98
Abz-**I**LR↓SSKQ-EDDnp	0.36	4.02	89.26
Abz-**F**LR↓SSKQ-EDDnp	0.57	6.70	85.47
Abz-**V**LR↓SSKQ-EDDnp	0.25	4.67	54.62
Abz-**Q**LR↓SSKQ-EDDnp	0.14	5.87	24.21
Abz-**L**LR↓SSKQ-EDDnp	0.20	8.52	23.47
Abz-**R**LR↓SSKQ-EDDnp	0.13	5.80	23.17
Abz-**A**LR↓SSKQ-EDDnp	0.07	3.34	22.01
Abz-**N**LR↓SSKQ-EDDnp	0.06	3.39	17.80
Abz-**H**LR↓SSKQ-EDDnp	0.08	4.90	15.82
Abz-**D**LR↓SSKQ-EDDnp	0.07	4.67	15.61

Table 11: Kinetic parameters for the hydrolysis of the FRET peptide series Abz-KLRXSKQ-EDDnp by serine-protease from *Myceliophthora* sp. for the characterization of its S_1' subsite specificity.

Substrate	k_{cat} $(s)^{-1}$	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Abz-KLR↓**F**SKQ-EDDnp	0.02	0.45	40.526
Abz-KLR↓**I**SKQ-EDDnp	0.02	0.57	33.46
Abz-KLR↓**G**SKQ-EDDnp	0.16	5.70	27.70
Abz-KLR↓**Q**SKQ-EDDnp	0.05	2.00	27.63
Abz-KLR↓**E**SKQ-EDDnp	0.17	7.10	23.54
Abz-KLR↓**H**SKQ-EDDnp	0.15	7.72	19.63
Abz-KLR↓**R**SKQ-EDDnp	0.07	4.81	14.20
Abz-KLR**P**SKQ-EDDnp		No hydrolysis	

Table 12: Kinetic parameters for the hydrolysis of the FRET peptide series Abz-KLRSXKQ-EDDnp by serine-protease from *Myceliophthora* sp. for the characterization of its S_2' subsite specificity.

Substrate	k_{cat} $(s)^{-1}$	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Abz-KLR↓S**F**KQ-EDDnp	1.42	4.28	331.92
Abz-KLR↓S**H**KQ-EDDnp	0.58	2.24	260.55
Abz-KLR↓S**L**KQ-EDDnp	0.17	1.02	163.90
Abz-KLR↓S**A**KQ-EDDnp	0.77	7.30	105.92
Abz-KLR↓S**V**KQ-EDDnp	0.15	3.00	51.17
Abz-KLR↓S**G**KQ-EDDnp	0.03	0.66	49.58
Abz-KLR↓S**N**KQ-EDDnp	0.11	2.28	46.00
Abz-KLR↓S**R**KQ-EDDnp	0.05	1.2	39.23
Abz-KLRS**P**KQ-EDDnp		No hydrolysis	

Table 13: Kinetic parameters for the hydrolysis of the FRET peptide series Abz-KLRSSXQ-EDDnp by serine-protease from *Myceliophthora* sp. for the characterization of its S_3' subsite specificity.

Substrate	k_{cat} $(s)^{-1}$	K_m (μM)	k_{cat}/K_m (mM^{-1} s^{-1})
Abz-KLR↓SS**R**Q-EDDnp	0.04	0.8	51.54
Abz-KLR↓SS**V**Q-EDDnp	0.04	1.30	28.45
Abz-KLR↓SS**Q**Q-EDDnp	0.11	4.41	23.99

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CONCLUSÃO FINAL

O fungo termofílico *Myceliophthora* sp. foi capaz de produzir uma protease alcalina e termoestável através de FES utilizando farelo de trigo, um resíduo agroindustrial de baixo custo como substrato.

A caracterização da protease no extrato enzimático bruto mostrou que a enzima imobilizada em alginato manteve algumas propriedades da enzima livre, porém sua termoestabilidade foi aumentada exibindo uma atividade residual de 40 % após 25 horas de incubação a 65 °C. Além disso, a imobilização da protease bruta permitiu a reutilização de 9 ciclos.

A caracterização bioquímica e funcional sugeriu que a protease purificada se trata de uma serino-protease de peso molecular 28.2 kDa. A enzima pura apresentou pH ótimo alcalino com uma larga faixa de atividade e temperatura ótima em torno de 40-45 °C. A serino-protease não sofreu alterações significativas na sua atividade catalítica em função da força-iônica bem como agentes redutores indicando uma não dependência eletrostática e de grupos tiol. Por outro lado, estudos com inibidores indicaram sua completa inibição por PMSF, porém benzamidina não afetou sua atividade proteolítica. Esses resultados combinados com a preferência por aminoácidos hidrofóbicos observados no mapeamento de especificidade dos subsitios sugerem que esta serino-protease possui um sítio ativo hidrofóbico, o que justifica a falta de inibição da benzamidina que canonicamente depende de uma interação eletrostática de seu grupamento guanidina com o grupamento carboxílico da cadeia lateral do resíduo de aminoácido aspártico presente no subsítio S_1 de tripsina-símiles.

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