UNIVERSIDADE FEDERAL RURAL DE PERNAMBUCO
DEPARTAMENTO DE MORFOLOGIA E FISIOLOGIA ANIMAL
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOCIÊNCIA ANIMAL

ROMUALDO BRANDÃO COSTA JÚNIOR

Efeito do ultrassom na atividade enzimática de colagenase produzida por *Aspergillus sp.* UCP1276: Bioprospecção e potencial biomédico

Recife-PE
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Área de concentração: Biotecnologia

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Dedico este trabalho a minha FAMÍLIA!

RESUMO

Introdução: Nos últimos anos, o ultrassom tem sido usado com sucesso em um grande número de Bioprocessos na área da biotecnologia, tais como no melhoramento (*upregulation*) da hidrólise enzimática (biocatálise) e consequentemente na produção de peptídeos biologicamente ativos dentre esses, os obtidos pelas colagenases, que são proteases capazes de degradar o colágeno nativo e desnaturado, com aplicações relevantes na indústria farmacêutica e de cosméticos.

Objetivo: A presente pesquisa teve como objetivo estudar a potencial aplicação do ultrassom na atividade de uma colagenase purificada de *Aspergillus sp.* UCP1276 como uma ferramenta biotecnológica para produção de peptídeos bioativos.

Metodologia: A colagenase utilizada no projeto foi purificada utilizando cromatografia de troca iônica (DEAE-sephadex) seguida de cromatografia de exclusão molecular (Superdex-G75 HR 10/300 GL) utilizando o sistema FPLC AKTA Avant. O efeito do Ultrassom na atividade da enzima e na capacidade de degradar o Colágeno tipo I foi avaliado utilizando um Banho Ultrassônico (frequência 40kHz, 155W RMS) variando os tempos reação enzimática (0 - 200min), a 37ºC. O perfil da hidrólise enzimática do Colágeno tipo I nos tempos 5, 15 e 30min foi observada através de SDS-PAGE. Os peptídeos obtidos foram testados no ensaio de atividade anticoagulante utilizando plasma pobre em plaquetas para avaliar o efeito nos tempos de coagulação (TP, TTPA, TPAE, INR e TT).

Resultados: Os resultados demonstraram um real e significativo aumento na atividade da colagenase, cerca de 60%, sendo percebida pela degradação do colágeno tipo I associada a um aumento da atividade da enzima mesmo na presença de inibidores (PMSF). Além disso, quando a colagenase purificada foi submetida à exposição ao US, tornou-se mais eficaz para degradar o colágeno tipo I, dados observados em eletroforese SDS-PAGE, e produzir peptídeos biologicamente ativos em ensaios de atividade anticoagulante sendo eficaz em inibir cerca de 90% do tempo de protrombina quando comparada às amostras controle.

Conclusão: Dessa forma, os resultados indicam a efetividade do ultrassom de baixa frequência na atividade enzimática com uma visão de aplicabilidade comercial do bioprocesso.

Palavras-chave: Biocatálise; bioconversão; atividade enzimática; ultrassom

ABSTRACT

Introduction: In recent years, ultrasound (US) has been successfully used in many crucial bioprocesses such as upregulation of enzymatic hydrolysis (bio catalysis), and consequently in peptides bioproduction. Collagenases are proteases able to degrade native and denatured collagen, with relevant applications in leather, food, pharmaceutical and cosmetic industries, as well as in medical practice in the treatment of burns and wounds.

Objective: The present research shows the potential application of US on collagenase enzyme purified from *Aspergillus sp.* UCP1276 as a biotechnological tool to improve biomedical applications.

Methodology: In our work, the collagenase used in the design was purified using a purification protocol previously established by ion exchange chromatography (DEAE-sephadex) followed by molecular exclusion chromatography (Superdex-G75 HR 10/300 GL) using the AKTA Avant FPLC system. The effect of Ultrasound on enzyme activity and the ability to degrade Type I and Type V Collagens was evaluated using an Ultrasonic Bath (frequency 40kHz, 155W RMS), varying the exposure times of the enzyme to the US (5, 15 and 30min), as well as the effect of the US on the enzymatic reaction at times of 5, 15 and 30 min at 37 ° C. The peptides obtained through enzymatic hydrolysis were submitted to electrophoresis, followed by anticoagulant activity using platelet-poor plasma to evaluate the effect on coagulation times (TP, APT, TPAE, INR and TT).

Results: The results showed a significative increase in the activity of collagenase, about 60%, being detected by the degradation of type I collagen associated to an increase of enzyme activity even in the presence of inhibitors (PMSF). In addition, when purified collagenase was subjected to exposure to the US, it became more effective to degrade type I collagen, observed data on SDS-PAGE electrophoresis, and to produce biologically active peptides in anticoagulant activity assays being effective in inhibiting about 90% of the prothrombin time when compared to the control.

Conclusion: The results indicate the effectiveness of low frequency ultrasound in improving enzyme yields with a vision of commercial applicability of the process.

Keywords: Biocatalysis; Bioconversion; Enzyme activity; ultrasound.

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1. INTRODUÇÃO

O colágeno é uma proteína que devido a sua estrutura possui resistência a degradação da maioria das proteases comuns, dessa forma as colagenases vem a ser uma alternativa para a degradação do mesmo.

Colagenases são proteases com a capacidade de degradar o colágeno, e assim, as enzimas com essa capacidade são difíceis de encontrar devido às características estruturais do colágeno que o tornam resistente à maioria das proteases comuns. (WANDERLEY et al., 2017). Proteases colagenolíticas são enzimas que degradam o colágeno e podem ser agrupadas em: metalo-proteases e cisteíno proteases. Aproximadamente 30% das proteínas de mamíferos são colágeno e podem ser denominadas como proteínas da matriz. Sua degradação ocorre em condições fisiológicas e patológicas (FAYAD et al., 2017).

Colagenases podem ter diversas funções, apresentando-se como: (1) Fatores de virulência (a habilidade de degradar o colágeno possibilita a invasão no tecido de hospedeiros, sendo um importante fator na etiologia das doenças); (2) Ferramentas biotecnológicas (no amaciamento de carne, curtimento de couro e obtenção de peptídeos bioativos a partir da degradação do colágeno, e; (3) Medicamentos (a colagenase proveniente de *Vibrio proteolyticus* ATCC 53599 é usada no tratamento tópico de feridas como escaras, que são compostas de proteínas desnaturadas como o colágeno, elastina, fibrina, hemoglobina e proteínas coaguladas, além de possuir efeito imuno estimulador e ser utilizada em tratamentos de cicatrização de feridas e na doença de Dupuytren, como uma alternativa ao uso de métodos invasivos, como cirurgias (DABOOR et al, 2010)

O ultrassom é uma onda mecânica acústica produzida pelo movimento oscilatório das partículas de um meio. Por ser uma forma de energia mecânica tem como principais propriedades: reflexão, adsorção e dispersão e precisa de um meio físico para se propagar, como através de sólidos, líquidos ou gases. Diversos estudos têm apresentado o efeito dessas ondas sobre as propriedades das enzimas. Fatores como alterações moleculares e forças mecânicas da ação direta das ondas ultrassônicas seriam também responsáveis por melhorias na enzimólise (Wang et al., 2018). Nesse contexto, pesquisas tem demonstrado que a exposição ao ultrassom pode modificar o arranjo das estruturas secundárias e terciárias da pepsina e α-

amilase, promovendo aumento e inibição das atividades, respectivamente. A ação direta das ondas ultrassônicas na estrutura da enzima pode levar a mudanças de conformação que poderiam ativar ou inativar as enzimas

Sendo assim, o objetivo da presente pesquisa foi avaliar o efeito do ultrassom sobre a atividade de uma Colagenase previamente purificada de *Aspergillus sp* UCP 1276 utilizando colágeno tipo I como substrato, e em seguida avaliar as propriedades biológicas dos peptídeos formados a partir da hidrólise enzimática.

2. REVISÃO DE LITERATURA

2.1 Proteases

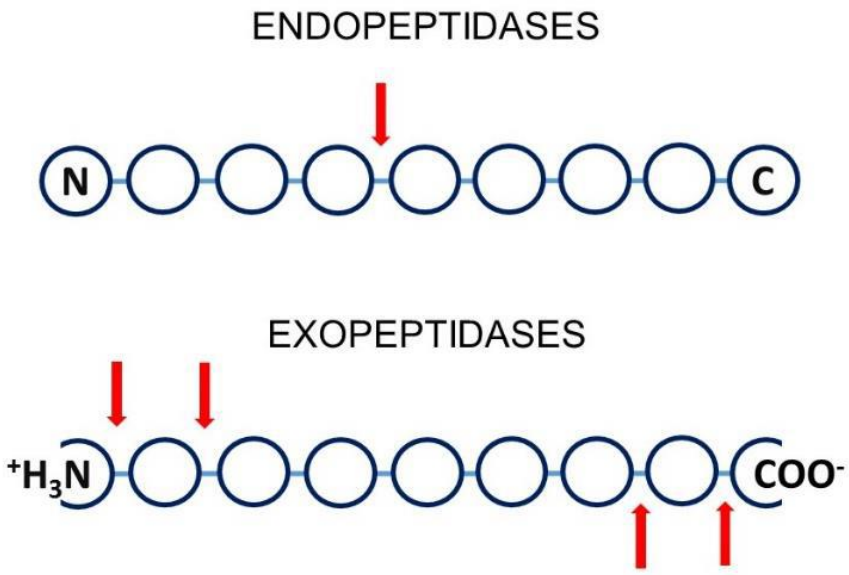
As proteases (ou enzimas proteolíticas) pertencem ao grupo das hidrolases e catalisam a quebra de ligações amida em moléculas proteicas. Apesar de também serem chamadas de proteinases e peptidases, as proteinases degradam as proteínas, enquanto que as peptidases estão restritas aos fragmentos de proteínas ou peptídeos (SAXENA, 2015; CAMARGO et al, 2012).

Essas enzimas são agrupadas de acordo com a especificidade com o substrato, podendo clivar as extremidades C-terminal e N-terminal (exopeptidades) ou sequências dentro da cadeia polipeptídica (endopeptidases) como demonstrado na Figura 1 A classificação das proteases pode ser feita, também, segundo a especificidade de sua cadeia lateral e da presença de grupo funcional no sítio ativo, agrupando-as em serino proteases, cisteíno proteases, aspartil proteases, treonino proteases, glutâmico proteases e metalo proteases (BARRET, 1999)

Nas serino-, treonino- e cisteíno- proteases a clivagem de ligações peptídicas ocorre através da cadeia lateral nucleofílica de um aminoácido. As aspartil-, glutâmico- e metalo- proteases, por sua vez, atuam nas ligações peptídicas através de uma molécula de água ativada (RAWLINGS; BARRETT, 2013a). As aspartil proteases possuem resíduos de aspartato como ligantes de moléculas de água ativadas, enquanto resíduos de glutamina são os ligantes das glutâmico-proteases. Todas as enzimas aspartil e glutâmico proteases são endopeptidases (RAWLINGS; BARRETT, 2013a). Nas metalo proteases o ligante normalmente é um íon di-valente ancorado por aminoácidos (zinco, cobalto, manganês, níquel ou cobre) (RAWLINGS; BARRETT, 2013b). As cisteíno proteases atuam nas ligações

peptídicas através dos grupos sulfidril de resíduos de cisteína, enquanto as serino proteases dependem de um grupo hidroxil de resíduos de serina. Esses dois tipos possuem mecanismo catalítico similar, onde um nucleófilo e um doador de prótons são requeridos.

Figura 1. Esquema do local de ação das proteases nas cadeias polipeptídicas.



Fonte: Adaptado de McDonald (1985).

Nos organismos vivos, as proteases estão relacionadas a processos regulatórios, regulando a localização e atividade de outras proteínas, criando novas moléculas bioativas, contribuindo para o processamento da informação celular e gerando, transduzindo e amplificando os sinais moleculares. Porém, também são responsáveis por uma variedade de condições patológicas, como o câncer, desordens neurodegenerativas e cardiopatias.

Na indústria, as proteases são utilizadas em diversos processos tecnológicos como no processamento de alimentos (atuando na clarificação de sucos e fermentação de bebidas e pães), papel (separando a celulose da lignina no processo de preparação da polpa), tecidos (no tratamento de lãs), formulação de detergentes (auxiliando a remoção de manchas), além de ter um importante papel no setor farmacêutico. Dentre as proteases de aplicação na área farmacêutica, um grupo de enzimas do tipo Colagenase se destacam nesse âmbito. A colagenase contribui para a formação de tecido de granulação e subsequente reepitelização de úlceras da pele.

O colágeno de tecido sadio ou do tecido de granulação recentemente formado não é afetado pela colagenase.

2.2 Colagenase

Colagenases são enzimas proteolíticas capazes de degradar a região helicoidal do colágeno em pequenos fragmentos. Em contraste com as colagenases de mamíferos, que clivam a hélice do colágeno em um único ponto, as colagenases microbianas atacam múltiplos sítios ao longo da hélice e por este motivo vem sendo largamente aplicadas na obtenção de peptídeos bioativos do colágeno (VILLEGAS et al., 2018). Devido ao uso potencial das colagenases microbianas na produção de peptídeos bioativos, existe um interesse em encontrar novas linhagens de fungos capazes de produzir estas enzimas com novas características e em um meio de produção com baixo custo industrial.

As colagenases verdadeiras são metalo-proteases zinco-dependentes encontradas em mamíferos e bactérias. Elas são diferenciadas das demais proteases colagenolíticas (serino-colagenases) por terem a capacidade de degradar a forma em tripla hélice do colágeno insolúvel, enquanto as outras degradam o colágeno desnaturado.

As principais colagenases encontradas são as isoladas de tecido do trato digestivo de vários peixes e invertebrados, incluindo: tailfin girino (GROSS e NAGAI, 1965; NAGAI et al, 1966)., Pele de coelho (FULLMER e GIBSON, 1966), tecido uterino de ratos Wistar (JEFFREY e GROSS, 1967), trato digestivo de caranguejos (ZEFIROVA et al, 1996), trato digestivo de camarões (BARANOWSKI et al, 1984), trato digestivo de lagostas (GARCIA-CARRENO et al, 1994), trato digestivo de camarão tropical (*Litopenaeus vannamei*) (VAN SELOS e WORMHOUDT, 1992) e peixe-gato (*Parasilurus asotus*).

Pesos moleculares relatados variam significativamente com base no tipo de enzima (serina ou metallocollagenase) e a fonte (microbiana ou tecido animal). HARPER et al. (1965) isolaram duas colagenases de *Clostridium histolyticum* com pesos moleculares de 57,0 a 105,0 kDa. BOND e VAN WART (1984) isolaram seis colagenases diferentes das mesmas espécies com pesos moleculares variando 68,0 a 128,0 kDa. Relataram a presença de colagenases isoladas do *Clostridium perfringens* apresentando pesos moleculares variando 80,0 a 120,0 kDa. As

colagenases bacterianas tem normalmente peso molecular > 55,0 kDa, enquanto pesos moleculares de colagenases obtidos a partir de tecidos animais tendem a ser inferior. Por exemplo, SAKAMOTO et al. (1972) isolaram colagenase com peso molecular de 41,0 kDa a partir de ossos do rato.

MCCROSKERY et al. (1975) relataram que o peso molecular da colagenase de músculo de coelho é entre 33 kDa a 35 kDa. Um número de investigadores (KRISTJANSSON et al, 1995; ROY et al, 1996; DABOOR et al, 2010).) isolaram a partir de glândulas digestivas uma serino-colagenase de organismo marinho com pesos moleculares <60 kDa. Sugeriram que algumas das variações nos pesos moleculares de colagenases podem ser simplesmente devido à proteólise de um precursor maior da colagenase. Para uso na indústria, essa variação será menos importante do que a atividade colagenolítica geral, mas o potencial de variação deve ser observado (NIRMAL et al 2011; DABOOR et al, 2010).

A produção de colagenases dos tecidos é estimulada na presença de colagenases microbiana que parecem servir como um fator-chave, semelhante à ação de enzimas exógenas produzida por alguns micro-organismos quando adicionado à alimentação humana ou animal (TAOKA et al., 2007; DABOOR et al, 2010). Eles são normalmente classificados como serino-colagenase ou metalo-colagenase, com base em suas diferentes funções fisiológicas.

Serino-Colagenases, como todas as serino-proteases contém um resíduo de serina em seus sítios catalíticos. Eles têm tipicamente pesos moleculares na faixa de 24,0 a 36,0 kDa (ROY et al., 1996). Eles são normalmente associados com o órgão digestivo (ZEFIROVA et al., 1996), são capazes de decompor a estrutura tripla hélice de colágeno tipo I, II e III, e estão frequentemente envolvidos com a produção de hormônios, a degradação protéica, coagulação do sangue e fibrinólise (NEURATH, 1984; NIRMAL et al 2011).

Metalo-colagenases são membros da família Matrix Met alo-proteinase (MMP), com pesos moleculares entre 30,0 a 150,0 kDa (HARRIS e VATAR, 1982). Como todos MMP, metalo-colagenases são enzimas dependentes de zinco e são inibidas por qualquer quelante que se liga a esses íons. Cálcio (íon divalente) é necessário para a estabilidade (STRICKLIN et al., 1977). Apenas MMP 1, 8, 13, 14 e 18 têm atividade contra os tipos de colágeno nativo de cadeia tripla I, II, III, VII e X (FREIJE et al., 1994). Metalo-colagenases são comumente recuperados de tecidos

animais como ossos, barbatanas, peles e de hepato-pâncreas de caranguejo marinho (SIVAKUMAR et al., 1999; NIRMAL et al 2011; DABOOR et al, 2010).

Como enzimas proteolíticas, colagenases têm um número de aplicações industriais. O colágeno é parcialmente responsável pela tenacidade em carnes vermelhas e usado como tenderizers na indústria de alimentos (FOEGEDING e LARICK, 1986; CRONLUND e WOYCHIK, 1987).

As colagenases têm aplicações em pele e couro (GOSHEV et al., 2005; KANTH et al., 2008). No entanto, as utilizações mais comuns destas enzimas parecem estar na medicina. Elas são usados para tratar queimaduras e úlceras (AGREN et al, 1992; PULLEN et al, 2002), para eliminar tecido cicatricial (SHMOILOV et al., 2006) e desempenham um papel importante no transplante bem sucedido de órgãos específicos (KLOCK et al, 1996; KIN et al, 2007).

Na Indústria farmacêutica existem várias aplicações para o uso das Colagenases, principalmente, quando envolve o tratamento de feridas. GYNO IRUXOL (colagenase e cloranfenicol) é um medicamento que envolve associação de colagenase com cloranfenicol e é utilizado como agente desbridante em lesões superficiais, promovendo a limpeza enzimática das áreas lesadas, retirando ou dissolvendo, enzimaticamente, tecidos necrosados e crostas. A cicatrização da ferida é acelerada se não houver tecido necrosado no ferimento.

2.3 Micro-organismos produtores de Colagenases

Colagenases microbianas são relatados a partir de bactérias, actinomicetos e fungos. Entre os fungossão relatados: *Aspergillus sclerotiorum* (KUNDU et al, 1974), *Aspergillus flavus* (SUKHOSVROVA et al 2003), *Coccidioides immitis* (LUPAN& NZIRAMASANGA 1986), *Arthrobotrys amerospora* um fungo de nematóides (SCHENCK et al 1980), fungo antárctico *Arthrobotrys tortor Jarowaja* (TOSI et al 2001) e *Lagenidium giganteum* um fungo parasita-mosquito (DEAN& DOMNAS 1983). TOSI et al (2001) rastrearam espécies fúngicas pertence Arthrobotrys para a produção de colagenase e descobriu que a colagenase a partir de estirpe do antárctico de *Arthrobotrys tortor* (ATCC 96018) era uma enzima constitutiva e produzia cerca de três vezes mais atividade do que outras espécies *Arthrobotrys tortor* B114, *Arthrobotrys conoides* CMM 1017 e *Arthrobotrys oligospora* CBS 280,86 (NIRMAL et al 2011).

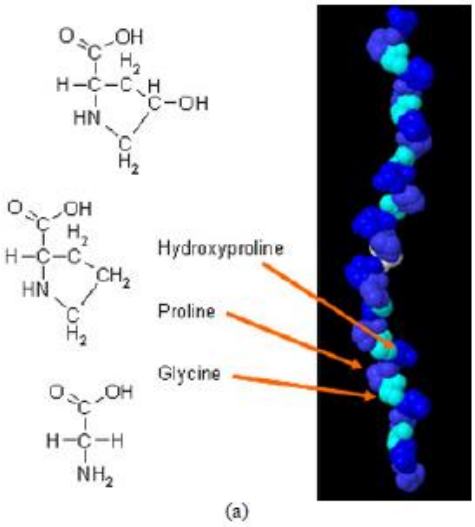
2.4 Colágeno

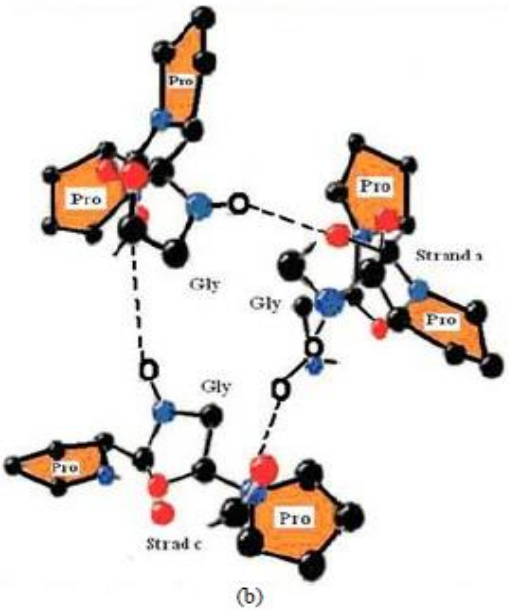
O colágeno é o substrato específico da colagenase e é encontrado nos tecidos conjuntivos de animais, tornando-se aproximadamente 30% da proteína no corpo humano (BHAGWAT et al., 2018). É composto por três cadeias peptídicas numa estrutura de hélice tripla (Figura. 2), que oferecem suporte a ambos as células e tecidos (DABOOR, 2010). Uma cadeia é sempre composta de vários tríletes de sequências Gly X-Y, nas quais X-Y podem ser qualquer aminoácido (porém, comumente X é uma prolina e Y uma hidroxipolina) e são geralmente estabilizadas por pontes de hidrogênio e por ligações cruzadas. Nos mamíferos, cerca de 21 tipos de colágeno foram identificados (KIELTY e GRANT, 2002; DABOOR et al, 2010), enquanto vinte e oito tipos distintos de colágeno, foram identificados no corpo humano (RASKOVIC et al., 2014).

O percurso helicoidal da estrutura do colágeno é destrógiro, sentido oposto ao enrolamento das hélices polipeptídicas individuais, que é levógira. Essas duas conformações permitem um enrolamento mais apertado possível das múltiplas cadeias polipeptídicas. O enrolamento da hélice tríplice fornece uma grande resistência às forças de tensão, sem nenhuma capacidade para o estriamento. Assim, o colágeno apresenta uma resistência mecânica que é aumentada pelo enrolamento helicoidal de múltiplos seguimentos em uma super-hélice, de uma forma muito parecida a cordões enrolados entre si e sobre si mesmo, para formar uma corda mais resistente.

Os aminoácidos hidroxilisina e hidroxiprolina, presentes na estrutura do colágeno, não são sintetizados, mas são formados por hidroxilação da prolina e lisina, respectivamente, que se inicia nos ribossomos durante a tradução do mRNA do colágeno. Assim, o processo de hidroxilação é concluído após a formação da estrutura linear.

Figura 2 – Estrutura de tripla hélice de colágeno: A (Adaptado de Morris e Gonsalves, 2010) e B (Adaptado de TQS, 2004). Fonte: Am. J. Biochem. & Biotech, 6 (4):. 239-263 de 2010p.





Colágenos foram classificados com base na expressão de genes diferentes durante a construção do tecido. Colágeno do tipo I é o tipo mais comum que se encontra nos ossos, tendões, ligamentos e pele, enquanto o colágeno tipo III, é a segunda mais comum e encontra-se em tecidos elásticos, tais como os vasos sanguíneos e vários órgãos internos (KIELTY e GRANT, 2002; DABOOR et al, 2010). A frequência dos tipos V e XI são baixos, mas eles são encontrados associado aos tipos I e II em osso e cartilagem, bem como em outros tecidos (PROCKOP e KIVIRIKKO, 1995; MYLLYHARJU e KIVITIKKO, 2001; KIELTY e GRANT, 2002; NIRMAL et al 2011).

2.5 Ultrassom: Princípios biofísicos

O ultrassom é uma onda mecânica acústica produzida pelo movimento oscilatório das partículas de um meio. Por ser uma forma de energia mecânica tem como principais propriedades, reflexão, adsorção e dispersão, e precisa de um meio físico para se propagar, como através de sólidos, líquidos ou gases (MASON et al., 2005). Ele emite frequências acima de 20 kHz, tendo em vista que o ser humano só consegue detectar frequências entre 20 Hz a 20 kHz. Frequências abaixo de 20 Hz são referidas como infrassom (DALAGNOL et al., 2017).

As ondas ultrassônicas são classificadas em dois grandes grupos, dependendo da sua frequência e intensidade. Ultrassons de baixa energia são de alta frequência (2-20 MHz) e baixa intensidade (<1 $W.cm^{-2}$) e não são destrutivos, tendo emprego em técnicas de maturação, concentração ou dispersão de partículas em fluidos (O'DONNELL et al., 2010). Ondas ultrassônicas de alta energia são de baixas frequências (20-100 kHz) e desenvolvem níveis de potência mais altos (10 - 1000 $W.cm^{-2}$), com energia suficiente para romper enlaces intermoleculares, sendo capazes de modificar algumas propriedades físicas e favorecer reações químicas (PAWAR et al., 2018).

O ultrassom é produzido a partir de um transdutor, que converte a energia elétrica em energia mecânica sonora em frequências ultrassônicas. Os sistemas de aplicação de ultrassom mais utilizados são os banhos e as sondas. No banho, o transdutor é diretamente unido à base ou às paredes do tanque e a energia ultrassonora é transmitida diretamente através de um líquido, ocorrendo muita dispersão de energia ultrassonora (SINISTERRA, 1992).

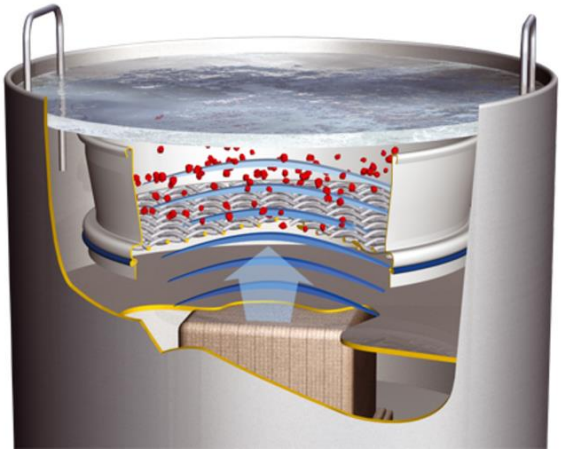
A utilização do ultrassom em tecnologia de alimentos tem evoluído muito com o passar dos anos, pois é uma técnica que tem apresentado efeitos benéficos no processamento, na preservação, maiores rendimentos e seletividade, tempos de processamento mais curtos, redução dos custos de operação e manutenção, melhoria dos atributos de qualidade, e redução de patógenos (PATIST; BATES, 2008). Além de melhorar a qualidade e segurança dos alimentos, o ultrassom possibilita a elaboração de novos produtos com propriedades distintas (AWAD et al., 2012). Uma das maiores vantagens do uso do ultrassom na indústria de alimentos comparado com outras técnicas é sua ação eficiente, sendo considerada uma técnica segura e não poluente (KENTISH; ASHOKKUMAR, 2011), devido ao fato de se tratar

de uma nova tecnologia, com sustentabilidade ambiental (CHEMAT; ZILLE-HUMA; KHAN, 2011).

Figura 3. Ultrassom de uso laboratorial. A) Banho ultrassônico termostatizado com frequência fixa em 40kHz. B) Mecanismo de ação das ondas através da água para gerar alteração na atividade da Enzima Colagenase.

A.

B.



2.6 Efeitos do ultrassom em propriedades de Enzimas

A utilização do ultrassom em reações de hidrólise pode ser uma ferramenta importante no controle sobre agregação ou dispersão de partículas, proporcionando mudanças conformacionais na estrutura das proteínas, assim como aumentando a transferência de massa entre substrato-enzima (YU; ZENG; LU, 2013). Tendo em vista, o colapso cavitacional ocasionado pelas ondas ultrassônicas, o qual proporciona aumento da pressão e temperatura em regiões determinadas, muitos estudos começaram a ser desenvolvidos, sugerindo que os efeitos mecânicos e químicos gerados durante a sonicação desempenham um papel importante na inativação de enzimas (ISLAM; ZHANG; ADHIKARI, 2014; O'DONNELL et al., 2010).

Em reações enzimáticas o ultrassom pode agir alterando as condições do meio, e perturbando as ligações fracas, induzindo mudanças conformacionais na estrutura das proteínas (WANG et al., 2018). Isso pode levar a inativação de muitas enzimas, devido às pequenas alterações na estrutura e nas condições ambientais, como temperatura, pressão, tensão de cisalhamento e pH (ISLAM; ZHANG; ADHIKARI, 2014). Os efeitos cavitacionais em combinação com a frequência de irradiação podem promover a formação de radicais livres, que atuam alterando as células e enzimas presentes no meio. No caso de proteínas globulares, como no caso das enzimas, frequências entre 0-100 kHz são fortemente absorvidas e podem levar ao rompimento da estrutura. Com a formação de radicais hidroxila (OH) e a geração de calor durante o colapso de bolhas, pode-se afetar a estabilidade do biocatalisador, sendo este um fator limitante para aplicações combinadas de ultrassom/enzimas (EASSON et al., 2018).

O fator limitante para o uso do ultrassom em reações bioquímicas é o aumento na temperatura promovido pela irradiação, uma vez que enzimas podem ser inativadas termicamente. Contudo, este não é de todo um fator negativo já que a inativação térmica de enzimas é importante em alguns processos biotecnológicos. É preciso destacar que a inativação não ocorre em todos os casos, pois o efeito do ultrassom pode ser destrutivo ou construtivo dependendo da intensidade das ondas (LUO et al., 2019).

Entretanto, o tratamento com ultrassom em frequência e intensidade adequada pode levar a um aumento da atividade enzimática, sendo utilizado como forma de ativar a enzima (YILMAZ, 2018). Em baixas frequências, o ultrassom favorece algumas reações enzimáticas, tanto com a melhora na transferência de massa,

quanto atuando sobre a estrutura da enzima (Wang et al., 2018), porém, os efeitos do ultrassom na ativação das enzimas ainda não estão bem claros.

3. OBJETIVO

3.1 Objetivo Geral

Avaliar o efeito do ultrassom na atividade colagenolítica de uma protease previamente purificada de *Aspergillus sp.* UCP1276, bem como estudar o potencial biomédico da enzima em ensaios de cicatrização e produção de peptídeos com atividade anticoagulante.

3.2 Objetivos Específicos

- Purificar a enzima com atividade colagenolítica através de métodos cromatográficos;
- Estudar o efeito do ultrassom na atividade enzimática da enzima colagenolítica durante a reação de hidrólise do Substrato Azocoll, na presença e ausência de inibidor PMSF e na cinética enzimática;
- Avaliar o potencial de hidrólise da colagenase frente ao substrato Colágeno Tipo I;
- Utilizar o Sistema de Eletroforese SDS-PAGE para observar o grau de hidrólise do Colágeno Tipo I sob ação do Ultrassom;
- Avaliar a produção de peptídeos gerados na hidrólise do Colágeno Tipo I e o seu potencial anticoagulante.
- Identificar o melhor tempo de ação do Ultrassom sobre a enzima colagenolítica e a concentração de substrato Colágeno tipo I na produção de peptídeos com atividade anticoagulante.
- Avaliar a atividade antiagregante plaquetária dos peptídeos obtidos da Hidrólise do Colágeno Tipo I utilizando a Colagenase previamente purificada.

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CAPÍTULO I

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Ultrasound-assisted enzyme catalyzed hydrolysis of collagen to produce peptides with biomedical potential: Purification process

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Abstract

Ultrasound is used for a growing variety of purposes in diverse areas because it is highly efficient and energy saving, has low instrument requirements and produces no pollution. The involvement of ultrasound in enzymatic reactions shows great potential for industrial applications. In this work, ultrasonic treatment used throughout the whole process of enzymatic reactions of a Collagenase previously purified from *Aspergillus sp.* UCP1276 was evaluated enhance the biodegradation of collagen and to produced peptides with anticoagulant activity. Results showed the enzyme yield was found to be enhanced by different factor for collagelotytic protease in presence of several inhibitors. Besides, after 30 minutes of incubation by ultrasound exposure was observed a high efficiency to produce peptides with biological properties concerning to anticoagulant activity as well as in prothrombin time. The results indicate the effectiveness of low frequency ultrasound in improving enzyme yields with a vision of commercial applicability of the process.

Keywords: anticoagulant activity; ultrasound incubation; catalytic degradation, collagenolytic activity

1. Introduction

Proteases represent a relevant class of enzymes which has applications in both physiological and commercial fields (Madhan et al. 2007). For catalyzing the hydrolysis of proteins, they are applied in various industrial sectors, such as detergent, food, pharmaceutical, chemical, leather and silk, in addition to the treatment of waste (Pillai et al. 2011; Vijayaraghavan et al. 2014).

Collagenolytic proteases are proteases that degrade the collagen molecule, involved in various physiological process, such as fetal bone development, wound repair and meat tenderization. They are classified into two types, namely, metallo-collagenase and serine collagenase, based on their physiological functions. These enzymes convert their substrates into small, readily soluble fragments, which can be easily removed from fabrics. All the proteolytic enzymes found in detergents are nonspecific serine endo-proteases (e.g. subtilisin) with a preferred cleavage on the carboxyl side of hydrophobic amino acid residues, but they are able to hydrolyze most peptide links (Daboor 2010).

Microbial sources are relevant for commercial purposes since proteases from plants and animals are not enough to supply the increasing market demands. In general, microorganisms are an excellent source of enzymes due to their biochemical diversity and susceptibility to be easily genetically manipulated (Rao et al. 1998).

Aspergillus are in the order Eurotiales also comprising the genera Penicillium, which are known to have a potential for producing various metabolites such as antibiotics, organic acids, medicines or enzymes, milk-clotting enzymes and extracellular proteases. Isolates of the so-called domesticated species, such as *A. oryzae*, *A. sojae* and *A. tamarii* are used in oriental food fermentation processes and as hosts for heterologous gene expression.

Ultrasound (US) treatment has been used in a positive way for enzyme technology (Bashari et al. 2016). Particularly low frequency ultrasound has been reported for improving biotechnological processes based mainly in the effects of the acoustic cavitation on the enzyme structure and on the enhancement of the mass transfer due local turbulences (Szabó and Csiszár 2013). Particularly, proteolytic activity, for example by pepsin, has been discussed as activated or improved by Ultrasound exposure due conformational changes in folding, binding and structure (Delgado-Povedano and Luque de Castro 2015), more precisely in secondary and tertiary structures (Yu et al. 2014).

This present work aimed, based in simple methods, to evaluate the biotechnological potential of a protease with collagenolytic activity previously purified from *Aspergillus sp UCP 1276*, and to characterize its activity under ultrasound exposure. Furthermore, the effects of ultrasonic exposure on the enzyme activity was studied to produce peptides for potential applications like in thrombolytic therapy as an anticoagulant.

2. Materials and methods

2.1 Microorganism and fermentation medium

The fungal species used in this study belonged to the genus *Aspergillus* (UCP 1276) and was obtained from the Catholic University of Pernambuco (RENNORFUN/SISBIOTA). The strain was isolated from Caatinga soil and kept in Czapek agar medium. For all the experiments, fungal spore suspensions with a final concentration of 10^4 spores/mL were used.

For production medium, the keratin-degrading microorganism was cultivated for 10 days at 30°C using a complex medium containing 0.025 (g/L) CaCl2, 0.005 (g/L) ZnSO4 .7H2O, 0.015 (g/L) FeSO4 .7H2O and 0.05 (g/L) MgSO4 .7H2O at pH7.8 in 500mL Erlenmeyer flasks. The feather fragments (0.5%) were weighed and added separately to each flask (Anbu, Gopinath, Hilda, Lakshmipriya, & Annadurai, 2007). The flasks were then autoclaved for 15min at 121°C, followed by inoculation with a fungal spore from the culture medium with shaking at 120rpm at 30°C for 10 days. Every day, aliquots of 10mL were collected from the cultures and centrifuged at 4°C at 10,000g for 20min to harvest the supernatant containing the protease. This supernatant was then used to determine protein dosage and collagenolytic activity. Since the target collagenase was an extracellular sample, the filtrate was analyzed to determine the final protein concentration and activity, then used as an enzyme source (referred to as the crude extract). All the collagenase purification experiments were performed using the crude extract.

2.2 Protease activity

The methods for protease activity determination were based on Ginther et al (1979). A volume of 0.15 mL of enzyme extract was added to a solution containing azocasein 1% w/v in 0.1 M Tris-HCl buffer, pH 7.4. The mixture was incubated for 1 hour at 37 °C in the dark. The reaction was stopped by the addition of trichloroacetic acid (TCA), 10% w/v, and was further centrifuged for 20 minutes at 3000 x *g*. A volume of 0.8 mL of the supernatant was collected and added to 0.2 mL of a 1.8 M NaCl solution. Absorbance was measured at 420 nm.

2.3 Collagenase activity

The collagenase activity was performed using the Azocoll method described by Chavira et al (1984). Azocoll was washed and suspended in a 0.2M Tris–HCl buffer (pH 7.8) containing 1.0 mM $CaCl_2$ up to a final concentration of 5mg/mL. Thereafter, 50 µL of sample and 950 µL of buffer were mixed with 5 mg of Azocoll suspension in a 1.0mL reaction tube and incubated at 37 °C in a water bath at 60-minute. After, samples were chilled in ice for 5 min to stop the reaction and centrifuged at 8000 g and 4 °C for 20min. The absorbance of the supernatant was measured at λ520 nm by means of a UV-VIS spectrophotometer. One unit of enzyme activity (U) was defined as the amount of enzyme per mL of crude extract that led, after 60 min of incubation, to an absorbance increase of 0.1 at λ520 nm, because of the formation of azo dye-linked soluble peptides. The specific activity was calculated as the ratio of the enzymatic activity to the total protein content of the sample, and expressed in U/mg.

2.4 Protein determination

The protein content of the samples was determined by the methods of Smith et al. (1985), using bovine serum albumin as standard.

2.5 Gel-filtration chromatography: Purification and determination of molecular weight

To obtain the collagenase used in this work, the method was previously described by Ferreira *et al*, (2016). Briefly, after use anionic chromatography on DEAE-sephadex, for the gel filtration method, an ÄKTA Avant purifier (GE Healthcare, Uppsala, Sweden) was used to separate protein fractions eluted by Tris–HCl buffer (pH 8) added by 0.15 M NaCl on a Superdex 75 (HR10/300GL) PC 3.2/30 column. The protein profile of separation was evaluated at 215 nm and 280 nm. Weights determination were achieved using Gel Filtration Markers Kit for Protein Molecular Weights 6,500-66,000 Da purchased from Sigma-Aldrich.

2.6 Ultrasound exposure

Technique was achieved according to Ovsianko et al., (2005) with modifications. Briefly, for the ultrasound treatment, microtubes (1000uL) containing the collagenase were distributed in a plastic shelve and placed in the center of ultrasonic water bath (Unique 1850A, Indaiatuba, Brazil), with internal dimension of 30x15x10 cm, ultrasound frequency of 40 kHz

and potency of 155 Watts RMS. The samples were completely immersed in the water (as shown in Figure 1) and the exposure time corresponded to 5, 15 and 30 min. The experiments comprised too the submission of enzyme activity in presence of PMSF (1mM) and follow to observed the reversed effect activity of US.

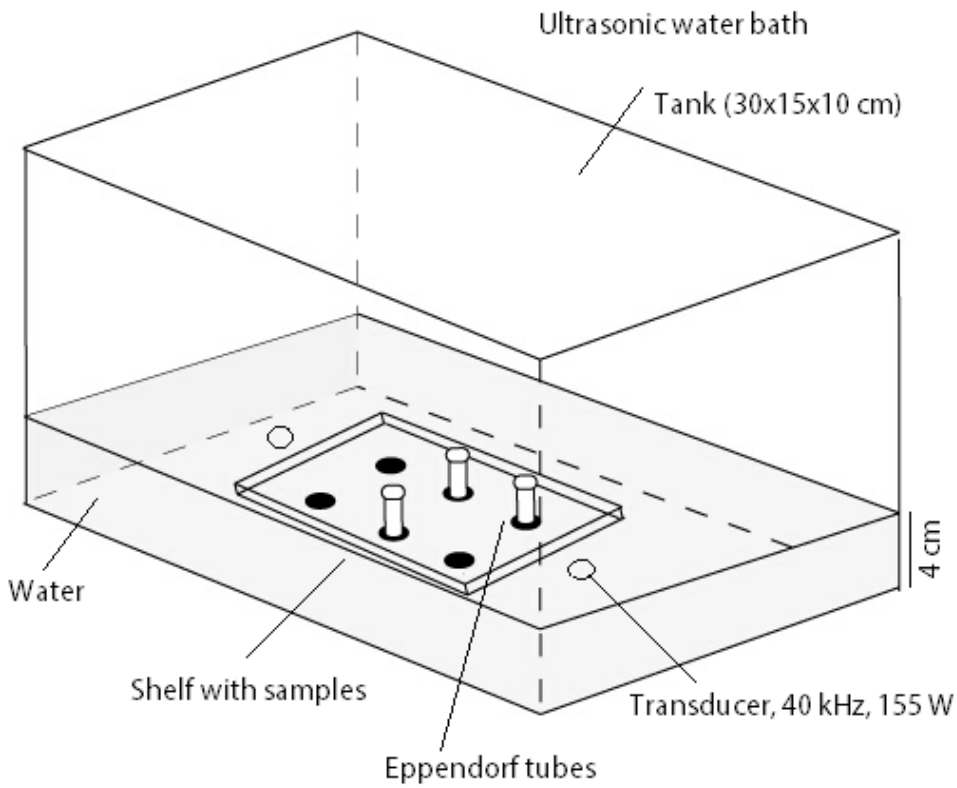

Figure 1. Spatial distribution of the samples in the ultrasonic water bath.

2.7 Collagen hydrolysis by the purified Collagenase and SDS-PAGE

To investigate the degradation ability of the collagenase on native proteins, purified collagenase was incubated with Type I collagen from bovine Achilles tendon (Sigma Chemical Co., St. Louis, MO). Type I collagen at 5.0 mg/mL was incubated with an appropriate amount of collagenase (45μg) in Tris-HCl buffer at 37 °C for 5, 15 and 30 min under Ultrasound effect using bath ultrasonic. Post the reaction, samples were precipitated with 20% TCA, to remove proteins, and the supernatants were neutralized with NaOH, lyophilized, and subject to SDS–PAGE, followed by staining. The peptide patterns generated by collagen hydrolysis were determined by SDS-PAGE, by separation in 12.5% gel and stacking in a 4% gel, according to the method of Laemmli (1970). The gel was stained and destained as described earlier. Molecular Weight Marker Kit, lyophilized powder, for Molecular Weights 14,000-69,000 was

purchased from Sigma-Aldrich. 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).

2.8 Anticoagulant activity in human platelet-poor plasma (PPP)

The method for obtaining the PPP used blood suspension obtained with sodium citrate anticoagulant centrifuged at 3000 × g and at 25◦C. A coagulometer BFT II (Dade Behring) was used to evaluate the anticoagulant effect of the collagen-degrading peptides, obtained by US conditions, by analysis of the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT). Different concentrations of the peptides (0; 30; 40 and 50 ug/mL) were used in the tests. The negative controls were prepared differently depending on the test: for PT and TT assays, 50 uL of PPP were added to 50 uL of 0.15 M NaCl (incubated at 37◦C for 1 min), followed by addition of 100 uL of Thromborel S and Thrombin(Dade Behring) reagents; for the APTT assay, 50 uL of PPP added to50 uL of 0.15 M NaCl and 50 uL of Dade actin activated cephaloplastin reagent-Dade Behring (incubated at 37◦C for 2 min), followed by the addition of 50 uL of 0.025 M $CaCl_2$. The control times for APTT, PT and TT were 33.7 s, 13.0 s and 23.0 s, respectively. The coagulation inhibition (CI) index was calculated as follows:

$$CI = \frac{CTt}{CTc}$$

Where CTt is the coagulation time for tests and CTc is the coagulation time for controls.

Samples were used under US effect for 30min.

2.9 Statistical analysis

Data are represented as the mean ± SEM of three replicates.

3. Results and Discussion

3.1 Purification process to obtain collagenase from Aspergillus sp UCP 1276

In the current study, before to apply the collagenase obtained from bioprocess using chicken degrading-feather, a protease with collagenolytic activity was purified from metabolic extract by Superdex G-75 HR/10/300GL column. The results of the purification procedure are

summarized in figure 2. The peak obtained in figure 2 shows 26.3 U/mg of activity against Azocool and was used in other assays.

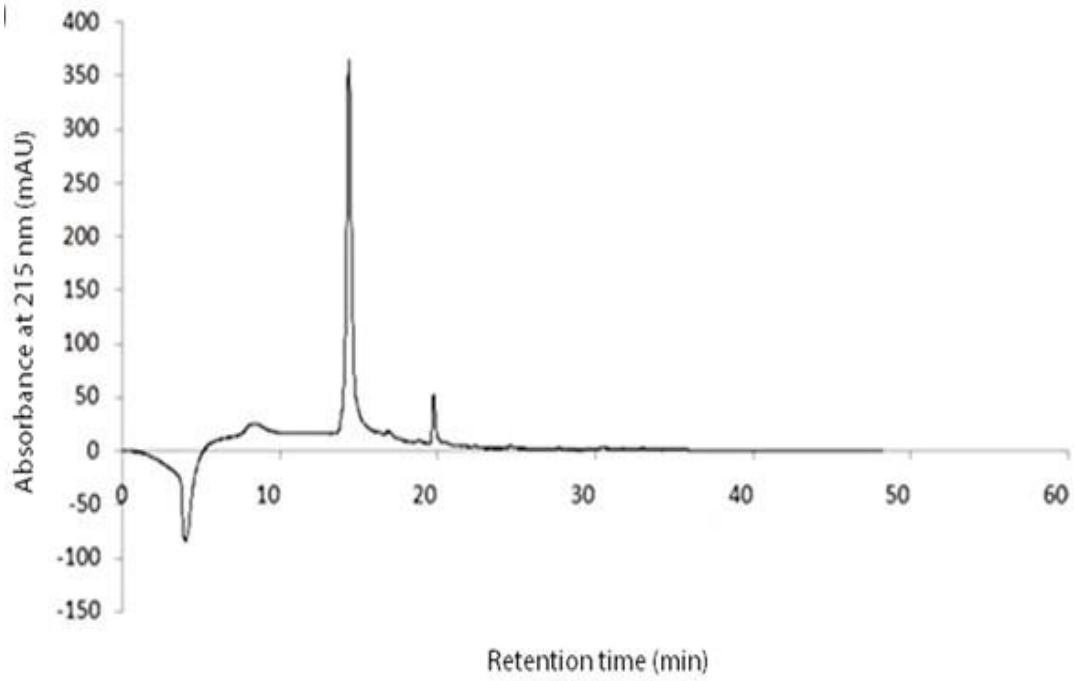

Figure 2. Elution profile of collagenase from *Aspergillus* UCP 1276 through gel filtration chromatography on Superdex 75 column (ÄKTA avant).

3.2 Ultrasound effects on the protease activity

For many years, ultrasound has been employed as an enzyme inactivation method, whereas some works have stated that ultrasound did not inactivate all enzymes under mild conditions. Ultrasound has positive effects on enzyme activity and can be used to accelerate enzymatic reactions. The involvement of ultrasound in enzymatic reactions shows great potential for industrial applications. In this work, ultrasonic treatment used throughout the whole process of enzymatic reactions will be demonstrated.

The ultrasound irradiation on the purified enzyme was studied in different situations of time of reaction and in presence or absence of inhibitor PMSF to verify a possible mechanism of action of the US treatment on the enzyme catalysis. Under exposure, the activity was enhanced, reaching more than 72.0% increase in relation to the non-exposed enzyme (Figure 3a), with activity stability during 30 minutes of exposure (Figure 3b). In relation to the influence of the US exposure time on the enzyme activity, Szabó and Csiszár (2013) observed a reduction

of the enzymatic activity of commercial cellulase after 65 minutes, while Zhou et al. (2013) found an increase in the activity after 20 minutes of enzyme reaction under US irradiation, with subsequent loss of activity.

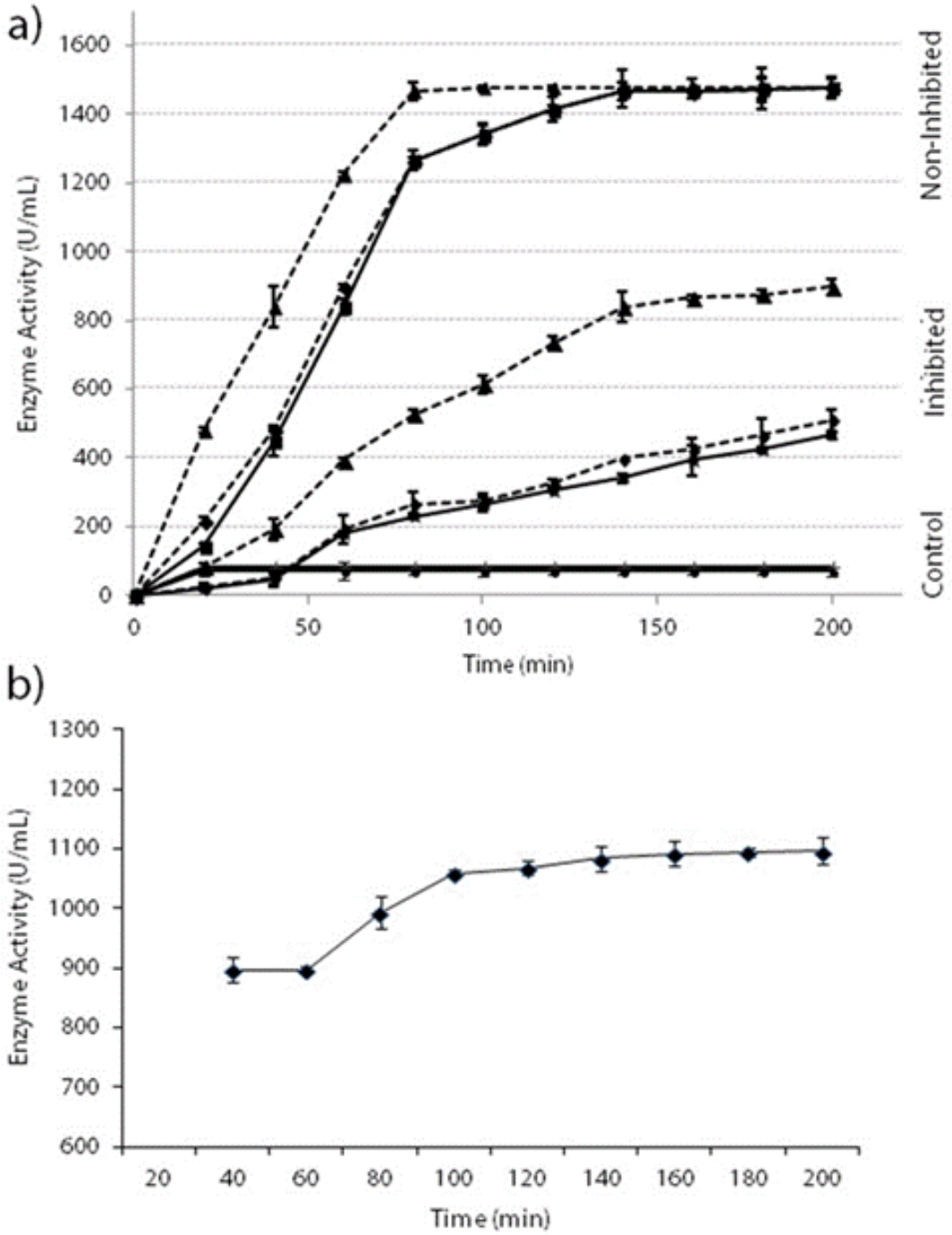

Figure 3. a) Effects of ultrasound exposure on the purified collagenase using Azocool as substrate. The reaction time was evaluated until 200 min. The assays were divided by reactions partially inhibited and non-inhibited by PMSF inhibitor (10 mM) and by the

US treatment (dashed line) or Absence (continuous line). They are also classified by the moment of US exposure: before the protease reaction (—▪—), during the protease reaction (—▲—), native enzyme (—●—), the control (○) and negative control (+). **b)** Collagenase previously purified without US conditions. Native collagenase obtained from gel filtration.

The test in the presence of inhibitor showed that the under US exposure, the inhibitor effects were relatively diminished, when compared with the enzyme activity in presence of inhibitor but non-exposed. At the same time, the inhibition by PMSF with posterior US exposure did not alter the collagenolytic activity, when compared to the non-exposed samples. That loss of the PMSF-inhibition due the US treatment could be probably related directly to the vibrational forces that would hinder the linkage inhibitor-enzyme or even indirectly by thermodynamic implications, which would affect the probability of interaction between inhibitor and enzyme by energetic reasons (caused by changes in the liquid pressure and temperature due the acoustic cavitation).

Many mechanisms about how ultrasound irradiation affects enzyme activity have been far discussed in the literature. The most likely mechanism reported is the acoustic cavitation (Islam et al. 2014), caused when the ultrasound waves promote implosion and fragmentation of gas bubbles in a liquid medium. Gębicka and Gębicki (1997) have discussed that the collapsing bubbles cause physical alterations in the medium leading to an increase in the temperature and pressure and consequently could influence the enzyme activity profile, although a limited number of studies have reported an enhanced *in vitro* enzyme activity (Barton et al. 1996). Islam et al. (2014) have commented that the combination of heat, pressure, and pressure plus heat induced by ultrasound could be greatly effective on enzyme activities. The author cite enzymes as peroxidase, lipase, amylase and also proteases (trypsin) as being inactivated by ultrasound, following a protein denaturation promoted by mechanical forces from the cavitating bubbles or by the action of free radicals formed by sonolysis of water.

On the other hand, other studies have proposed alternative theories to elucidate the sonic effect on enzymes. Schmidt et al. (1987) discussed that diffusion increases and substrate concentration alterations could result in improved enzyme activity stimulated by ultrasonic fields. Barton et al. (1996) have cited localized increases in temperature and enhanced mixing of substrate, enzyme and products to promote the enzyme activation. Johns (2002) have proposed a mechanical mechanism called frequency resonance hypothesis, which suggests that

ultrasound irradiation provides enough energy to induce transient conformational shifts in enzymes (turning conformational shapes on and off) altering their activity performance.

Proteases are controversially reported as affected by ultrasound irradiation. Studies have shown that the different possibilities to influence the enzymatic activity by ultrasound treatment, included the effects on both enzyme and substrates, determine whether the activity is increased or inhibited. Abadía-García et al. (2016) discussed about that high ultrasound densities would cause direct rearrangements in the protein structure as well as protein aggregate formation, resulting in alterations in the protein hydrolyze level. That would justify why inactivation of enzyme by ultrasound irradiation is often reported.

Figure 4 shows the ability of collagenase from *Aspergillus* to hydrolyze the collagen after Ultrasound exposure. We observed no significative differences on visual degradation through SDS-PAGE technique. Maybe, to use 2D-electrophoresis, the results may be visualized with more efficiency. Besides in figure 4, the collagen control was not submitted to SDS-PAGE for its insolubility.

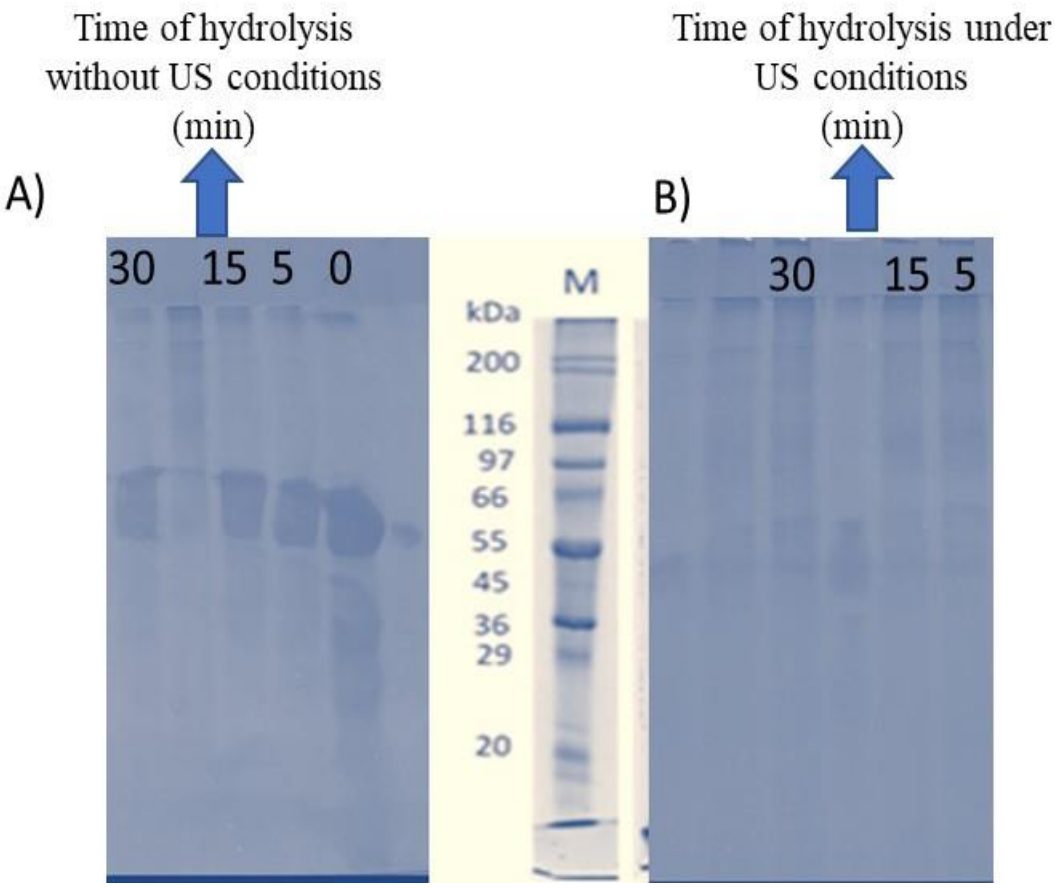

Figure 4. Hydrolysis of Collagen by collagenase protease over time**. A)** Hydrolysis of Collagen type I without US conditions at different times (0, 5, 15 and 30 min). **B)** Ultrasound exposure time 5, 15 and 30 minutes. During these time intervals the samples were analyzed by 12% SDS-PAGE. M – Markers.

Vercet et al. (2001) has observed inactivation of trypsin and α-chymotrypsin due their susceptible structure that does not allows them to resist to the disruption forces that are generated under sonic irradiation. Ovsianko (2005) studied the effects of an ultrasound exposure on the proteolytic activity and on the activation of chymotrypsinogen and trypsinogen and observed a significant decrease (at a potency of 26.4 W/cm^2) in the activity of trypsin and chymotrypsin and an inhibition of the protein's activation.

At the same time, Kim and Zayas (1991) found to be necessary a higher-energy ultrasound exposure to inhibit the proteolytic activity of chymosin. Following the authors, the prolonged exposure to high ultrasound intensities could inactivate directly enzyme active sites. In contrast, lower ultrasound intensities could enhance the enzyme activity, based in the mechanisms of substrate diffusion and mechanical forces (considering treatment of both enzyme or substrate), what has been reported in fact for enzymes in general (Schmidt et al. 1987) and also particularly for proteases (Abadía-García et al. 2016; Cherniavsky et al. 2011; Jyothi and Suneetha 2010) what corroborated the results in the present study.

Wang et al. (2016) reported that ultrasound treatment improved the reduction capacity and iron chelating potential of hydrolysates of soybean protein fractions [7S (b-conglycinin), and 11S(glycinin)]. The authors justified the ultrasound effects by an enhancement in the contact of the protein fractions with enzymes and by activation of soybean antioxidant peptides. At the same time, Ajmal et al. (2016) found that the low-frequency ultrasound was able to improve by 22 times the activity of the free enzyme --- when compared with non-exposed reactions, and additionally no molecular damages were caused by the treatment. Similar results were found according Uluko et al. (2018) and George et al (2014).

Ultrasound is an acoustic wavewith a frequency N20 kHz that needs a medium to propagate [20]. Accompanied by the spread of an ultrasonicwave, a series of alternating cycles of compression and rarefaction emerge in the liquid medium. During the rarefaction cycle, microbubbles are formed because of the reduced pressure. Consequently, acoustic cavitation is generated owing to the formation and subsequent dynamic life of microbubbles. During

cavitation, the pressure and temperature inside the bubble can rise to N1000 atm and 5000 K, respectively. Acoustic cavitation can be classified into stable and transient cavitation based on whether the microbubbles break up.

Ultrasonic cavitation causes changes in the protein conformation, herefore influencing the enzymatic activity. Many reports focused on the inactivating effect of ultrasound on enzymes, especially this effect on some undesirable enzymes in food processing. However, in recent years, an increasingamount of research has found that not all enzymes are inactivated by ultrasound. Many experimental results indicated that different enzymes had different degrees of tolerance and sensitivity to ultrasound. Generally, a low-intensity, shortduration ultrasonic treatment is more beneficial to improve enzyme activity, while prolonged exposure may result in progressive loss of the stability and activity of enzymes.

3.3 Collagen-degrading peptides for anticoagulant activity

Our results demonstrated the high efficiency of collagenase to hydrolysate the collagen at different times. Marchetti et al. (2018) have shown that CALB, a lipoenzyme, is a very effective catalyst for esterification reactions. However, the time required for achieving reaction equilibrium is around 72 hours. It will be of great benefit if the time to reach the equilibrium is reduced as it will have a positive impact on production rate of industrial processes.

Figure 5 presents the results of peptides produced by collagen hydrolysis on APTT (Activated Partial Thromboplastin Time). We observed an increase of the anticoagulant activity together to increase the concentration of peptides and associated to time of US exposure. To understand the intensification phenomena of collagenase catalyzed reactions, the effect of low frequency US on enzyme was studied by taking a model reaction. From the comparison of reaction time behavior, it is obvious that more time of US exposure has an improvement of the conversion rate.

Most enzymes are proteins whose structure is easily affected by physical (heating, irradiation, microwave, etc.) and chemical (acid, alkali, organic solvent, etc.) factors. Ultrasound is also reported to have an activation effect on membrane enzymes. However, previous papers reported that the active site of tyrosinase from most biological sources was located in a four-helix-bundle. This finding proved that the secondary structure had changed after the ultrasonic treatment. Moreover, the active site of tyrosinase was probably influenced due to radical or mechanical attack; thus, the tyrosinase activity was enhanced.

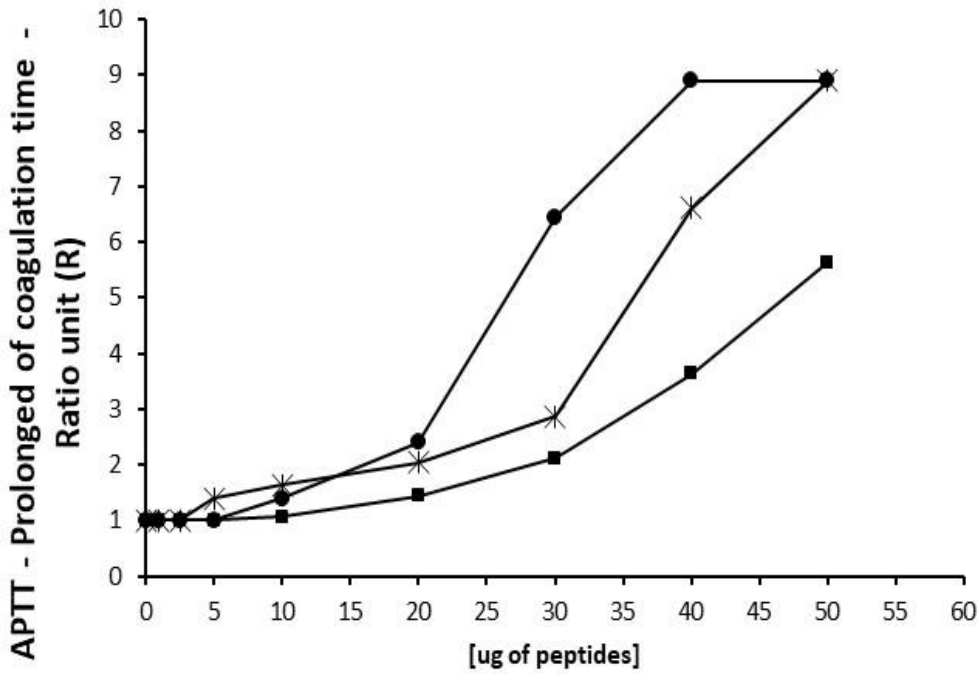

Figure 5. The effect of peptides produced by collagen hydrolysis on anticoagulant activity was assayed. Time of US exposure were determined and assayed according to: (●) 30min; (*) 15 min; (■) 5min of exposure.

Enzyme instability would reduce the reaction efficiency and increase the output cost. Reaction stability, thermal stability and storage stability play central roles in enzyme applications. Enhancing the enzyme stability during its reaction is therefore an urgent task.

Ultrasonic treatment affects the surfacemorphology of proteins and stimulates the combination of enzymes and substrates. Ultrasonic treatment unfolds protein molecules and creates micropores on the surface. As a result, the protein molecules tend to be disordered, and their surface areas increase, thus improving the affinity between enzymes and substrates and accelerating enzymatic hydrolysis [69]. It was found that wheat gluten treated by ultrasound had a looser network. Compared with untreated samples, the surface morphology of ultrasoundtreatedwheat gluten had fewer folds, and the protein particleswere significantly enlarged [70]. Yang et al. [71] studied the effect of ultrasonic pretreatment on the enzymolysis of defatted wheat germ protein catalyzed by alcalase. The hydrophobic amino acid content of hydrolysate from the pretreated wheat germ protein increased significantly over that of untreated samples. The results proved that ultrasonic pretreatment loosened the protein

structure and exposed more hydrophobic amino acid residues, causing the proteins to be more vulnerable to lactase. The effect of ultrasonic pretreatment on the kinetics of gelatin hydrolysis catalyzed by collagenase was investigated. The results revealed that pretreating gelatin by ultrasound increased the reaction rate and reduced the hydrolysis activation energy. Ultrasonic pretreatment also improved the gelatin hydrolysis degree owing to the change in the structure of gelatin induced by ultrasound.

4. Conclusion

The present work has established a process intensification approach using intermittent application of ultrasonic waves to hydrolysate the collagen and produce peptides with biological applications. The beneficiary effect observed in the current work suggests that the effect is strongly dependent on operating ultrasound parameters suggesting the importance of present study. The protease herein purified, as well as the method of ultrasound exposure, would have high potential for applications in the pharmaceutical and medical industry, as for example in the production of collagen peptides with biological activity.

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Figure Captions

Figure 1. Spatial distribution of the samples in the ultrasonic water bath.

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Figure 5. The effect of peptides produced by collagen hydrolysis on anticoagulant activity was assayed. Time of US exposure were determined and assayed according to: (●) 30min; (*) 15 min; (■) 5min of exposure.

CONSIDERAÇÕES FINAIS

A presente pesquisa traz um processo inovador na área da biodegradação de colágeno utilizando o Ultrassom como ferramenta na área da Biotecnologia. Os resultados demonstraram que o tempo de contato durante o processo de sonicação das reações envolvendo a enzima previamente purificada de *Aspergillus sp* UCP1276 foram essenciais para produzir peptídeos bioativos de interesse na área médica, uma vez que eles apresentaram atividade anticoagulante frente ao teste TTPA. Este ensaio é extremante importante para avaliar a via intrínseca da cascata de coagulação. Nos ensaios de eletroforese SDS-PAGE, a visualização do grau de hidrólise não pode ser totalmente acompanhada visualmente, uma vez que os peptídeos formados apresentaram uma não conformidade na separação. Dessa forma, sugere-se então, realizar técnica de separada por eletroforese bidimensional para visualização e determinação das frações peptídicas.

ANEXOS

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Can Ultrasonic Waves Improve Enzymolysis for Biomedical Applications?

Abstract

In recent years, ultrasound (US) has been successfully used in a large number of crucial bioprocesses such as upregulation of enzymatic hydrolysis (biocatalysis). Ultrasound treatment or pretreatment are supposed to activate/accelerate enzyme catalysis enhancing product formation. Such methods might offer high efficiency of enzymatic bioconversion and production of new biologically active peptides. We present here a short overview of the possible effects of ultrasound exposure to improve enzymatic processes and to minimize operational costs in biotechnological applications aiming to biomedical field.

Keywords: Biocatalysis; Accelerate; Upregulation; Bioconversion; Enzymatic; α-amylase; Immobilized; Diffusion

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Introduction

Acoustic cavitation has long been reported as the main responsible for changes in the physical properties of a liquid medium exposed to ultrasonic waves, giving rise to the formation of collapsing bubbles which lead to small pressure and temperature alterations. Since enzyme-substrate interactions are naturally favored by the high molecular diffusion rates in liquids, consequent changes in free energy of the system would directly alter the catalytic potential of the reactions [1].

On the other hand, the diffusion of substrates and direct mechanical forces are also reported for alterations in the enzyme conformation, which leads to enzyme activation [2].

The discussion of the use of US exposure as tool for improving biocatalysis is providential to the application in biotechnological processes. This study aims therefore to provide an insight of the possibilities that the US effects on enzymolysis offers to further researches and biotechnological processes [3].

Effect of Ultrasound on the Activity and Conformation of Enzymes

Factors such molecular alterations and mechanical forces from the direct action of ultrasonic waves would also be responsible for improvements on the enzymolysis. Researches have verified that the ultrasound exposure affected contrarily on the secondary and terciary structures of pepsin and α-amylase, with enhanced and inhibited activity respectively [4]. The author discussed that direct action of the ultrasonic waves on the enzyme structure lead to conformations changes that could activate or inactivate the enzymes [5]. That brings the idea that the enzyme structure is crucial to determine whether an enhancement will be provided.

On the other hand, the activity of immobilized enzymes have been described as affected by US exposure for changes in the concentration gradient and diffusion constants of the substrate and such activities presented increases in the reaction rate of 200% [6].

Enhanced Production of Bioactive Products by Ultrasound Stimulation

Bashari et al. [7] discuss that ultrasonic irradiation was used to improve the enzyme activity both by isolated action or combined with other agents: they verified that the kinetic activity of dextranase and the hydrolysis rate were improved and that the combined action of high frequency ultrasounds with high hydrostatic pressure enhances the enzymatic hydrolysis of dextran catalyzed by dextranase. Challenges associated with the introduction of new peptide products, for example, include proteolytic degradation methods like US, fast clearance in the body, low solubility in water, immunogenicity and regulatory hurdles [8].

Applications of the US as Tool for Biocatalysis

Activities of fibrinolytic enzymes, α-amilase, pepsin, dextranase and other enzymes are reported as enhanced under US exposure [9,10]. Thus the applications of the US devices in clotting processes, food industry are interesting for biotechnological applications. Besides, have studied the US effect upon the process of proteolytic autoactivation of the serine proteases precursors--chymotrypsinogen and trypsinogen. Ultrasound has also proposed as an economically feasible pretreatment alternative [11,12]. The effects of ultrasound on sludge includes particle size reduction,