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Lysobacter sp. A03: uma abordagem genômica e funcional para a busca de peptidases ativas em baixas temperaturas

Tese de Doutorado

JAMILE QUEIROZ PEREIRA

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JAMILE QUEIROZ PEREIRA

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Orientador: Prof. Dr. Adriano Brandelli

Co-orientador: Prof. Dr. Diego Bonatto

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Sumário

1 Lista de Abreviaturas

- AIDS Acquired ImmunoDeficiency Syndrome
- BLAST Basic Local Alignment Search Tool
- CFU Colony Forming Units
- EDTA Ethylenediamine Tetraacetic Acid
- ESI- MS Electrospray ionization Mass Spectrometry
- FMB Feather Meal Broth
- GST Glutathione S-transferase
- HIV Human Immunodeficiency virus
- HSAF Heat-stable Antifungal Factor
- IPTG Isopropyl β-D-1-thiogalactopyranoside
- kDa kilodaltons
- LB Luria Bertani
- MWCO Molecular Weight Cut-Off
- NCBI National Center for Biotechnology Information
- NC-IUMB Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
- ORF Open Reading Frame
- PC Protein-Convertase
- PDB Protein Data Bank
- PMSF Phenylmethanesulfonyl Fluoride
- PPC Pre-Peptidase C-terminal
- QMEAN Qualitative Model Energy ANalysis

- Q-TOF Quadrupole Time of Flight
- SDS-PAGE Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
- SMA Skimmed Milk Agar
- TCA Trichloroacetic Acid
- TSA Tryptic Soy Agar
- TSB Tryptic Soy Broth
- UHT Ultra High Temperature
- UTM Universal Transversa de Mercator

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4 Resumo

Peptidases são responsáveis pela hidrólise de ligações peptídicas em cadeias polipeptídicas e, além do seu papel vital em todos os organismos vivos, possuem uma longa história de utilização pela humanidade, principalmente no processamento de alimentos. Estima-se que o mercado global de enzimas chegue a US$7,1 bilhões em 2018, dos quais 60% correspondem ao comércio de peptidases. Porém, a maioria das peptidases disponíveis foi isolada de organismos mesofílicos e termofílicos. As enzimas psicrofílicas são uma alternativa valiosa, pois são ativas em baixas temperaturas, reduzindo os custos energéticos da sua utilização e são facilmente inativadas, sendo úteis em reações que necessitam de condições amenas. Com o objetivo de buscar por peptidases com atividade queratinolítica em baixas temperaturas, linhagens bacterianas antárticas foram isoladas e, de acordo com a sua habilidade de degradar resíduos queratinosos, três foram selecionadas e tiveram seus parâmetros bioquímicos avaliados. Considerando sua maior atividade queratinolítica, a bactéria *Lysobacter* sp. A03 foi escolhida para ter o seu genoma sequenciado e para a construção de uma biblioteca genômica a fim de isolar peptidases com potencial industrial e biotecnológico. Através dessa estratégia, uma serino-endopeptidase, denominada A03Pep1, foi encontrada, sendo funcional e estruturalmente caracterizada. Seu precursor, de 72,5 kDa, foi composto por 5 domínios que, através do auto-processamento, originam a forma madura da enzima, estimada em 35 kDa. A expressão heteróloga foi realizada no vetor pGEX-4T-2 e a peptidase A03Pep1 recombinante foi recuperada principalmente no sobrenadante dos cultivos da hospedeira *E. coli ArticExpress*. A atividade ótima da peptidase ocorreu em pH 9,0 e 40°C, sendo acentuada na presença de Ca^{2+} até a concentração de 25 mM e na presença de 10 mM de $Na^{2+,}$ $NH^{4+,}$ Mg^{2+} e Ba^{2+}, e foi inibida por 10mM de Zn^{2+}. Através da modelagem 3D, foi predito um sítio de ligação ao substrato mais amplo na peptidase A03Pep1 quando comparada à sua homóloga mesofílica AprV2, provavelmente devido ao encurtamento de uma alça situada próxima ao sítio ativo da enzima, presumivelmente como uma forma de aumentar a probabilidade de ligação ao substrato em baixas temperaturas. Através da discussão das características funcionais e estruturais da peptidase psicrofílica A03Pep1, os resultados possibilitam abordagens para o desenvolvimento de enzimas biotecnologicamente relevantes ativas em temperaturas baixas e moderadas.

5 Abstract

Peptidases are responsible for the hydrolysis of peptide bonds in a polypeptide chain and in addition to their critical roles in all living organisms, they have a long history of use by the humankind, mainly in the processing of food. The global enzymes market was estimated to be worth US$7.1 billion in 2018, of which the peptidases account for approximately 60%. However, the majority of the commercially available peptidases were isolated from thermophilic and mesophilic organisms. Psychrophilic enzymes are a valuable alternative as they are active at low temperatures, reducing the energy costs of its utilization and as they can be easily inactivated, being useful in reactions that have to be performed under mild conditions. Aiming to search for peptidases with keratinolytic activity under low temperatures, Antarctic bacterial strains were isolated and based on its ability of degrading keratinous waste, three of them were chosen and had their biochemical parameters evaluated. Considering its higher keratinase activity, the bacterium *Lysobacter* sp. A03 was selected to have its genome sequenced and for the construction of a genomic library to search for keratinolytic peptidases with biotechnological and industrial potential. Through this strategy, one serine-endopeptidase, called A03Pep1, was found and structurally and functionally characterized. Its precursor, of 72.5 kDa, was composed by five domains that, by means of autoprocessing, originate the mature enzyme, estimated in 35 kDa. The heterologous expression was performed in pGEX-4T-2 vector and the recombinant peptidase A03Pep1 was recovered mainly in the supernatants of *E. coli* ArcticExpress cells cultures. The optimal activity of the enzyme was at pH 9.0 and 40°C and was enhanced in the presence of Ca^{2+} until 25 mM and in the presence of 10 mM Na^{2+}, NH^{4+}, Mg^{2+} and Ba^{2+} and was inhibited by 10mM of Zn^{2+}. Based on the 3D structure modelling, was predicted a wider substrate binding site in A03Pep1 when compared with its mesophilic homologous AprV2, probably due to the shortening of a loop region located next to the active site of the enzyme, presumably as a way to enhance the probability of binding of substrates at low temperatures. By discussing the functional and structural characteristics found in the psychrophilic peptidase A03Pep1, the results provide possible approaches in developing new biotechnologically relevant enzymes active under low to moderate temperatures.

6 Introdução

6.1 Peptidases

As peptidases, também chamadas proteases, proteinases ou enzimas proteolíticas (RAWLINGS *et al.*, 2014), são um grupo de hidrolases que realizam a clivagem das ligações peptídicas em cadeias polipeptídicas e proteínas. São indispensáveis para a sobrevivência de todos os organismos vivos, sendo responsáveis por uma grande variedade de funções em todos os níveis de organização celular, envolvendo-se em tarefas como a digestão do alimento, a manutenção da hemostasia, a resposta inflamatória e a reprodução. Tamanha importância é refletida na quantidade de enzimas proteolíticas existentes, uma vez que a análise de genomas completos evidenciou que aproximadamente 2% de todos os produtos gênicos são peptidases, fazendo deste um dos maiores grupos de proteínas funcionais (RAO *et al.*, 1998, RAWLINGS & SALVESEN, 2013)

As enzimas proteolíticas possuem uma longa história de utilização pela humanidade, havendo registros do uso de renina (quimosina), uma peptidase retirada do quarto estômago de animais ruminantes, para coagular o leite usado na produção de queijos há mais de 8000 anos (NEELAKANTAN *et al.*, 1999). Além da manufatura de queijos, as peptidases são utilizadas no amaciamento de carnes, na redução do conteúdo proteico na farinha utilizada em pães, no melhoramento do malte durante a produção de cervejas, na fabricação de detergentes para roupas, para a remoção de corantes de base proteica, e na depilação de peças de couro.

Atualmente, as peptidases vêm ganhando espaço nas indústrias de cosméticos, de diagnóstico e na produção de fármacos, onde destacam-se as enzimas proteolíticas com atividade anti-inflamatória e digestiva. Adicionalmente, já vêm sendo realizados testes clínicos para o uso de peptidases de origem vegetal e bacteriana na remoção de tecidos necrosados em queimaduras. Entre elas, está uma protease obtida de *Vibrio proteolyticus* que mostrou-se efetiva contra proteínas desnaturadas como aquelas encontradas na pele queimada (LI *et al.*, 2012; GURUNG *et al.*, 2013).

Devido ao envolvimento em diversas condições patofisiológicas, as peptidases também são alvos em potencial para agentes terapêuticos contra doenças como hipertensão,

câncer, AIDS, malária e doença de Chagas. Como exemplos de sucesso, pode-se citar o medicamento captopril, que atua inibindo a ação da metalo-peptidase conversora da angiotensina (IECA), levando à redução da pressão arterial, que constitui a primeira escolha no tratamento de doenças cardiovasculares; diversos inibidores de aspartato-peptidases do vírus HIV já licenciadas como drogas para o combate à AIDS; assim como compostos que são efetivos contra peptidases de micro-organismos patogênicos como *Aspergillus* e *Candida* e de parasitas como *Plasmodium falciparum* (VANDOOREN *et al*., 2016; COOMBS *et al*., 2001).

De acordo com o local de clivagem, as peptidases são agrupadas como exopeptidases ou endopeptidases. As exopeptidases atuam clivando um ou alguns aminoácidos da porção N- ou C-terminal de uma cadeia polipeptídica, sendo chamadas de amino-peptidases, quando removem um único resíduo de aminoácido N-terminal, dipeptidil-peptidases, quando removem dois resíduos e tripeptidil-peptidases quando removem três resíduos de aminoácidos da porção N-terminal (Figura 1-a). De forma semelhante, as exopeptidases que agem na extremidade C-terminal, são denominadas carboxipeptidases, quando clivam apenas um resíduo ou peptidil-dipeptidases, quando removem dois resíduos na porção C-terminal da cadeia polipeptídica (Figura 1-b). As endopeptidases atuam clivando ligações peptídicas internas na cadeia polipeptídica e, de acordo com o seu mecanismo catalítico, o banco de dados de peptidases MEROPS as divide em aspartato-endopeptidases, glutamato-endopeptidases, asparagino-endopeptidases, cisteíno-endopeptidases, metalo-endopeptidases, treonina-endopeptidases e serino-endopeptidases, existindo ainda aquelas cujo mecanismo catalítico ainda é desconhecido (BARRET *et al.*, 2012; RAWLINGS *et al.*, 2014).

As serino-endopeptidases são a classe mais comum de peptidases, sendo encontradas em todos os domínios da vida e também nos vírus. Elas estão envolvidas em muitos processos biológicos, atuando na digestão de substratos proteicos utilizados como alimento, regulando o desenvolvimento dos organismos, atuando na resposta imunológica e participando na infecção viral às células. São caracterizadas pela presença da tríade catalítica Histidina-Serina-Aspartato (His-Ser-Asp), cuja serina ativa é responsável pelo ataque nucleofílico do átomo de carbono do grupo carbonila da ligação peptídica do peptídeo suscetível à hidrólise (Figura 2-a) (VOET &VOET, 2013). As serino-endopeptidases estão

divididas em tipo-tripsina e tipo-subtilisina, baseadas na sua estrutura. As enzimas Tipo-tripsina incluem serino-endopeptidases de mamíferos como quimiotripsina, tripsina, elastase e trombina. Em animais, atuam no processo de digestão alimentar, hemostasia, resposta imunológica e reprodução. As enzimas do Tipo-subtilisina são encontradas em plantas, fungos, protozoários, bactérias e vírus, havendo poucos representantes entre os animais. Geralmente possuem a tríade catalítica His-Ser-Asp e são alcalifílicas (RAWLINGS *et al.*, 2014).

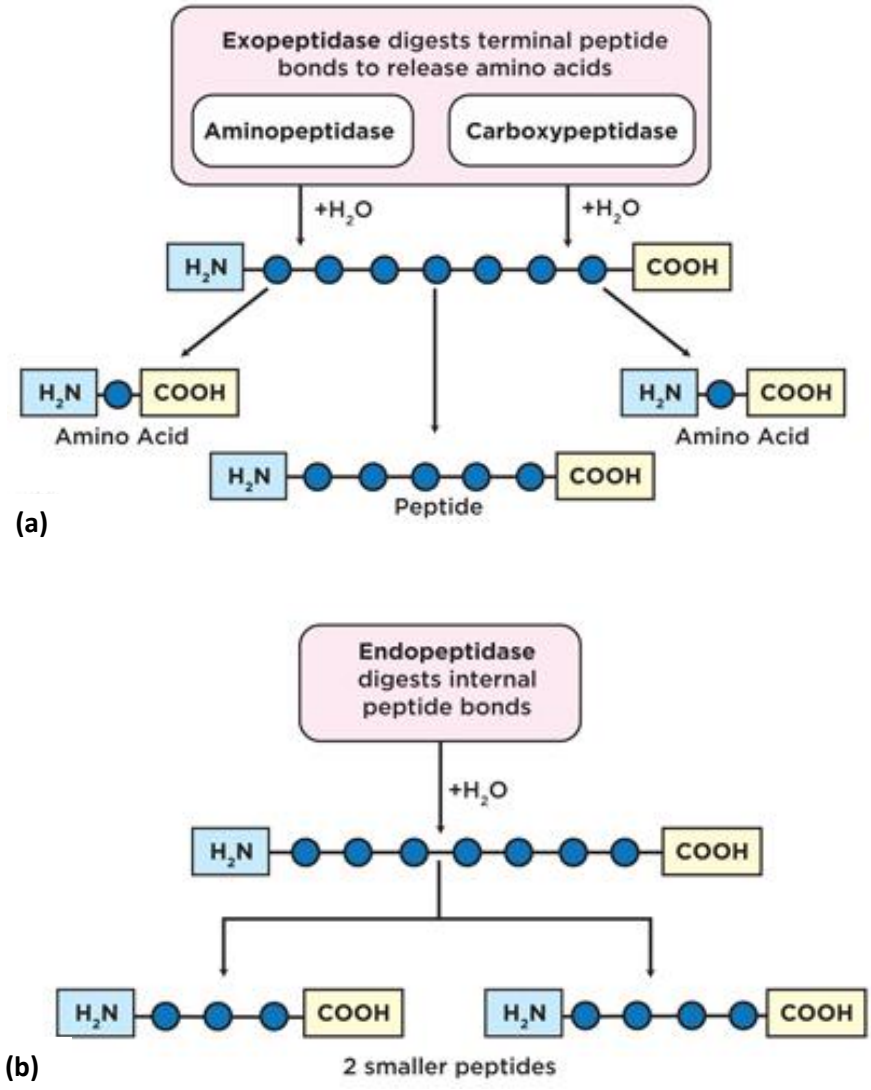

Figura 1: Mecanismos de ação das aminopeptidases e carboxipeptidases, os dois grupos principais de exopeptidases (a); mecanismos de ação das endoproteases (b). (Imagem modificada a partir de Silverthorn & Hill, 2012).

As peptidases nas quais o ataque nucleofílico à ligação peptídica suscetível a clivagem ocorre por intermédio de um grupo sulfidrila de um resíduo de cisteína são denominadas cisteíno-endopeptidases (Figura 2-b). Nelas, o doador do próton é sempre um resíduo de histidina, como na maioria das serino-endopeptidases identificadas. Em algumas famílias, somente a dupla Cis-His parece ser essencial para a catálise, enquanto em outras há evidência de que um terceiro resíduo é necessário. Um dos exemplos mais conhecidos de cisteíno-endopeptidases é a papaína, extraída do látex do mamoeiro (*Carica papaya*), utilizada no amaciamento de carnes, depilação de couros, processamento da lã e na indústria farmacêutica em medicamentos para auxiliar no processo de cicatrização da pele, preparo de derivados de tirosina para o tratamento da doença de Parkinson e no preparo de vacinas contra o tétano e amostras de imunoglobulina para injeções intravenosas (POLAINA & MACCABE, 2007; BARRET *et al.*, 2012).

As aspartato-endopeptidases, assim como as metalo-endopeptidases, diferem das serino-endopeptidases pelo fato de que o ataque nucleofílico à ligação suscetível do peptídeo é ativado por uma molécula de água, ao invés da cadeia lateral nucleofílica de um aminoácido. Os aminoácidos que estão envolvidos na catálise incluem dois resíduos de aspartato que atuam diretamente como ligantes para a molécula de água ativada. O exemplo mais conhecido de aspartato-endopeptidases é a pepsina, uma enzima digestiva produzida pelas células do epitélio estomacal (Figura 2-c).

Metalo-endopeptidases estão entre as hidrolases nas quais o ataque nucleofílico a uma ligação peptídica é mediada por uma molécula de água. Esta é uma característica compartilhada com as aspartato-endopeptidases, com a diferença de que nas metalo-endopeptidases um cátion metálico divalente, geralmente zinco, mas às vezes cobalto, manganês, níquel ou cobre, é responsável pela ativação da molécula de água. O íon metálico é mantido no lugar por aminoácidos ligantes, geralmente histidina, glutamato, aspartato ou lisina. As metalo-endopeptidases podem ser divididas em dois grandes grupos, de acordo com o número de íons metálicos necessários para catálise. Em várias metalo-endopeptidases, somente um íon zinco é necessário, mas em algumas famílias há dois íons metálicos que atuam juntos. As metalo-endopeptidases são amplamente distribuídas, existindo mais de 23.000 sequências identificadas nos bancos de dados, muitas das quais ainda não tiveram a sua importância fisiológica estabelecida (Figura 2-d).

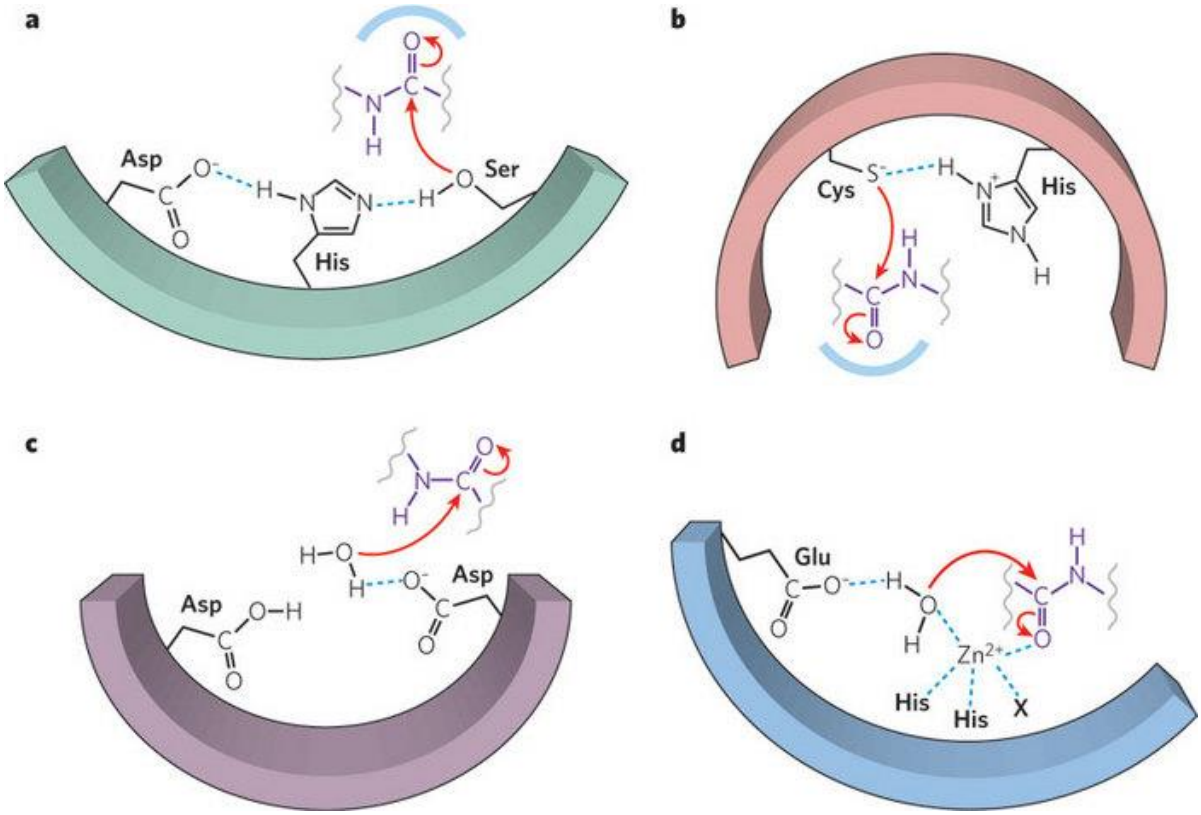

Figura 2: Endopeptidases classificadas de acordo com o seu sítio catalítico. Serino-endopeptidase (a); Cisteíno-endopeptidase (b); Aspartato-endopeptidae (c); Metalo-endopeptidase (d). As glutamato, asparagino treonino-endopeptidases não estão representadas nesta figura. (Figura extraída de Neitzel, 2010).

As treonino-endopeptidases são um grupo de enzimas proteolíticas caracterizadas pela presença de um único resíduo de treonina N-terminal no seu sítio ativo, que é o nucleófilo responsável pela clivagem da ligação peptídica. As treonino-endopeptidases foram descritas pela primeira vez em 1995, quando a estrutura do proteassoma foi resolvida. Ele é um dos sistemas proteolíticos mais importantes em eucariotos, uma vez que representa um importante ponto de checagem de qualidade das proteínas expressas em um organismo. As proteínas que estão danificadas, mal processadas, ou que já terminaram de desempenhar sua função são reconhecidas e degradadas no proteassoma (LOWE *et al.*, 1995; RAWLINGS & SALVESEN, 2013).

As glutamato-endopeptidases são um grupo de proteases recentemente descritas, compondo a sexta família de endopeptidases conhecidas. O mecanismo catalítico depende de um resíduo de ácido glutâmico no seu sítio ativo. Elas são encontradas em fungos filamentosos patógenos de animais e plantas, em bactérias e arquéias (RAWLINGS *et al.*, 2014).

O último grupo de endopeptidases classificadas é o das asparagino-peptidases, enzimas que têm a capacidade de clivar a si próprias em uma reação em que o nucleófilo é a asparagina. Elas incluem o precursor da enzima Tsh (*Temperature-sensitive hemagglutinin*) de *Escherichia coli*, na qual o grande pró-peptídeo C-terminal age como um auto-transportador, certas proteínas da cápsula viral e precursores que sofrem *splicing* proteico, como é o caso da subunidade A da DNA girase de *Mycobacterium leprae* (MADINGAN *et al*.2010; RAWLINGS *et al*., 2011).

6.2 Inibidores de Peptidases

Considerando que a maquinaria proteolítica é a peça chave na mobilização dos recursos proteicos celulares, ela dever ser altamente seletiva e rigorosamente regulada, já que o aumento da destruição de proteínas fundamentalmente importantes ou o atraso na degradação de proteínas regulatórias de vida curta pode alterar significativamente as funções celulares (VOET &VOET, 2013).

Devido ao seu potencial para causar danos se fora de controle, os organismos desenvolveram diversas estratégias para a regulação positiva e/ou negativa da atividade enzimática que incluem a existência de microambientes que permitem que as peptidases ajam de forma localizada e discreta, a produção de inibidores específicos que propiciam o término da ação enzimática e a síntese de peptidases na forma de precursores inativos denominados pró-proteínas ou zimogênios (LAZURE, 2002). Por consequência, grupos altamente diversificados de moléculas inibitórias evoluíram com a função de inativar reversível ou irreversivelmente as proteases e, até o momento, o banco de dados MEROPS soma um total de 81 famílias de inibidores de peptidases, agrupadas de acordo com a similaridade entre as sequências de aminoácidos, semelhança com o inibidor tipo (ou

holótipo) da família ou outra proteína que seja homóloga ao inibidor tipo daquela família (RAWLINGS *et al.*, 2014).

Diversos precursores de enzimas são ativados a partir da clivagem pós-traducional de ligações peptídicas através da autocatálise realizada por um domínio com ação de peptidase presente no próprio precursor (figura 3-b superior) ou através da clivagem executada por outras enzimas (figura 3-b inferior), levando a remoção de pró-sequências inibitórias que podem ser formadas por poucos resíduos de aminoácidos até estruturas com multidomínios (BASAK & LAZURE, 2003).

Nas peptidases que são sintetizadas na forma de zimogênios, as proteínas inibitórias frequentemente fazem da parte da própria cadeia polipeptídica da enzima, como é observado nos representantes bacterianos da Família I9 (figura 3-a). A fim de prevenir qualquer ativação indevida do zimogênio, visto que a tradução ocorre concomitantemente à transcrição, geralmente a pró-sequência precede a unidade catalítica, sendo encontrada na região N-terminal logo após o peptídeo sinal, no caso das peptidases secretadas. No entanto, pró-regiões C-terminais (PPC) também já foram descritas, por exemplo, na subtilase de *Pseudoalteromonas* sp. SM9913, na metaloprotease VchC de *Vibrio cholerae* e nas queratinases KerSMF e KerSMD de *Stenotrophomonas maltophilia* BBE11-1 (YAN *et al.*, 2009; FANG *et al.*, 2014, PARK *et al.*, 2015). Foi demonstrado que em muitos casos, esses domínios atuam ocupando o sítio de ligação de forma semelhante ao substrato, inibindo a ação da enzima. Porém, o papel dos domínios PPC vai além da simples inibição enzimática, tendo sido documentada a sua atuação como chaperonas intramoleculares auxiliando no dobramento correto da própria peptidase de que fazem parte. Nesse último caso, os domínios PPC podem atuar tanto diretamente, reduzindo a barreira de energia necessária para a transição do zimogênio para a sua forma madura como estabilizando o intermediário durante seu dobramento (LAZURE, 2002).

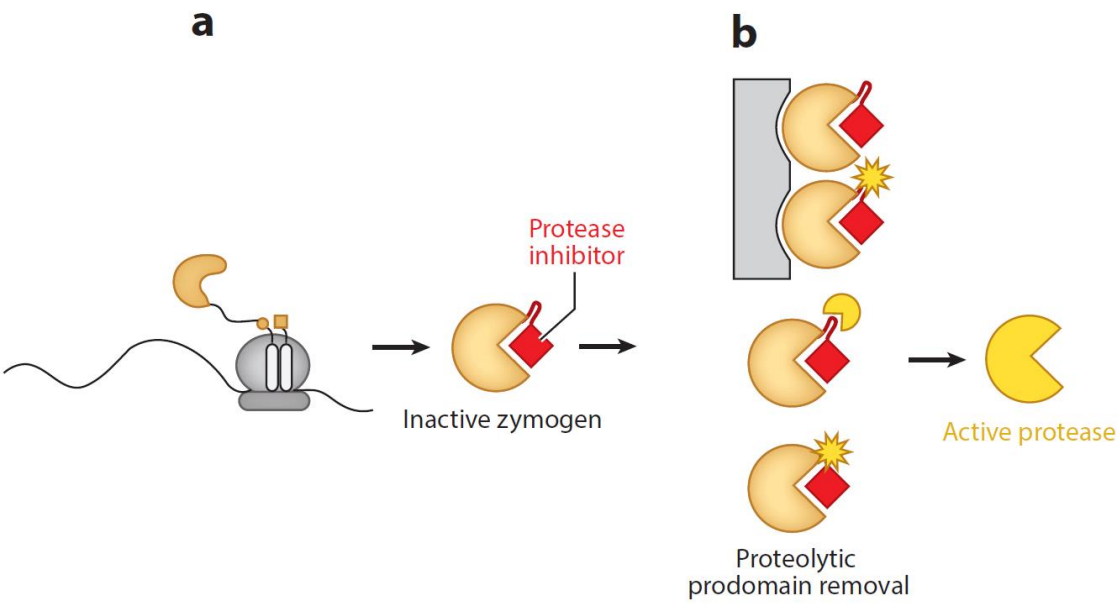

Figura 3: Síntese de peptidases como precursores inativos (a). Entre os mecanismos de ativação, está a remoção proteolítica do pro-domínio por outra peptidase ou através da autocatálise (b) (Figura extraída e modificada de Sanman & Bogyo, 2014).

6.3 O mercado mundial de enzimas

No começo do século XX, o farmacêutico e químico alemão, Dr. Otto Rohm, elaborou o primeiro preparado enzimático para uma aplicação comercial. Ele isolou tripsina pancreática e a adicionou a detergentes para a lavagem de roupas, para a degradação de proteínas. No entanto, foi somente a partir dos anos de 1960 que a biocatálise enzimática tornou-se industrialmente viável, com a produção em massa de peptidases microbianas para o uso em detergentes pela empresa dinamarquesa Novozymes (SARMIENTO *et al*., 2015). Desde então, o mercado de enzimas industriais evoluiu, movimentando uma quantia de US$ 2,3 bilhões de dólares anuais, dos quais 60% correspondem ao comércio de peptidases. (LI *et al.*, 2012).

De acordo com projeções de profissionais da indústria biotecnológica, a demanda mundial por enzimas tem aumentado em 7% ao ano, devendo chegar à quantia de US$ 7,1

bilhões em 2018, conforme a companhia de pesquisa de mercado americana *BCC Research* (BCC RESEARCH, 2014).

Adicionalmente, a catálise enzimática contribui para que os processos industriais sejam mais eficientes, pois as enzimas são altamente seletivas e rápidas; ambientalmente sustentáveis, uma vez que as enzimas são completamente biodegradáveis, reduzindo a geração de resíduos tóxicos; e utilizam menos energia nas reações (SARETHY *et al.*, 2011).

O aumento da renda per capita em países em desenvolvimento como Índia e China deve alavancar as aplicações industriais de biocatalisadores em setores como o de alimentos, de bebidas, de alimentação animal e de produtos de limpeza, pois, conforme as pessoas elevam o seu padrão de consumo, aumenta a necessidade do uso de enzimas na manufatura de produtos de maior qualidade. A elevação no consumo de carne, por exemplo, deverá levar ao aumento da demanda por enzimas utilizadas para melhorar a digestibilidade da ração, uma vez que os produtores rurais buscam maximizar o ganho de peso animal para satisfazer o mercado crescente (BEAUCHEMIN *et al.*, 2003; AMERAH *et al.*, 2011). Outro exemplo são as enzimas utilizadas para melhorar o sabor, a textura e o valor nutricional de pães, de bebidas e de queijos, produtos cada vez mais consumidos pela classe média em ascensão (MOLLER *et al.*, 2013; YARLAGADDA *et al.*, 2014). Também ressalta-se o crescimento do setor de cosméticos, onde enzimas estão crescentemente sendo utilizadas na elaboração de cremes, por exemplo, com agentes antioxidantes e fotoprotetores para a pele, como o ácido felúrico, obtido através da reação com a enzima feruloyl esterase (AURILIA *et al.*, 2008).

No Brasil, existe uma clara defasagem na área da chamada Tecnologia Enzimática, prevalecendo o uso da catálise química em detrimento da biocatálise nos processos industriais, fator que eleva os custos de produção, principalmente devido aos gastos energéticos, levando à produção de efluentes com alta carga de resíduos químicos (POLITZER & BON, 2006; LI *et al.*, 2012). No entanto, apesar de pequeno, o mercado enzimático brasileiro movimentou, de acordo com dados de 2005, cerca de US$ 147 milhões, dos quais quase US$ 127 milhões corresponderam a importações, deixando visível a desvantagem do país como produtor de enzimas comerciais, ainda mais quando se observa a importância do Brasil na produção mundial de alimentos e de biocombustíveis, setores estes que fazem grande uso de biocatalisadores (POLITZER & BON, 2006). O mercado

nacional precisa importar aproximadamente 86% das enzimas empregadas industrialmente, grande parte delas pelo setor de alimentos. Dessa forma, há uma necessidade urgente de incentivo a trabalhos focados na identificação de enzimas microbianas de interesse industrial a fim de reduzir a carência do mercado brasileiro visando a sua produção interna (FELLER & GERDAY, 2003; POLITZER & BOM, 2006; CAVICCHIOLI *et al*, 2011; SARETHY *et al.*, 2011).

6.4 Micro-organismos e produção de enzimas

Os micro-organismos, especialmente fungos e bactérias, são a fonte preferencial para a prospecção de enzimas, devido ao seu rápido desenvolvimento, pequeno espaço requerido para cultivo, ampla diversidade bioquímica e facilidade de manipulação genética, o que possibilita a criação de novos biocatalisadores com propriedades desejadas com vista a várias aplicações (RAO *et. al.*, 1998; ADRIO & DEMAIN, 2014).

Os contínuos avanços biotecnológicos, principalmente no que se refere à manipulação de DNA, sequenciamento de última geração e ferramentas de bioinformática que permitem a busca minuciosa por produtos de interesse em genomas inteiros, estão levando a uma procura incessante, por parte das indústrias, por enzimas customizadas para a otimização de processos químicos específicos, como catalisadores que atuem sob determinado pH, temperatura e salinidade (LI *et al.*, 2012). Por exemplo, os estudos de metagenômica de micro-organismos de habitats tão diferentes e inesperados como fendas vulcânicas, tundra ártica, rúmen de gado, estômago de cupins e ambientes marinhos têm identificado hidrolases microbianas com potencial para aplicações biocatalíticas como lipases, oxirredutases, amidases, amilases e proteases (KASANA, 2011). Adicionalmente, o sucesso recente dos programas de sequenciamento genômico tem resultado em uma explosão de informação disponível nos bancos de dados, criando assim a oportunidade de encontrar novas enzimas, entre outros produtos, através da mineração de dados no genoma de micro-organismos lá depositados (ADRIO & DEMAIN, 2014).

6.5 Enzimas de Micro-organismos psicrofílicos

A maior parte da biosfera terrestre é ocupada por ecossistemas de clima frio, como as águas profundas dos oceanos, e as regiões polares e alpinas que, apesar de aparentemente inóspitas, foram colonizadas com sucesso por micro-organismos adaptados a baixas temperaturas, conhecidos como psicrófilos. Esses extremófilos são caracterizados por apresentarem uma temperatura ótima de desenvolvimento entre 0 e 20°C, no entanto, micro-organismos cujo crescimento ocorre em temperaturas abaixo de -12°C já foram descritos. Eles apresentam fluxos metabólicos que são mais ou menos compatíveis com aqueles exibidos por mesófilos estreitamente relacionados que vivem em temperaturas moderadas, demonstrando o envolvimento de mecanismos de adaptação à temperatura. Os organismos psicrófilos, muito mais do que uma curiosidade biológica, representam uma fonte para estudos que buscam novos produtos de interesse biotecnológico como, por exemplo, enzimas ativas em temperaturas reduzidas. (CAVICCHIOLI *et al.*, 2002; KASANA, 2011; STRUVAY & FELLER, 2012; FELLER, 2013).

As enzimas ativas no frio são caracterizadas por uma alta eficiência catalítica em temperaturas baixas a moderadas, nas quais suas homólogas mesofílicas apresentam pouca ou nenhuma ação (Figura 4). Nas enzimas adaptadas ao frio, a baixa energia cinética das moléculas em reação é compensada pela flexibilidade das estruturas, conseguida pela combinação de características estruturais, que incluem a redução da hidrofobicidade do *core* enzimático, redução das interações iônicas e eletrostáticas, aumento da carga dos resíduos superficiais, promovendo o aumento da interação com o solvente; alças superficiais adicionais, substituição de resíduos de prolina por glicina nas alças superficiais, baixa relação arginina:lisina, interações inter-domínios e inter-subunidades mais fracas, menos interações aromáticas e um número reduzido de pontes dissulfeto. Como resultado, o sítio ativo e regiões adjacentes dessas enzimas permanecem flexíveis, e o aumento da flexibilidade conformacional é acompanhado pelo aumento da sua termolabilidade (CAVICCHIOLI *et al.* 2011). Porém, observa-se que a adaptação a baixas temperaturas não é perfeita e, mesmo que a atividade específica de enzimas psicrofílicas seja muito alta, ela permanece mais baixa do que aquela de enzimas mesofílicas atuando a 37°C (STRUVAY & FELLER, 2012).

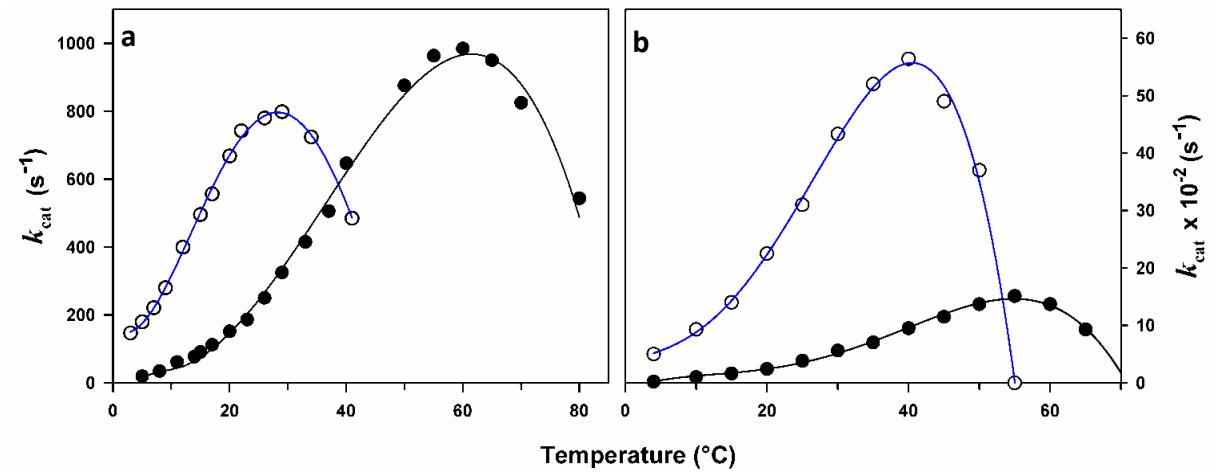

Figura 4: A atividade de enzimas psicrofílicas (círculos abertos, linha azul) quando comparada a mesofílicas (círculos fechados, linha preta) em diversas temperaturas ilustra as duas principais propriedades de enzimas adaptadas ao frio: atividade em temperaturas inferiores àquelas de enzimas mesofílicas e labilidade térmica. Amilases (a). Celulases (b). (Figura extraída e modificada de Struvay & Feller, 2012).

Apesar do conhecimento acerca das estruturas tridimensionais das enzimas adaptadas a baixas temperaturas ter aumentado consideravelmente nas últimas duas décadas, o número de estruturas cristalográficas disponíveis ainda é limitado. Acredita-se que um dos principais motivos para isso seja a dificuldade na obtenção de cristais de enzimas psicrofílicas, devido a sua maior flexibilidade. Uma forma encontrada para contornar o problema é a modelagem estrutural por homologia, que tem auxiliado na elucidação da forma 3D de diversas proteínas de organismos psicrofílicos e que, juntamente com os dados cristalográficos, tem demonstrado que cada enzima psicrofílica adota a sua própria estratégia adaptativa à temperatura ajustando-se para aumentar a resiliência global ou local da estrutura proteica (GEORLETTE *et al.*, 2004).

Os estudos estruturais em enzimas psicrofílicas têm revelado que a flexibilidade pode ser encontrada tanto uniformemente quanto restrita à região ao redor do sítio ativo e/ou fazendo parte dele, de forma que em determinadas enzimas, apenas uma pequena mudança

no sítio de ligação do substrato aumente a sua eficiência catalítica quando comparada a uma enzima mesofílicas homóloga. Por exemplo, uma metaloprotease dependente de zinco encontrada em uma linhagem de *Pseudoalteromonas* isolada do Oceano Ártico, demostrou ser globalmente flexível, como resultado da redução das ligações de hidrogênio em toda a enzima (XIE *et al.*, 2009). Ao contrário, D'Amico e colaboradores (2003), trabalhando com a α-amilase AHA de uma linhagem psicrofílica de *Pseudoalteromonas haloplanktis* demonstraram que a flexibilidade da enzima estava localizada na região do seu sítio ativo, que parecia desnaturar em temperaturas mais baixas em comparação aos demais componentes estruturais. Comparando-se a estrutura da α-amilase AHA com aquele da α-amilase pancreática suína, constatou-se que na enzima psicrofílica os loops que margeiam o sítio ativo eram marcadamente mais curtos, favorecendo a acessibilidade do substrato (Figura 5). Esses exemplos ilustram as formas específicas através das quais as enzimas adaptadas ao frio podem apresentar flexibilidade e o seu conhecimento pode ser de grande importância na prospecção e na manipulação genética de enzimas de microrganismos psicrofílicos de interesse biotecnológico (SHERIDAN *et al.*, 2010).

Vários trabalhos possibilitaram a descoberta de enzimas adaptadas ao frio que são utilizadas comercialmente, como a fosfatase alcalina da empresa New England Biolabs, a lipase 435 da empresa dinamarquesa Novozymes e a celulase IndiAge da Genecor, utilizada no processamento de *jeans* pela indústria têxtil. Dentre os fungos, um dos exemplos mais conhecidos de utilização de micro-organismos psicrofílicos é o da levedura *Candida antarctica*, produtora de duas lipases, uma delas propriedade da Novozymes, atualmente aplicada no processamento de alimentos, na indústria farmacêutica e de cosméticos. Além disso, o número de patentes requeridas para essas peptidases têm aumentado nos últimos anos, como para uma beta-galactosidase que hidrolisa a lactose do leite em baixas temperaturas, uma característica interessante para o processamento de laticínios para indivíduos com intolerância a lactose, sem alteração nas propriedades organolépticas do produto (HOYOUX *et al.*, 2001), e uma peptidase alcalina extracelular obtida de uma bactéria marinha relacionada com o gênero *Pseudomonas* cujo gene foi clonado em *E. coli*, expressando com sucesso a enzima recombinante (WANG *et al.*, 2010).

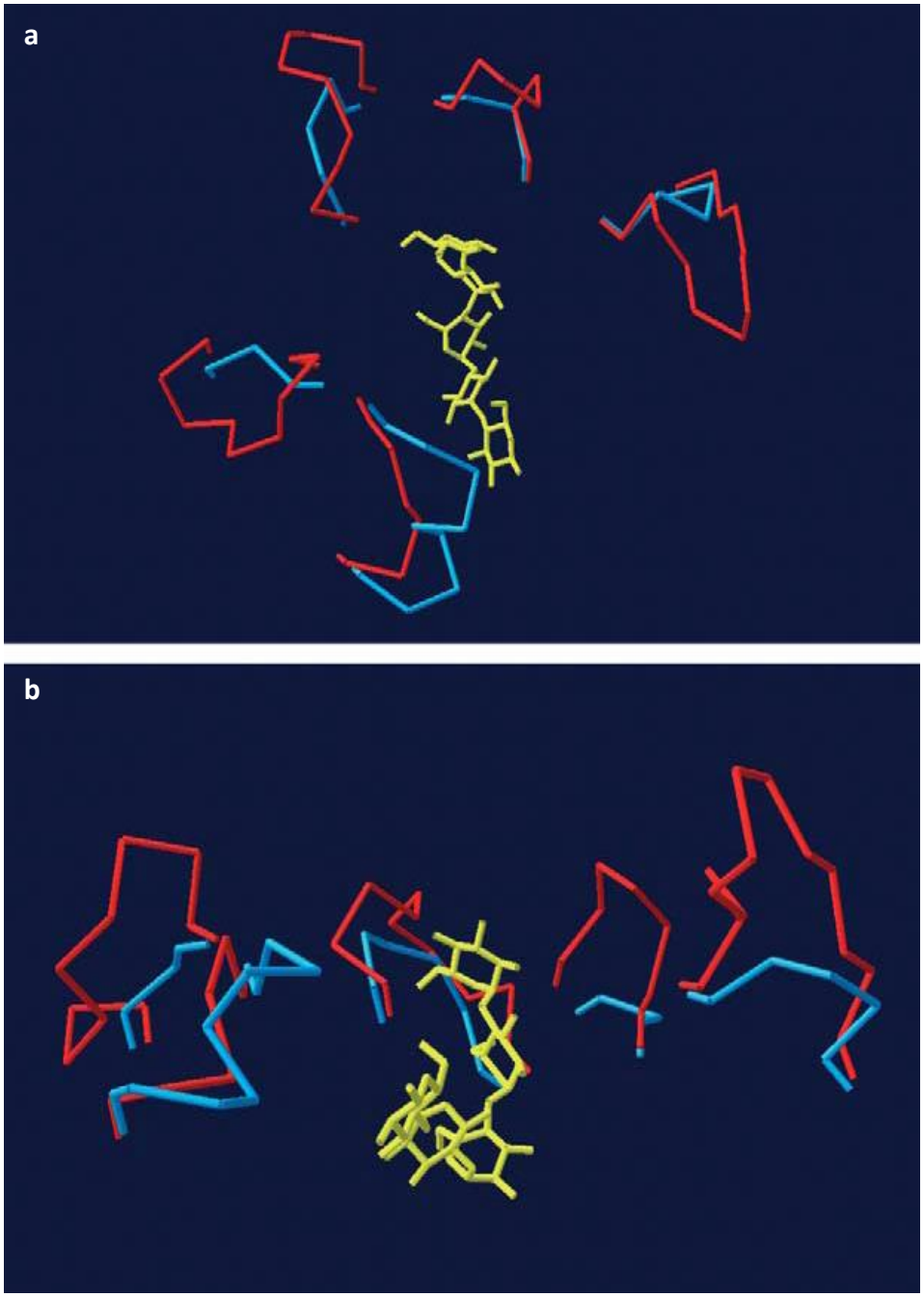

Figura 5: Flexibilidade local observada no sítio ativo da α-amilase psicrofílica AHA (azul) quando comparada a sua homóloga mesofílica suína (vermelho) (a). O substrato é mostrado no centro da figura (amarelo). Vista tangencial da superimposição das duas estruturas (b). (Figura extraída de Georlette *et al.*, 2004).

A expressão heteróloga de genes de enzimas obtidas de micro-organismos psicrofílicos tem se mostrado viável em linhagens comerciais de *E. coli*, tendo como vantagem adicional a formação de menos corpos de inclusão e um aumento na recuperação de proteína solúvel, como visto na protease extracelular PRO-2127 da linhagem antártica de *Pseudoalteromonas* sp. QI-1(YING *et al.*, 2011) e na lipase adaptada ao frio da linhagem IFO 3458 de *Pseudomonas fragi*, que reteve grande parte da atividade em baixa temperatura encontrada na enzima nativa quando expressa na linhagem hospedeira (ALQUATI *et al.*, 2002).

As características estruturais únicas das enzimas adaptadas ao frio fazem com que elas possam ter aplicações tão diversas quanto na indústria de detergentes domésticos, de alimentos, de tecidos, na área de biologia molecular e de análises laboratoriais. Por exemplo, elas podem ser utilizadas para a transformação de alimentos em baixas temperaturas, evitando assim a sua deterioração e a perda das propriedades nutricionais e sensoriais que poderia ocorrer com o aquecimento. Na indústria cosmética, a síntese de compostos voláteis e sensíveis ao calor, como fragrâncias, é facilitada em baixas temperaturas. Na biologia molecular, enzimas termolábeis são vantajosas pela sua inativação por tratamento térmico mais suave e sem interferência nas reações subsequentes. Na indústria têxtil, as enzimas adaptadas ao frio podem ser utilizadas para a otimização do processo de lavagem de tecidos em temperaturas mais baixas, poupando energia (CAVICCHIOLI *et al.*, 2002; FELLER, 2013).

Enzimas proteolíticas são rotineiramente adicionadas à composição de detergentes de roupas de uso doméstico. No entanto, devido aos hábitos dos consumidores, que tendem a preferir a lavagem das roupas em temperaturas acima dos 30°C, a maioria das proteases contidas nesses produtos são termofílicas ou termotolerantes. Essa mentalidade vem sendo modificada ao longo das últimas décadas, já que a preocupação com o aquecimento do planeta, causado pelas ações humanas, tem atingido parte da população, que agora tem optado por produtos que possam reduzir a sua "pegada ecológica" sobre a Terra, medida, entre outros parâmetros, pelas emissões de carbono de cada indivíduo (WACKERNAGEL & REES, 2004).

A relação entre a redução na temperatura e a diminuição do consumo de energia, com consequente impacto nas emissões de carbono, foi reconhecida pelos fabricantes de

detergentes que, ao combinarem um detergente padrão contendo 0,4% da enzima Savinase (uma enzima mesofílica) com outras derivadas de micro-organismos adaptados ao frio, observaram que ao baixar a temperatura de lavagem de roupas de 40°C para 30°C conseguiu-se uma economia de 30% na eletricidade utilizada, mantendo o mesmo desempenho obtido a 40°C, o que significou uma redução de 100g nas emissões de carbono por lavagem (NIELSEN, 2005; PROCTER & GAMBLE, 2009).

6.6 Queratinases

A queratina é uma proteína insolúvel que constitui o principal componente da estrutura de penas, unhas, cabelos, cascos, lãs e chifres. Ela é classificada em α- e β-queratina de acordo com a extensão e distribuição das ligações dissulfeto internas que ligam os resíduos de cisteína. A α-queratina é encontrada em todos os vertebrados, notoriamente em mamíferos, e sua estrutura secundária é formada principalmente por duplas de α-hélices paralelas superenroladas que se unem até formarem uma fibra de 32 unidades. A β-queratina é encontrada exclusivamente em répteis e aves e é constituída principalmente por folhas-β "pregueadas" e ligadas entre si, que são estabilizadas e enrijecidas por ligações dissulfeto (KESSEL & BEN-TAL, 2010).

A consequente rigidez da queratina faz com que esta proteína seja altamente recalcitrante e não degradável por peptidases como tripsina e papaína. No entanto, observa-se que a queratina não se acumula no ambiente, devido à atividade de peptidases especializadas denominadas queratinases. Geralmente, elas são enzimas extracelulares classificadas principalmente como serino-endopeptidases com grande similaridade com as sequências de subtilisinas, mas seu mecanismo de ação não está completamente elucidado, de forma que, de acordo com o Comitê de Nomenclatura da União Internacional de Bioquímica (NC-IUMB), elas são classificadas como um grupo de peptidases de mecanismo desconhecido. No entanto, sabe-se que a degradação da queratina envolve a ação cooperativa entre a redução das ligações dissulfeto e a quebra das ligações peptídicas (BANERJEE *et al.*, 2014).

Dados os vários tipos de substratos queratinosos presentes no ambiente e seus arranjos moleculares distintos, é razoável a afirmativa de que existam diferentes tipos de enzimas para agir sobre cada um deles. De fato, os estudos têm revelado que existe uma maior similaridade no nível de gênero entre as sequências de amino ácidos nas queratinases bacterianas que pode indicar a sua especificidade ao substrato de acordo com o tipo de queratina (BANERJEE *et al*., 2014)

Enzimas queratinolíticas são encontradas principalmente em bactérias do gênero *Bacillus*, embora algumas bactérias Gram-negativas como *Chryseobacterium*, *Pseudomonas* e *Burkholderia* tenham sido reportadas como linhagens produtoras de enzimas queratinolíticas, assim como alguns fungos dos gêneros *Aspergillus* e *Trichophyton*. A maioria das queratinases são ativas em pH neutro e alcalino e são isoladas predominantemente de organismos mesofílicos e termofílicos, com temperaturas ótimas de atividade variando entre 40°C em algumas espécies de *Bacillus* e 100°C em *Fervidobacterium islandicum*, ficando clara a falta de representantes psicrofílicos entre elas (BRANDELLI *et al.*, 2010; GOPINATH *et al*, 2015) (Tabela 1).

As queratinases possuem um amplo potencial para aplicação industrial e biotecnológica, como na formulação de detergentes domésticos e industriais, preparo de suplementos proteicos, na manufatura do couro, na indústria têxtil e no processamento de resíduos de penas que podem ser utilizados como fertilizantes e na ração de animais. (BRANDELLI *et al.*, 2010). Além disso, foi demonstrado recentemente o potencial para utilização de enzimas queratinolíticas em formulações para a degradação de proteínas infecciosas, as chamadas príons (OKOROMA *et al.*, 2013), para melhorar a acessibilidade de drogas para o tratamento de micoses nas unhas (SHIVAKUMAR *et al.*, 2014) e na indústria cosmética, principalmente em produtos para o cuidado capilar (VILLA *et al*, 2013).

Microrganismo	Tipo Catalítico	Massa molecular (kDa)	pH ótimo	Temperatura ótima (°C)
Bacillus sp. SCB-3	Metallo	134	7	40
Bacillus cereus DCUW	Serine	80	8.5	50
Bacillus licheniformis FK14	Serine	35	8.5	60
Bacillus licheniformis K-508	Thiol	42	8.5	52
Bacillus licheniformis MSK103	Serine	26	9–10	60–70
Bacillus licheniformis PWD-1	Serine	33	7.5	50
Bacillus licheniformis RPk	Serine	32	9.0	60
Bacillus pumilis	Serine	65	8.0	65
Bacillus subtilis KD-N2	Serine	30.5	8.5	55
Bacillus subtilis KS-1	Serine	25.4	7.5	–
Bacillus subtilis MTCC (9102)	Metallo	69	6	40
Bacillus subtilis RM-01	Serine	20.1	9	45
Clostridium sporogenes	–	28.7	8	55
Chryseobacterium sp. kr6	Metallo	64	8.5	50
Chryseobacterium indologenes TKU014	Metallo	P1: 56	P1: 10	P1: 30–50
Chryseobacterium indologenes TKU015	Metallo	P2: 40	P2: 7–8	P2: 40
Chryseobacterium indologenes TKU016	Metallo	P3: 40	P3: 8–9	P3: 40–50
Fervidobacterium islandicum AW-1	Serine	>200	9	100
Fervidobacterium pennavorans	Serine	130	10	80
Kocuria rosea	Serine	240	10	40
Kytococcus sedentarius	Serine	30–50	7–7.5	40–50
Lysobacter sp. NCIMB 9497	Metallo	148	–	50
Microbacterium sp. kr10	Metallo	42	7.5	50
Nesternkonia sp. AL-20	Serine	23	10	70
Nocardiopsis sp. TOA-1	Serine	20	>12.5	60
Stenotrophomonas maltophilia	Serine	35.2	7.8	40
Streptomyces sp. S7	Serine-metallo	44	11	45
Streptomyces sp. strain 16	Serine	KI: 203.2	KI: 9	KI: 50
Streptomyces sp. strain 17	Serine	KII: 100.8	KII: 9	KII: 50
Streptomyces sp. strain 18	Serine	KIII: 31.8	KIII: 9	KIII: 50
Streptomyces sp. strain 19	Serine	KIV: 19.2	KIV: 9	KIV: 60
Streptomyces albidoflavus	Serine	18	6–9.5	40–70
Streptomyces pactum	Serine	30	7–10	40–75
Streptomyces gulbagensis DAS 131	–	46	9	45
Streptomyces thermoviolaceus	–	40	8	55
Thermoanaerobacter sp. 1004-09	Serine	150	9.3	60
Thermoanaerobacter keratinophilus	Serine	135	8	85
Xanthomonas maltophilia	Serine	36	8	60

Tabela 1: Principais linhagens bacterianas produtoras de queratinases destacando o seu tipo catalítico, massa molecular, pH e temperatura ótimos de atividade. (Tabela modificada a partir de Brandelli *et al.*, 2010).

6.7 *Lysobacter*

Lysobacter é um gênero de bactérias Gram-negativas com formato de bastonete pertencente à família *Xhantomonadaceae*, dentro da classe *Gammaproteobacteria*. Atualmente, são reconhecidas 30 espécies dentro do gênero, descrito em 1978, sendo a espécie tipo *Lysobacter enzymogenes*, conhecida pela vasta produção de enzimas (CHRISTENSEN & COOK, 1978). As espécies de *Lysobacter* são isoladas principalmente do solo e água e têm como características distintivas a sua motilidade por deslizamento, um alto conteúdo de G+C e a produção de enzimas líticas ativas contra outros micro-organismos, incluindo outras bactérias, fungos e nematódeos (KRASOVKAYA *et al.*, 2013). A protease α-lítica, isolada de *L. enzymogenes*, foi a primeira enzima bacteriana a apresentar homologia com serino-endopeptidases pancreáticas, sendo extensivamente estudada através de cristalografia de raios-X devido a facilidade de purificação, o que possibilitou o seu estudo espectroscópico por Ressonância Magnética Nuclear e identificação de um único resíduo de histidina localizado no seu sítio ativo (EPSTEIN & WENSINK, 1988). Recentemente, foi evidenciada a produção de produtos bioativos por linhagens de *Lysobacter*, como ciclodepsipeptídeos ativos contra linhagens de *Staphylococcus* aureus resistentes à meticilina (XIE *et al.*, 2012).

Linhagens de *L. enzymogenes* foram identificadas como potenciais agentes para controle biológico. Por exemplo, foi comprovada a ação do isolado *L. enzymogenes* C3 no biocontrole de fungos causadores da podridão em raízes de trigo (EKEN & YUEN, 2014). A mesma espécie foi identificada como fonte de metabólitos secundários bioativos, como o Fator Antifúngico termoestável (HSAF), com ação contra fungos patogênicos de plantas e animais (QIAN *et al.*, 2013) . Também para o controle biológico, a linhagem *L. antibioticus* 13-1 foi utilizada no combate da bactéria *Xanthomonas oryzae* pv. *oryzae* causadora da mancha foliar em arroz, com eficiência na supressão da doença de quase 70% (JI *et al.*, 2008).

Até recentemente, somente linhagens mesofílicas e uma termofílica (*L. thermophilus* – WEI *et al.*, 2012) de *Lysobacter* haviam sido descritas, mas o trabalho de Fukuda e colaboradores (2013) descreveu pela primeira vez uma nova espécie, chamada *L. oligotrophicus*, isolada de um lago da Antártida. Em seguida, a publicação de Kimura *et al.*

(2015) descreveu-a como produtora de um pigmento marrom escuro semelhante à melanina, porém solúvel, com potencial aplicação industrial.

Membros do gênero *Lysobacter* também são conhecidos por degradar quitina e outros polissacarídeos, além de apresentarem uma alta atividade proteolítica, através da produção de proteases extracelulares de interesse ecológico, médico e agrícola, como a enzima AlpB produzida pela linhagem *Lysobacter* sp. XL1 e a serino protease LepB produzida pela linhagem *Lysobacter* sp. IB-9374, esta última disponível comercialmente (CHOHNAN *et al.*, 2004; KRASOVKAYA *et al.*, 2013). Como representante psicrofílico, o isolado *Lysobacter* sp. A03, isolado do ambiente Antártico, mostrou-se produtor de proteases extracelulares capazes de degradar a queratina proveniente de resíduos da indústria avícola em temperaturas abaixo de 20 °C (PEREIRA *et al*,. 2014).

7 Objetivos

O objetivo deste trabalho foi a identificação, clonagem e expressão de peptidases queratinolíticas de interesse industrial e biotecnológico ativas em temperaturas reduzidas produzidas pela linhagem psicrofílica *Lysobacter* sp. A03 através do *screening* funcional de uma biblioteca genômica.

7.1 Objetivos Específicos

- Construção de uma biblioteca genômica de *Lysobacter* sp. A03;

- *Screening* funcional da biblioteca em busca de peptidases ativas em baixas temperaturas,

- Clonagem dos genes de peptidases;

- Expressão e purificação das peptidases,

- Caracterização funcional das peptidases;

- Caracterização estrutural da peptidase através de modelagem por homologia;

-Sequenciamento do genoma de *Lysobacter* sp. A03 para a busca de novas enzimas de interesse biotecnológico.

8 Resultados – Capítulo 1

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Isolation of three novel Antarctic psychrotolerant feather-degrading bacteria and partial purification of keratinolytic enzyme from *Lysobacter* sp. A03☆

Jamile Queiroz Pereira[a], Fernanda Cortez Lopes[a], Maria Virginia Petry[b], Luis Fernando da Costa Medina[c], Adriano Brandelli[a,*]

[a] Laboratório de Bioquímica e Microbiologia Aplicada, Departamento de Ciência de Alimentos (ICTA), Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, CEP 91501-970 Porto Alegre, Brazil
[b] Laboratório de Ornitologia e Animais Marinhos, Universidade do Vale do Rio dos Sinos, Av. Unisinos 950, CEP 93022-000 São Leopoldo, Brazil
[c] Laboratório de Biologia Molecular, Universidade do Vale do Rio dos Sinos, Av. Unisinos 950, CEP 93022-000 São Leopoldo, Brazil

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ABSTRACT

Three psychrotolerant bacteria (designated as A03, A08 and A17U) were isolated from penguin feathers collected in the Elephant Island, Antarctic. They were able to grow in feather meal as sole carbon and nitrogen source at 9 °C, 20 °C and 30 °C. Based on 16S rDNA sequencing, the strains were identified as belonging to the genera *Lysobacter*, *Arthrobacter* and *Chryseobacterium*, respectively. The isolate *Lysobacter* sp. A03 was capable to degrade feather meal almost completely in 7 days of cultivation at 20 °C, which was also the optimal growth temperature for *Arthrobacter* sp. A08 and *Chryseobacterium* sp. A17U. Despite the optimum growth temperature of the isolates was in the range of psychrotolerant microorganisms, the enzyme extracts showed maximum caseinolytic and keratinolytic activities under temperatures between 15 and 20 °C above that point, which is expected when enzymes are isolated from their source organism. For *Lysobacter* sp. A03, proteolytic activity was strongly inhibited by serine protease inhibitor PMSF, while for *Arthrobacter* sp. A08 and *Chryseobacterium* sp. A17U the enzymatic activity was inhibited by EDTA and partially inhibited by PMSF and 1,10-phenantroline. These results were in agreement with those found in zymograms. The protease of *Lysobacter* sp. A03, partially purified by two-step ion exchange chromatography, was only inhibited by PMSF. The peptide bands were analyzed by mass spectrometry resulting in the absence of significant homology to other *Lysobacter* proteins or microbial proteases of NCBI database. The production of keratinolytic proteases by these cold-adapted bacteria can be valuable for the reduction of energy costs in the application of enzymes in industrial processes as an alternative to mesophilic and thermophilic microorganisms.

1. Introduction

The Antarctic continent has a great potential for prospecting new cultivable microorganisms with biotechnological relevance. A growing number of works have been focused on the diversity of bacterial taxa from this environment (Bowman et al., 1997; Shivaji et al., 2004; Cary et al., 2010; Teixeira et al., 2010), but fewer have been interested on biotechnological characteristics, such as enzyme production by psychrophilic and psychrotrophic strains.

The South Shetland Islands and Antarctic Peninsula is the nest-site of various birds, including two penguin species (*Pygoscelis papua* and *Pygoscelis adeliae*) that, after the breeding season, go through one complete molt (del Hoyo et al., 1992). Their feathers, although highly recalcitrant, do not accumulate in the environment, suggesting the action of the soil microbiota (Lucas et al., 2003; Park and Son, 2009). Furthermore, keratin wastes are generated by poultry industry on the order of millions of tons per year (Park and Son, 2009; Agrahari and Wadhwa, 2010). Such feathers can be processed by high-pressure cooking and milling to generate a valuable additive in animal feed as a protein supplement. However, this approach has nutritional limitations because it

☆ Scientific relevance: The present article describes the efficient degradation of keratin by three novel psychrotolerant Antarctic bacteria isolated from penguin feathers. These bacteria can be useful for development of energy-saving bioprocesses for the management of recalcitrant keratin wastes, and as a viable option for obtaining cold-active keratinolytic enzymes. The keratinases showed interesting properties for application in the enzymatic hydrolysis of proteins, particularly feather keratin.

* Corresponding author. Fax: +55 51 3308 7048.
E-mail address: abrandi@ufrgs.br (A. Brandelli).

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leads to a loss of essential amino acids and to a poor digestibility (Onifade et al., 1998; Brandelli, 2008). Thus, enzymatic digestion by microorganisms has been used as an alternative to convert keratin waste in a valuable and environmental-friendly product.

Keratinases are the enzymes that target the hydrolysis of keratinous substrates such as wool, nails and feather. These enzymes are produced by microorganisms belonging to the three domains of life (Brandelli et al., 2010). The majority of keratinases described to date originate from mesophilic and thermophilic organisms (Riessen and Antranikian, 2001; de Azevedo et al., 2006; Park and Son, 2009) and, despite their advantages, the application of these enzymes may be restricted due to high energy consumption, increased possibility of byproducts formation and degradation of thermolabile substrates and products. The production of keratinases by psychrophilic and psychrotrophic strains has been poorly reported and can provide a good alternative to transform substrates at low energy cost (Joshi and Satyanarayana, 2013).

Thus, the aim of this work was the identification of feather degrading bacteria, isolated from penguin feathers collected in Elephant Island, Antarctic, besides the characterization of proteases produced by these isolates, in order to find novel enzymes with potential biotechnological applications.

2. Materials and methods

2.1. Isolation of microorganisms

The microorganisms were isolated from decomposing penguin feathers collected in Elephant Island (coordinates UTM 588094, 3210090, zone 21), in December 2009. Fragments of feathers were collected aseptically, flooded in 90 mL of sterile saline solution (8.5 g/L NaCl) and serially diluted to 10^{-9}. A volume of 100 μL of each dilution was streaked on feather meal agar (FMA) (10 g/L feather meal, 0.5 g/L NaCl, 0.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4 and 12 g/L agar) as unique source of carbon and nitrogen (Riffel et al., 2003). After periods of incubation at room temperature (20–25 °C) that ranged from one to four weeks, the single colonies were picked and transferred to Tryptic Soy Agar (TSA) and maintained at 4 °C. The long term storage of bacterial strains was performed in TSB (Tryptic Soy Broth) containing 20% (v/v) glycerol followed by freezing storage at −20 °C (Laboratório de Biologia Molecular, Unisinos, São Leopoldo, Brazil).

2.2. Screening of proteolytic activity

For primary screening of proteolytic activity, isolates were inoculated in skimmed milk agar plates (SMA) (5 g/L peptone, 3 g/L yeast extract, 100 mL/L sterile UHT skimmed milk, and 12 g/L agar) and incubated at temperatures that ranged from 9 to 30 °C and pH between 5.0 and 11.0, for periods that ranged from one to seven days. The diameter of the clearing zones around the colonies was used as a criterion for selection of the strains and the optimal temperature and pH of proteolytic activity was used for cultivation in feather meal broth (Pillai and Archana, 2008).

2.3. Molecular identification

Total DNA of isolates able to grow both in FMA and in SMA plates was extracted and the partial 16S rDNA was amplified with universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′). The PCR products were sequenced with forward primers 27F and 518F (5′-CCAGCAGCCGCGGTAATACG-3′) at least three times from independent reactions to avoid artifacts introduced by the infidelity of Taq polymerase during amplification. The sequences obtained were submitted to the BLAST search algorithm present on GenBank database (www.ncbi.nlm.nih.gov/BLAST), edited using Bioedit software and aligned with Clustal X using default parameters (Thompson et al., 1997) to create consensus sequences that were deposited in the GenBank database.

2.4. Growth conditions

The selected isolates were cultivated in feather meal broth (FMB) (10 g/L feather meal, 0.5 g/L NaCl, 0.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4) at 125 rpm for seven days, at the temperatures of 20 °C, 30 °C and 40 °C and optimal pH, according to results of SMA plates. Culture supernatants obtained after centrifugation at 10,000× *g* for 10 min were used as crude extracts for the subsequent analysis. The bacterial growth was determined in the temperature of maximum enzymatic activity achieved, using serial dilutions of the cultures until 10^{-9} in saline solution (8.5 g/L NaCl) followed by plating on TSA for CFU counting after 48 h incubation at 20 °C.

2.5. Evaluation of feather meal degradation

The medium alkalinization was measured daily as an indicative of keratin hydrolysis (Riffel et al., 2003). The soluble proteins were determined in the supernatants according to the Folin phenol reagent method (Lowry et al., 1951), using bovine serum albumin as standard.

2.6. Proteolytic assay

Enzymatic assays were performed using azokeratin and azocasein as substrates (Daroit et al., 2009) with modifications. Azokeratin was synthesized according to the method described by Riffel et al. (2003). Assays were conducted for 1 h by incubating 100 μL of enzymatic supernatant in 300 μL of a 10 mg/mL suspension of azokeratin in phosphate buffer, pH 7.0 at 30 °C. The reactions were stopped by adding 500 μL of 10% (w/v) trichloroacetic acid (TCA). The tubes were centrifuged at 10,000 rpm for 5 min and the enzymatic activity was measured by the increase of the absorbance at 440 nm. The assays were performed in triplicate and the controls were prepared by adding 10% TCA before incubation. One unit of keratinolytic activity was defined as the amount of enzyme that resulted in an increase in absorbance at 440 nm of 0.01 after reaction with azokeratin for 1 h (Daroit et al., 2009). To determine the proteolytic activity, a similar protocol was used with azocasein (Sigma, St. Louis, USA) as substrate, except for the addition of 200 μL of 1.8 M NaOH to the reaction after centrifugation followed measurement at 420 nm (Daroit et al., 2009).

2.7. Effect of temperature and pH on proteolytic activities

To determine the best conditions of protease activity in the supernatant of isolates cultivated in FMB at the optimal temperature for feather degradation measured in Section 2.4, enzymatic assays were carried out at temperatures of 10, 20, 30, 40, 50, 60 and 70 °C, according to above protocol for azocasein, and at pH that ranged from 5.5 to 9.5 in 0.1 M sodium citrate (pH between 5.5 and 7.0) and 0.1 M Tris–HCl (pH between 7.5 and 9.5) buffers.

2.8. Effect of protease inhibitors

The protease inhibitors phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), 1–10-phenanthroline, iodoacetamide and pepstatin A were used to investigate the type of proteases present in the supernatants. The enzyme preparations were incubated for 10 min with the inhibitors before adding the substrate. The inhibitors were used at a final concentration of 1 mM, excepting for pepstatin A that was used at 5 μM.

2.9. Zymography

Zymograms were carried out under non-reducing conditions with the samples of the optimum time of cultivation in FMB by loading 15 μL of the supernatants in 12% polyacrilamide gels containing gelatin (1 mg/mL). After electrophoresis, gels were washed twice for 30 min in a solution of 2.5% (v/v) Triton X-100 in a 0.1 M Tris–HCl buffer, pH 8.0 to remove SDS, incubated in development buffer (0.1 M Tris–HCl, pH 8.0; 5 mM $CaCl_2$, 200 mM NaCl) overnight at 37 °C, both without and in the presence of proteases inhibitors PMSF (10 mM) or EDTA (10 mM), stained with Commassie Brilliant Blue R-250 and destained with a solution of 10% acetic acid and 40% methanol in distilled water. Proteolytic bands appeared as clear spots in the blue background of gels (Lopes et al., 2011).

2.10. Partial purification of Lysobacter proteases

A partial purification protocol was developed for *Lysobacter* sp. A03 strain, which achieved the best results in terms of keratin waste degradation. The culture supernatant obtained in FMB was concentrated 8-fold using an Amicon Ultra-15 30K centrifugal filter unit (Millipore). The concentrated supernatant was applied to a CM-Sephadex CL-6B cation exchanger column equilibrated with 50 mM phosphate buffer pH 8.0 and eluted with the same buffer followed by a linear gradient from 0 to 2 M NaCl. The fractions were collected and those that showed proteolytic activity on azocasein were pooled and dialyzed. The sample was then subjected to an anion exchanger chromatography on Q-Sepharose column eluted with a linear gradient from 0 to 2 M NaCl in the same buffer. The fractions showing proteolytic activity were pooled and tested in the presence of the same protease inhibitors as described in item 2.8. The sample was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel stained with Coommassie blue G-250. Protein bands were excised from gels, extracted by in-gel digestion with trypsin (Shin et al., 2006) and analyzed by electro-spray ionization (ESI)-MS/MS in a quadrupole acceleration time-of-flight (Q-TOF) mass spectrometer. An MS/MS ion search was performed using the Mascot search engine (http://www.matrixscience.com). The default search parameters used were enzyme = trypsin, fixed modification = carbamidomethyl (C), mass values = monoisotopic, peptide and fragment mass tolerances = ±0.5 Da and maximum missed cleavages = 2. Proteins with a Mascot score of more than 58 ($p < 0.05$) were considered reliable for using in the study.

3. Results

3.1. Characterization of proteolytic strains

Six morphologically distinct isolates named as A01, A03, A04, A05, A08 and A17U were able to grow on feather meal agar plates as unique source of carbon and nitrogen at 9, 20 and 30 °C. After seven days, all isolates grew in all temperatures and pH tested. However, only three strains (A03, A08 and A17U) produced clear zones, indicative of proteolysis on skimmed milk agar plates, preferably at temperatures around 20 °C and pH between 5.0 and 9.0. In pH 11.0, colony growth was observed for all strains, but clear zones of proteolytic activity were not visualized. The largest clear zone was observed for isolate A17U, with a diameter of 17.4 mm, followed by A08 (9.5 mm) and A03 (8.0 mm).

3.2. Bacterial identification

The strains A03, A08 and A17U, which showed proteolytic activity, were submitted to 16S rDNA sequencing. The sequences containing the hypervariable 16S rDNA regions V2, V3 and V6 were used to construct the data matrix together with sequences from related genera compiled from NCBI data bank. As seen in Table 1, the isolate named as A03 was identified, with 99% of similarity with the species of *Lysobacter* sp. (family Xhantomonadaceae); the strain A08 was associated with *Arthrobacter* sp. (family Actinomycetes), with 98% similarity, and the strain A17U was close to the species of *Chryseobacterium* sp., which belongs to the Flavobacterium family, with a similarity of 99%. The sequences obtained from Antarctic isolates were deposited in the GenBank database under accession numbers JQ995768, JQ995769 and JQ995770.

Table 1
Molecular identification of Antarctic isolates based on 16S rDNA gene sequencing.

Isolate	Sequence identity	Sequence length (bp)	Similarity (%)	GenBank accession number
A03	*Lysobacter* sp.	1150	99	AM111012
A08	*Arthrobacter* sp.	1224	98	FR682677
A17U	*Chryseobacterium* sp.	1278	99	AY553293

3.3. Keratin degradation

The three strains identified as proteolytic in SMA plates were tested for their capability to degrade feather meal. The isolate *Lysobacter* sp. A03 was able to degrade the substrate within a range of temperatures between 20 °C and 30 °C, with a maximum proteolytic activity at 20 °C (Fig. 1). After seven days at 20 °C, feather meal was almost all degraded and the visual result was corroborated by the increase in the amount of soluble protein that reached 2.35 mg/mL (Fig. 2).

The strain *Arthrobacter* sp. A08 showed a similar result, with a maximum release of proteins of 2.05 mg/mL (Fig. 2), preferably at temperatures below 30 °C (Fig. 1). *Chryseobacterium* sp. A17U showed minor keratin degradation during cultivation, with amounts of soluble proteins in the range of 0.65–1 mg/mL (Fig. 2), but also showing increased activity at 20 °C (Fig. 1). The increase of initial pH of the culture media from 7.0 to 8.0–8.5 was verified for all bacteria growing in feather meal broth.

3.4. Proteolytic and keratinolytic activities

Proteolytic and keratinolytic activities of the three isolates were evaluated during growth in FMB for 7 days at optimum growth

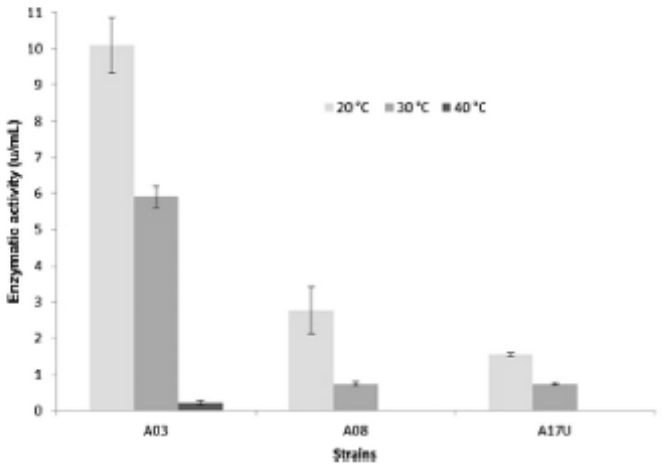

Fig. 1. Protease production by cultivation of *Lysobacter* sp. A03, *Arthrobacter* sp. A08 and *Chryseobacterium* sp. A17U in feather meal broth. Proteolytic was determined with azocasein as substrate in the optimal time of proteases production. Bars are the means ± sem of three independent experiments.

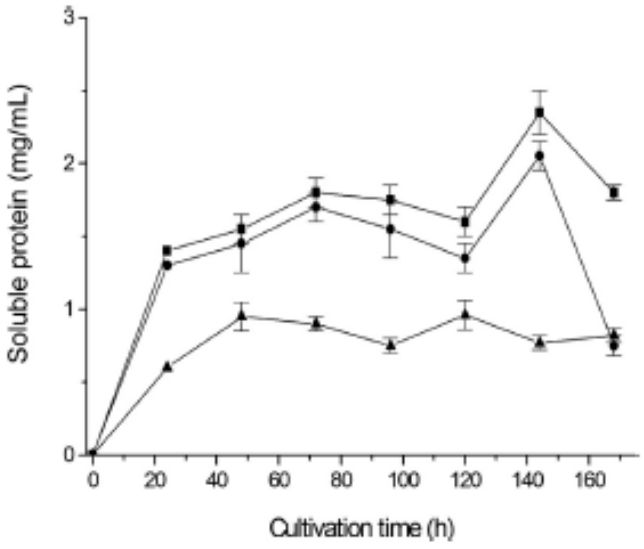

Fig. 2. Soluble protein produced by bacteria cultivated in feather meal broth for 7 days at 20 °C, 125 rpm (■) *Lysobacter* sp. A03; (●) *Arthrobacter* sp. A08; (▲) *Chryseobacterium* sp. A17U. Values are the means ± sem of three independent experiments.

temperature of 20 °C. The assays on both azocasein and azokeratin demonstrated that the three bacteria produce proteases with keratinolytic activity. The strain *Lysobacter* sp. A03 showed an increase in the proteolytic activity on azocasein over the first days, with a peak in the fifth day (Fig. 3A). This result was in agreement with a major release of soluble protein, in the day after the peak of proteolytic activity, and was coincident with the maximum bacterial counts for the isolate. The optimal keratinolytic activity on azokeratin was achieved in the sixth day. For the isolate *Arthrobacter* sp. A08, both proteolytic and keratinolytic activities reached the maximum in the third day during the exponential growth phase (Fig. 3B), with the peak of soluble proteins in the supernatant registered two days after. Similarly to *Lysobacter* sp. A03, the optimal proteolytic activity in *Chryseobacterium* sp. A17U strain was coincident with the maximum CFU counts, occurred in the third day of incubation, while the keratinolytic activity was observed during the decline phase. The amount of soluble proteins in supernatants of strain A17U remained stable during the seven days of cultivation (Fig. 3C).

3.5. Influence of temperature and pH on proteolytic activity

The optimal temperature of proteolytic activity in azocasein and azokeratin was about 40 °C, that is, 20 °C above the optimum cultivation temperature for *Lysobacter* sp. A03 and *Chryseobacterium* sp. A17U, while for *Arthrobacter* A08 the maximum proteolytic activity was achieved around 35 °C, 15 °C above the optimum for cultivation (Fig. 4A). For all three Antarctic isolates, the enzymes retained approximately 60% of the maximum activity in the range of 30–50 °C during 1 h of incubation. The effect of pH was evaluated in the optimal temperature for each tested bacteria (Fig. 4B). The proteases of the isolates were active in neutral and alkaline conditions. For *Lysobacter* sp. A03 the optimal activity was achieved at pH 9.5, although the enzymatic extract was stable over a wide range of pH values between 7.0 and 9.5. Similarly, *Arthrobacter* sp. A08 and *Chryseobacterium* sp. A17U showed maximum

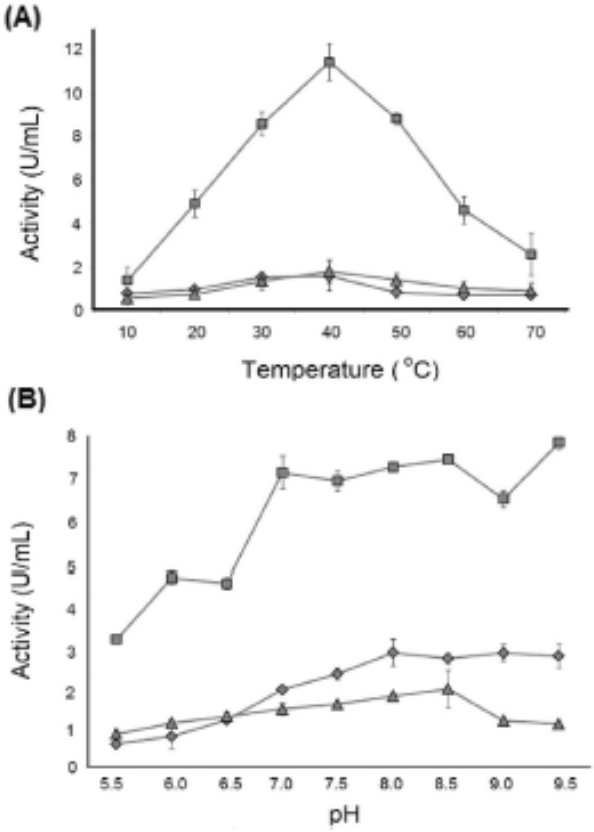

Fig. 4. Effect of temperature and pH on the proteolytic activity of (■) *Lysobacter* sp. A03, (◆) *Arthrobacter* sp. A08 and (▲) *Chryseobacterium* sp. A17U. Assays were carried out using azocasein as substrate. Values are the means ± sem of three independent experiments.

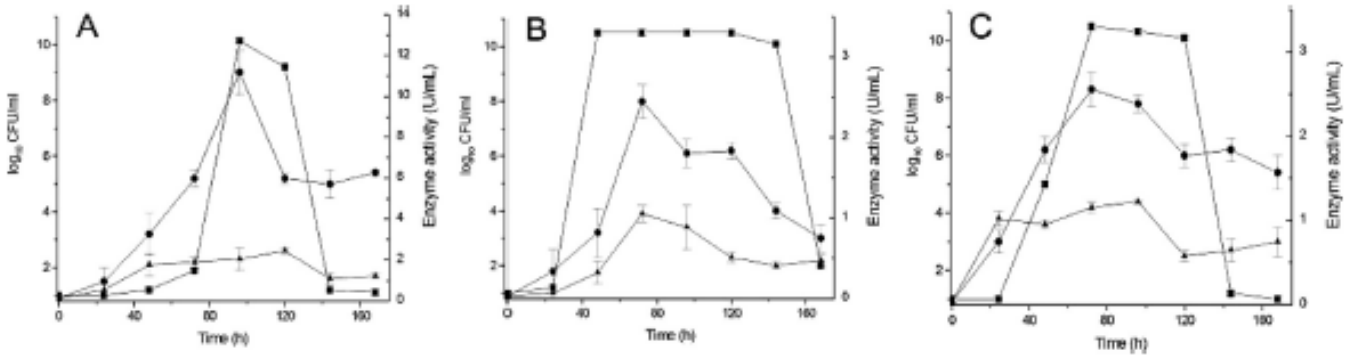

Fig. 3. Growth and protease production profile of *Lysobacter* sp. A03 (A), *Arthrobacter* sp. A08 (B) and *Chryseobacterium* sp. A17U (C). Strains were incubated during 7 days in FMB at 20 °C, initial pH 7.0, 125 rpm (■) Log CFU/mL, (●) activity on azocasein, (▲) activity on azokeratin. Values are the means ± sem of three independent experiments.

Table 2
Effects of protease inhibitors on the proteolytic activity of three Antarctic bacteria.

Inhibitor	Concentration (mM)	Residual activity (%)		
		A03	A08	A17U
Control	–	100	100	100
PMSF	1	23	75	73
EDTA	1	55	36	18
1,10-Phenanthroline	1	95	82	75
Iodoacetamide	1	98	103	110
Pepstatin A	0.005	89	106	100

The results are the average of three independent experiments and are expressed as percentages.

activity at alkaline pH, with an optimal at 8.5. However, for *Chryseobacterium* sp. A17U, at higher pH, the proteolytic activity decreased. At pH 5.5–6.5, the three isolates showed minor proteolytic activities.

3.6. Effect of inhibitors on enzymatic activity

The effect of inhibitors is shown in Table 2. The crude extract from *Lysobacter* sp. A03 was inhibited by the serine protease inhibitor PMSF in 77% and in a minor degree by the chelating agent EDTA. Nevertheless, the specific metalloprotease inhibitor 1,10-phenanthroline had virtually no effect on the enzymes activity as well as the inhibitors iodoacetamide and pepstatin A, suggesting the presence of serine protease-type enzymes. On the other hand, the strains *Arthrobacter* sp. A08 and *Chryseobacterium* sp. A17U had the proteolytic activity inhibited by EDTA, and partially inhibited by both 1,10-phenanthroline and PMSF, while the other inhibitors do not affect the enzymatic activity.

3.7. Zymography

The zymography of the enzyme extracts of the three bacteria, cultivated under the optimal conditions in FMB, are shown in Fig. 5. *Lysobacter* sp. A03 presented five clear zones of proteolytic activity. Two bands were inhibited by incubation of the zymogram gel with 10 mM PMSF, while no inhibition was observed by 10 mM EDTA, indicating the presence of serine proteases. For *Arthrobacter* sp. A08, a single proteolytic band was identified (Fig. 5), which was inhibited by the addition of either PMSF or EDTA. The strain *Chryseobacterium* sp. A17U showed a similar result. The three bands of proteolytic activity were partially inhibited by incubation of the gel with PMSF or EDTA (Fig. 5).

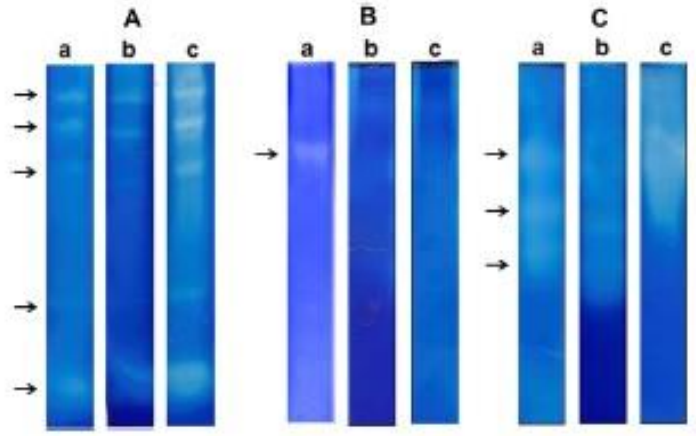

Fig. 5. Zymogram analysis of keratinolytic proteases secreted by *Lysobacter* sp. A03 (A), *Arthrobacter* sp. A08 (B) and *Chryseobacterium* sp. A17U (C). Arrows indicate bands of proteolytic activity. (a) Control, (b) 1 mM PMSF and (c) 1 mM EDTA.

Table 3
Purification steps of *Lysobacter* sp. protease.

Step	Protein (mg/mL)	Total activity (U/mL)	Specific activity (U/mg)	Purification factor	Recovery (%)
Concentrated supernatant	14.34	15.5	1.08	1	100
CM-Sephadex	1.36	4.8	3.53	3.27	31
Q-Sepharose	0.41	1.5	3.66	3.4	10

3.8. Partial purification of Lysobacter proteases

The partial purification of *Lysobacter* sp. A03 proteases is summarized in Table 3. Starting from concentrated supernatant, a yield of 10% and a 3.4 purification fold were achieved by the two-step chromatographic procedure. The proteolytic assay with protease inhibitors resulted in a decrease of 73% in the enzymatic activity in presence of PMSF, whereas the inhibitors EDTA, 1–10-phenanthroline, iodoacetamide and pepstatin A had no effect on the activity (data not shown). The partially purified sample showed five protein bands in SDS-PAGE, with molecular masses of approximately 110, 50, 45 40 and 20 kDa (Fig. 6). These bands were excised from the gel and analyzed by ESI-MS/MS. The results of Mascot search are summarized in Table 4. No significant matches were observed among the amino acid sequences of the *Lysobacter* peptides and non-redundant protease sequences of NCBI database. The sequences of four peptides matched with proteins of the

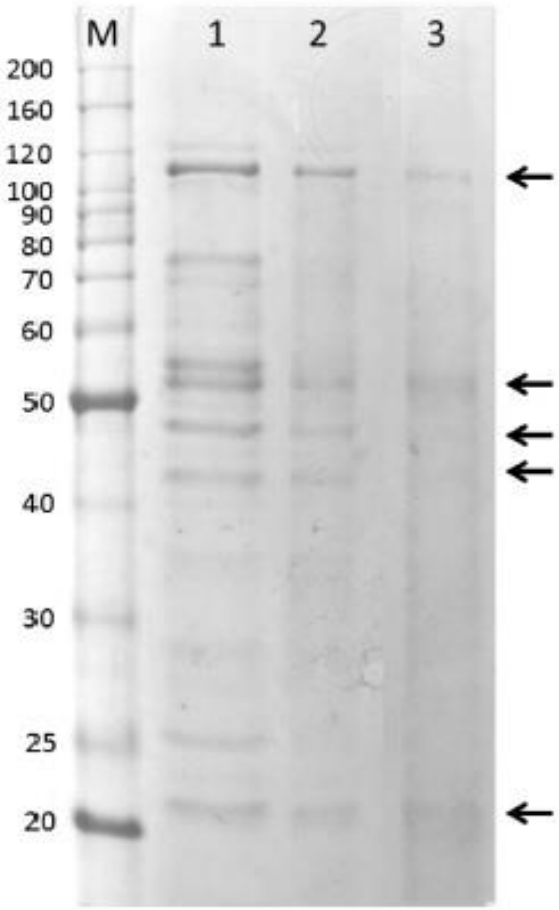

Fig. 6. SDS-PAGE analysis of keratinolytic protease secreted by *Lysobacter* sp. A03. (M) 20–200 kDa Protein Marker, (1) Concentrated by ultra-filtration supernatant (2) CM-Sephadex chromatography (3) Q-Sepharose chromatography. Arrows indicate protein bands that were subject of mass spectrometry analysis.

Table 4
Results of the Mascot MS/MS ion search for digested protein bands of partially purified protease from *Lysobacter* sp. A03.

Estimated protein mass (kDa)	Protein name	NCBI accession	Species	Mascot score	MS/MS peptide sequence
20	Hypothetical protein	WP_021817662	*Halomonas* sp.	74	GSDAYNQALSER
40	Hypothetical protein	WP_019184662	*Stenotrophomonas maltophilia*	58	DGASAQYGSDAIAGVINIVLR
45	Hypothetical protein	WP_017172960	*Xanthomonas axonopodis*	58	DGASAQYGSDAIAGVVNIVLK
50	Catalase	ABX56931	*Staphylococcus epidermidis*	102	TQQGIENYTDEEAAK
110	Nitrous oxide reductase	ACN97387	*Halomonas gudaonensis*	59	GNAYTTLFIDSQIVK

Gammaproteobacteria class, one of them corresponding to a nitrous oxide reductase protein from *Halomonas gudaonensis* and the other three to hypothetical proteins from *Halomonas* sp., *Stenotrophomonas maltophilia* and *Xanthomonas axonopodis*, these two last belonging to the same order of *Lysobacter* (Xanthomonadales). The exception was the peptide obtained from the band of 50 kDa, which showed similarity with the catalase of *Staphylococcus epidermidis*.

4. Discussion

Six bacterial strains isolated from penguin feathers collected in Elephant Island were able to grow in FMA plates, and three of these isolates produced clear zones of up to 17 mm in SMA plates at psychrophilic temperatures. Riffel and Brandelli (2006) reported colony diameter/halo diameter ratios of 10 mm and 50 mm at 22 °C and 30 °C, respectively, for *Chryseobacterium* sp. kr6, after 24 h growth. Working with a psychrophilic strain of *Flavobacterium limicola*, Tamaki et al. (2004) observed an optimum growth temperature of about 15–20 °C, while maximum proteolytic activity was 5 °C.

On the basis of 16S rDNA sequencing, the proteolytic isolates were identified as *Lysobacter* sp. A03, *Arthrobacter* sp. A08 and *Chryseobacterium* sp. A17U. The genus *Lysobacter* was related with the production of extracellular keratinases only by the study of Allpress et al. (2002), which reported the first metalloprotease with keratinolytic activity. Species of *Arthrobacter* have been largely associated with cold environments like Antarctic soils and ice sheets (Bowman et al., 1997; Wang et al., 2009). However, the genus was only associated with the production of keratinases by the studies of Lucas et al. (2003) and Nilegaonkar et al. (2002). The proteolytic potential of *Chryseobacterium* spp. has been previously described (Wang et al., 2008), which lead to the identification of three distinct metalloproteases with keratinase and elastase activities in the isolate *Chryseobacterium indologenes* TKU014. In addition, the high capacity for degradation of keratinous wastes by bacteria belonging to this genus has been described (Brandelli, 2005; Riffel and Brandelli, 2006).

The measurements of the release of soluble protein during cultivation on feather meal indicate that the Antarctic isolates have a high potential for biotechnological purposes. Moreover, all bacteria caused the alkalinization of the medium, which can be used as an important indicative of proteolysis, due to deamination reactions, that generally increases with keratinolytic activity (de Azeredo et al., 2006).

In this study, the crude extract of the three isolates showed maximum protease activity in azocasein and azokeratin at 15–20 °C above the optimal cultivation temperature, which was in the range of psychrotrophic microorganisms. Since the optimal growth temperature is the result of several parameters, enzymes may have an optimal activity from 10 to 20 °C higher than the optimal growth temperature of their origin organisms (Sheridan et al., 2000). For *Lysobacter* sp. A03 and *Chryseobacterium* sp. A17U maximum protease activity was achieved at 40 °C, whereas for *Arthrobacter* sp. A08 the optimum temperature for proteolysis was determined at 35 °C. Cold-active proteases of *Pseudomonas* and *Flavobacterium* isolates from Antarctic also showed activity at higher temperatures (Martínez-Rosales and Castro-Sowinski, 2011). This shift toward higher temperatures of activity than that of cultivations can be explained by the fact of, once isolated from cells that have a lower resilience against temperature changes, the extracellular enzymes of psychrophilic and psychrotrophic microorganisms have the possibility to adjust their catalytic parameters to achieve the optimization of catalysis before a point where the protein denaturation occurs (Feller and Gerday, 2003).

The three bacteria presented alkaliphilic features, a common characteristic in keratinases that can be valuable for its use in detergent industries, since activity and stability at high pH are requirements for compatibility with the chelating and oxidizing agents used in its production (Rao et al., 1998). Furthermore, keratinases can be useful in situations where other enzymes, such as trypsin, papain and pepsin, fail to act (Xie et al., 2010).

Most of the keratinases described belongs to the extracellular family of serine proteases (Gradisar et al., 2005), but examples of metalloproteases with keratinolytic activity have been increased, particularly among Gram-negative bacteria (Brandelli et al., 2010). In this work, the proteolytic activity of *Lysobacter* sp. A03 was inhibited mainly by serine protease inhibitor PMSF and in a minor degree by metalloprotease inhibitor EDTA. In the zymograms, five proteolytic bands were visualized, suggesting that the keratinolysis is due the action of an enzyme consortium, with the contribution of both serine proteases and metalloproteases. A similar result was obtained by Xie et al. (2010), which described four keratinases from *Streptomyces* sp. 16. The proteolytic activities of *Arthrobacter* sp. A08 and *Chryseobacterium* sp. A17U were largely inhibited by EDTA. According to zymogram analysis, only one band of proteolysis could be detected in *Arthrobacter* sp. A08, suggesting that a single metalloprotease is associated to the keratinolytic activity of this bacterium. For *Chryseobacterium* sp. A17U, three proteolytic bands were identified and inhibited by EDTA, confirming the production of extracellular metalloproteases by this strain.

The partial purification of *Lysobacter* sp. A03 protease confirmed that serine proteases are associated with the proteolytic activity. The absence of correspondence of the peptides obtained by Mascot search with those of the genus *Lysobacter* might be explained by the deficiency of studies about proteolytic enzymes other than the already investigated Alpha-lytic, Beta-lytic and Lys-C proteases from the species *Lysobacter enzymogenes* and the Lysil-endopeptidase from *Lysobacter* sp. IB-9374 (Jekel et al., 1983; Chohnan et al., 2002, 2004; Ahmed et al., 2003; Lapteva et al., 2012). A search for the *Lysobacter* proteases in the Protein Data Bank (www.rcsb.org) results in only 48 hits, all of them related to the Alpha-lytic protease structure. Similarly, a search for the same terms at the NCBI protein data bank returns 99 results, all of them associated with Alpha-lytic and Lysil-endopeptidase from *Lysobacter* species. Thus, there is an urgent need for novel information on *Lysobacter* proteases, once the genus is very interesting as a source for biotechnologically relevant enzymes and other compounds. Further work of purification and cloning of the proteases

from *Lysobacter* sp. A03 are in progress to elucidate their sequences and catalytic characteristics.

In conclusion, three novel keratinolytic bacteria collected from Antarctic samples were isolated by cultivation in feather meal agar. On the basis of sequence and phylogenetic reconstruction, they were identified as *Lysobacter* sp. A03, *Arthrobacter* sp. A08 and *Chryseobacterium* sp. A17U. These strains produce a variable array of proteolytic enzymes that may be useful to bioconversion of keratin-rich waste. The production of keratinolytic proteases by cold tolerant microorganisms might be attractive as an alternative for the reduction of energy input for biotechnological processes and its industrial utilization.

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9 Resultados – Capítulo 2

9.1 Artigo Publicado - Whole-Genome Shotgun Sequence of the Keratinolytic Bacterium *Lysobacter* sp. A03, Isolated from the Antarctic Environment. *Genome Announcements*, v. 3, n. 2, 2015.

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Whole-Genome Shotgun Sequence of the Keratinolytic Bacterium *Lysobacter* sp. A03, Isolated from the Antarctic Environment

Jamile Queiroz Pereira,[a,b] Adriana Ambrosini,[a] Fernando Hayashi Sant'Anna,[a] Michele Tadra-Sfeir,[c] Helisson Faoro,[c] Fábio Oliveira Pedrosa,[c] Emanuel Maltempi Souza,[c] Adriano Brandelli,[b] Luciane M. P. Passaglia[a]

Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil[a]; Instituto de Ciências e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul (ICTA-UFRGS), Porto Alegre, RS, Brazil[b]; Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná (UFPR), Curitiba, PR, Brazil[c]

***Lysobacter* sp. strain A03 is a protease-producing bacterium isolated from decomposing-penguin feathers collected in the Antarctic environment. This strain has the ability to degrade keratin at low temperatures. The A03 genome sequence provides the possibility of finding new genes with biotechnological potential to better understand its cold-adaptation mechanism and survival in cold environments.**

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Address correspondence to Luciane M. P. Passaglia, luciane.passaglia@ufrgs.br.

Lysobacter is a genus of Gram-negative bacteria first described in 1978 (1) that belongs to the family *Xanthomonadaceae*, within the *Gammaproteobacteria*. They are characterized by gliding motility, a high G+C content, and the production of a broad range of proteases and antibiotics, thus representing a source of biocontrol agents (2).

The Antarctic strain A03 was isolated from decomposing-penguin feathers collected on King George Island, Antarctica, and was identified as a *Lysobacter* sp. by both 16S rRNA and 16S-23S rRNA intergenic transcribed spacer gene sequencing. The isolate was able to grow preferentially in feather meal broth (FMB) substrate and showed high proteolytic activity at temperatures of approximately 20°C, within the range of psychrophilic microorganisms (3). Considering its biotechnological potential, mainly due to its production of various extracellular proteases, the genome sequence of the *Lysobacter* sp. A03 strain was obtained and the preliminary analysis is presented here.

A03 whole-genome shotgun sequencing was performed on the MiSeq Illumina platform using the MiSeq reagent kit, version 2. A total of 109,889 paired-end reads were obtained, with an average length of 240 bp and approximately 18-fold coverage. The assembly was performed using CLC Genomics Workbench (http://www.clcbio.com/products/clc-genomics-workbench/), A5-miseq (4), CISA (5), and SPADES (6), and considering the lower N_{50} value (51,277) and the smaller number of contigs (101), the assembly constructed by SPADES was chosen. The CheckM (7) program was used to assess the quality of the microbial genome, and the automatic annotation of the genome sequence was performed in the RAST server (8).

The draft genome sequence of strain A03 comprised 2,873,548 bp representing approximately 99.1% of the genome size, with a G+C content of 65.79%. A total of 2,615 coding sequences (CDSs), 46 tRNA genes, and 2 rRNA genes were predicted. As expected, many peptidase-coding genes were found, including a sequence coding for an extracellular keratinase; furthermore, three genes involved in cold shock response (2 *cspA* and 1 *cspG*) and six beta-lactamase resistance genes were predicted.

The availability of the genome sequence from *Lysobacter* sp. A03 may provide methods for searching for new biotechnologically relevant enzymes and might increase the understanding of the possible mechanisms that lead to the cold adaptation of life.

Nucleotide sequence accession numbers. This whole-genome shotgun project has been deposited at GenBank under the accession no. JXSS00000000. The version described in this paper is version JXSS01000000.

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Supporting Information

Figure S1: *Lysobacter* sp. A03 genome assembly evaluation using the Quality Assessment Tool QUAST showing the statistics of the four programs used. Despite of the lower number of *contigs* generated by CISA, the SPADES assembler was chosen due to its lower N50 and absence of mismatches per 100 Kb.

QUAST report

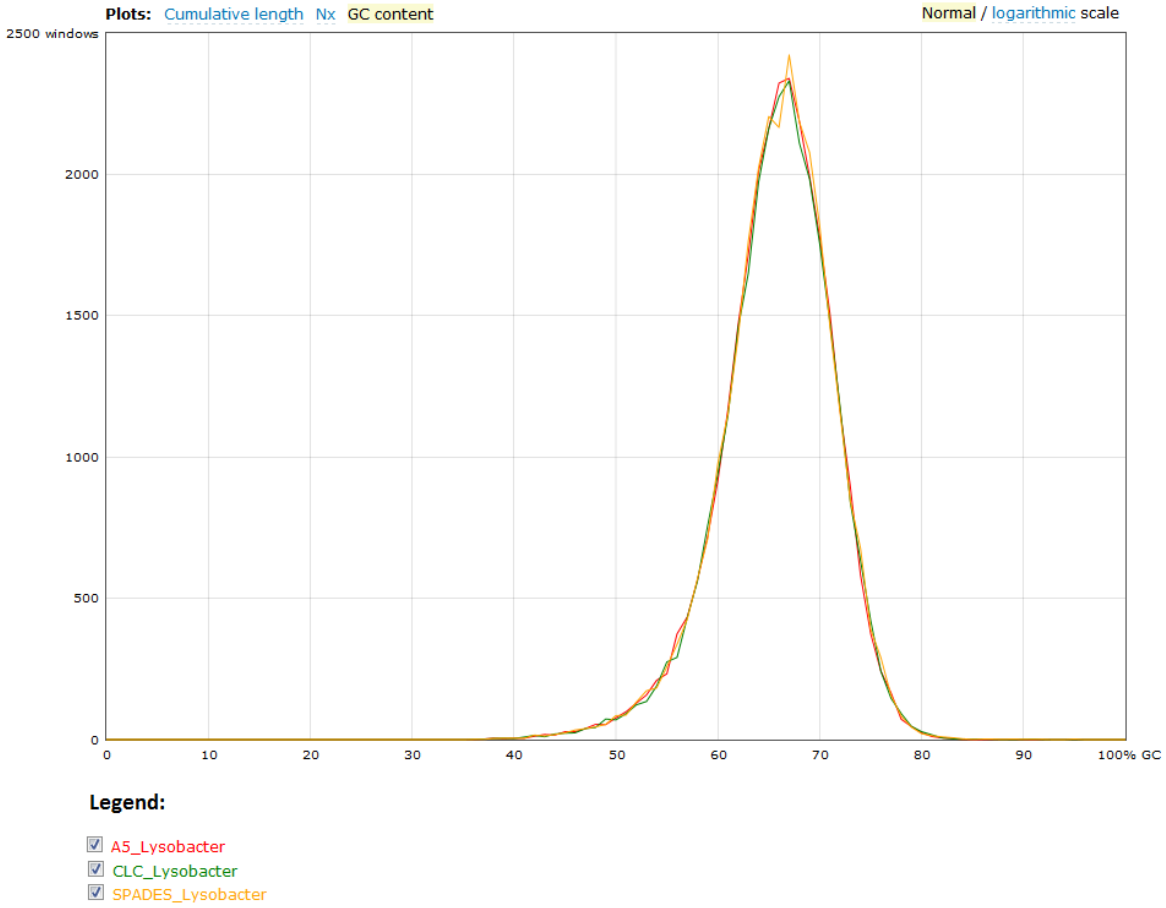
15 December 2014, Monday, 14:18:22

All statistics are based on contigs of size >= 500 bp, unless otherwise noted (e.g., "# contigs (>= 0 bp)" and "Total length (>= 0 bp)" include all contigs.)

Extended report worst.....best

Statistics without reference	A5_Lysobacter	CISA_Lysobacter	CLC_Lysobacter	SPADES_Lysobacter
# contigs	132	53	274	101
Largest contig	240 136	204 117	76 302	202 722
Total length	2 844 265	2 950 465	2 813 107	2 862 096
N50	43 307	71 521	21 192	51 277
Mismatches				
# N's per 100 kbp	60.54	5.8	49.91	0

Figure S2: *Lysobacter* sp. A03 genome G+C content estimated by the assembler programs A5, SPADES and CLC WorkBench. *Contigs* are broken into nonoverlapping 100 bp windows. Plot shows numbers of windows for each GC percentage.



10 Resultados – Capítulo 3

10.1 **Artigo submetido ao periódico Journal of Molecular Biology** - Functional and structural analysis of a new cold-active keratinase isolated from the Antarctic bacterium *Lysobacter* sp. A03

PEREIRA, J. Q., AMBROSINI, A, PASSAGLIA, L. M. P, BRANDELLI, A.

Functional and structural analysis of a new cold-active keratinase isolated from the Antarctic bacterium *Lysobacter* sp. A03

Jamile Pereira [1, 2] ; Adriana Ambrosini [2].; Luciane Passaglia [2].; Adriano Brandelli [1*]

1 Laboratório de Bioquímica e Microbiologia Aplicada, Instituto de Ciências e Tecnologia de Alimentos, Universidade Federal do Rio Grande do Sul (UFRGS), Brazil

2 Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil

* Corresponding autor: Dr. Adriano Brandelli

E-mail: abrand@ufrgs.br

Running Title: *Lysobacter* sp. A03 cold-active keratinase

10.1.1.1 ABSTRACT

Functional genomic library using fosmids as vectors was constructed to search for keratinolytic peptidases produced by the psychrophilic strain *Lysobacter* sp. A03, isolated from decomposing penguin feathers at the Antarctic environment. After subcloning in pGEM-T easy vector, one proteolytic clone was selected, its insert was sequenced and the enzyme, belonging to S8 family of subtilisin-like peptidases was structurally characterized. The A03Pep1 peptidase precursor has an estimated molecular mass of 72.5 kDa and showed to be composed of five domains: an N-terminal signal peptide, a propeptide inhibitor belonging to I9 family, the catalytic domain, a bacterial pre-peptidase (PPC) sequence and a pro-protein convertase (PC) domain. The enzyme was overexpressed in pGEX-4T-2 vector and showed optimal activity at pH 9.0 and 40°C. The SDS-PAGE analysis showed that the mature enzyme has approximately 35kDa, a result in agreement with that predicted by the bioinformatics tools. The activity of the A03Pep1 keratinase was enhanced in the presence of Ca^{2+} metal ions until 25 mM and in the presence of 10 mM Na^{2+}, NH^{4+}, Mg^{2+} and Ba^{2+} and was inhibited by 10mM of Zn^{2+}. Based on the 3D structure modelling, was predicted that the shortening of a loop next to the catalytic site might be responsible for the enlargement of the substrate binding pocket in A03Pep1 when compared with its mesophilic homologous AprV2, presumably as a way to enhance the probability of binding of substrates at low temperatures. By discussing the functional and structural characteristics found in the psychrophilic peptidase A03Pep1, the results provide possible approaches in developing new biotechnologically relevant peptidases active under low to moderate temperatures.

Keywords: fosmid library, functional screening, peptidases, psychrophilic, 3D Modelling

10.1.1.2 INTRODUCTION

Enzymes are used in many industries, from textile production and bakery products to animal feeding, biofuels, laundry detergents, pharmaceuticals and as a basic biological research tool [1]. When compared with chemical catalysis, enzymes exhibit indisputable advantages, as they work under mild reaction conditions, have a long half-life and produce much less pollutants [2-3]. Peptidases are the largest class of commercial enzymes, accounting for approximately 60% of the global enzyme market. Microorganisms are the main source of proteolytic enzymes, as they can be easily cultivated, produce large amounts of the desired product, can be selected according to the application, and can be genetically modified to enhance their properties [2].

Among peptidases, keratinases are the group of biocatalysts responsible for the degradation of the recalcitrant protein keratin, found mainly in structures such as feathers, wool, hair, nails and horns, and have a broad biotechnological and industrial applications as in detergent formulation, preparation of protein supplements, leather manufacture, textile processing, feather meal processing for animal feed and fertilizer production and waste management [4]. Furthermore, it was demonstrated the potential for using keratinolytic enzymes in formulations for prion degradation [5] and to enhance ungual drug delivery [6]. Keratinases are found mainly in bacteria of the genus *Bacillus*, although some Gram-negative bacteria such as *Chryseobacterium, Pseudomonas* and *Burkholderia* were reported as keratinase-producing strains. Most keratinases are classified as serine- and metallo-proteases, are active at neutral to alkaline pH and are isolated predominantly from mesophilic and thermophilic organisms, with optimal activity at temperatures ranging from 40°C in some *Bacillus* species to 100°C in *Fervidobacterium islandicum*. [4-7].

In recent years, it has increased the number of studies showing the advantages of using enzymes coming from psychrophilic microorganisms, i.e. those organisms able to live in permanently cold environments as the Polar Regions, the depths of the oceans and the high altitudes [8]. Cold active enzymes showed to have features that make them very attractive for use in biotechnology: they have a high activity at lower concentrations, reducing the amount of enzyme needed in a reaction, they are heat labile, being easily and selectively inactivated under moderate heat input and they remain efficient at ambient temperature, saving energy both at domestic and industrial levels [8-9]. It was demonstrated that such characteristics of cold active enzymes are due to their flexible structure, that compensates for the low kinetic energy at cold environments, achieved by some features as decreased core hydrophobicity, while there are an increased surface hydrophobicity, weaker interactions inter-domains and inter-subunits, decreased secondary structure content and a bias toward some amino acid residues rather than others. Nevertheless, there are still many questions about structure-function relationships in cold adapted enzymes to be answered [10].

Lysobacter is a genus of Gran-negative gliding bacteria that belongs to the Gammaproteobacteria Class, generally isolated from soil and freshwater habitats. Its species are known by the production of many biotechnologically relevant compounds as biocontrol agents with activity against plant pathogens [11], antibiotics as lysobactin [12] and enzymes, giving up attention to those produced by strains of *L. enzymogenes*. Recently, a psychrophilic strain of *Lysobacter* named as *Lysobacter* sp. A03, isolated from penguin feathers in Antarctica, was identified as a keratinase producing strain [13]. It was able to grow preferentially at temperatures around 20 °C and to not grow above 35 °C. The strain showed maximum peptidase production after 96 h of cultivation in feather meal broth as sole source

of nutrients, producing a wide range of enzymes. The enzymatic activity was high at temperatures between 20 and 30 °C, but presented an optimal temperature of incubation of 40 °C at pH9.5 and was demonstrated to gradually loss its activity at temperatures above 50°C. Considering the ability of A03 strain to degrade keratin as other sources of proteins under lower temperatures in comparison to its mesophilic homologues, it was selected for the construction of a genomic library to search for cold-active peptidase genes of biotechnological and industrial interest and, by the functional analysis and comparative structural modelling, provide new insights on the molecular mechanisms responsible for enzyme activity at low temperatures.

10.1.1.3 MATERIALS AND METHODS

Bacterial strain and plasmids. The genomic library samples were prepared using *Escherichia coli* EPI300 (Epicentre, Madison, WI, USA) and *E. coli* JM109 (Promega, Fitchburg, WI, USA) as hosts for Fosmid pCC1FOS (Epicentre, Madison, WI, USA) and plasmid pGEM-T easy (Promega, Fitchburg, WI, USA), respectively. The *a0301* gene cloning was made using *E. coli* BL21 (New England Biolabs, MA, USA) as hosts for pGEM-T easy vector. The enzyme expression was made using *E. coli* ArcticExpress (Agilent Technologies) as hosts for pGEX-4T-2 vector.

DNA extraction and fosmid library construction. The Antarctic bacterial strain *Lysobacter* sp. A03 was isolated from decomposing penguin feathers and cultivated in Feather Meal Agar 1% (FMA) as sole source of carbon and nitrogen [13]. The genomic DNA was extracted using the Wizard Genomic DNA Purification kit (Promega) according the manufacturer's protocol and was quantified by using a NanoDrop spectrophotometer

(NanoDrop Technologies, Inc.). Approximately 30 µg of DNA was randomly sheared by pipetting to generate fragments of about 30 Kb which were gel-purified, their ends were repaired and then ligated to fosmid pCC1FOS (Epicentre). After phage-packaging, fosmids were propagated in *E. coli* EPI300, which was then plated on Luria Bertani (LB) Agar containing 12.5 µg/mL of chloramphenicol. A negative control was made transforming pCC1FOS in *E. coli* EPI300 without DNA insert. The resulting clones were resuspended in 2 mL of LB media and stored at -70 °C in glycerol at a final concentration of 20% until use.

Library screening. The clones from *Lysobacter* sp. A03 library were replicated in Luria Bertani (LB) plates amended with Chloramphenicol 12.5µg/mL and 10% skimmed milk and were incubated during 24- to 48-h at 28 °C. The proteolytic activity was verified by the presence of a clear halo around the colonies, as an indicative of the milk casein hydrolysis. The positive clones were isolated and cultivated in LB broth containing 12.5µg/mL chloramphenicol plus 0.1% arabinose for fosmid extraction and partial sequencing to select unique clones.

Sub-cloning of the protease-encoding gene. The unique fosmids, positive for proteolytic activity, were cleaved with different combinations of EcoRI, NsiI, PstI and SphI (Promega), enzymes, then fragments around 3-5kb were ligated into pGEM-T easy vector (Promega) cleaved with the same pair of enzymes. The resulting sub-cloned vectors were used to transform *E. coli* JM109 cells that were screened for proteolytic activity in LB plates containing ampicillin 100 µg/mL, 0.5 mM IPTG, 80 µg/mL X-Gal and 10% skimmed milk. The positive clone, chosen also based on its stable and higher protease production was isolated and cultivated in LB broth containing ampicillin 100 µg/mL for plasmid extraction and insert sequencing using M13 Forward and Reverse primers (Promega), as well as

internal specific primers designed to obtain the full sequence of the protease-containing fragment.

Sequence analysis of gene coding for *a0301* peptidase. To define the Open Reading Frame of the *Lysobacter* sp. A03 keratinase gene, named *a0301*, and its amino acid sequence, the ORF Finder tool was used and the search for homologous sequences was made using the nucleotide-nucleotide Basic Local Alignment Search Tool (BlastN) and the protein–protein basic local alignment search tool (BlastP), from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The amino acid composition was evaluated using the Composition Profile web tool [14], by comparing the sequence of the peptidase with the SwissProt databank. The enrichment or depletion of a particular amino acid was considered significant with $p< 0.05$. To predict the presence and cleavage site of an N-terminal signal peptide, was used the program SignalP 4.1, with a cutoff of 0.8 (http://www.cbs.dtu.dk/services/SignalP/). The prediction of conserved domains was performed by the BLAST analysis of conserved domains, also implemented at the NCBI website and through the Pfam database of protein families [15]. The classification of the A03Pep1 protease was performed by comparing the sequence to the MEROPS peptidase database (http://merops.sanger.ac.uk) [16]. To predict the secondary structure of A03Pep1 polypeptide, was used the PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/) and the phyre2 protein folding recognition server [17].

Heterologous expression of the gene coding for *a0301* peptidase. Based on the ORF sequence defined by the Orf Finder tool, the gene encoding the *a0301* peptidase was entirely amplified using two primers containing EcoRI and XhoI (Promega) restriction sites. The amplified product was gel-purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and inserted into a pGEM-T easy vector used to transform *E. coli* JM109 cells.

To facilitate the selection of the peptidase-bearing plasmids, the proteolytic colonies were screened in LB plates amended with ampicillin 100 µg/mL, IPTG 0.5 mM, X-Gal 80 µg/mL and skimmed milk 10%. After the confirmation of the insert sequence, the plasmid containing the peptidase gene was digested with EcoRI and XhoI and then ligated into the pGEX-4T-2 expression vector, cleaved with the same restriction enzymes and then used to transform *E. coli* ArcticExpress (Agilent Technologies) cells. The selection was made in LB plates containing ampicillin at 100 µg/mL and gentamycin at 20 µg/mL and the resistant clones were isolated and cultivated in LB broth containing the same antibiotics at 37 °C until the OD_{600} value reaches 0.6, then the temperature was reduced to 15 °C and the expression of the recombinant protein was induced by the addition of 0.5 mM IPTG following cultivation for additional 24 h. The purified peptidase was analyzed by SDS PAGE 12% and by zymography under non-denaturing conditions.

Effect of pH and temperature on enzymatic activity. The expressed keratinase and azocasein solution (Sigma, St. Louis, USA) 1% (w/v) were prepared over a pH range of 6.0 – 10 by using 0.1 M sodium phosphate buffer (pH 6.0-8.0) and 0.1 M carbonate-bicarbonate buffer (pH 9.0-10). Assays were conducted by incubating 100 µL of enzyme in 200 µL of a 10 mg/mL suspension of azocasein in each pH, at 37 °C for one hour. The reactions were stopped by adding 500 µL of 10 % (w/v) trichloroacetic acid (TCA). The tubes were centrifuged at 10,000 rpm for 5 min and the supernatants were added to 200 µL of a 1,8 M NaOH solution. The enzymatic activity was measured by the increase of the absorbance at 440 nm. The assays were performed in triplicate and the controls were prepared by adding 10 % TCA before incubation. One unit of proteolytic activity was defined as the amount of enzyme that resulted in an increase in absorbance at 440 nm of 0.01 U after reaction with azocasein for 1 h [18].

The effect of temperature on the protease activity was examined at 10, 20, 30, 40, 50 and 60 ◦C for one hour at the optimum pH. The thermo-stability was determined by measuring the residual activity in azocasein at the optimal pH and temperature after incubating the enzyme at 37, 55 and 70°C for 5, 15, 30, 60 and 120 minutes. The assays were conducted by incubating 100 µL of the treated keratinase in azocasein as above and the initial activity was defined as 100%.

Effect of metal ions and inhibitors on the enzymatic activity. In order to investigate the effects of metal ions on the enzymatic activity, Na^{+}, NH^{4+}, Zn^{2+}, Mg^{2+}, Ba^{2+} and Ca^{2+} were added individually into the reaction mixture to the final concentration of 10 mM. The effect of the specific inhibitor of serine-peptidases phenylmethylsulfonyl fluoride (PMSF) 5 mM, metallopeptidase inhibitor ethylene diamine tetraacetic acid (EDTA) 5 mM and organic solvent isopropyl alcohol 5% (v/V) was also evaluated. The activity was measured at the optimum pH and temperature in azocasein and the activity of the control without any additive was defined as 100%.

To further investigate the effect of Ca^{2+} on the peptidase activity, the enzymatic assays were performed in increasing concentrations among 0 and 40 mM of Ca^{2+} in the same conditions as above mentioned.

3D structure prediction. The prediction of the 3D structure of the A03Pep1 peptidase by homology modeling was performed using Modeller [19], and the online protein folding recognition servers Phyre2 [17] and Swiss-Model [20]. The refinement of the predicted model was done with ModRefiner [21]. To validate the stereochemical quality of the predicted model, was used the PROCHECK [22] program and the Molprobity [23] web service tool, by analyzing the overall structure and residue-by-residue geometry of proteins,

that was then plotted as Ramachandran diagrams. To identify the binding cavities and to calculate the volume of the active site cavity in the structure of A03Pep1, was used the Computed Atlas of Surface Topography of proteins (CASTp) server, with a probe radius of 1.4 Å [24]. The graphical representations where visualized and edited using the Pymol software version 1.8 (Schrödinger, LLC).

10.1.1.4 RESULTS

Genomic library screening. Using pCC1FOS to construct the genome library were generated approximately 28,000 insert-bearing *E. coli* clones, with insert sizes of 30 Kb on average. Through the functional screening in LB agar plates amended with skimmed milk, were detected 12 potentially protease-positive clones after 48 h of incubation at 28 °C. Controls made with *E. coli* EPI 300 carrying pCC1FOS without DNA insert did not shown the presence of haloes even after prolonged times of incubation. Starting from the 12 initial protease positive fosmid clones, which were sequenced in order to search for those probably bearing different peptidase genes, four of them were selected to carry out the subcloning in the pGEM-T easy vector. From them, only one, cleaved with the combination of EcoRI and SphI enzymes, was able to retain its proteolytic activity when incubated in LB + skimmed milk agar plates, being selected for sequencing and the subsequent analysis (Fig. S1).

DNA and Protein sequence analysis. The insert sequence of 6 Kb obtained by subcloning was submitted to the Orf Finder tool and the putative peptidase gene, comprising 2,133bp, was denominated *a0301*. The search for homologous sequences performed at the BLAST tool implemented on the NCBI website showed an identity of 78% with the keratinase *kerF* from *Stenotrophomonas maltophilia* YHYJ-1, which has an ORF of 1,818 bp (GenBank

accession number HM590650) and the same homology with the keratinase *KerSMF* from strain BBE11-1, encompassing an ORF of 1,743 bp, also from a strain of *S. matophilia* (GenBank accession number KC763971) [25-26].

As many biologically important enzymes, the A03Pep1 peptidase was shown to be synthesized as a large inactive precursor composed by 710 amino acids with a deduced molecular weight of 72,5 kDa, that consists in five domains: a signal peptide, a propeptide inhibitor, the catalytic domain, a bacterial pre-peptidase (PPC) sequence and a pro-protein convertase (PC) domain (Fig. 1). The amino acid sequence was deposited in Genbank under the accession number WP_043958332. The comparison between the amino acid composition of A03Pep1 and its closest homologue, KerSMF, showed a bias toward more flexible amino acid residues in the psychrophilic enzyme, remarkably alanine and threonine and the reduction in the content of less flexible residues, mainly glutamic acid and lysine (Fig. 2)

In the A03Pep1, the signal peptide was predicted to be 28 amino acid residues in length, with the cleavage site between Ala(28) and Ala(29) in a region composed by an alpha-helix “interrupted” by a small turn exactly at the putative cleavage site (Fig. S2). The propeptide domain, belonging to the I9 family, was formed by five β-sheets and two α-helices surrounded by loops which, in the 3D structure modelling (data not shown) appeared to be a very disordered region.

Alignments of A03Pep1 with homologous sequences of subtilisin-like peptidases performed using T-Coffee tool [27] allowed predicting the mature sequence of the enzyme, which consists in 334 amino acids residues with a molecular weight of approximately 33 kDa (Figure 3). The major sequence identity of the predicted mature peptidase was with the

subtilisin-like serine peptidases S8 from *Stenotrophomonas maltophilia*, with which A03Pep1 shared 72% of similarity. The overall secondary structure of the catalytic domain is very similar to that of serine-endopeptidases. The proposed catalytic triad (Asp41, His98 and Ser270) was found in conserved regions of the enzyme and, next to the His residue, between positions 90 and 91, was observed a deletion of 7 amino acids in relation to its homologous AprV2 subtilisin-like peptidase, where that region correspond to a loop that partially occludes the substrate binding site and that is presumed to mediate enzyme-substrate interactions [28]. A small deletion, of 4 amino acid residues, at that same region was observed when comparing A03Pep1 with KerSMD and KerSMF.

Following the catalytic domain, at the A03Pep1 C-terminus, were predicted a PPC (Bacterial Pre-peptidase C-terminal) domain and a Proprotein convertase P-domain (PC) both composed almost exclusively by β-sheets surrounded by loops, assuming a beta-roll conformation. In A03Pep1, the PPC structure was shown to be similar to that at the C-terminal of KerSMD keratinase, also from *S. maltophilia* BBE11-1, with 67% of sequence identity. At the C-terminal of A03pep1 peptidase was found an unusually large PC domain. The alignment between the A03Pep1 PC domain with those from others related enzymes showed a very low sequence identity among them. In addition, was found a slightly higher asparagine content in the convertase domain of A03Pep1, a feature commonly seen in psychrophilic proteins that can be related to the heat sensibility of such amino acids [29].

Heterologous expression of A03Pep1 keratinase. The construction pGEX-4T-2-A03Pep1 vector was expressed in *E. coli* ArcticExpress, but the attempts to purify the peptidase from the cell lysate were unsuccessfully, maybe due to the auto-processing of the N-terminal domains of the precursor protein leading to the cleavage of the GST tag. Thereby, once a large amount of the target protein was expressed in the supernatant and the SDS-PAGE

showed that after ultrafiltration with an Amicon Ultra-15 10,000 MWCO filter (Millipore) followed by an another step of ultrafiltration with an Amicon Ultra-15 30,000 MWCO the enzyme was purified almost to homogeneity (Fig. 4-a), we decided to perform the enzymatic assays assuming that the activity observed in that fraction was exclusively due to the A03Pep1 peptidase, as showed by the zymogran (Fig. 4-b). The A03Pep1 molecular mass observed was of approximately 35 kDa, a result in agreement whit that predicted by the sequence alignments and domain analysis. The enzyme showed the highest activity at pH 9.0 (Fig. 5-a) and 40°C (Fig. 5-b), losing about 60% and 90% of its original activity at 55°C and 70°C, respectively after 15 minutes of heat treatment (Fig. 5-c). The activity of A03Pep1 peptidase was measured in the presence of metal ions. As shown in table 2, the enzymatic activity only was inhibited by 10 mM $Zn^{2+}$and was enhanced in the presence of 10 mM Ca^{2+} Na^{2+}, NH^{4+}, Mg^{2+} and Ba^{2+}. As the Ca^{2+} showed the highest positive effect among the metal ions on the enzymatic activity, assays in increasing concentrations were performed. As seen in figure 6, Ca^{2+} enhanced in 60% the activity of A03Pep1 at the maximum of 25 mM. From table 3, it has been noticed that the activity of A03Pep1 was completely inhibited by PMSF, was strongly inhibited by the presence of EDTA and was enhanced by isopropyl alcohol.

Predicted 3D structure of A03Pep1. The three dimensional structure of the catalytic domain of *Lysobacter* sp. A03Pep1 was predicted using Modeller v. 9.15, Swiss Model and Phyre2. Among the three models, that predicted by Modeller was the best with 98% of the residues found in the allowed region of the Ramachandran plot and a QMEAN score of 0.73. Among the models available, the highest identity was with the crystal structure of the subtilisin-like protease AprV2, from *Dichelobacter nodosus* (PDB accession number 3LPA), with 56% of similarity and a coverage of 46% [28] which was also used to model KerSMD keratinase, with which AprV2 share 47% of similarity [26]. The overall 3D

structure of A03Pep1 was shown to be very similar to that of the majority of serine-peptidases, with six β-sheets involving the central α-helices and a hairpin situated next to the catalytic center, with the catalytic triad found at the end of the central α-helices (Fig. 7-a). The enzyme has two disulfide bridges, between residues Cys-89 and Cys-134 and between residues Cys-176 and Cys-213, being that the first was shown to form a loop which is a part of the predicted substrate binding site. Interestingly, the superimposition of the 3D structure of A03Pep1, AprV2 and AprB2, this last also a serine peptidase from *D. nodosus*, (Fig. 7-b) showed the shortening of this loop in the psychrophilic enzyme and, apparently, this feature facilitates the access to the substrate binding site. Furthermore, according to CASTp server (Fig. 8) the predicted substrate binding site of A03Pep1 was showed to be wider than that of AprV2, once the shorter loop in A03pep1, with the aid of the disulfide bridge between residues Cys-89 and Cys-134, seems to pull back the structure, facilitating the access to the catalytic site. This feature was the main structural difference observed between the mature A03Pep1 and its mesophilic homologous enzymes, being commonly found in others psychrophilic hydrolases [8].

10.1.1.5 DISCUSSION

Starting from a genomic library, we found a cold-active peptidase gene with keratinolytic activity. The enzyme, named A03Pep1 was classified as a Subtilisin-like serine protease, belonging to family S8, according to MEROPS peptidase database. The amino acid sequence showed that the enzyme is closed related to the keratinases from *Stenotrophomonas maltophilia* strains YHYJ-1 and BBE11-1. These two keratinolytic strains have been target of recent researches aiming the heterologous cloning and expression

of their keratinases genes and, for strain BBE11-1, was shown a great potential for industrial application of their peptidases KerSMD and KerSMF when expressed in *E. coli*, showing optimal enzymatic activity under mesophilic to thermophilic temperatures and alkaliphilic conditions [26].

The biochemical parameters of the recombinant A03Pep1 showed a cold-tolerant to mesophilic character, with the optimal enzymatic temperature of 40°C and pH 9.0, and a thermal stability below 50°C, although the optimal temperature of *Lysobacter* sp. A03 cultures were situated around 20°C. This shift towards higher optimal temperatures of enzymatic activity in psychrophilic enzymes was demonstrated to be the result of the kinetic effect of heat under the reaction rate, and the studies showed that the optimal temperature of activity is generally 10 to 20 °C above that of maximal growth [30]. Furthermore, the temperature is not the only factor controlling the existence of psychrophilic organisms and most strains from cold environments can grow at temperatures well above what they encounter in their habitats, reflecting simply the highest temperature that they can tolerate [31].

The properties of A03Pep1 in terms of effect of pH, metal ions and inhibitors, indicated that the enzyme is an alkaliphilic serine peptidase whose activity is enhanced by the presence of some metal ions, manly Ca^{2+} at an optimum concentration of 25 mM. A similar result was observed for the keratinase from *Bacillus* sp. P45, in which the presence of increasing concentration of Ca^{2+} and Mg^{2+} enhanced the enzymatic activity. Maximum activity was recorded with 3 mM and 4 mM of Ca^{2+} and Mg^{2+}, respectively [32]. It was demonstrated that in the keratinase produced by *Purpureocillium lilacinum* LPSC #876, the presence of Ca^{2+} increases the enzyme thermal stability significantly, with 10 mM of Ca^{2+} being the ideal concentration in the assays at 55°C for 1 h [33]. According to the SDS-PAGE

analysis, the molecular mass of the mature A03Pep1 peptidase was about 35 kDa, very similar to the keratinase KerSMF (36 kDa), from *Stenotrophomonas maltophilia*, which was demonstrated to degrade keratin at lower temperatures than its homologue KerSMD ant to possess a low thermostability [34].

The domain prediction showed that the precursor protein has some structural features that make them unusually larger than its mesophilic homologous *KerF* and *KerSMF* but more similar to the halophilic cold-adapted subtilase MCP-03, from *Pseudoalteromonas* sp. SM9913, an enzyme structurally closely related to A03Pep1, with an ORF of 2,130bp and an estimated molecular mass of 72.6 kDa that also consists of five domains: a signal peptide sequence, an N-terminal prosequence, a catalytic domain and two C-terminal extensions [35]. The propeptide domain, belonging to the I9 family, also called activation peptide, is found in subtilisin-like serine peptidases of family S8 being, as in A03Pep1, synthesized as part of a larger precursor protein which, during enzyme maturation, is responsible for the modulation of the folding of the pro-enzyme, but also acting as a temporary inhibitor by protecting the substrate binding site of the enzyme until its releasing by cleavage [36]. The serine peptidase Alpha Lytic (α-LP), from *Lysobacter enzymogenes*, also showed a signal sequence and a pro-region unusually large. The pro-region act as a very strong inhibitor of α-LP, but it is necessary for protease folding. Cloning experiments in *E. coli* demonstrated that the precursor is translocated into the periplasmic space, where it is rapidly folded and processed through the cleavage between the pro-region and the catalytic domain followed by secretion across the outer membrane [37]. The A03Pep1 pro-region could play an important role in the correct folding of the peptidase at low temperatures, as the structures of cold-active peptidases have a lower number of interactions such as hydrogen and disulfide bonds, which may difficult its maturation in these conditions. Furthermore, the insertion of

the propeptide into the active site of A03Pep1 peptidase might protect that region from denaturation until the release of the mature enzyme, once the active site of psychrophilic enzymes is known to be more flexible and more hydrophilic than their mesophilic [38].

Next to the putative substrate binding site was observed the deletion of 7 and 4 amino acids residues in relation to its homologous AprV2, from *Dichelobacter nodosus* and KerSMF/KerSMD, from *Stenotrophomonas maltophilia*, respectively. It was revealed that in AprV2, that region corresponds to a loop tethered by the disulfide bond between residues Cys89-Cys141, next to the primary substrate binding site and it was demonstrated that this structure is required for optimal proteolytic activity [28]. In psychrophilic enzymes as the α-amylase AHA from *Alteromonas halopanctis* A23, were observed small deletions in loops bordering the catalytic site that was believed to be one way to enlarge that region, as a strategy to reduce de energy required for substrate accommodation and release of the product [39].

Additionally to the N-terminal domains, A03Pep1 exhibit two C-terminal domains. The PPC domains are found at the C-terminal of secreted metallopeptidases from families M4, M9 and M28 as well as in the serine peptidase family S8 in bacteria and archaea and was inferred that they contribute to protein folding, being cleaved off after secretion, but previous to activation of the peptidase [36]. The actual function of them is not clear but it was shown that removing or replacing the PPC C-terminal domain seems to aid the expression of recombinant bacterial serine proteases. For example, when the PPC domain from the keratinase KerSMF, from *S. maltophilia* BBE11-1, was used to replace the N- and C-terminal domains of its homologue KerSMD, the resultant mutant protein exhibited high activity to degrade feather waste under mesophilic conditions [34]. The extracellular serine protease HP70, also from a strain of *S. maltophilia* was successfully overexpressed in *E. coli*

after a structural modification that removed part of the C-terminal domain. The enzyme was most active at 40 °C and pH 7-11 and has a potential application in the industry of detergents [40]. Similarly, through cloning and deletion mutagenesis experiments, was shown that the subtilase MCP-03 PPC domains, from the psychrotolerant strain *Pseudoalteromonas* sp. SM9913 are unnecessary for enzyme secretion through the *E. coli* membrane, as both native and mutants enzymes could be purified directly from fermented broth. In addiction, was shown that the PPC domains had inhibitory effects on MCP-03 catalytic efficiency and might play a role increasing the enzyme thermostability [35]. The subtilisin-like extracellular peptidase AprV2, which crystal structure was used to make the comparative modelling of A03Pep1, also was shown to be synthesized as an inactive precursor with an N-terminal pre-pro-region, a serine peptidase domain and a C-terminal domain of unknown function. The active form of the enzyme is produced by cleavage of the N- and C-terminals and, by targeted mutagenesis studies, was demonstrated that a disulphide-tethered loop located next to the primary substrate binding site act mediating the interaction between the enzyme and the subtract [28].

At the A03Pep1 C-terminal, was found a pro-protein convertase domain that makes the keratinase longer them its mesophilic counterparts. Convertases are endopeptidases associated to peptidases from family S8B, the kexin subfamily, and have been identified in all eukaryotes, in Cyanobacteria, *Streptomyces avermitilis* and in members of the Gammaproteobacteria class [41]. It was demonstrated that they can act as cold shock chaperones, avoiding the misfolding of the enzyme when submitted to low temperatures, as seen in others psychrophilic proteins that act both at the transcription and in the translation levels [42]. The presence of such domain could explain the enhancement of the activity of A03Pep1 in the addition of Ca^{2+}, as the convertases are $Ca2^{+}$ dependents. Therefore, as the

I9 inhibitor and the PPC domains block the peptidase activity, the convertase could act removing those structures and aiding in the correct folding of the enzyme, which might be valuable to the folding of A03Pep1, as the hydrophobic forces, which are crucial for protein folding and stability, are weakened under low temperatures[8].

The most striking feature found in A03Pep1 peptidase was a wider substrate binding site when comparing the 3D structure of the enzyme with that of AprV2 crystal. Considering that the substrate binding is one of the most temperature-sensitive steps in an enzymatic reaction, this characteristic is frequently found in cold-adapted enzymes, as above mentioned by the α-amylase AHA, once a large active site facilitates the binding of the substrate at low temperatures, where the reaction rates are slowly. Additionally, was demonstrated that non-specific psychrophilic enzymes can accept a broad range of substrates than the mesophilic homologues, because substrates slightly different can fit in the active site [9]. In the other hand, such characteristic lead to a decrease in the stability of the interaction enzyme-substrate, giving rise to higher K_m values, which represents a measure of the affinity of the enzyme for the substrate.

In summary, the subtilisin-like serine peptidase A03Pep1, identified by genomic library screening of the Antarctic bacterial strain *Lysobacter* sp. A03, showed to have some remarkable characteristics commonly associated to cold-loving enzymes, highlighting its high activity under low to mesophilic temperatures, its relatively low thermostability and the wider predicted substrate binding site when compared to mesophilic homologous proteins. We presume that such features could account for the high keratinolytic and overall proteolytic activities observed in the A03Pep1 enzyme at low temperatures. Our analyses improve the knowledge about psychrophilic peptidases and their mechanisms of activity, providing new insights to the development of new biotechnologically relevant enzymes.

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10.1.1.6 REFERENCE

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10.1.1.7 FIGURE AND TABLES LEGENDS

Tables

Table 1. Effect of metal ions on the activity of A03Pep1 peptidase.

Table 2. Effect of inhibitors on the activity of A03Pep1 peptidase.

Figures

Fig. 1 Schematic diagram of A03Pep1 precursor showing the domains found and the position of the residues of the catalytic triad (Asp, His, grey lozenge , Ser red lozenge).

Fig. 2 Comparison between the amino acid composition of A03Pep1 and KerSMD against the SwissProt database. More flexible residues are shown in green and less flexible are shown in red. A particular enrichment or depletion is statistically significant when $p < 0.05$.

Fig. 3 Alignment of the predicted mature sequence of A03Pep1 with the serine-peptidase of known 3D structure 3LPA and the keratinases KerSMF and KerSMD. The identical residues are shaded in black boxes and the catalytic triad is indicated by the bottom black arrows. The secondary structure of the A03Pep1 peptidase is shown on the top.

Fig. 4. 12% SDS-PAGE (a) and zymography (b) analysis of the purified A03Pep1 Keratinase. Lane 1, protein molecular mass marker; lane 2 induced cell lysate of *E. coli* Arctic Express harboring pGEX-4T-2; lane 3, supernatant of the induced culture with activity; lane 4, purified A03Pep1 keratinase after ultrafiltration.

Fig. 5 Effect of pH (a), temperature (b) and thermal stability (c) of the purified A03Pep1 peptidase on azocasein. The enzyme activity without pre-treatment was taken as 100%.

Fig. 6 Effect of Ca2+ concentration on the A03Pep1 keratinase activity. The activity of the control without Ca2+ was defined as 100%.

Fig. 7 Modeled structure of the predicted mature form of A03Pep1 keratinase showing the catalytic triad (Asp, His, Ser) and the two disulfide bridges (a); superimposition of the 3D structures of A03Pep1 (blue), AprV2 (light pink) and AprB2 (dark pink). The grey arrow indicates the location of the main structural difference among the three molecules, where A03Pep1 showed a shorter loop at the entrance of the putative substrate binding site (b).

Fig. 8 Comparison of the binding pockets of A03Pep1 (A) and its homologue subtilisin-like peptidase AprV2 (PDB 3LPA) (B). The volumes of the binding pockets were calculated using the CASTp server and a probe radius of 1.4 angstroms.

10.1.1.8 Tables

Table 1

Table 1. Effect of metal ions on the activity of A03Pep1 peptidase.

Metal Ion	Final Concentration (mM)	Relative activity (%)
None	0	100
Na^{2+}	10	108.06 ± 1.12
NH^{4+}	10	110.07 ± 4.5
Mg^{2+}	10	117.13 ± 3.2
Zn^{2+}	10	67.63 ± 1.18
Ba^{2+}	10	106.38 ± 4.4
Ca^{2+}	10	131.91 ± 2.1

Relative activity represents the mean ± SD (standard deviation) of three individual experiments at optimum condition, pH 9.0, temperature 40°C.

Table 2

Table 2. Effect of inhibitors on the activity of A03Pep1 peptidase.

Inhibitor	Final concentration	Residual activity (%)
PMSF	5 mM	0± 0.00
Isopropyl alcohol	5% (v/v)	115.3± 0.26
EDTA	5mM	48,79± 3.48

Relative activity represents the mean ± SD (standard deviation) of three individual experiments at optimum condition, pH 9.0, temperature 40°C.

10.1.1.9 Figures

Figure 1.

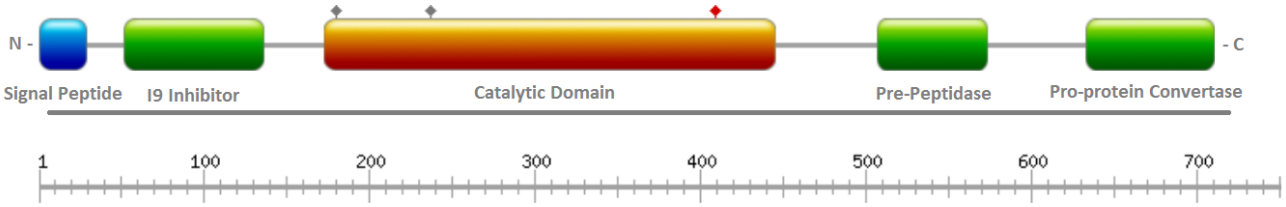


Figure 2.

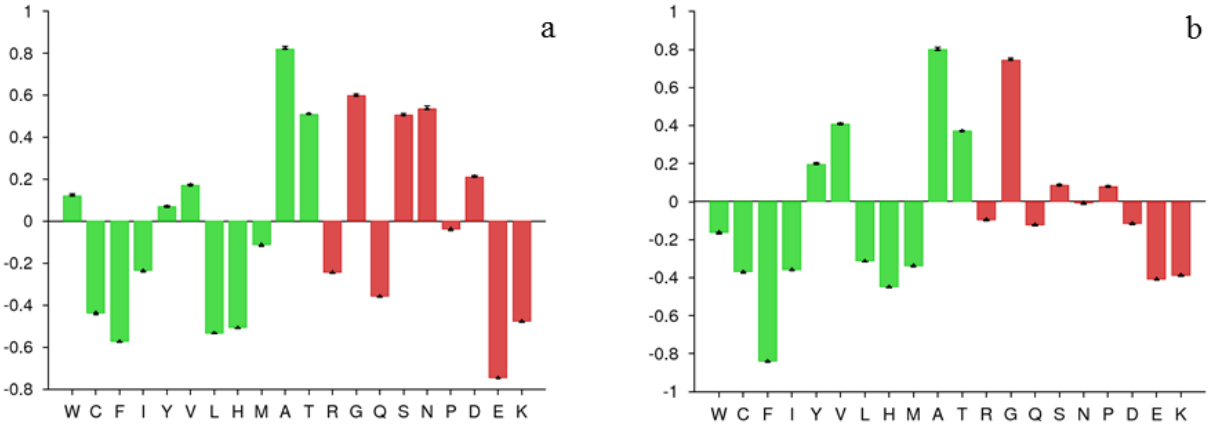
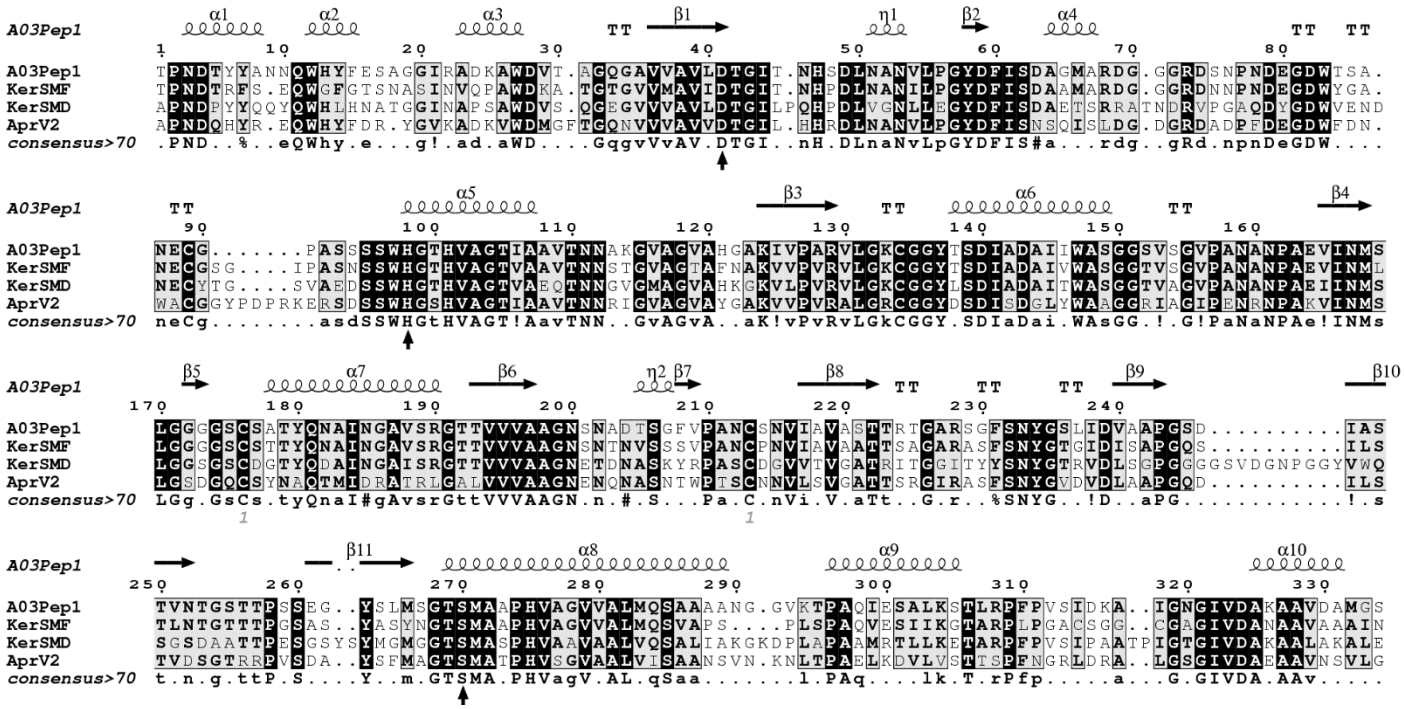

a
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
W C F I Y V L H M A T R G Q S N P D E K
b
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
W C F I Y V L H M A T R G Q S N P D E K

Figure 3



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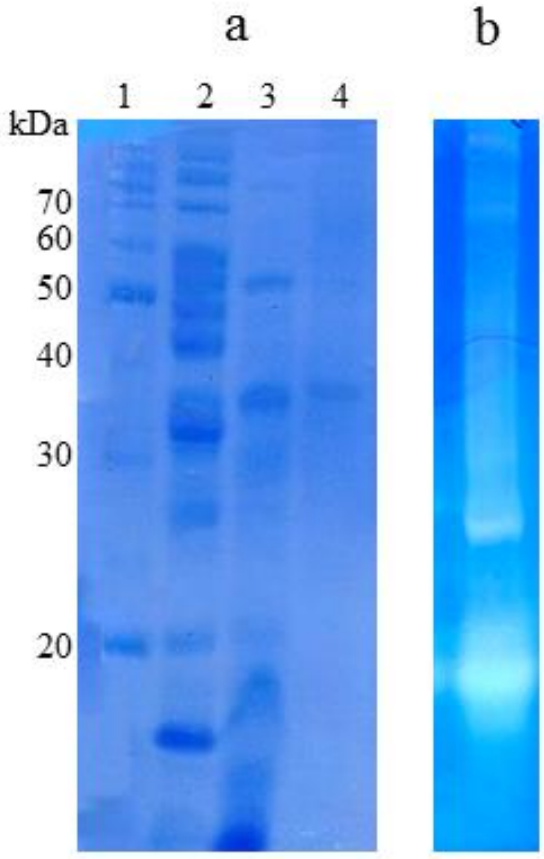
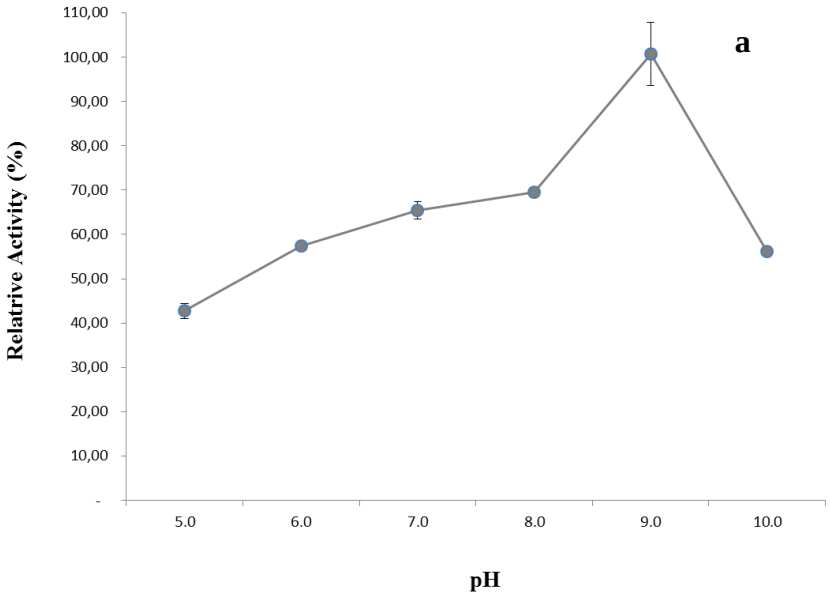
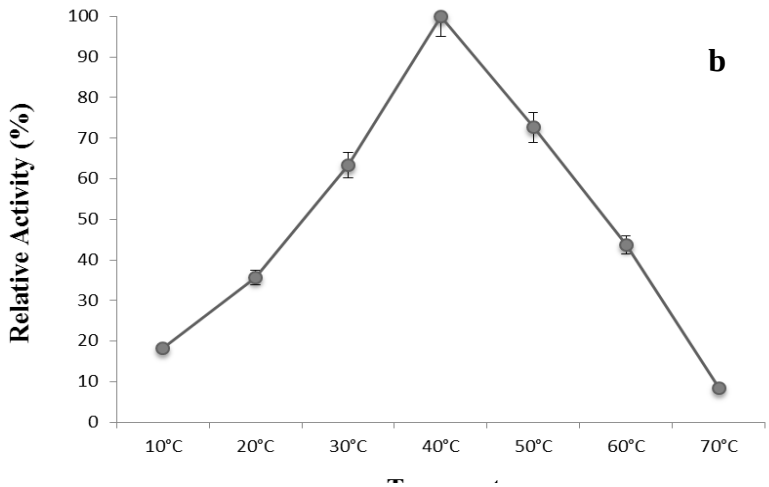


Figure 5


a
Relatrive Activity (%)
110,00
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
-
5.0
6.0
7.0
8.0
9.0
10.0
pH


b
Relative Activity (%)
100
90
80
70
60
50
40
30
20
10
0
10°C
20°C
30°C
40°C
50°C
60°C
70°C

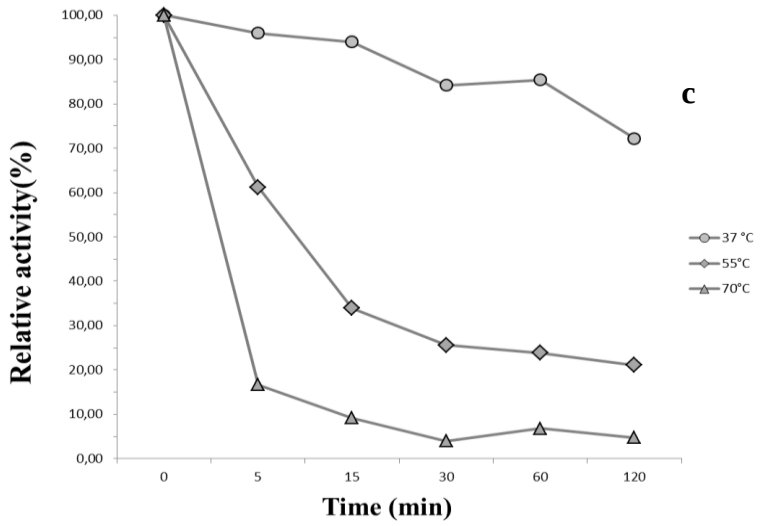

c
Relative activity(%)
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
0
5
15
30
60
120
Time (min)
37 °C
55°C
70°C

Figure 6

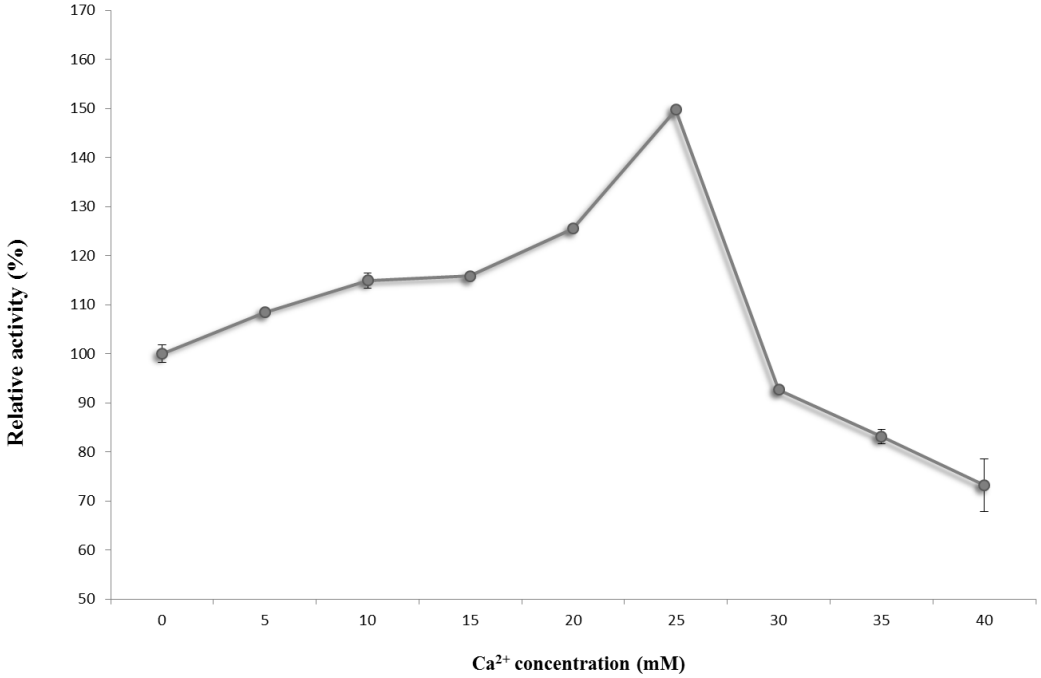

Relative activity (%)
170
160
150
140
130
120
110
100
90
80
70
60
50
0
5
10
15
20
25
30
35
40
Ca2+ concentration (mM)

Figure 7

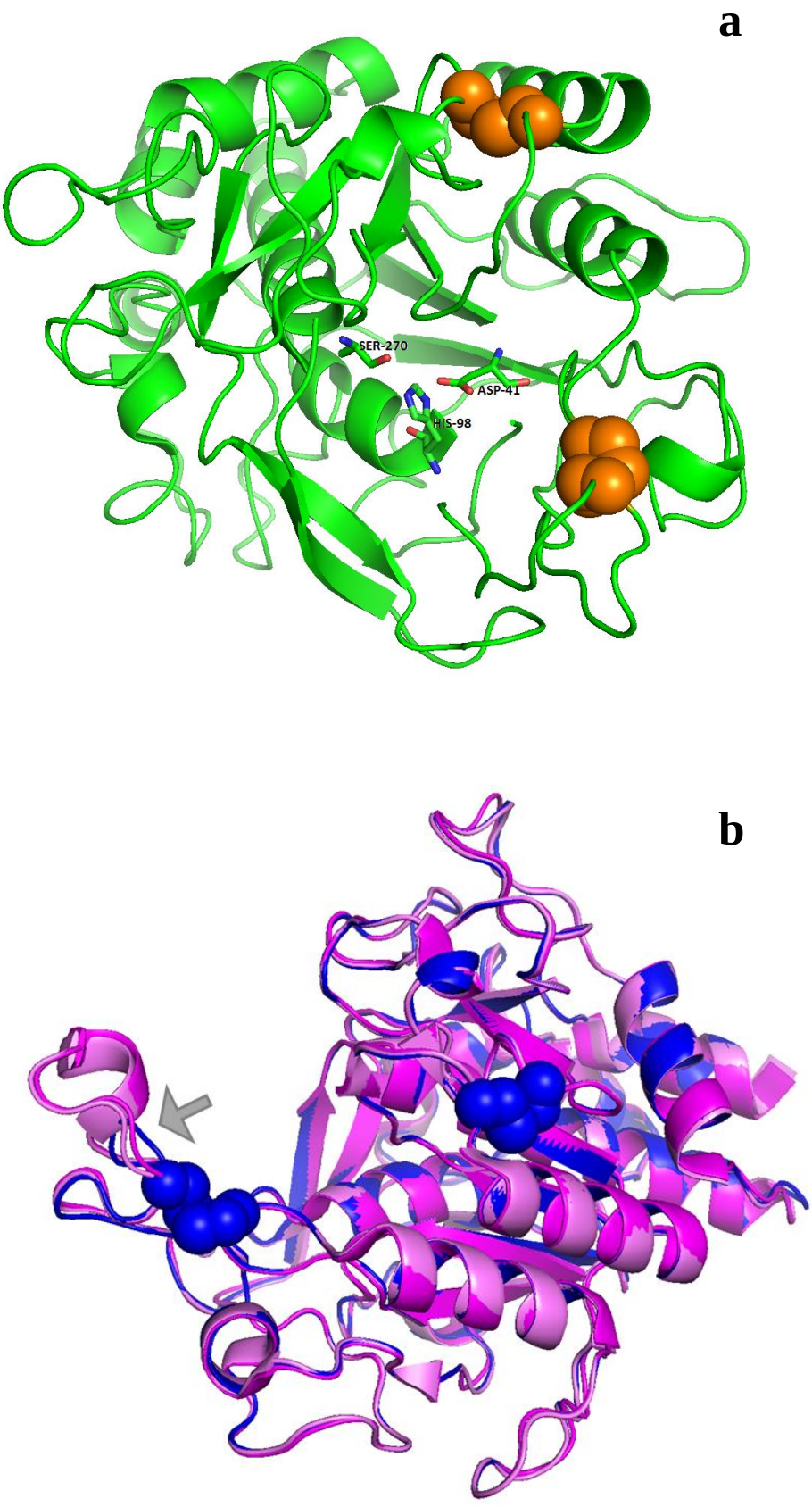
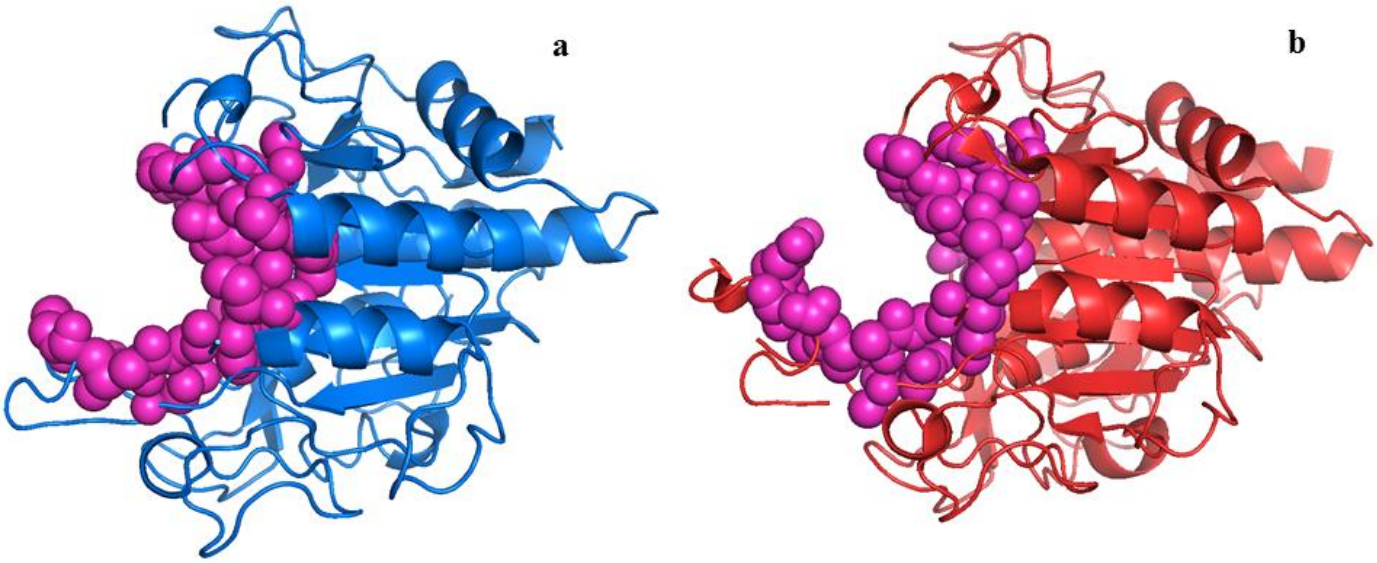


Figure 8



10.1.1.10 SUPPLEMENTAL MATERIAL

Fig. S1 Skimmed milk agar plate of the transformation of the peptidase gene in pGEM-T easy vector after 48 h of incubation at 28°C. The haloes around the colonies are indicative of proteolytic activity.

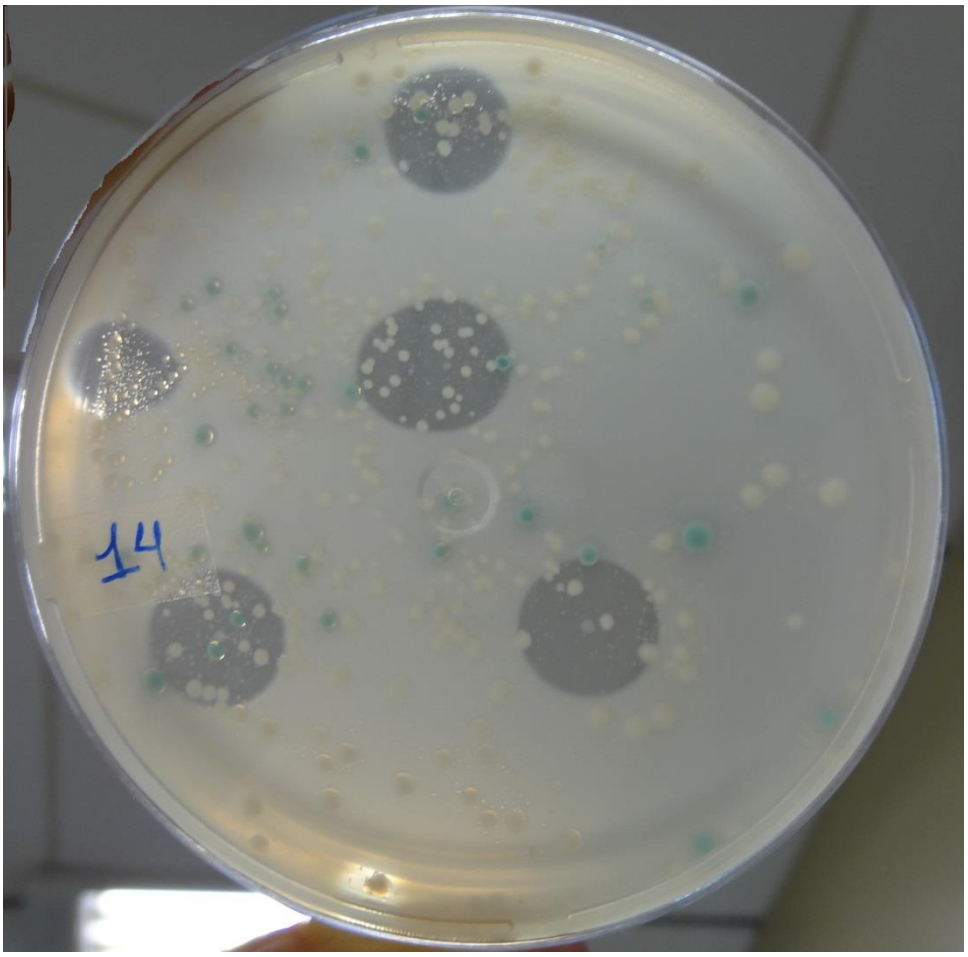
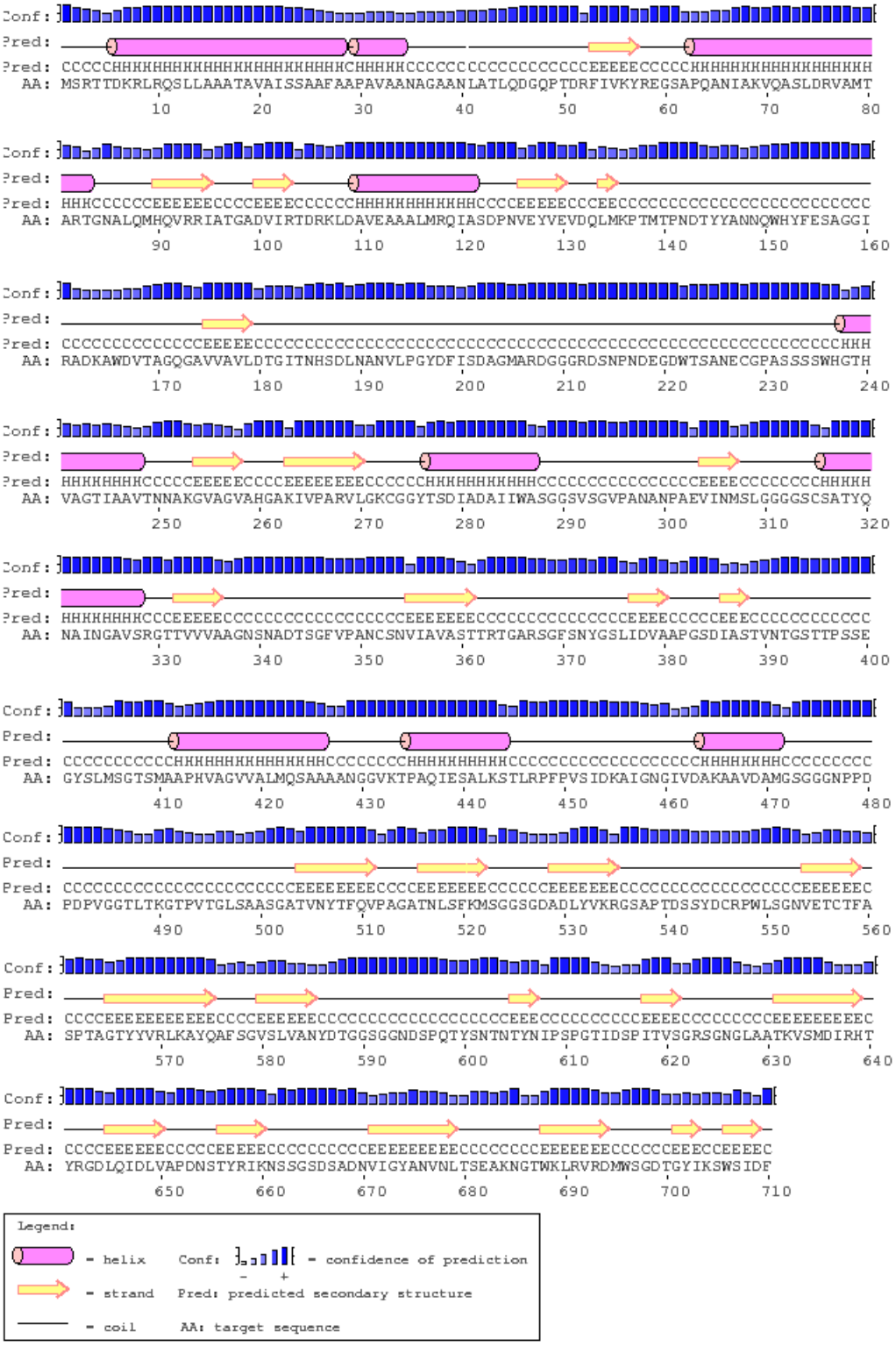


Fig. S2. Secondary structure prediction of the A03Pep1 precursor calculated using PsiPred. The blue bars indicate the confidence of the prediction.



11 Discussão Geral

A demanda industrial por preparações altamente ativas de biocatalisadores com estabilidade e especificidade apropriadas em termos de pH, temperatura, íons metálicos e surfactantes continua a estimular a busca por novas fontes de peptidases. Ainda assim, a prospecção de enzimas proteolíticas esteve, durante muito tempo, focada na identificação de proteases provenientes de micro-organismos cujo desenvolvimento ótimo situa-se na faixa de temperatura mesofílica e termofílicas (FELLER, 2013).

No entanto, nos últimos 20 anos, têm crescido o interesse na descoberta, isolamento e investigação de micro-organismos provenientes de ambientes extremos, os chamados extremófilos. Entre eles, os organismos psicrófilos são de particular interesse, pois suas enzimas são capazes de agir e permanecer estáveis em temperaturas nas quais suas homólogas mesofílicas apresentam pouca ou nenhuma função. Em habitats permanentemente frios, as baixas temperaturas obrigaram os organismos a desenvolver mecanismos enzimáticos únicos que tornassem as taxas metabólicas compatíveis com a vida. Estas proteínas são de especial interesse comercial, tanto diretamente quanto como modelos para a engenharia de biocatalizadores com características específicas para um dado processo industrial (BAE & PHILLIPS, 2004). Além disso, elas proporcionam uma oportunidade única para estudos acadêmicos focados nas relações entre estabilidade, dinâmica e função de proteínas.

A bactéria *Lysobacter* sp., A03, isolada de penas de pinguim coletadas na Antártida juntamente com outras linhagens bacterianas, foi selecionada com base na sua capacidade de degradar resíduos de penas preferencialmente em temperaturas entre 15 e 20°C, ou seja, dentro da faixa de crescimento de organismos psicrofílicos. Apesar disso, a temperatura ótima para a atividade proteolítica extracelular foi de 40°C, refletindo o resultado do efeito cinético do aquecimento sobre a taxa de reação, de forma que os estudos demonstram que a temperatura ótima de atividade de uma enzima encontra-se geralmente entre 10 e 20°C acima daquela de crescimento máximo (SHERIDAN *et al.*, 2000). No entanto, nos ensaios em azocaseína, verificou-se que a atividade enzimática foi rapidamente reduzida em temperaturas superiores a 50°C, ressaltando a característica de termolabilidade dos biocatalizadores de organismos psicrófilos (CAVICCHIOLI *et al.*, 2011).

A habilidade de enzimas adaptadas ao frio de catalisar reações em temperaturas baixas e moderadas é de grande interesse industrial e biotecnológico, ainda mais considerando-se a crescente preocupação com o aquecimento global e os esforços que vêm sendo realizados para economizar energia (STRUVAY & FELLER, 2012). Por exemplo, o uso de linhagens bacterianas produtoras de queratinases ativas em temperaturas baixas a moderadas seria de grande relevância para a indústria de processamento do couro, não somente pela economia de energia, mas também pela redução dos impactos de produtos químicos utilizados normalmente no processo de depilação das peças (SHERIDAN *et al.*, 2000, BRANDELLI *et al.*, 2010). Além disso, o uso de linhagens de micro-organismos queratinolíticos psicrofílicos seria uma alternativa promissora na indústria avícola, onde os resíduos queratinosos, principalmente penas, são convertidos em complementos para ração animal através de moagem e cocção sob pressão. Esse processo, além de energeticamente custoso, produz suplementos com baixa digestibilidade e reduzido valor nutricional, dada a insolubilidade da queratina e a degradação de alguns aminoácidos durante o processo (GOPINATH *et al.*, 2015).

Em adição aos processos industriais, em alguns dos quais é possível o uso direto de linhagens proteolíticas não patogênicas ou de seus extratos brutos, a purificação enzimática é necessária nos demais processos biotecnológicos que envolvem química fina, como na produção de fármacos, cosméticos e reagentes utilizados em laboratório. Dessa forma, o desenvolvimento de meios para a purificação rápida e de baixo custo de enzimas constitui uma parte importante no estudo de biocatalizadores comercialmente relevantes. Muitos trabalhos desenvolvem técnicas cromatográficas para chegar a esse objetivo. No entanto, em muitas circunstâncias a expressão para purificação da enzima de interesse diretamente no organismo de origem pode ser dificultada, seja pela presença de fatores de virulência que inviabilizam sua utilização em larga escala ou pela expressão em níveis insatisfatórios da proteína alvo. Assim, a clonagem e expressão heteróloga é uma das melhores alternativas disponíveis.

Considerando a relevância do isolado A03 como produtor de peptidases extracelulares e a sua capacidade de degradar resíduos queratinosos, foi realizada a construção de uma biblioteca genômica para o *screening* funcional de enzimas proteolíticas ativas em baixas temperaturas. A partir dessa abordagem, uma peptidase foi isolada e

subclonada com sucesso em vetor pGEM-t Easy o que permitiu a determinação do *Open Reading Frame* do gene *a0301* e sua expressão em *E.coli* a partir do vetor pGEX-4T-2. A enzima, denominada A03Pep1, apresentou maior identidade com as queratinases de linhagens de *S. maltophilia*, uma bactéria filogeneticamente próxima de *Lysobacter*, também pertencente à Ordem Xanthomonadales, com linhagens reconhecidamente produtoras de queratinases em condições mesofílicas de temperatura (FANG *et al.,* 2014).

Apesar de a expressão heteróloga ter sido realizada com sucesso, não foi possível a purificação da enzima A03Pep1 fusionada à glutationa S-transferase (GST) através de cromatografia por afinidade, observando-se que a atividade enzimática foi quantificada em grande quantidade no sobrenadante dos cultivos. Um dos motivos seria a autólise da proteína precursora da queratinase e remoção de GST juntamente com os domínios N-terminais, durante o processamento que dá origem a forma madura da enzima. Fenômeno semelhante foi relatado por Fang e colaboradores (2014) durante o estudo da queratinase KerSMD, de *Stenotrophomonas maltophilia* BBE11-1, na qual a tentativa de purificação por cromatografia de afinidade ao níquel, através da adição de uma cauda de histidina à porção C-terminal da enzima não foi possível. Os autores argumentam que a autólise dos domínios PPC C-terminais de KerSMD seria responsável pela remoção da etiqueta de histidina durante a maturação da enzima. Uma estratégia para contornar esse problema, seria a adição da cauda de histidina imediatamente após o término da sequência madura da queratinase, no entanto, a ausência dos domínios C-terminais poderia comprometer o dobramento correto da enzima e por consequência a sua atividade. Ainda assim, foi possível a purificação da peptidase A03Pep1 diretamente dos sobrenadantes dos cultivos para indução da expressão através de duas etapas de ultracentrifugação, tornando possível a avaliação dos parâmetros bioquímicos da enzima.

Nos ensaios enzimáticos realizados com o substrato azocaseína, verificou-se que a peptidase A03Pep1 foi mais ativa a 40°C e pH9,0, apresentando alta atividade na faixa entre 30 e 40°C, resultado bastante similar àquele observado nos ensaios realizados com os sobrenadantes brutos do cultivo de *Lysobacter* sp. A03. Uma possibilidade seria o fato de que a maior parte da atividade enzimática extracelular da bactéria seja devida à peptidase A03Pep1, uma vez que ela pode desempenhar o papel principal na degradação dos substratos queratinosos tornando-os acessíveis às enzimas secundárias.

O efeito de inibidores de peptidases e íons metálicos confirmou que a enzima A03Pep1 é de fato uma serino-peptidase, pois sua atividade foi completamente perdida na presença do inibidor específico PMSF. No entanto, a atividade foi reduzida a menos de 50% pelo inibidor de metalo-peptidases EDTA. Esse resultado aparentemente contraditório é explicável pelo fato de que o EDTA é um forte quelante de íons metálicos, os quais influenciam fortemente a estabilidade de serino-peptidases. Adicionalmente, verificou-se o aumento da atividade da queratinase na presença de diversos íons, ressaltando-se Ca^{2+}. Daroit *et al.* (2011), trabalhando com a queratinase produzida por *Bacillus* sp. P45 demonstraram o efeito positivo do Ca^{2+} sobre a estabilidade térmica da enzima na concentração ótima de 3 mM. Na protease alcalina PAP da linhagem psicrofílica de *Pseudomonas* sp. TAC 18 foi relatado que a ligação de um íon cálcio no domínio proteolítico é responsável por uma mudança conformacional de 13-Å em um loop que torna o sítio ativo da enzima mais acessível na comparação com a forma mesofílica da mesma protease (AGHAJARI *et al.*, 2003).

A fim de avaliar a estabilidade térmica da queratinase A03Pep1, a enzima foi incubada em diferentes temperaturas por até 120 minutos. Observou-se que após 15 minutos de tratamento térmico a 55 e 70 °C, a enzima perdeu aproximadamente 60 e 90 % da atividade original, respectivamente. A característica de maior termolabilidade das enzimas adaptadas ao frio pode representar uma vantagem quando do seu uso em substratos sensíveis ao calor, como alimentos e medicamentos, por exemplo. No entanto, essa característica também pode trazer desvantagens para o emprego industrial direto de biocatalizadores provenientes de micro-organismos psicrofílicos, uma vez que sua baixa estabilidade dificulta a sua estocagem e incorporação às formulações. Dessa forma, o conhecimento da estrutura e mecanismos de ação das enzimas psicrofílicas também pode fornecer uma alternativa indireta para o desenvolvimento, através da engenharia genética, de enzimas que aliem uma maior estabilidade, tolerância em pH elevados e atividade em temperaturas reduzidas. Esse foi o caminho encontrado pela indústria de detergentes, onde as subtilisinas que compõe formulações ativas em baixas temperaturas foram “engenheiradas” para apresentarem essa característica baseando-se na estrutura da subtilisina de linhagens Antárticas de *Bacillus* (NARINX *et al.*, 1997).

No entanto, enquanto diversos estudos estruturais têm sido realizados a fim de identificar as bases moleculares da adaptação ao calor das proteínas dos organismos termofílicos, principalmente devido à facilidade de obtenção de cristais, os mecanismos moleculares da adaptação ao frio permanecem relativamente desconhecidos, devido ao número limitado de estruturas resolvidas de proteínas de psicrófilos, provavelmente por causa da sua termolabilidade e flexibilidade que dificultam os estudos cristalográficos (CAVICCHIOLI *et al*, 2011). Os métodos de modelagem comparativa oferecem uma alternativa e podem auxiliar na elucidação das bases moleculares da atividade de enzimas ativas no frio. Dessa forma, a estrutura tridimensional da peptidase A03Pep1 foi inferida através de modelagem por homologia. Apesar da estrutura geral da enzima ter se mostrado bastante semelhante àquela da homóloga mesofílica AprV2, utilizada como modelo, observou-se que o encurtamento de um loop próximo à região do sítio ativo pode facilitar o acesso do substrato ao sítio de ligação, o que pode responder pela alta atividade catalítica em baixas temperaturas. Na enzima AprV2, acredita-se que esta alça encubra parcialmente o sítio ativo, apesar da argumentação dos autores de que ela auxiliaria na ligação do substrato (KENNAN *et al.*, 2010). Vários trabalhos têm demonstrado que a estrutura das enzimas adaptadas ao frio é bastante similar àquela das enzimas mesofílicas e que em muitos casos pequenas substituições em alguns aminoácidos, principalmente próximos ao sítio ativo, auxiliam na flexibilização da estrutura e maior eficiência em temperaturas reduzidas (FELLER, 2013).

Este estudo permitiu identificar uma nova peptidase com atividade queratinolítica em temperaturas reduzidas, a partir do *screening* funcional do genoma da bactéria psicrofílica *Lysobacter* sp. A03. Essa enzima representa uma nova alternativa ao uso daquelas provenientes de micro-organismos mesofílicos e termofílicos, principalmente em termos de economia de energia em processos industriais como também contribui para o conhecimento dos mecanismos de adaptação das hidrolases provenientes de organismos de climas frios.

12 Conclusões

- Micro-organismos produtores de queratinases extracelulares ativas em baixas temperaturas foram isolados com sucesso do ambiente antártico;

- Eles foram capazes de degradar principalmente o substrato farinha de penas 1% a 20°C sob condições alcalifílicas;

- O isolado *Lysobacter* sp. A03 mostrou-se superior aos demais na sua capacidade de degradar queratina, sendo capaz de degradar farinha de penas quase completamente em 96 h;

- A construção de uma biblioteca genômica do isolado *Lysobacter* sp. A03 permitiu a identificação de um novo gene de uma peptidase queratinolítica denominado *a0301*;

- A maior identidade da sequência do gene foi com as serino-peptidases queratinolíticas KerSMF e KerSMD de *Stenotrophomonas maltophilia*;

- A análise dos domínios funcionais revelou que a enzima é sintetizada na forma de um precursor inativo formado por cinco regiões, sendo elas um peptídeo sinal, um inibidor N-terminal pertencente à Família I9, o domínio catalítico pertencente à Família S8 de serino-peptidases, um domínio PPC e um domínio PC relacionado às convertases da família da *kexina*;

- O gene foi expresso com sucesso no vetor pGEM-T Easy e no vetor pGEX-4-T 2 e a enzima, denominada A03Pep1 foi recuperada principalmente no sobrenadante dos cultivos;

- Não foi possível a purificação da enzima expressa a partir de cromatografia por afinidade, uma vez que possivelmente a etiqueta GST tenha sido clivada durante o processamento da peptidase, dessa forma, uma nova estratégia de purificação através de ultrafiltração foi empregada com sucesso para a obtenção da peptidase;

- Os ensaios enzimáticos revelaram que a peptidase foi mais ativa a 40°C, em condições alcalifílicas e sua atividade foi aumentada na presença de íons cálcio;

- A peptidase A03Pep1 apresentou termoestabilidade abaixo dos 50°C, perdendo atividade rapidamente em temperaturas superiores;

- A modelagem por homologia da estrutura 3D mostrou que a peptidase é bastante semelhante às serino-peptidases mesofílicas da família S8 e que a maior diferença encontrada foi a deleção de alguns resíduos de aminoácidos em uma região de alça situada próxima ao sítio ativo que aparentemente facilita o acesso do substrato ao seu sítio de ligação, podendo ser responsável pela atividade da enzima A03Pep1 em temperaturas reduzidas.

13 Perspectivas

- Realizar a superexpressão da peptidase A03Pep1 através da clonagem em vetor pET23a para a purificação da enzima utilizando-se cromatografia por afinidade;

- Avaliar, através de Ressonância Magnética Nuclear (RMN) a estrutura da peptidase A03Pep1 a fim de obter novas informações estruturais sobre a enzima e sua atividade em baixa temperatura;

- Continuar a prospecção de hidrolases de interesse biotecnológico e industrial através de *Genome Mining* a partir do genoma já sequenciado de *Lysobacter* sp. A03, visando a sua clonagem e expressão;

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15 CURRICULUM VITAE

PEREIRA, J. Q.

Nome: Jamile Queiroz Pereira

Nascimento: 23/03/1984 - Sapucaia do Sul/RS - Brasil

Endereço Profissional: Avenida Bento Gonçalves, 9500, prédio 43.212. Laboratório 218, Campus do Vale, CEP: 91501-970.

Telefone profissional: +55 51 3308-6249

E-mail: jamile.qp@gmail.com

Formação Acadêmica:

- 2012 – 2016: Doutorado no Programa de Pós-Graduação em Biologia Celular e Molecular. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil Orientador: Adriano Brandelli. Bolsista da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

- 2010 - 2012: Mestrado (*stricto senso*) no Programa de Pós-Graduação em Biologia Celular e Molecular. Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil Orientador: Adriano Brandelli. Bolsista da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

- 2001 – 2008: Graduação em Ciências Biológicas. Universidade do Vale do Rio dos Sinos, UNISINOS, São Leopoldo, Brasil. Orientador: Victor Hugo Valiati

Formação complementar

- 2008 – 2008: Extensão universitária em Sistemática Molecular. Universidade do Vale do Rio dos Sinos, UNISINOS, São Leopoldo, Brasil.

Estágios:

- 2006 - 2009: Estágio no Laboratório de Biologia Molecular. Bolsista FAPERGS. . Carga horária: 20 horas semanais. Orientador: Victor Hugo Valiati.
- 2004 - 2006 Projeto MONALISA: Monitoramento Ambiental Local de Impactos Sobre Arroios da bacia do rio dos Sinos. Bolsista CORSAN. Carga horária: 20 horas semanais. Orientador: Uwe Shultz.

Monitorias:

- 2010: Monitora do curso de Férias, edição de inverno: "Você conhece a Célula?" Carga Horária: 60 horas. Orientadora: Célia R. Carlini.
- 2005 - 2005: Monitora das disciplinas de Biologia Animal I, II e III. Carga horária: 8 horas semanais. Orientador: Gelson Fiorentin.

Vínculo institucional

- 2009 – 2010: Professora de Ciências Biológicas e Biologia. Escola Técnica Estadual Portão, Escola Estadual de Ensino Fundamental Portão Velho. Carga horária: 40. Regime: Integral.

Idiomas

- Inglês: Compreende razoavelmente, fala razoavelmente, escreve bem, lê bem.
- Espanhol: Compreende bem, fala pouco, escreve razoavelmente, lê bem.
- Francês: Compreende razoavelmente, fala razoavelmente, escreve pouco, lê razoavelmente.
- Português: Compreende bem, fala bem, escreve bem, lê bem.

Produção bibliográfica

Artigos completos publicados em periódicos

1. **PEREIRA, J. Q**.; AMBROSINI, A.; SANT'ANNA, F.H.; TADRA-SFEIR, M.; FAORO, H.; PEDROSA, F. O. SOUZA, E. M.; BRANDELLI, A.; PASSAGLIA, L. M. P. Whole-Genome Shotgun Sequence of the Keratinolytic Bacterium *Lysobacter* sp. A03, Isolated from the Antarctic Environment. *Genome Announcements*, v. 3, p. e00246-15, 2015.

2. **PEREIRA, J. Q**.; LOPES, F. C.; PETRY, M. V.; MEDINA, L. F. C.; BRANDELLI, A.. Isolation of three novel Antarctic psychrotolerant feather-degrading bacteria and partial purification of keratinolytic enzyme from *Lysobacter* sp. A03. *International Biodeterioration & Biodegradation*, v. 88, p. 1-7, 2014.

3. HERMANNS, G.; FUNCK, G. D.; SCHMIDT, J. T**.; PEREIRA, J. Q**.; BRANDELLI, A.; RICHARDS, N. S. P. S.. Evaluation of Probiotic Characteristics of Lactic Acid Bacteria Isolated from Artisan Cheese. *Journal of Food Safety*, v. 34, p. 380-387, 2014.

4. PIENIZ, S.; ANDREAZZA, R.; **PEREIRA, J. Q.**; OLIVEIRA CAMARGO, F. A.; BRANDELLI, A.. Production of Selenium-Enriched Biomass by *Enterococcus durans*. Biological Trace *Element Research*, v. 155, p. 447-454, 2013.

5. LOPES, F. C.; TICHOTA, D. M.; PEREIRA, J. Q.; SEGALIN, J.; O., RIOS, A.; BRANDELLI, A.. Pigment Production by Filamentous Fungi on Agro-Industrial Byproducts: an Eco-Friendly Alternative. *Applied Biochemistry and Biotechnology*, v. 171, p. 616-625, 2013.

6. LOPES, F. C., SILVA, L. A. D. E., TICHOTA, D. M., DAROIT, D.J., VELHO, R. V., **PEREIRA, J. Q.**, BRANDELLI, A.. Production of proteolytic enzymes by a keratin-degrading *Aspergillus niger*. *Enzyme Research*. , v.2011, p.1 - 9, 2011.

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1. PEREIRA, J. Q., LOPES, F. C., TICHOTA, D. M., MEDINA, L. F. C., BRANDELLI, A. Isolamento de microrganismos queratinolíticos provenientes do continente antártico com potencial utilização pela indústria avícola. In: XX Congreso Latinoamericano de Microbiología IX Encuentro Nacional de Microbiólogos, 2010, Montevidéo. Libro de resúmenes. , 2010. p.68.

2. LOPES, F. C., TICHOTA, D. M., VELHO, R. V., PEREIRA, J. Q., RIOS, A. O., BRANDELLI, A. Padronização de técnicas moleculares para a identificação de fungos produtores de pigmentos In: IV Simpósio de Microbiologia Aplicada I Encontro Latino-Americano de Microbiologia Aplicada, 2010, Porto Alegre.

3. TICHOTA, D. M., LOPES, F. C., PEREIRA, J. Q., RIOS, A. O., BRANDELLI, A. Utilização de resíduos para a produção de metabólitos ativos de Penicillium chrysogenum. In: XX Congreso Latinoamericano de Microbiología IX Encuentro Nacional de Microbiólogos, 2010, Montevidéo. Libro de resúmenes. , 2010. p.55 – 55.

4. JUNG, D. M. H., PEREIRA, J. Q., CHRISTOFF, A. U., VALIATI, V. H. Avaliação filogenética das amostras de *Oxymycterus nasutus* do Rio Grande do Sul In: II Mostra de Pesquisa e iniciação científica da ULBRA de Gravataí, 2008, Gravataí.

5. PEREIRA, J. Q., JUNG, D. M. H., CHRISTOFF, A. U., VALIATI, V. H. Estruturação genética e Geográfica em *Oxymycterus nasutus* (Rodentia: Sigmodontinae) no Rio Grande do Sul In: IX Salão de Iniciação Científica da PUCRS, 2008, Porto Alegre.

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8. SILVEIRA, L. K., PEREIRA, J. Q., ZARDO, D., LACERDA, P. S. Delimitação de áreas de preservação permanente no município de Araricá através dos programas livres Spring e Google Earth. In: Mostra UNISINOS de Iniciação Científica, 2007, São Leopoldo.

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