UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS BIOLÓGICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

Purificação e Caracterização de Protease com Atividade Colagenolítica produzida por *Actinomadura* sp.

Aluno: Luciana Lopes Silveira
Orientador: Ana Lúcia Figueiredo Porto
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Recife, fevereiro de 2015.

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Purificação e Caracterização de Protease com Atividade Colagenolítica produzida por *Actinomadura* sp.

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como requisito final para a obtenção do grau de Mestre em Ciências Biológicas, área de concentração Biotecnologia, linha de pesquisa Biomateriais e Microbiologia Básica e Aplicada.

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Seleção de meio líquido para a produção de antibiótico por linhagens *Streptomyces* spp. isoladas da rizosfera da planta xique-xique (*Pilosocereus gounellei)* do bioma da Caatinga/ Luciana Lopes Silveira – Recife: O Autor, 2012.

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DEDICATÓRIA

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—[…] E nunca considere seu estudo uma obrigação, mas sim como uma oportunidade invejável de aprender, sobre a influência libertadora da beleza no domínio do espírito, para seu prazer pessoal e para o proveito da comunidade à qual pertencerá seu trabalho futuro.‖ Albert Einstein.

RESUMO

As proteases são enzimas proteolíticas, as quais apresentam grande interesse pelas indústrias em diversas áreas. As proteases que possuem a capacidade de degradação da hélice de colágeno tem sido estudadas para a aplicação dos peptídeos de colágeno, no tratamento de doenças como: hipertensão, inibindo a enzima conversora de angiotensina II (ECA), assim como da própria enzima pelas indústrias farmacêuticas em tratamentos de feridas com necrose ou no tratamento da doença de Dupuytren. As proteases com atividade colagenolítica, capazes de degradar o colágeno, podem ser obtidas através de diversas fontes, e dentre estas, os micro-organismos são escolhidos devido à sua diversidade bioquímica e susceptibilidade para a manipulação genética. Devido ao potencial dessas enzimas, existe uma procura de novas fontes microbianas, as quais seja possível obter a protease com rapidez e baixo custo no processo de purificação. O presente trabalho teve como objetivos purificar e caracterizar protease com atividade colagenolítica obtida através da Actinobactéria *Actinomadura* sp., isolada do solo *Licania rígida* BETH. O microorganismo foi cultivado em meio a base de farinha de soja, líquido metabólico foi concentrado através do processo de precipitação com acetona (70%), seguida foi purificado parcialmente pelo processo de cromatografia de troca iônica em resina de DEAE-sephadex (G-50). A protease com atividade colagenolítica foi eluida, utilizando gradiente de concentração, com solução de NaCl 0,1M em tampão Tris-HCl, pH 8,0. O cromatograma foi obtido através da leitura no comprimento de onda de 280nm. A tabela de purificação foi obtida através da realização da atividade proteásica, da colagenolítica e da concentração proteica em cada etapa de purificação. Para a determinação do peso molecular e da pureza foi realizado a eletroforese em gel (SDS-page). A caracterização da amostra purificada foi realizada através de ensaios de: termoestabilidade, a enzima foi submetida às termperaturas de 50° - 80°C durante 30 minutos; temperatura ótima, nas temperaturas de 30°C – 80°C; pH ótimo, variando o pH5-6 (0,1M do tampão de ácido acético- acetato de sódio) e 7-9 (0,1M do tampão Tris- HCl); efeito de inibidores, utilizando os inibidores fluoreto de fenilmetanosufonil, dodecil sulfato de sódio, beta- mercaptoetanol e pepstatina em contato com a enzima durante 60 minutos; o efeito de íons metálicos, foi utilizado soluções com ions divalentes (Fe 2+, Mg 2+, Ca 2+, Zn2+) em contato com a enzima durante 60 minutos; e efeito de frequência ultrassônica, enzima submetida a frequência ultrassônica (40 kHz), em diferentes intervalos de tempo. Os resultados obtidos demonstraram que a protease é possível purificar utilizando o gradiente de de NaCl 0,1M em tampão Tris-HCl, pH 8,0, assim como uma atividade colagenolítica específica de 31.094,89 U/mg de proteína, com um fator de purificação de 9,81. Na eletroforese foi possível observar duas bandas, com pesos moleculares de 20 KDa e 45,0 KDa. A enzima apresentou termoestabilidade até 60°C, a temperatura ótima de atividade em 50°C. Sob condições de variação de pH, a atividade enzimática apresentou maiores atividade colagenolítica em faixa de pH 7 a 8, reduzindo bruscamente em pH 9,0. Quando submetida ao efeito da frequência ultrassônica, a enzima após 10 minutos apresentou aumento de 50% de sua atividade colagenolítica inicial. Sendo assim, essa nova biomolécula apresenta potencial biotecnológico, uma vez que o processo de produção envolve um substrato, a farinha de soja, que é amplamente encontrado no Brasil, e o protocolo de purificação desenvolvido foi eficaz para a obtenção da protease com atividade colagenolítica.

Palavra-chave: *Actinomadura* sp., colagenase, protease, purificação, caracterização

ABSTRACT

Proteases are proteolytic enzymes, which have high interest by industries in various fields. Protease having the helix collagen degradation capacity has been studied for application of the collagen peptides in the treatment of diseases such as hypertension, inhibiting the angiotensin II converting enzyme (ACE), the enzyme itself as well as by pharmaceutical industries in treatment of wounds with necrosis or treatment of Dupuytren's disease. Proteases with collagenolytic activity capable of degrading the collagen can be obtained from several sources, and among them, the micro-organisms are chosen because of their biochemical diversity and susceptibility to genetic manipulation. Because of the potential of these enzymes, there is a demand for new microbial sources, which can be obtained with the protease speed and low cost in the purification process. This study aimed to purify and characterize protease with collagenolytic activity obtained by actinobacteria *Actinomadura* sp., Isolated from soil of *Licania rigida* BETH. The microorganism was grown in medium based on soybean flour, metabolic liquid was concentrated by precipitation process with acetone (70%), then was partially purified by ion exchange chromatography procedure resin DEAE-Sephadex (G-50). The protease collagenolytic activity was eluted with using a concentration gradient with 0.1M NaCl solution in Tris-HCl buffer, pH 8.0. The chromatogram was obtained by scanning the wavelength of 280nm. The purification table is obtained by performing the protease activity of collagenolytic and the protein concentration in each purification step. To determine the molecular weight and the purity was conducted gel electrophoresis (SDS-PAGE). Characterization of the purified sample was conducted through tests: thermal stability, the enzyme was subjected to termperaturas 50 ° - 80 ° C for 30 minutes; optimum temperature at temperatures of 30 ° C - 80 ° C; optimum pH, varying the pH5-6 (0.1 M sodium acetate acetic acid-buffer) and 7-9 (0.1 M of buffer Tris- HCl); effect of inhibitors using the inhibitors fenilmetanosufonil fluoride, sodium dodecyl sulfate, beta-mercaptoethanol and pepstatin contact with the enzyme for 60 minutes; The effect of metal ions, was used solutions with divalent ions (Fe 2+, Mg 2+, Ca 2+, Zn 2+) in contact with the enzyme for 60 minutes; ultrasonic frequency and effect, subject enzyme ultrasonic frequency (40 kHz) at different time intervals. The results obtained demonstrated that the protease can be purified using gradient of 0.1 M NaCl in Tris-HCl buffer, pH 8.0, as a specific collagenolytic activity 31094.89 U / mg protein with a factor of purification 9.81. The electrophoresis was observed two bands with molecular weights of 20 KDa and 45 KDa. The enzyme showed thermostability at 60 ° C, the optimum temperature for activity at 50 ° C. Under conditions of varying pH, the enzyme activity showed higher collagenolytic activity in the pH range 7 to 8, reducing sharply at pH 9.0. When submitted to the effect of ultrasonic frequency, the enzyme after 10 minutes increased by 50% from its initial collagenolytic activity. Therefore, this presents new biotechnological potential biomolecule, since the production process involves a substrate, soybean flour, which is widely found in Brazil, and purification protocol was developed to obtain effective protease with collagenolytic activity.

Keyword: Actinomadura sp, collagenase, protease, purification, characterization.

.

LISTA DE FIGURAS

LISTA DE FIGURAS DO ARTIGO

LISTA DE TABELAS

LISTA DE TABELAS DO ARTIGO

LISTA DE ABREVIATURAS

IUB :União Internacional de Bioquímica

AA: Ácido Ascórbico

PEG: Polietilenoglicol

CCD: Cromatografia de camada delgada

CGS: Cromatografia gás-sólido

CLS: Cromatografia líquido-solido

CSS: Cromatografia supercrítica com fase estacionária solida

CP: Cromatografia em papel

CGL: Cromatografia gás-liquido

CLL: Cromatografia liquido-liquido

CTI: Cromatografia por troca iônica

CB: Cromatografia por bioafinidade

CE: Cromatografia por exclusão

D_R: Distancia de Migração do Soluto

D_M: Distância de Migração da Fase Movel

W_b: Distancia entre o picos

SDS- PAGE: Eletroforese em gel de poliacrilamida contendo dodecil sulfato de sódio

Sumário

1. INTRODUÇÃO

O bioma da Caatinga é caracterizado por altas temperaturas, estação seca durante cerca de seis meses, umidade baixa e precipitação anual em torno de 250 a 500 mm. Na estação das chuvas, a qual é chamada de inverno, as temperaturas são mais amenas e sua duração é curta..

A *Licania rigida* BENTH, pertence à família Chysobalanaceae, conhecida como Oiticica, é uma planta do Nordeste do brasileiro, com uso na medicina popular, suas folhas são utilizadas no tratamento de diabetes e inflamações.

A rizosfera é região do solo com grande diversidade microbiana que varia de acordo com o terreno, clima, a oferta de nutrientes e os microrganismos ali presentes.

As actinobactérias são abundantes no solo e ocorrem em média um milhão de células por grama de solo, são heterotróficos importantes na decomposição da matéria orgânica, podendo ocorrer em ambientes aquáticos e também colonizando plantas.

Os gêneros mais conhecidos de actinobactérias são: *Streptomyces*, *Actinomadura*, *Nocardiopsis*, *Olindenses*.

Algumas actinobactérias podem causar enfermidades no homem como: doenças pulmonares (*Nocardia, Rhodococcus, Mycobacterium*), infecções sistêmicas (*Nocardia, Rhodococcus, Mycobacterium*), micetoma (*Actinomadura, Nocardiopsis, Streptomyces* e *Nocardia*), outras infecções cutâneas (*Nocardia*, *Dermatophilus*), bem como infecções oportunistas.

Os metabólitos secundários das actinobactérias podem apresentar atividade antitumoral; antifúngica; bactericida; propriedades proteolíticas, decomposição de queratinas, quitinas, amido, além de auxiliar também no ciclo do nitrogênio e aminoácidos, atuando também como inibidor enzimático; além de degradar matéria orgânica.

As proteases são enzimas proteolíticas que estão presentes em todos os organismos. Portanto, para estudo, os micro-organismos são a fonte preferida devido à sua diversidade bioquímica e susceptibilidade para a manipulação genética.

As colagenases são enzimas proteolíticas que podem hidrolisar o colágeno nativo (região helicoidal) e o colágeno desnaturado, em pequenos fragmentos. O mecanismo de ação destas enzimas depende de sua origem, as colagenases oriundas de bactérias, clivam a ligação peptídica entre um aminoácido neutro e glicina na região não polar da molécula do colágeno.

A fonte mais comum de colagenases é *Clostridium hystolyticum*, microorganismo patogénico; sendo, fontes alternativas mais seguras dessas enzimas são esperadas, incluindo, entre outras, espécies de, *Bacillus*, *Streptomyces*, *Penicillium* (ROSSO et al., 2012), e *Cândida*.

As proteases específicas possuem um papel fundamental na economia, pois representam o terceiro maior grupo de enzimas industriais. As proteases com capacidade de degradar o colágeno são

importantes pois os peptídeos biologicamente ativos gerados tem diversas aplicações biotecnológicas e industriais e terapêuticas, como conservantes não alérgicos para medicamentos, ingredientes para alimentação parenteral, tratamento da diabetes, além de apresentarem atividade antioxidante, antimicrobiana e anti-hipertensiva.

2. OBJETIVOS

2.1.Geral:

- Purificar e caracterizar a protease com atividade colagenolítica obtida através de *Actinomadura* sp.

2.2.Específicos:

- Purificar a protease com atividade colagenolítica através de métodos cromatográficos convencionais.
- Avaliar o grau de Pureza através da eletroforese SDS-PAGE.
- Caracterizar a enzima quanto ao pH ótimo, termoestabilidade, temperatura ótima, efeito de íons e inibidores na atividade enzimática colagenolítica.
- Analisar o efeito da frequência ultrassônica na atividade colagenolítica.

CAPÍTULO 1
REVISÃO BIBLIOGRÁFICA

3. REVISÃO BIBLIOGRAFICA

3.1.Oiticica (Licania rígida BENTH)

A *Licania rigida* BENTH, pertence à família Chysobalanaceae, popularmente conhecida como Oiticica, é uma planta do Nordeste do brasileiro (DINIZ et al., 2008), caracterizada por ser uma planta perene, de grande longevidade e porte. Geralmente, as plantas são encontradas nas marginais de rios e riachos.

Figura 1- Oiticica (*Licania rigida* BENTH) (VALE VIVA VERDE)

A partir das espigas racemosas, acontece a inflorescência, entre o mês de Junho até outubro. Suas flores apresentam um caráter hermafrodito. A abertura das flores coincide com a época mais seca do ano (SILVA et al., 2010).

Suas sementes são usadas para extrair óleo pelas indústrias, nas décadas de 30 a 50, servindo de fonte de renda. Em algumas regiões utilizam as suas folhas no tratamento medicinal, contra a de diabetes e inflamações (DINIZ et al., 2008; ALVES & NASCIMENTO).

Segundo Silva et al.(2010) as flores de oiticica são atrativas para as abelhas para a coleta de néctar, uma vez que esta planta apresenta potencial considerável para produção de mel.

3.2.Actinobactéria

3.2.1. Características Gerais

As actinobactérias compreendem um grupo de bactérias Gram positivas que tem um alto conteúdo de G+C, ou seja, alto teor de guanina e citosina. As semelhanças entre os actinomicetos e os fungos vêm de observações que mostraram o micélio filamentoso e as colônias com diversas colorações surgindo o nome actinomicetos que significava actino= radiado e micetos = fungos, ou seja, “fungos radiados”

(CUNHA et al., 2009; JEFFREY, 2008; FLÄRDH & BUTTNER,2009). Apresentam diferentes tipos morfológicos, que varia de acordo com o gênero (FLÄRDH & BUTTNER, 2009).

A partir do esgotamento de nutrientes pode ser observada a produção de metabólitos secundários, a diferenciação morfológica é desencadeada formando diversos tipos de esporóforos que podem ser espiralado, verticilado, ondulado, etc (FLÄRDH & BUTTNER, 2009).

O micélio é formado por células procarióticas longas com vários nucleóides que podem penetrar ou aderir a diferentes tecidos, além de ajudar na secreção de enzimas e outros metabólitos. A proximidade entre o substrato e os esporos, torna necessária uma menor quantidade de enzima ser secretada para atingir níveis efetivos de crescimento (EMBLEY & STACKEBRANDT, 1994).

As actinobactérias são abundantes no solo importantes na decomposição da matéria orgânica, podendo ocorrer em ambientes aquáticos e também colonizando plantas (CASTRO et al., 2010; RAHMAN et al., 2011). Embora em baixa frequência algumas actinobactérias podem causar enfermidades no homem como: doenças pulmonares (*Nocardia*, *Rhodococcus*, *Mycobacterium*), infecções sistêmicas (*Nocardia*, *Rhodococcus*, *Mycobacterium*), micetoma (*Actinomadura*, *Nocardiopsis*, *Streptomyces* e *Nocardia*), outras infecções cutâneas (*Nocardia*, *Dermatophilus*).

As actinobactérias produzem enzimas intracelulares, em menor quantidade quando comparadas com as extracelulares, as quais têm a função principal de degradar macromoléculas presentes no meio ambiente, como celulose, amido, e muitos outros componentes orgânicos encontrados no solo, para que possam ser absorvidos como nutrientes por essas bactérias filamentosas (CHATER, 2006; TORTORA et al., 2003; MADIGAN et al., 2003; PEREIRA JR., 2008).

O processo biotecnológico envolvendo micro-organismos é utilizado em diversas áreas como na indústria, onde o objetivo é produzir compostos que possam ser utilizadas para a comercialização; na agropecuária que é utilizada para o controle biológico de praga e vetores em plantas e animais; no setor alimentício, onde pode ser empregado na produção de bebidas, pães, queijos, enzimas; além da sua aplicação na recuperação ambiental (OLIVEIRA *et al.*, 2006).

Os produtos que são sintetizados por microrganismos podem ser classificados de três formas, levando em consideração diferentes níveis de complexidade molecular, tais como: produtos finais do metabolismo - etanol, ácido lático, ácido butírico, e outros compostos derivados de processos anaeróbicos; produtos intermediários do metabolismo primário - aminoácidos, nucleotídeos, vitaminas e enzimas; e produtos de metabolismo secundário - antibióticos e toxinas. Os metabólitos secundários das actinobactérias podem apresentar atividade antitumoral; antifúngica; bactericida; propriedades proteolíticas, decomposição de queratinas, quitinas, colágeno e amido, além de auxiliar também no ciclo do nitrogênio e aminoácidos, atuando como inibidor enzimático (KITOUNI et al., 2005, WANG & VINING, 2003); além de degradar matéria orgânica (CHATER, 2006; KENNEDY, 1999; VERNEKAR et al., 2001; VODA et al., 2003; UKUZI et al., 2005).

3.2.2. Actinomadura sp.

O gênero *actinomadura* sp., pertence a família das actinobactérias, sendo o mais frequente após o gênero *Streptomyces* sp. No entanto, os gêneros *Micromonospora*, *Actinomadura*, *Streptoverticillium*, *Actinoplanes*, *Nocardia*, *Saccharopolyspora* e *Streptosporangium* spp. possuem cada vez mais um papel importante na produção de um amplo espectro de metabolitos secundários, sejam eles produção de antibióticos, antitumorais, xilanases, além de atividades como antimalárica (Jayaprakashvel, 2012; Manivasagan, 2013)

Esse gênero é muito estudado a produção de metabólitos secundários, tais como antibióticos e antitumorais, como pode ser observada na tabela 1.

Tabela 1 - Principais espécies de Actinomadura e seus produtos de interesse biotecnológico.

Linhagem	**Produto Biotecnológico**	**Referência**
Actinomadura hibisca	Antiviral	NAPAN et al 2012
Actinomadura hibisca	Antifungico	NAPAN et al 2012
Actinomadura keratinilytica strain Cpt29	Queratinase	HABBECHEM et al., 2014
Actinomadura namibiensis	Antibióticos	KRAWCZYK et al., 2013
***Actinomadura* sp.**	Anticancer	HAN et al. ,2003
***Actinomadura hibisca* P157-2.**	Antifungico	PAUDELA et al, 20011
***Actinomadura* sp. 007**	Anticancer	HAN et al, 2005
***Actinomadura* sp. BCC27169**	Antibióticos	INTARAUDOM et al, 2014
***Actinomadura* sp. S14**	Xilanase	SRIYAPAI et al, 2011
***Actinomadura* sp. SBMs009**	Anti-inflamatório	SIMMONS et al, 2011
Actinomadura mexicana	Protease	QUINTANA et al, 2003
***Actinomadura meyerii* sp.**	Proteases	QUINTANA et al, 2003

Embora seja muito frequente entre as actinobactérias, existem raros estudos sobre a sua capacidade de produção de enzimas protésicas com atividade colagenolítica.

3.3.Protease

Tradicionalmente as proteases ou peptidases são enzimas que catalisam a hidrólise de ligações peptídicas em proteínas ou peptídeos, liberando peptídeos de tamanho variável ou aminoácidos livres. Na nomenclatura internacional de classificação de enzimas (EC), as peptidases pertencem à classe 3 e subclasse 3.4, que ainda está dividida em dois grandes grupos: as exo e as endopeptidases.

As exopeptidases clivam ligações peptídicas nas extremidades N ou C terminal das cadeias polipeptídicas e podem ser denominadas de aminopeptidases e carboxipeptidases, respectivamente.

As endopeptidases atuam preferencialmente nas regiões internas das cadeias polipeptídicas, e as proximidades dos grupos N ou C terminais têm um efeito negativo na atividade enzimática (SILVA-LÓPEZ, 2010).

Representam o terceiro grupo de enzima utilizado na indústria. Esse interesse é devido a sua atuação utilizada em diversas atividades industriais tais como processamento de alimentos, bebidas, formulação de detergentes, processamento de couro e pele, no amaciamento de carne e desenvolvimento de agentes terapêuticos (CHANALIA, 2011; SILVA NEVES et al., 2006).

A capacidade de produção de proteases por vegetais e animais, não é capaz de suportar a demanda industrial, por isso, nos últimos anos, houve um aumento de interesse sobre a produção de proteases a partir de microrganismos, como fonte alternativa. A protease microbiana tem como vantagem uma diversidade bioquímica e susceptibilidade para manipulação genética (CHANALIA, 2011).

Ha, Bekhit & Carne (2014) estudaram os efeitos das formas L- e iso do ácido ascórbico (AA) sobre a atividade de quatro proteases vegetais e três proteases microbianas e observaram a capacidade de regular a atividade das proteases investigadas através da utilização de uma concentração adequada da isoforma de AA.

Yariswamy et al. (2013) investigar o potencial de cicatrização proteases produzidas de *Wrightia tinctoria*, no final do estudo, concluiu que as serinoproteases purificadas são capazes de promover diretamente a cicatrização nos animais testados.

3.3.1. Aplicação de Proteases

Dentre o grupo mais importante de enzimas industriais, as proteases possuem um papel importante por está presente em processos como o processamento do couro (VIJAYARAGHAVAN,LAZARUS & VICENTE,2013), detergente (CASTRO et al., 2004), de preservação de alimentos(WALSH, 2002) e como agente terapêutico (MICKELSON et al., 2014; SALAS-CANSADO et al.,2013).

Durante o processamento do couro, as proteases são usadas para auxiliar a remoção da epiderme e do pêlo, durante o tratamento do couro nos curtumes, para melhorar o produto final. (VIJAYARAGHAVAN,LAZARUS & VICENTE, 2013)

As proteases também estão presentes na composição de detergentes, sendo responsável pela remoção de mancha de óleo e gorduras.(CASTRO et al., 2004).

A utilização de proteases em alimentos pode acontecer para melhorar as características nutricionais, retardar a deteorização, modificação de propriedades funcionais, prevenção da mudança de sabores e odores (PARDO ET AL., 2000).

Uma das aplicações da protease como agente terapêutico, foi observado em estudos veem sendo realizados em países como a Espanha, Estados Unidos, onde utilizam a aplicação da protease colagenolítica para o tratamento da síndrome de Dupuytren, sendo possível a conclusão de que o tratamento com a aplicação da protease, além de eficaz, é mais econômico, menos invasivo, do que a cirurgia aconselhável.(MICKELSON et al., 2014; SALAS-CANSADO et al., 2013)

3.4.Colagenase

Por definição, as colagenases são proteases capazes de degradar o colágeno nativo e desnaturado. São produzidas por vegetais (KIM, GUDDAT & OVERALL, 2007), animais (WU et al., 2010) e Microrganismos (ROSSO et al, 2012). Assim como as proteases, os microrganismos têm sido mais estudados para a obtenção de colagenases (SANDHYa et al, 2005).

Podem ser classificadas em metalocolagenases e serinocolagenases. As metalocolagenases tem em sua estrutura o íon zinco, e em geral necessitam do íon cálcio para obter estabilidade e uma melhor atividade (GROSS & LAPIERE, 1962; PETERKOFSKY, 1982).

Acredita-se que o mecanismo de ação de cada enzima varia de acordo com a sua origem. As colagenases produzidas pelo fungo (*Clostridium histolyticum*) são capazes de clivar em diversos sítios. As colagenases produzidas por fibroblastos humanos são capazes de clivar apenas um sítio (KANTH et al., 2008, HUEBNER et al., 2010).

A colagenase pode ser usada para tratamento de doenças como o tratamento da Doença de Dupuytren, estudos revelam que o tratamento além de eficaz, também mostra um custo menor que o método cirúrgico, além de haver uma menor necessidade de acompanhamento com fisioterapeutas e médicos após o procedimento (MEHTA & BELCHER, 2014; MICKELSON et al., 2014; SALAS-CANSADO et al.,2013).

Há relatos da utilização da colagenase para o tratamento de ulceras nos pés de pacientes diabéticos. A pesquisa concluiu que o uso de pomadas com a colagenase é eficiente, além de apresentar um menor custo para o tratamento (TALLIS et al., 2013).

A colagenase presente no corpo humano pode atuar em diversas funções de acordo com tecido. A colagenase presente nos ossos auxilia no processo de absorção dos osteoclastos, como descrito por Søe, Merrild & Delaissé (2013). Está presente também nos fibroblastos, atuando como regulador das fibras de colágeno.

3.5.Colágeno

O colágeno, além de ser o principal componente de pele, ossos, tendões, cartilagem e dentes, também está presente na matriz extracelular do tecido dos vertebrados (UITTO et al., 2008). Essa proteína pertence a superfamília de proteínas fibrosas estruturais e insolúveis encontradas em organismos multicelulares. (RAVANTI & KÄHÄRI, 2000; UITTO et al, 2008, CHUNG & UITTO, 2010; DABOOR et al., 2010)

O colágeno é uma proteína extracelular, a qual tem sua forma molecular de bastão, tendo cerca de 3000Å de comprimento e 15Å de diâmetro. (BERG, TYMOCZKO & STRYER, 2007).

É chamada de tropocolágeno, a unidade do colágeno é composto por três cadeias peptídicas formando uma tripla hélice, chamada de cadeia alfa (α), a qual contem cerca de 1000 aminoácidos. (DABOOR et al, 2010; BERG, TYMOCZKO & STRYER, 2007). A hélice é estabilizada pela repulsão estérica de anéis de pirrolidina dos radicais de prolina e hidroxiprolina (Figura 2)

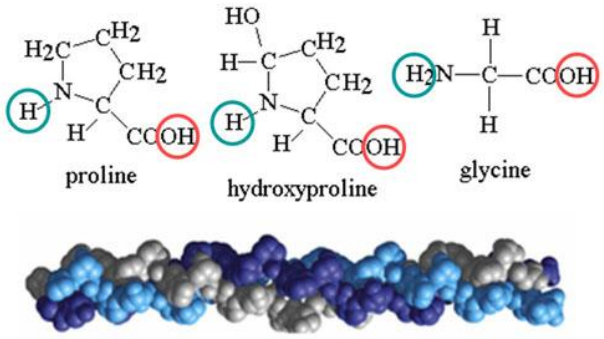

Figura 2- Composição da hélice de colágeno (BERG, TYMOCZKO & STRYER, 2007)

As presenças desses anéis se repelem entre si, gerando a forma helicoidal, que possui cerca de três aminoácidos por volta.

A tripla hélice de colágeno é formada pelo enovelamento das cadeias α na conformação helicoidal, o qual será determinado de acordo com a sequência primária da cadeia peptídica. A ligação dessas hélices se dá através de pontes de hidrogênio que se formam entre o grupamento peptídico NH da glicina e os CO em outras cadeias. (UIITO et al., 2008; BERG, TYMOCZKO & STRYER, 2007). (Figura 3a e 3 b)

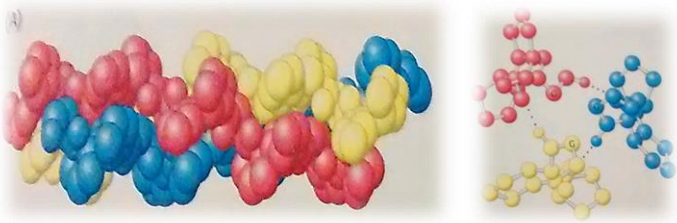

Figura 3- Estrutura da tripla helice de colágeno. (A) Modelo compacto da estrututra. (B) Secção transversal da fita de colágeno. (BERG, TYMOCZKO & STRYER, 2007)

Aproximadamente 30% das proteínas do corpo humano, já foram descritas cerca de 30 tipos diferentes de colágenos. Os tipos de colágeno são classificados baseados na diferente expressão gênica da cadeia peptídica durante a formação do tecido (DI LULLO et al., 2002).

Veeruraj et al. (2015) descreveu em seu trabalho a caracterização e identificação de colágenos extraídos a partir da pele de Lula, como sendo colágenos do tipo I, devido ao tipo de cadeia presente.

3.6. Produção de Enzimas

3.6.1. Fermentação

Existem basicamente dois tipos de fermentações, a fermentação submersa, a mais comum para a produção de enzimas em escalas industriais, a fermentação semi-sólida, utilizada para a fermentação industrial em alguns países orientais (SANT'ANNA JR, 2001).

Para a produção enzimática, poucos são os processos prévios necessários, sendo o de maior importância o pré- inóculo. O qual nada mais é do que um cultivo, sob agitação inoculada com a cultura estoque durante um determinado tempo, o seu valor para inoculo no fermentador principal pode variar de 1 a 10% (p/v). O processo de fermentação pode ser resumido pela figura 4. Durante a transferência devem ser observados diversos aspectos, entre eles a presença de contaminantes e os aspectos macroscópicos do microorganismo para determinar se houve algum tipo de mutação (SANT'ANNA JR, 2001).

Outro fator que pode influências a fermentação de um microorganismo, é o meio de cultura utilizado. Os meios de cultura podem ser o meio de cultura sintético e o natural.

Porém para as indústrias, tem-se buscado a utilização de meios com a maior quantidade de matéria primas naturais, devido ao elevado custo dos meios sintéticos (SANT'ANNA JR, 2001).

Cultura estoque

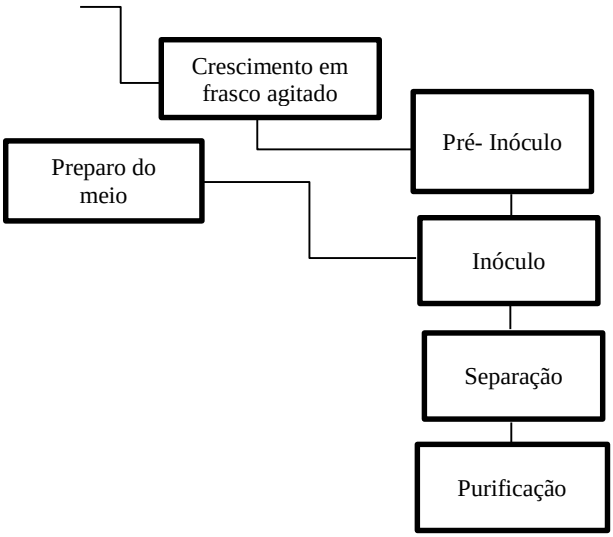

Figura 4 - Etapas no processo de fermentação (SANT'ANNA JR, 2001)

3.6.2. Separação e Concentração de Enzimas

Os processos usados para a separação e concentração da enzima, variam de acordo com a sua localização, sendo ela intracelular ou extracelular (SANT'ANNA JR, 2001).

Nas enzimas extracelulares, elas estão presentes no líquido metabólico, sendo assim, são menos complexos para a sua obtenção. É necessário ser feita a separação do líquido metabólico da massa celular processo que pode ser realizado através de centrifugação ou filtração. Com posterior processo de concentração enzimática. Esse processo pode ser através de filtração em meio filtrante de celulose ou diluir o preparado enzimático a níveis satisfatórios e acondicionado com estabilizantes SANT'ANNA JR, 2001; GE Healthcare Life Sciences).

As enzimas intracelulares, a separação da biomassa já é por si só uma etapa de concentração enzimática. Porém os processos necessários para a ruptura celular, como moagem com esferas de vidro, homogeneização em alta pressão, sonicação e congelamento, além de tratamentos químicos e enzimáticos, acabam por aumentar o custo na produção dessas enzimas (SANT'ANNA JR, 2001; ABRAHÃO NETO, 2001).

3.6.3. Precipitação

Para eliminar impurezas e aumentar a concentração da enzima alvo, normalmente utiliza-se da técnica de precipitação, sendo uma forma rápida e eficiente (ABRAHÃO NETO, 2001).

A precipitação de força iônica utiliza de altas concentrações de salinas, sendo os sais mais usados o sulfato de amônio e sulfato de sódio, para a precipitação, pois os mesmo tem a capacidade de promover a remoção da água de hidratação da proteína, tornando-a insolúvel. Essa técnica tem se

difundido devido a sua fácil execução e por obter bons rendimentos. Porém apresenta baixa seletividade, por provocar corrosão em equipamentos, além de não haver recuperação para reuso dos reagentes (SANT'ANNA JR, 2001).

A escolha do sal é determinada por sua eficácia, sendo determinada de acordo com a natureza do ânion, sendo os polivalentes mais eficazes no aumento da força iônica (ABRAHÃO NETO, 2001).

Durante a precipitação com sal, o mesmo é adicionado ao sobrenadante até que a porcentagem (%) de saturação em que a enzima precipite seja alcançada. A quantidade vai variar de acordo com a composição do extrato. A adição do sal deve ser feita de forma lenta e gradual, sob agitação para favorecer a homogeneização. Em seguida, deve ser centrifugado para a formação do "pellet", o qual com o auxilio de um tampão é redissolvido. Geralmente devido a grande quantidade sal usado para a precipitação é necessário a adiciona uma etapa para a dessalinização, como a dialise (ABRAHÃO NETO, 2001).

A precipitação com solventes é a mais utilizada na indústria, devido a sua capacidade de obter rendimentos adequados, seletividade mediana e a viabilidade de reuso dos solventes (SANT'ANNA JR, 2001).

A adição de solvente orgânico miscível, como a acetona, etanol, metanol, acarreta na diminuição da constante elétrica dielétrica da solução formada, levando a diminuição da solubilidade e o aumento da agregação das proteínas por interações eletrostáticas. Em geral, maior o tamanho da molécula, menor é a quantidade de reagente necessário para que ocorra a precipitação. (ABRAHÃO NETO, 2001).

Para que não ocorra à desnaturação durante o processo deve acontecer em baixas temperaturas, assim como o solvente deve estando refrigerado e ser adicionado de forma bem lenta. Assim como na precipitação de força iônica, é necessário que o material seja centrifugado, para que haja a formação do "pellet", para retirar o solvente, pode recorrer a uma etapa de evaporação, de preferencia em baixas temperaturas para que seja evitada a desnaturação da enzima (ABRAHÃO NETO, 2001).

Precipitação isoelétrica é frequentemente utilizada para a precipitação de proteínas indesejáveis, pois a probabilidade de ocorrer à desnaturação das proteínas é alta. A precipitação ocorre quando se a distribuição de cargas na superfície da enzima é neutra, de forma que a quantidade de cargas negativas é equivalente às positivas, as anulando. Com isso forma uma atração eletrostática entre as moléculas gerando um precipitado (ABRAHÃO NETO, 2001).

As proteínas também podem ser precipitadas com o auxilio de polímeros orgânicos, como o PEG. Nessa precipitação não é necessário grandes quantidades de polímeros para que ocorra a precipitação O mecanismo de precipitação é semelhante ao do solvente orgânico (ABRAHÃO NETO, 2001).

3.7.Purificação de Enzimas

3.7.1. Aplicação de Metodos Cromatograficos

Para obter um maior grau de pureza, empregam-se técnicas cromatográficas. Como a cromatografia de troca-iônica, cromatografia de troca em gel e a cromatografia de afinidade (SANT'ANNA JR, 2001).

O grau de purificação desejado está associado à aplicação do produto. Para uso técnico e industrial, as disponíveis no mercado são preparações pouco purificadas, mas para uso farmacêutico e analítico é necessário obter preparações com alto grau de pureza (SANT'ANNA JR, 2001).

A escolha das técnicas a serem empregadas está vinculada às propriedades moleculares inerentes de cada enzima. Dessa forma, deve ser feito uma combinação de várias etapas para cada enzima de forma a explorar suas propriedades. (ABRAHÃO NETO, 2001)

Em geral, as primeiras etapas da purificação, o objetivo é reduzir o volume, como o uso das técnicas de precipitação. Em seguida, são utilizadas técnicas que exploram as interações eletrostáticas, como a cromatografia de troca iônica. E como etapa final, tem-se como objetivo o aumento da resolução, onde são empregadas técnicas como a cromatografia de afinidade (ABRAHÃO NETO, 2001).

Entre as etapas cromatográficas, geralmente, é necessária uma concentração, pois um volume menor facilita a etapa subsequente. Além de que dependendo do mecanismo cromatográfico escolhido, pode diminuir a sua resolução (ABRAHÃO NETO, 2001).

3.7.2. Cromatografia

Cromatografia pode ser definida como uma separação diferencial dos componentes entre uma fase móvel e uma fase estacionária (BERG, TYMOCZKO & STRYER, 2007).

Os métodos cromatográficos podem ser classificados em diferentes modalidades, como a técnica empregada, o mecanismo de separação envolvido, e aos diferentes tipos de fase utilizados. Considera-se que a classificação mais importante é em relação ao mecanismo de separação (processos físicos, químicos ou mecânicos) (COLLINS, 2006).

Baseados, principalmente, em atrações dipolares (força de Van der Walls), ou coulômbicas (incluído a força de hidrogênio), os processos físicos são de adsorção e absorção (Figura 5) (COLLINS, 2006; BERG, TYMOCZKO & STRYER, 2007).

O mecanismo cromatográfico da adsorção baseia-se no soluto ser adsorvido, entra a fase estacionária e a fase móvel, por grupos ativos na sua superfície. A dessorção (volta do soluto a fase móvel) pode acontecer por volatilidade ou solubilidade da fase móvel. Esse mecanismo é comumente encontrado em cromatografia de camadadelgada (CCD), cromatografia gás-sólido (CGS), cromatografia líquido-

solido (CLS) e cromatografia supercrítica com fase estacionária solida (CSS) (COLLINS, 2006; BONATO, 2006; LOPES, 2006) .

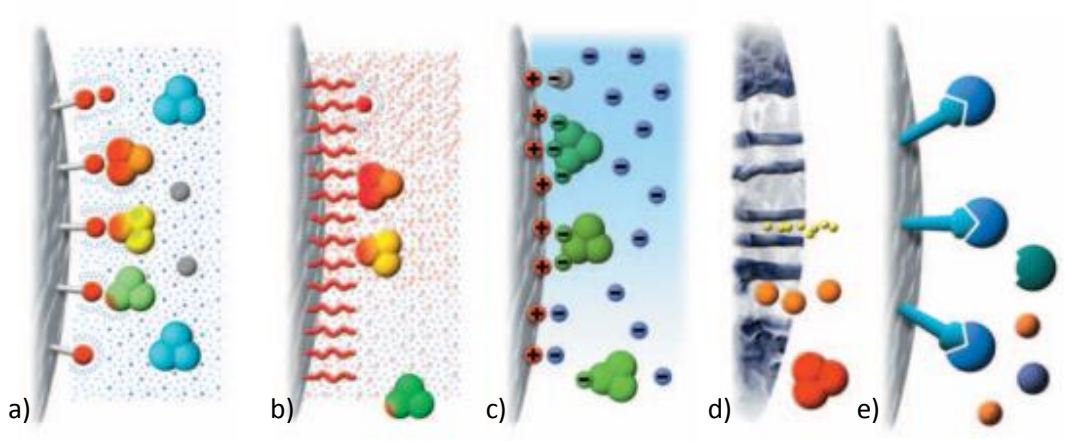

Figura 5 - Esquema dos mecanismos cromatográficos (a) adsorção; (b) partição; (c)troca ionica; (d) exclusão; (e) bioafinidade (GE Healthcare Life Sciences)

O mecanismo de partição é um processo interfacial, quando a fase estacionária é um liquido, espalhado na superfície de um suporte sólido e inerente. Baseia-se na diferença de solubilidade dos componentes da amostra na fase estacionária. Para que ocorra a volta do componente a fase móvel, depende da volatilidade (fase móvel gasosa) ou solubilidade nessa fase (fase móvel líquida). A cromatografia em papel (CP), na cromatografia gás-liquido (CGL), e na cromatografia liquido-liquido (CLL) são baseadas nesse mecanismo (BRAGA, 2006).

O mecanismo de troca iônica baseia-se em uma retenção de íons, em uma fase estacionária, a qual é adicionada grupos funcionais ionizáveis, cátions ou aníons. A fase móvel em geral, é uma solução iônica tamponante compatível com o grupo funcional ionizável. Através de processos de eluições, ou deslocamento, a fase móvel compete pelos grupamentos iônicos com os grupamentos da amostra. A representação do processo do mecanismo de cromatografia por troca iônica (CTI) (COLLINS, 2006; ABRAHÃO NETO,2001; BERG, TYMOCZKO & STRYER, 2007; SPADARO,2006).

O mecanismo de bioafinidade utiliza grupos com especificidade biológica, os quais são ligados a um suporte (fase estacionária). Dessa forma, só acontecerá a ligação dos componentes complementares, presentes na fase móvel, aos grupos ligados a fase estacionária. Na cromatografia por bioafinidade (CB), a eluição do componente, pode acontecer de duas formas, através da mudança de propriedades da fase móvel, como o pH, ou mudança do componente ligado a fase estacionária, utilizando outro componente que possui uma atração maior pelo grupamento da fase estacionária (SPADARO & FONSECA, 2006).

A cromatografia por exclusão (CE) é um mecanismo puramente físico, onde não há interação entra a fase móvel e a estacionária. A fase estacionária usada é uma matriz de forma, tamanho e porosidade uniformes, as moléculas presentes na amostra (fase móvel) são separadas de acordo com o seu

tamanho, sendo as moléculas menores, capazes de atravessar a fase estacionária com maior rapidez, e as maiores com mais lentidão (ROTHSCHILD, 2006).

Na cromatografia em coluna, geralmente ocorre com um fluxo contínuo da fase móvel, até que todos os componentes tenham saído da coluna e sua presença seja detectada, e indicada graficamente .

O cromatograma construído, onde a linha de base representa a passagem da fase móvel através do detector. Os componentes eluidos apresentam-se em forma de picos (figura 6).

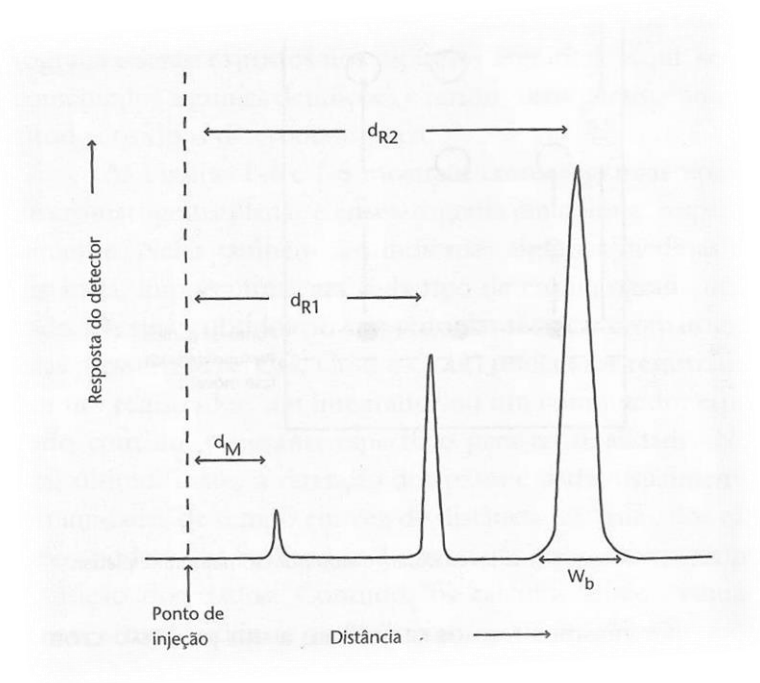

Figura 6 - Esquema de cromatograma (COLLINS, 2006)

Denomina-se D_R a volume percorrido entre a injeção da amostra até o pico traçado, e D_M a volume percorrido por uma molécula, a qual não interage com a fase estacionária, entre o ponto de injeção até o detector.

A resolução de uma coluna, pode ser calculada a partir da distancia que separa os pontos máximos dos picos e da media das larguras de suas respectivas bases (W_b). O valor de resolução é diretamente proporcional à qualidade da separação (COLLINS,2006) .

3.7.2.1.Cromatografia por Troca Iônica

Na cromatografia por troca iônica, a fase estacionária é altamente carregada, e o soluto com cargas e sinais contrários a esta.

Esse tipo de cromatografia vai ser determinado pela diferença no grau de afinidade eletrostática entre o trocador e os íons da fase móvel A diferença entre os íons da fase móvel e da matriz, se dá pela diferença de carga, a qual pode ser controlada pelo pH, por trocadores iônicos fracos e médios, e a força iônica. Como pode ser observado na figura 7, onde o equilíbrio inicial é alterado com a adição

de íons, os quais tem afinidade maior que o adsorvido na coluna. Para a reutilização da coluna, basta ser equilibrada com o eluente inicial, voltando ao equilíbrio.

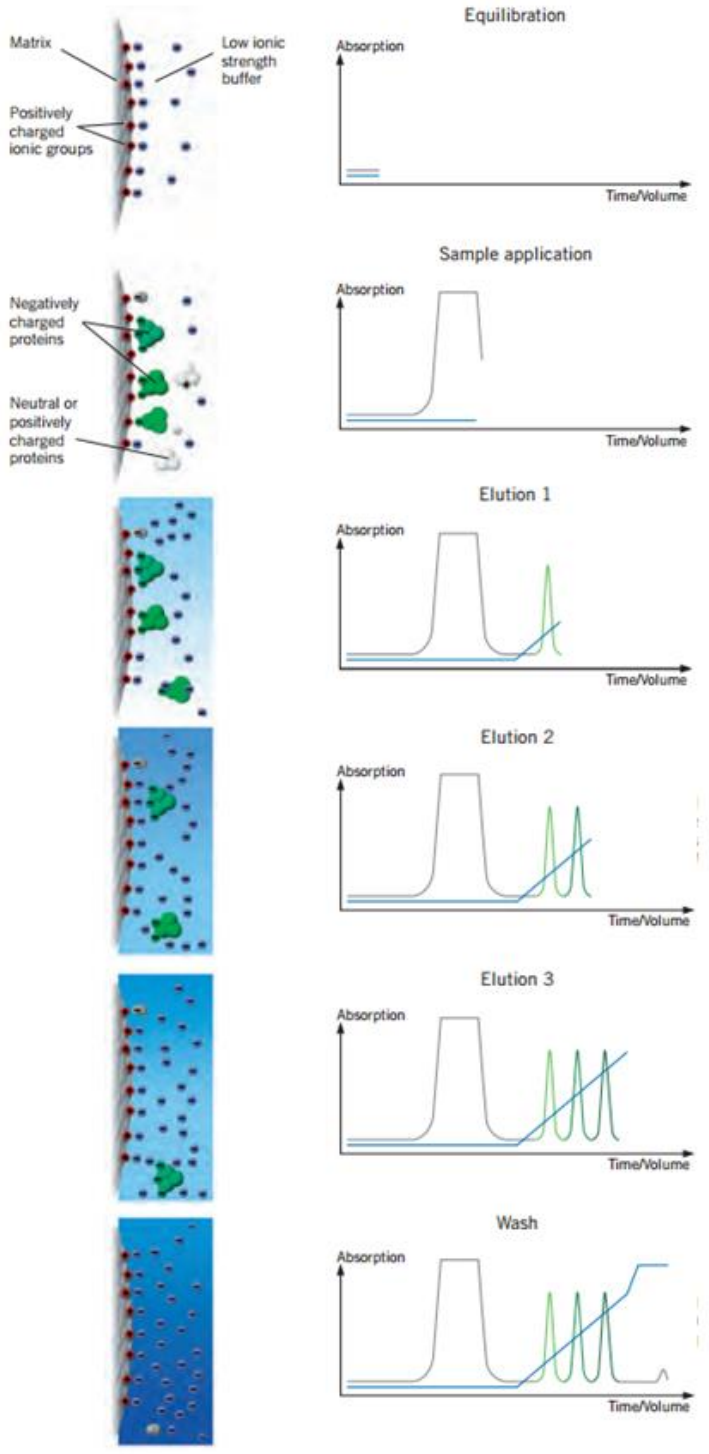

Figura 7- Esquema do mecanismo de eluição do soluto por força iônica na coluna de troca iônica. (GE Healthcare Life Sciences)

A matriz do trocador é constituída por um material poroso, inerte, insolúvel em água e em solventes orgânicos. Podem ser classificadas de acordo com o material que as formam, sendo natural ou sintética, orgânica ou inorgânica. Podem ser classificadas também de acordo com o trocador ligado a matriz, em trocadores catiônicos (com sítios carregados negativamente que retem cátions) e em

trocadores aniônicos (com sítios carregados negativamente que retem anions) (COLLINS,2006; SPADARO,2006).

A capacidade de um trocador é uma medida de troca de ions que pode ocorrer entre a matriz e os ions presentes na fase móvel. Essa medida é dependente de fatores como a força iônica, o pH e a temperatura do eluente (COLLINS,2006).

A seletividade de um trocador é dependente do grau de ligações cruzadas na matriz, da carga dos íons (quanto maior a carga, mais fortemente se liga) e o tamanho dos íons (solvatados) (SPADARO,2006).

A cromatografia por troca iônica pode ser influenciada pela escolha do trocador iônico e da fase móvel, que devem ser feita de acordo com o objetivo final, pela amostra, o excesso de material causa a perda na resolução e a escassez pode causar em dificuldade para quantificar. (BERG, TYMOCZKO & STRYER, 2007).

A eluição da cromatografia pode ser feita de duas formas: a primeira é utilizando o próprio tampão como eluente, esse processo é considerado lento e geralmente usado para moléculas com a mesma carga; a segunda é utilizando uma mudança discreta de pH e/ou força iônica (SPADARO, 2006).

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CAPÍTULO 2

ARTIGO A SER SUBMETIDO – PROCESS BIOCHESMISTRY

PARTIAL PURIFICATION OF A PROTEASE WITH COLLAGENOLYTIC ACTIVITY FROM *ACTINOMADURA* SP.

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SHORT COMMUNICATION

ABSTRACT

Proteases with collagenolytic activity, capable of degrading collagen, may be obtained from several sources, and among them, the microorganisms are the resources of choice because of its biochemical diversity and susceptibility to genetic manipulation. This work had as objectives purification and characterization of a protease with collagenolytic activity obtained from *Actinomadura* sp using a medium soy flour as substrate. The purification process involved precipitation with acetone, followed by ion exchange chromatography. Results showed an efficient elution by gradient with 0.1M NaCl solution in Tris-HCl buffer, pH 8.0. A partial characterization demonstrated this novel biomolecule as thermostable up to 60 ° C in 60 minutes, presenting an optimal temperature and pH at 50°C range 7-8, respectively. When submitted to the ultrasonic effect on different frequency intervals, after 10 minutes where about an increase was observed in the range of 50% on collagenolytic activity. Thus, this new biomolecule has biotechnological potential. Since the production process involves a substrate, soybean flour, which is widely found in Brazil, and partial purification protocol was developed to obtain effective protease with collagenolytic activity.

Keyword: *Actinomadura* sp., protease; purification; characterization

1. Introduction

Proteases are a unique class of enzymes that occupy an important position as regards their applications, both in physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins. The proteases are degradative enzymes that catalyze the hydrolysis of proteins.[1] Proteases are a group of enzymes used in various industrial sectors, such as detergent, food, pharmaceutical, chemical, leather and silk, in addition to the treatment of waste, hence its great commercial importance [2,3].

Microorganisms are an excellent source of enzymes due to their diversity and their susceptibility to genetic manipulation. Proteases from microbial origins are relevant since they have almost all the desired characteristics for their biotechnological applications are the most used.[4] Among the microbes capable of producing biomolecules of interest, class Actinobacteria represents a wide range of valuable and important sources of pharmaceutically active metabolites. Actinomycetes are responsible for much of the secondary metabolites discovery [5], including antibiotics [6], antitumor agents [7] e enzymes [8]. The actinomycetes are abundant in the soil may occur in aquatic environments and also colonizing plants.[9] The production of bioactive compounds can be influenced by the interaction of the plant with the microflora present in the region of the roots.[10].

Collagenase is used in medicine for the treatment of diseases such as Peyronie's disease[11], Dupuytren's Disease[12,13]. As with treating necrotic wounds, pressure sores, post-operative scars. In addition to the collagenase application, the product coming from the hydrolysis of collagen, collagen peptide, has been studied for the treatment of various diseases such as hypertension and diabetes[14], and may have antimicrobial activity[15].

The objective of this research was to evaluate the production of collagenolytic proteases by *Actinomadura* sp. and the best conditions for purification, characterization the protease with collagenolytic activity.

2. Materials and methods

2.1.Microorganism and Growth

The strain L09, *Actinomadura* sp., was obtained by the collection of the Department of Antibiotics (UFPEDA), Federal University of Pernambuco. The microorganism was isolated from the soil samples rhizosphere of *Licania rigid Benth*. (Oiticica).

The isolate was cultivated on a sterile Soy bean flour medium containing (g/L): soy bean flour, 10; Glicose, 1.0; K_2HPO_4, 1.0; at pH 7.5. As inoculum, 10% spore suspensions were used after 48 h of cultivation in the same media. Flasks were incubated for 72h at 37°C with constant shaking at 180 rpm. The cell-free supernatants were analyzed for protein content and enzyme activities.[16]

2.2.Proteolytic activity assay

For the determination of protease activity [17], 0.15 mL of sample was added to a solution containing azocasein 1% w / v in 0.1 M Tris-HCl pH 7.4 buffer, the mixture was incubated for 1 hour at 37 ° C protected from light. The reaction was stopped by the addition of trichloroacetic acid (TCA) to 10% w / v, and centrifuged for 20 minutes at 12500 rpm, 0.8 ml supernadant was added to 0.2 ml of 1.8 M NaCl. The reading of absorbance was performed at the spectrophotometer at a wavelength of 420nm. One unit of protease activity was defined as the amount of enzyme required producing an absorbance change equal to 0.01 in 60 minutes and is expressed as U mL-1

2.3.Assay for collagenase activity

To determine the collagenolytic activity used Azo Dye-impregnated collagen (Azocoll) (Sigma Chemical Co. St. Louis, MO) at a concentration of 5 mg / ml in 0.1 M Tris-HCl pH 7.2 buffer. After successive washings, was added 0.05ml of sample, and incubation was for 3 hours under 37 ° C with constant stirring (100 rpm). The reading of absorbance was performed at the spectrophotometer at a wavelength of 520nm.[18]

One unit of protease activity was defined as the amount of enzyme required producing an absorbance change equal to 0.01 in 60 minutes and is expressed as U mL-1

2.4.Protein determination

The amount of protein was determined by the method of Smith[19], using bovine serum albumin as a standard.

2.5.Gel chromatography

The supernadant was submitted to organic solvent (70% acetone) precipitation, and stirring it for 10 minutes. After, the samples were centrifuged at 3500 rpm for 20 minutes. The protein determination and evaluation of proteolytic activities were carried out.

Samples were applied to the Sephadex- DEAE column (G50 10x80mm) for the purification of proteins. The column was equilibrated with 0.1M Tri-HCl buffer pH 8.0 and eluted with NaCl at 22°C at concentrations of 0.1 M, 0.2 M and 0.3 M buffer in which the column was equilibrated. The flows used of 0.5 ml / min, the absorbance monitored at 280 nm and the eluate with gradient was collected in 1 ml fractions. The fractions were collected, pooled and concentrated. The protein determination and proteolytic and collagenolytic activities were checked.

2.6.SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970)[20] using a 12,5% crosslinked polyacrylamide gel. Silver staining was performed to visualize the protein bands. Molecular weights are estimated by using the protein standards: bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), glyceraldehyde 3-phosphate dehydrogenase (36.0 kDa), carbonic anhydrase (29.0 kDa), trypsinogen (24.0 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.2 kDa).

2.7.Partial Characterization

2.7.1. Temperature Stability

The purified samples were subjected to temperatures of 50-80 ° C for 30 minutes, followed by the dosage of the activities performed, with subsequent determination of collagenolytic activity.

2.7.2. Effects of temperature on collagenolytic activity

The collagenolytic protease activity was assayed under at different temperatures between 30 - 80 °C for 60 minutes.

2.7.3. Effects of pH on collagenolytic activity

To determine the optimum pH of the reaction with protease with collagenolytic, the pH of the reaction mixture containing 5 mg/mL Azocoll was varied in the range 5.0-9.0. The buffers used were 0.1 M sodium acetate buffer (pH 5–6), 0.1 M Tris–HCl (pH 7–9).

2.7.4. Effect of Metal Ions

The purified enzyme was added to 10 mM of salt solution containing the divalent ions (Fe^{+2}, Mg^{+2}, Ca^{+2}, Zn^{+2}) for 60 minutos at 37 ° C, with subsequent measurement of collagenolytic activity.

2.7.5. Effect of Inhibitors

The purified collagenase was incubated with each reagent at room temperature for 60 min in 10 mM Tris-HCl buffer (pH 7,2) and collagenase activity was assayed as described above. Phenylmethanesulphonyl fluoride (PMSF), SDS, beta-mercaptoethanol, Pepstatin A were used in this experiment as protease inhibitors. The residual activities were determined as a percentage of the activity in control sample. The influence of different metal ions on collagenase activity at 10 mM final concentration were also determined using native collagen as substrate, as described above for protease inhibitors.

2.8.Effect of the Ultrasonic frequency

The purified collagenase was submitted for a ultrasonic frequency (40 Hz) in room temperature during a different times and the and collagenase activity was assayed as described above.

3. Results and Discussion

The crude extract containing the active protease enzyme with a specific protease activity of 396.81U mg/ml. In the first purification step by protein precipitation using acetone,was obtained approximately 64.31% of the protease and the 11.41 times fold purified.

The protease precipitated with acetona was passed through by ion exchange chromatography Sephadex- DEAE. Although this fraction contained different proteins with different molecular weights, three peaks P1, P2 and P3(Figure 01), which were eluted with 0.1 M NaCl, 0.2 M and 0.3 M, showed specific protease activity partly purified with 68.175,18U/mg protein, 155.513,20U/mg protein, 3.496,97U/mg protein and 17.17%, 3.91% and 0.88% of yield, respectively. The partially purified collagenolytic specific activity obtained was 31.094,89U/mg protein , 12.140,76U / mg protein 2.461,33U / mg protein and 17.17%, 3.91% and 0.88% yield, respectively (Table 1).

The figures presented show that P1 obtained a higher activity both protease and collagenolytic. Identifying the concentration gradient of 0.1M NaCl for this protease is optimal for the enzyme eluted with a smaller amount of contaminants and higher purity.

Higher activity found by Petrova et al., [19] which used the Streptomyces sp. Strain 3B to produce protease with collagenolytic activity by successive filtration processes (precipitation, chromatography and ion exchange and affinity), obtained a specific collagenolytic activity of 3600U / mg protein.

The fractions obtained from the DEAE-Sephadex column were subjected to electrophoresis, could be observed in the fraction eluted with 0.1 M NaCl two bands with the molecular weight with approximate 20 Kda and 45,0 kda(Figure 2).

The presence of this two band on the P1, we could observe the partial purification of the protease with collagenolytic activity using just a ionic chromatography column.

Asker et al.,[21] showed in their paper two fractions purified proteases with weight of 25 KDa and 28 KDa from *Bacillus megaterium* with recovery of 30.54% and 11.00% after precipitation with ammonium sulfate, and Sephadex-DEAE column and Sephadex G -200.

Waghmare et al.,[22] was purified alkaline protease from *Stenotrophomonas maltophilia* strain SK by precipitation with ammonium sulfate and ion exchange chromatography on DEAE-cellulose

column was obtained 22,22U/mg specific protease activity, exhibit less activity that obtained by the purification of the produced protease by *Actinomadura* sp. of 68175,18U/mg.

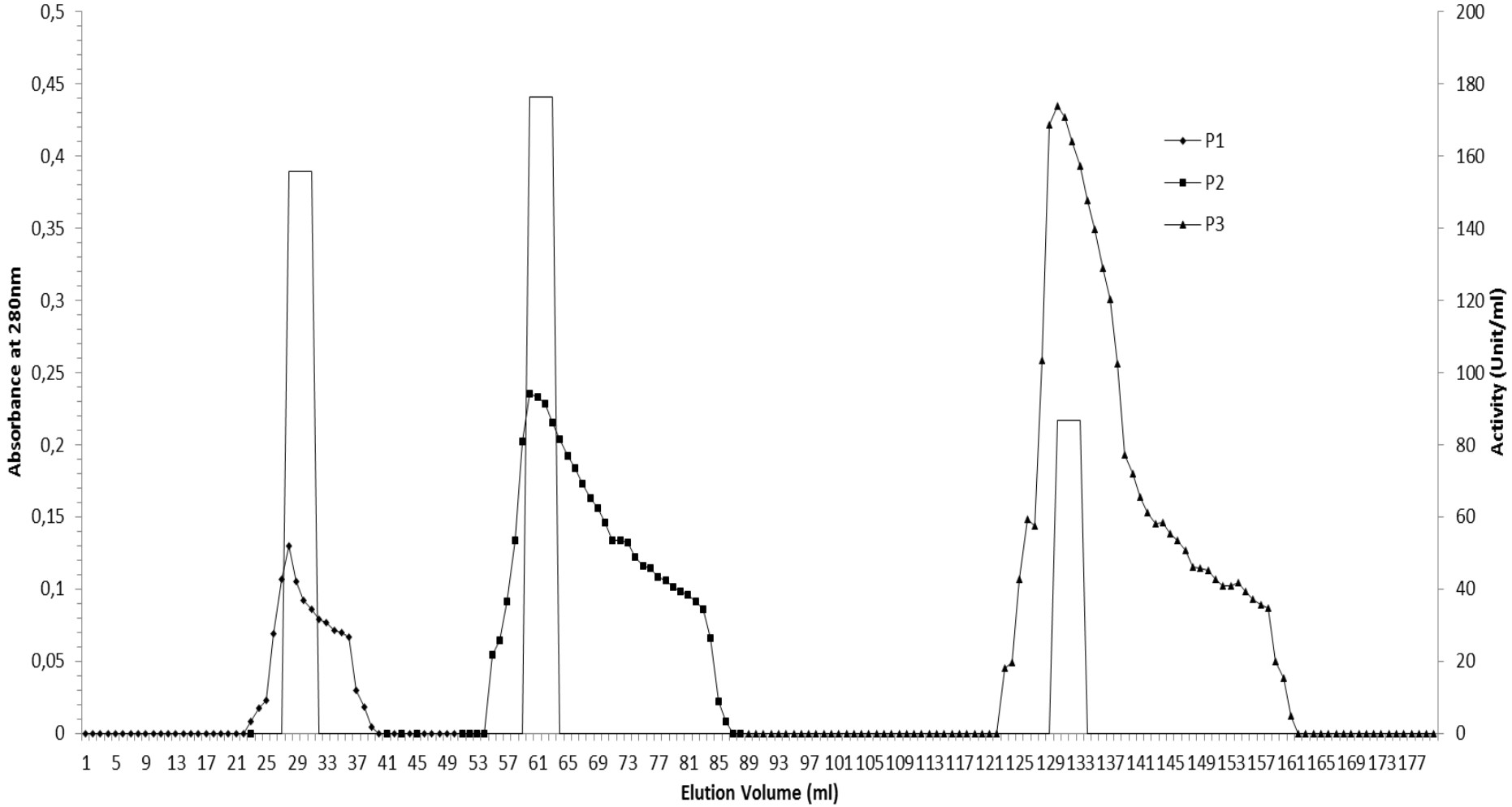

Figure 1- Chromatography profile of the elution with NaCl with different concentration of protease with collagenolytic activity using the column Sephadex- DEAE . P1(—●—). Elution with 0.1M NaCl. P2.(—■—) Elution with 0.2M NaCl. P3.(—▲—) Elution with 0.3M NaCl.

Table 2 Purification of protease with collagenolytic activity from *Actinomadura* sp.

	Protein (mg/ml)	**Total activity (U)**		**Specific activity (U/mg protein)**		**Purification fold**		**Yield (%)**	
		Protease	Collagenase	Protease	Collagenase	Protease	Collagenase	Protease	Collagenase
Crude extract	0,060	236,67	189,00	3969,81	3170,25	1,00	1,00	100	100
P0	0,011	507,33	358,00	45297,62	31964,29	11,41	10,08	64,31	56,82
P1	0,002	155,67	71,00	68175,18	31094,89	17,17	9,81	0,66	0,37
P2	0,011	176,33	138,00	15513,20	12140,76	3,91	3,83	0,75	0,73
P3	0,025	86,67	61,00	3496,97	2461,33	0,88	0,78	0,37	0,32

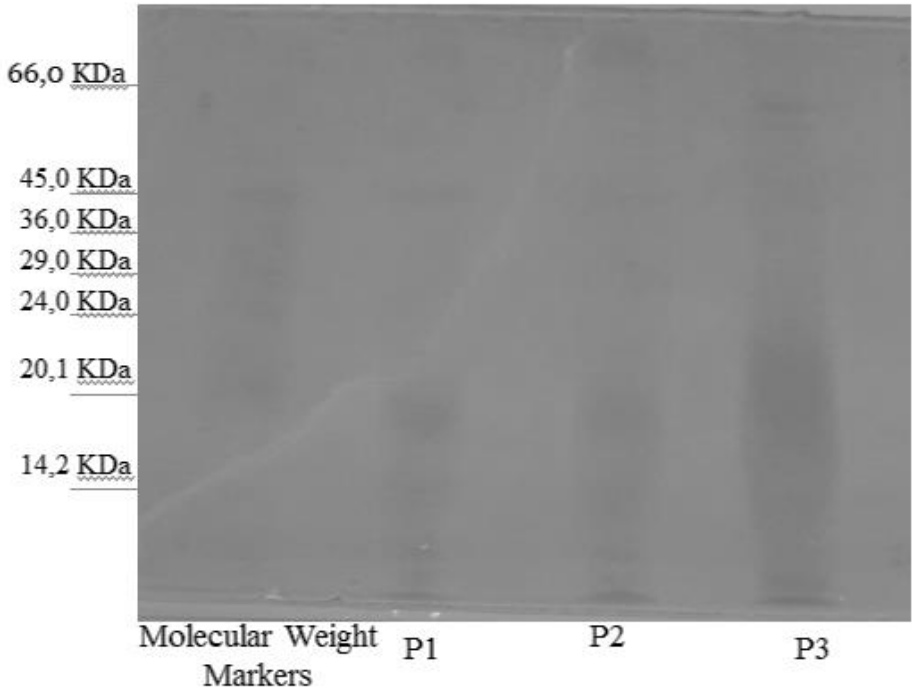

Figura 2 - SDS–PAGE analysis of purified protease. The eluted of protease from *Actinomadura* sp. using Sephadex- DEAE with different NaCl concentration. Lane 1: molecular weight markers, Lane 2: Sephadex- DEAE eluted with the 0.1M NaCl, Lane 3: Sephadex- DEAE eluted with the 0.2M NaCl, Lane 4: Sephadex- DEAE eluted with the 0,3M NaCl.

The partial characterization of effects of pH ,temperature stability, optimum temperature, metal ions , inhibiotors and ultrasonic frequency was realized using the P1 fraction.

The protease with collagenolytic activity showed maximum relative collagenolytic activity at pH 7.0 and 8.0 (Figure 2).

As observed by Harris et al. [23] the structure of the triple helical conformation of collagen when submitted to acid and basic pHs, the structure is significantly altered compared to the native structure. This modification may become unable the active site of the protease and as a consequence the decrease of activity on pH in acid and base.

The optimum pH range corroborates those submitted Lima et al., [24] and Lima et al., [25] with bacterial and fungal collagenase.

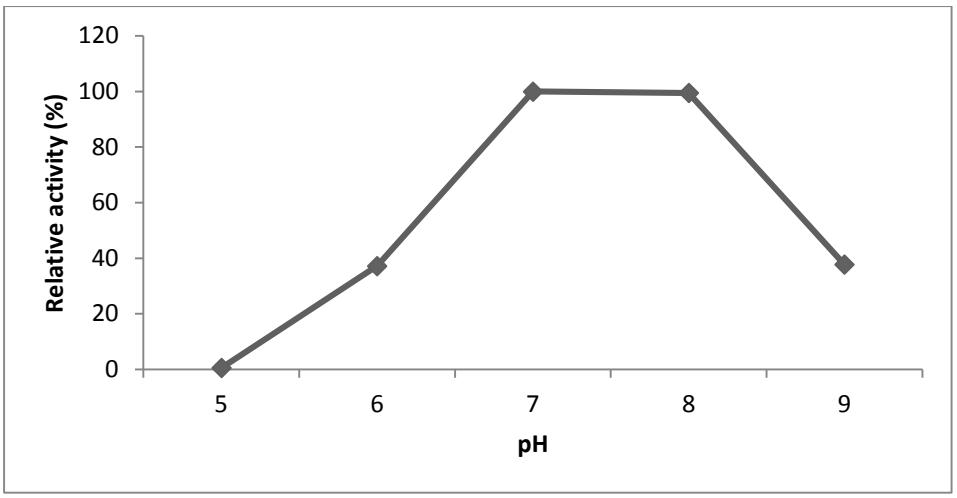

Figure 3- Effects of pH on the relative activitiy of collagenase from *Actinomadura* sp. after purification with Sephadex-DEAE column.

The maximum activity on the effect of temperature was obtained with the 50 ° C (Figure 3). The increase of the reaction temperature changes the solubility of collagen molecules and may expose new active site for the protease. The higher the temperature, the greater the unfolding of the protein, so that the foregoing become inaccessible sites hydrolysis, decreasing the collagenolytic activity.

The same temperature as described by Lima et al. [24] with collagenase from Bacillus stearothermophillus.

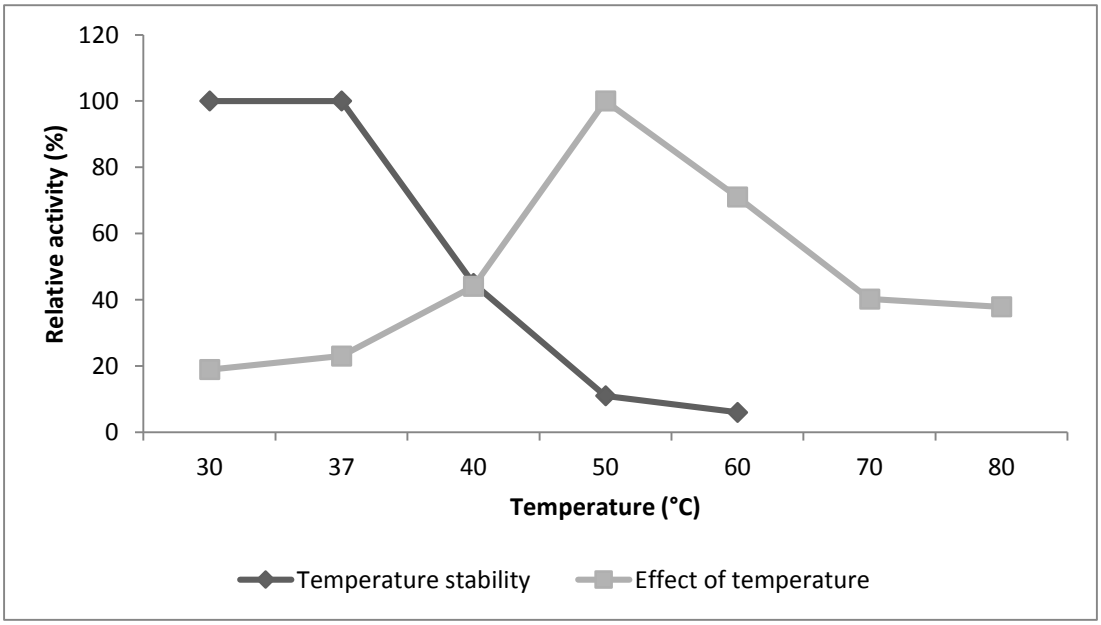

Figure 3 - Effects of temperature and temperature stability on the relative activity of collagenase from *Actinomadura* sp. after purification with Sephadex-DEAE column.

It has been observed more than 40% relative activity in the 40-70 ° C range in this work. George et al. [26] was purified alkaline protease from *Vibrio metschnikovii* NG 155, which had more than 40% of the activity at the temperature of 30-70 ° C, however the maximal activity at pH 9-10 range, superior from that observed work.

As may be observed in Figure 4, the enzyme has no stability at temperatures above 40°C. This effect can be explained so that the temperature rise above 40 ° C there is probably the unfolding of the protease. After removal of the temperature, the enzyme is not able to return the shaping appropriately, decreasing or losing activity.

Was observed in collagenolytic enzyme purified latex fig (Ficus carica var. Brown Turkey) which has a high stability enzyme as a function of temperature [27].

In the assay with effect of metal ions, was observed the ions calcium and potassium showed slight decrease in control activity, magnesium ion showed a significant decrease, as well as the zinc ion (table 2).

The works of Petrova et al.,[16,28] zinc showed an inhibition of maximum activity, but the calcium and magnesium ions, have not suffered such inhibition.

The assay of the inhibitory effect on the activity (Table 2), the inhibitors observed β-mercaptoethanol and PMSF showed inhibition. The β-mercaptoethanol is a reactant capable of breaking the disulfide binding present in the enzyme, irreversibly denaturing. The PMSF is also an irreversible inhibitor of the serine residue acting protein serving as serine proteases identifier. Wu et al.[29] Also observed a decrease in collagenase activity by β-mercaptoethanol. And Roy et al.[30] described serine protease collagenase was strongly inhibited by PMSF.

The SDS and Pepstatin A are reagents whose ability to change the protein conformation, the change in conformation of the protease when in contact with the reagent, increased collagen degradation by the enzyme capacity.

Table 2 - Effect of metal ions and inhibitors on activity of collagenase from *Actinomadura* sp.

Inhibitor and Metal Ions	Concentration mmol/L	Residual collagenase activity (%)
PMSF	10	77,61
Beta Mercaptoetanol	10	79,64
SDS	1	113,83
Pepstatina	10	134,59
CaCl	10	95,79
KCl	10	94,70
ZnCl	10	35,00
MgSO4	10	79,24

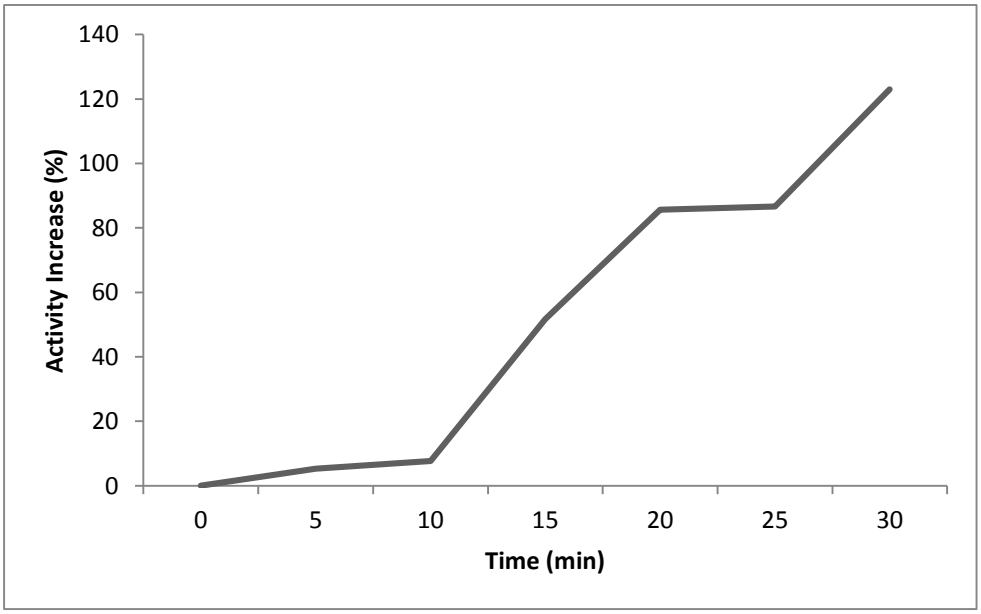

Figure 4- Effects of sonication (40Hz) on the increase activity of collagenase from *Actinomadura* sp. after purification with Sephadex-DEAE column.

It was observed that the enzyme upon being subjected to the effect of the Ultrasonic frequency, the activity increased, reaching more than 100% increase, with no decrease in exposure of 30 minutes.(Figure 4).

Due to the purification process is partial and extracellular bacterial source, some contaminants are still present in P1. The effect of interference from contaminants in collagenolytic activity is the protease called quenching effect. While undergoing the frequency of ultrasound, the "shaking" of the molecules leads to a separation of protease impurities increasing the collagenolytic activity.

As opposed to what was observed by Zhou et al., [31] which there was an increase in the first 20 minutes on the enzyme activity Neutrase, with subsequent loss of the activity.

4. Conclusion

This article describes a strategy to extract and partially purify the protease with collagenolytic activity of crude extract from Actinomadura sp. Due existed a few works about this microorganism produced a protease with collagenolytic activity. The purified protease has higher specific collagenolytic activity found in the literature, using just a one-step purification. Collagen hydrolysis results suggested that the Actinomadura sp. could be used to produce collagen peptides with biological activity potential industrial and medical interest.

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