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PROTEASE FIBRINOLÍTICA DE *Mucor subtilissimus* UCP 1262: PRODUÇÃO, PURIFICAÇÃO, CARACTERIZAÇÃO BIOQUÍMICA E ESTRUTURAL

AMANDA EMMANUELLE SALES

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TESE de doutorado apresentada ao Programa de Pós-Graduação em Biociência Animal da Universidade Federal Rural de Pernambuco, como pré-requisito parcial para obtenção do grau de DOUTOR em Biociência Animal. Área de Biotecnologia.

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Tese de doutorado

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À Deus e aos meus pais Elvo e Maria.

“Curious fact that those who never fail are also those who never truly succeed”.

Neil deGrasse Tyson

@neiltyson 5 de jun

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LISTA DE ABREVIATURAS E SÍMBOLOS

ANOVA- Análise de variância

BSA- Albumina de soro bovino

CGJ- Cheonggukjang

DCVSs - Doenças cardiovasculares

EC- Comissão Internacional de Enzimas

EDTA- Ácido etilenodiaminotetra-acético

K- Coeficiente de partição

Y- Recuperação

FP- Fator de purificação

KDa- Quilodaltons

FDA-Food and Drug Administration

PEG- Polietileno glicol

PMSF- Fluoreto de metilfenilsulfonil.

RPM- Rotações por minuto

SDFA- Sistema de duasfases aquosas

SDS- Dodecil sulfato de sódio

PAGE- Eletroforese gel de poliacrilamida

t-PA - Fator tecidual ativador de plasminogênio

Tm- Temperatura de desnaturação térmica

UK – Uroquinase

dH- Entalpia de desnaturação

dS- Entropia de desnaturação

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SUMÁRIO

RESUMO

Proteases fibrinolíticas são enzimas que degradam a fibrina, o principal componente dos coágulos sanguíneos. O acúmulo da fibrina nos vasos sanguíneos leva a trombose, fenômeno responsável por doenças cardiovasculares. Uma alternativa promissora para a terapia trombolítica tem sido a produção dessas enzimas por micro-organismos que promovem baixo custo, alta eficiência e capacidade de produção em larga escala. Produzir proteases fibrinolíticas por linhagens de fungos filamentosos por fermentação submersa e desenvolver o processo de purificação utilizando Sistemas de Duas Fases Aquosas (SDFA) e cromatografia líquida, além de caracterizar bioquímico e estruturalmente a enzima. Dentre as 36 espécies estudadas, 58% apresentaram atividade fibrinolítica acima de 100 U/mL A espécie com maior atividade foi *Mucor subtilissimus* UCP 1262 com 415 U/mL. Foram realizados processos fermentativos que resultaram na produção de 1075 U/mL de atividade fibrinolítica, com capacidade de degradação do coágulo sanguíneo de 16,7% *in vitro*. A extração da protease fibrinolítica produzida por fermentação submersa foi realizada utilizando o sistema de duas fases aquosas (SDFA) com Polietileno glicol (PEG) e sulfato de amônio. O PEG 8000 (g/mol) a 15% e sulfato de amônio a 25% foi selecionado como a condição mais eficiente para a extração da enzima na fase do sal, apresentando 345 U/mL de atividade, coeficiente de partição K=0,65; Recuperação Y=253,1% e Fator de purificação FP=8,8. A protease fibrinolítica produzida por *Mucor subitilissimus* UCP 1262 foi também pré-purificada utilizando fermentação extrativa com SDFA (PEG e sulfato de amônio), onde a espécie fúngica foi capaz de crescer mesmo em altas concentrações de sal, produzir e extrair simultaneamente para a fase do PEG do sistema. A protease fibrinolítica foi purificada também através de métodos cromatográficos utilizando um protocolo de purificação com dois passos. O extrato bruto inicial com a enzima foi pré-clarificado utilizando precipitação com acetona e adsorção em cromatografia de troca-iônica em DEAE-sephadex G50, o qual foi capaz de aumentar a pureza em 5,30 vezes com a recuperação de 36, 31%. O sistema de eletroforese bidimensional 2DE acoplado ao SDS-PAGE mostrou uma banda única de aproximadamente 15,3 kDa e a focalização isoelétrica apresentou o ponto isoelétrico no pH 3,9, exibindo uma natureza de enzima ácida. Adicionalmente a enzima foi significativamente inibida por PMSF e alta afinidade catalítica para o substrato sintético amidolítico N-Succinyl-Ala-Ala-Pro-Phe p-nitroanilide (SAApNA) e azocaseína. Sugerindo ser uma serino-protease semelhante à quimotripsina. Desdobramento proteico induzido por pH e temperatura foram aplicados para estudar as mudanças conformacionais da enzima e mostraram através da curva de desnaturação térmica, mudança da elipticidade a 222 nm, indicando um Tm (Temperatura de desnaturação) da proteína de 58,14°C. O dicroísmo circular no UV distante (far UV CD) da protease fibrinolítica mostrou a estrutura secundária da proteína com maior teor de α-hélix. Estes resultados demonstram um protocolo de purificação de enzimas eficiente. E o estudo da enzima purificada estabeleceu bases para elucidar mecanismos responsáveis pelas mudanças de conformação de uma nova enzima fibrinolítica sob a variação de condições variadas de temperatura e pH. Esta enzima fibrinolítica pode representar uma nova fonte de agente terapêutico no tratamento de doenças trombolíticas.

Palavras-chave: SDFA, *Mucor subtilissimus*, Protease fibrinolítica, purificação, dicroísmo circular.

ABSTRACT

Fibrinolytic proteases are enzymes that degrade fibrin, the main component of blood clots. The accumulation of this protein leads to thrombosis responsible for cardiovascular disease including myocardial infarction. A promising alternative to thrombolytic therapy has been the production of these enzymes by microorganisms which promotes low cost, high efficiency and capacity for large scale production. This study aimed to select species of filamentous fungi isolated from Caatinga soil samples - Pernambuco - Brazil and assess their potential for production of proteases with fibrinolytic activity. Among the 36 isolates studied, 58% showed fibrinolytic activity above 100 U/mL. The microorganism with the higher activity in terms of enzyme production was *Mucor subtilissimus* UCP 1262 with 415 U/mL. Further optimization of the fermentation process resulted in the production of 1075 U/mL of enzymatic activity. The fibrinolytic enzyme had a capacity of enzymatic degradation of the blood clot of 16.7 % *in vitro*. Extraction of fibrinolytic protease produced at submerged fermentation was carried out using a PEG/ammonium sulphate aqueous two-phase system (ATPS). PEG 8000 15% and 25% ammonium sulphate were selected as the most appropriate components for extraction with Fibrinolytic Activity in salt phase: 345 U/mL; K: 0.65; Y: 253.1 % and FP: 8.8. The fibrinolytic enzyme from *Mucor subitilissimus* UCP 1262 was pre-purified using extractive fermentation in PEG and ammonium sulphate ATPS, in which the fungal strain was able to grown even in high salt concentration, produced and extracted simultaneously to the PEG phase. A novel protease with fibrinolytic activity was purified also by chromatographic methods using a two-step purification protocol. Compared to the crude enzyme extract, the specific activity of the enzyme increased 5.30 fold with a recovery of 36.31%. The initial crude extract with the enzyme was pre-purified using acetone precipitation and adsorbed by ion exchange chromatography on DEAE-sephadex G50. The two-dimensional electrophoresis system (2DE) coupled with SDS-PAGE showed a single protein band of approximately 15.3 kDa and isoelectric focusing point of 3.9, exhibiting a nature as an acidic enzyme. Additionally, the activity was slightly inhibited by EDTA, but significantly inhibited by PMSF and also had a higher affinity for the N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (*SAApNA*) and azocasein substrates, suggesting to be a chymotrypsin-like protease. Protein unfolding induced by pH and temperature were applied to study the protein conformational changes and showed from the thermal denaturation curve, change in ellipticity at 222 nm, indicated *Tm (Melting temperature)* of the protein to be 58.14°C. The far UV circular dichroism (CD) of the fibrinolytic protease showed the secondary structure with most content percentage of α-helix. These results demonstrate an economical, viable enzyme purification protocol. And studying the purified fibrinolytic enzyme have established basis for elucidating mechanisms responsible for the changes in conformation of the new fibrinolytic enzyme under varying conditions of temperature and pH. This novel fibrinolytic enzyme may represent a new source of therapeutic agents to treat thrombosis diseases.

Keywords: ATPS, *Mucor*, fibrinolytic protease, purification, circular dichroism.

1. INTRODUÇÃO

O custo da produção e purificação de enzimas na indústria é o maior obstáculo para a aplicação com sucesso de proteases na medicina. Para a produção de enzimas fibrinolíticas muitas tentativas têm sido feitas para aumentar a expressão da enzima, incluindo a seleção de um meio de cultura ideal e otimização das condições ambientais. Uma vez que diferentes micro-organismos possuem diferentes características fisiológicas, sendo necessário otimizar os componentes do meio e condições para o crescimento celular e produção da enzima (KOTB, 2014).

Existem mais de vinte enzimas no corpo que contribuem com a coagulação do sangue enquanto que apenas uma, a plasmina, pode quebrar e remover o coágulo. A plasmina (EC 3.4.21.7) mantém o fluxo sanguíneo em lesões nos vasos (CHOI et al, 2009; WANG et al, 2011). É uma serino protease terminal acionada por ativadores de plasminogênio que rompe a rede de fibrina proteoliticamente (DEEPAK et al, 2010; ROVATI et al, 2010). As enzimas dependentes de plasminogênio para a sua atividade são enzimas que inicialmente precisam ativar o plasminogênio sanguíneo formando consequentemente a plasmina que hidrolisa as redes de fibrina. Estas enzimas têm sido utilizadas na terapia atual, porém possuem alto custo para aquisição e têm apresentado efeitos colaterais como: fibrinogenólise sistêmica acompanhada de sangramento, como também complicações neurológicas como hemorragia intracraniana (CHITTE et al, 2011).

Ainda há necessidade de se investigar enzimas fibrinolíticas específicas que superem as desvantagens dos efeitos colaterais. Desta forma, enzimas fibrinolíticas semelhantes a plasmina têm atraído o interesse médico nas últimas décadas. A pesquisa científica tem buscado como alternativa trombolíticos que agem diretamente na degradação do coágulo. Como resultado essas enzimas fibrinolíticas semelhantes a plasmina têm sido descobertas (KOTB, 2014).

Doenças cardiovasculares do sistema arterial e venoso são um problema que têm crescido rapidamente em todo o mundo e afetando indivíduos de todas as classes socioeconômicas. Dentre as diversas doenças circulatórias, a trombose, o infarto do miocárdio, e o tromboembolismo pulmonar são as de maior ocorrência. A terapia trombolítica com agentes fibrinolíticos é uma abordagem padrão para o tratamento de doenças

cardiovasculares na fase aguda, usualmente aplicado para conter de forma rápida a carga do trombo (KIM et al., 2015).

Para a obtenção de agentes terapêuticos enzimáticos, são necessários estudos para descoberta de novas fontes enzimáticas, bem como do processo fermentativo. Proteases fúngicas são secretadas extracelularmente e em alguns casos os metabólitos fúngicos refletem as condições de cultivo. A nutrição e o ambiente físico podem ser um fator dominante para o crescimento fúngico e para os níveis de produção de proteases extracelulares em sistemas de cultura submersa (LU et al., 2010).

O principal ecossistema da região Nordeste do Brasil é o Bioma "Caatinga", palavra de origem indígena que significa "floresta aberta", nomeado assim devido a sua aparência durante a estação seca. É constituída por uma extensiva planície semi-árida encontrada principalmente na Região Nordeste do País, do Piauí ao norte de Minas Gerais, com exceção do estado do Maranhão, que não possui "Caatinga" (FÁTIMA AGRA, et al., 2007).

O objetivo deste trabalho foi selecionar espécies fúngicas isoladas do Bioma Caatinga (PE-Brasil) produtoras de proteases fibrinolíticas, estudar as condições de produção, purificação e caracterizar estruturalmente a enzima purificada.

2. OBJETIVOS

2.1. Objetivo Geral

Produzir proteases fibrinolíticas por linhagens de fungos filamentosos por fermentação submersa e desenvolver o processo de purificação utilizando Sistemas de Duas Fases Aquosas (SDFA) e cromatografia líquida, além de caracterizar bioquímico e estruturalmente a enzima.

2.2. Objetivos Específicos

- Selecionar linhagens de fungos filamentosos com potencial para produção de enzimas com atividade fibrinolítica;
- Produzir proteases fibrinolíticas por fermentação submersa em diferentes condições e estudar a influência de variáveis do processo de produção utilizando um planejamento fatorial;
- Caracterizar bioquimicamente a protease fibrinolítica em relação ao pH e temperatura ótimos, estabilidade ao pH e a temperatura, inibidores e íons metálicos;
- Determinar as condições mais adequadas por meio de planejamento fatorial para obter a purificação da enzima com sistemas de duas fases aquosas (SDFA) e por sistema integrado de produção e purificação (Fermentação extrativa);
- Purificar a enzima utilizando métodos cromatográficos;
- Realizar o perfil eletroforético da enzima e ponto isoelétrico;
- Caracterizar estruturalmente a enzima através de espectroscopia de dicroísmo circular no UV distante.

3. REVISÃO BIBLIOGRÁFICA

3.1. Proteases

Proteases são enzimas que catalisam a quebra de proteínas por hidrólise das ligações peptídicas. Estão presentes na maioria dos sistemas biológicos e executam diversas funções vitais, tais como, a regulação do processamento de proteínas e níveis de proteínas intracelulares, remoção de proteínas anormais ou danificadas da célula, além do desenvolvimento, fisiologia, defesa e resposta ao estresse (BI et al, 2011).

Protease é uma das principais enzimas extensivamente utilizadas como detergentes, na indústria de couros, alimentos e indústria farmacêutica. Elas perfazem entre 60 e 65% do mercado mundial de enzimas o que dá significado à sua importância industrial. São enzimas que possuem um papel importante no metabolismo de organismos e podem ser isoladas de plantas (látex do *Artocarpus heterophyllus*) (SIRITAPETAWEE et al., 2012) (SIRITAPETAWEE et al., 2012)e látex de *Calotropis procera* (RAWDKUEN et al., 2011); a partir de animais (pepsina de estômago bovino) (IMELIO et al., 2008) e tripsina de pâncreas bovino (TUBIO et al., 2009); invertebrados marinhos (*Urechis unicinctus*) (BI et al., 2013) e a partir de micro-organismos: *Pseudomonas aeruginosa* (MEENA et al., 2013), *Aspergillus oryzae* (SANDHYA et al., 2005), *Bacillus cereus* SIU1 (SINGH et al., 2010), *Streptomyces* (UESUGI et al., 2011).

Proteases são aplicáveis industrialmente por funcionarem em condições mais simples (ex. temperatura, pH, condições atmosféricas), não necessitam proteção dos grupos funcionais dos substratos, possuem meia-vida maior do que catalizadores químicos. Além disso, enzimas podem ser selecionadas geneticamente e modificadas quimicamente para aumentar suas propriedades: estabilidade, especificidade ao substrato e atividade específica. (ADRIO; DEMAIN, 2014).

Cerca de 150 processos industriais utilizam enzimas ou células microbianas para catálises. O mercado industrial de enzimas é muito competitivo sendo a empresa mais importante a Novozymes (Dinamarca), seguida por DSM (Holanda), e DuPont (EUA) entre outras. A América do Norte e Europa são os maiores consumidores de enzimas industriais, entretanto, a região da Ásia irá aumentar a demanda por enzimas na China, Japão e Índia, refletindo o tamanho e força da economia desses países (ADRIO; DEMAIN, 2014).

Devido à grande especificidade ao substrato, as proteases possuem ampla variedade de aplicações, como no processamento de couro, formulações de detergentes, indústrias de processamento de alimentos, síntese de peptídeos, preparações de hidrolisados proteicos, indústria farmacêutica, recuperação de prata de filmes fotográficos e em ferramentas de análise em pesquisa básica (SHANKAR et al, 2011; AGREBI et al, 2010). As proteases estão entre as mais importantes enzimas industriais respondendo por quase 60% do total mundial de vendas (RAMAKRISHNA et al, 2010; TRIPATHI et al, 2011).

A crescente demanda por proteases com propriedades específicas tem levado a intensa busca por novas fontes de proteases (BI et al, 2011). Além disso, muitas dessas enzimas têm sido descobertas a partir de diferentes micro-organismos, como bactérias, actinomicetes, fungos e algas (LU et al, 2010). Com sua eficiência no uso e várias implicações biotecnológicas, as proteases alcalinas com alta especificidade e estabilidade frente a variações de pH, sais, temperatura, solventes orgânicos, íons de metais e surfactantes têm aumentado a sua demanda. Selecionar micro-organismos de ambientes naturais inexplorados irá facilitar significativamente a busca por diferentes enzimas com características peculiares (RAVAL et al., 2014).

Um estudo realizado no ano de 2014 acerca dos produtos biológicos aprovados pelo FDA (Food and Drug Administration), revelou que produtos de origem biológica (enzimas, modificadores de receptores e anticorpos monoclonais) têm sido objeto de dinamismo considerável em termos de atratividade pela indústria farmacêutica. A razão disso tem sido talvez pela inovação da tecnologia e pela preocupação com a segurança (ex. Imunogenicidade), além dos custos elevados das mercadorias (incluindo as patentes para modificação e ou produção de medicamentos de origem biológica) (KINCH, 2015).

A alfimeprase foi submetida a testes clínicos pela Nuvelo, Inc. (San Carlos, CA), que licenciou a enzima da Amgen. A alfimeprase teve sucesso nas Fases I e II dos testes clínicos para obstrução da artéria periférica e obstrução do dispositivo de acesso venoso central. Entretanto, nos testes da fase III a alfimeprase não alcançou as expectativas e pontos para ambas obstruções PAO e CVAD e na fase II do teste de acidente vascular cerebral e a Nuvelo decaiu o desenvolvimento em 2008 (MARKLAND, SWENSON, 2010).

Um aumento do interesse por estes produtos ocorreu com o aumento da experiência com produtos biológicos, avanço da engenharia de proteínas e melhorias na fabricação. Consequentemente, os últimos anos testemunharam a aquisição de empresas de biotecnologia

de alto perfil para a fabricação de produtos de origem biológica (ex. Genentech, MedImmune entre outros) cujos agentes em destaque foram os fibrinolíticos. Os ativadores de plasminogênio *Alteplase* (Genentech em 1996) e *Reteplase* (Boehringer-Mannheim em 1996); estão entre os cinco produtos biológicos aprovados em uma das três principais categorias (Moduladores enzimáticos) (KINCH, 2015).

3.2. Proteases fibrinolíticas

A terapia trombolítica com agentes fibrinolíticos é uma abordagem padrão para o tratamento de doenças cardiovasculares na fase aguda, usualmente aplicado para conter de forma rápida a carga do trombo. No entanto, agentes fibrinolíticos ativadores de plasminogênio normalmente possuem efeitos colaterais indesejáveis, incluindo sangramento gastrointestinal e reações alérgicas. Desta forma, a pesquisa por enzimas fibrinolíticas seguras a partir de uma variedade de fontes estão em andamento (KIM et al., 2015).

Existe a necessidade de se investigar enzimas fibrinolíticas específicas semelhantes a plasmina que têm atraído muito mais interesse médico nas últimas décadas. As enzimas dependentes de plasminogênio para a sua atividade são enzimas que inicialmente precisam ativar o plasminogênio sanguíneo formando consequentemente a plasmina que hidrolisa as redes de fibrina (CHITTE et al, 2011).

O sistema fibrinolítico, conhecido por sua habilidade em regular a ativação do plasminogênio em plasmina ativa, tem sido inicialmente associado com a remoção de fibrina e coágulos sanguíneos. O ativador de plasminogênio tecidual foi utilizado como agente terapêutico contra doenças tromboembólicas há mais de 30 anos, enquanto que a inibição desse sistema tem se provado efetivo contra alguns distúrbios hemorrágicos. Entretanto, recentemente, novas e inesperadas funções para esse sistema têm sido identificadas, principalmente em relação ao sistema nervoso central onde não existe relação com a degradação de fibrina e remoção de coágulos. Assim, parece razoável questionar se os agentes usados para modificar componentes ou atividades do sistema fibrinolítico possuem quaisquer consequências clínicas não relacionadas com a sua utilização prevista na hemostasia (DRAXLER; MEDCALF, 2014).

Desta forma, investigadores têm buscado soluções para complicações com o uso de ativadores de plasminogênio como: fibrinogenólise sistêmica acompanhada de sangramento, como também complicações neurológicas como hemorragia intracraniana. A pesquisa

científica tem buscado como alternativa trombolíticos que agem diretamente na degradação do coágulo. Como resultado essas enzimas fibrinolíticas têm sido descobertas e estas são enzimas semelhantes à plasmina (KOTB, 2014).

3.2.1. Fontes alternativas de enzimas fibrinolíticas

O aproveitamento de fontes naturais para a descoberta de micro-organismos com potencial biotecnológico pode ser uma importante força motriz para o desenvolvimento dos setores bioindustriais.

Explorar regiões com grande biodiversidade é uma estratégia para a descoberta de novas fontes de biomoléculas de interesse industrial como também é uma forma de se obter mais informações acerca da biodiversidade desses locais. A região do leste da Índia é considerada uma das regiões de maior biodiversidade do mundo. MUKHERJEE e RAI (2011) realizaram um estudo nessa região, onde isolaram bactérias promissoras para a produção de proteases fibrinolíticas através de amostras de solo da região de Assam, Nordeste da Índia. Este é um dos trabalhos pioneiros na otimização estatística para a produção de protease fibrinolítica por uma espécie de *Bacillus* sp. Gram-negativo.

Uma das regiões contendo grande biodiversidade da Argentina, a floresta tropical "Las Yungas" foi objeto de estudo para a exploração de fontes de enzimas fibrinolíticas (GRAU e DIEGO BROWN, 2000). Como outras florestas tropicais, Las Yungas têm sofrido risco ambiental devido às diferentes atividades humanas que têm afetado a biota nativa, incluindo a microbiota fúngica. Uma forma de restauração da diversidade e de descoberta da microbiota com potencial biotecnológico, em particular a microbiota fúngica, é uma forma de alertar e incentivar a preservação da biodiversidade desses locais (ROTAVI et al., 2010). No Brasil, o bioma Caatinga, representa uma das maiores áreas que é coberta por um único tipo de vegetação e cerca de 60% da região nordeste do Brasil. Este bioma se constitui de florestas secas com considerável diversidade de arbustos, ervas, trepadeiras e árvores aromáticas com flora amplamente conhecida (ADELINO et al., 2015). Trabalhos sobre diversidade de fungos têm sido publicados com o objetivo de caracterizar espécies que constituem novos registros para o continente americano e o Bioma Caatinga (CRUZ, 2009; SANTA et al., 2011).

Devido à promessa de benefícios biológicos do consumo de alimentos que são fontes de enzimas fibrinolíticas, novas fontes dessas enzimas têm sido investigadas (DEEPAK et al.,

2008; YUAN et al., 2012; RADNAABAZAR et al., 2011), a partir de alimentos fermentados orientais. O estudo do uso de produtos fermentados tradicionais orientais e do isolamento de micro-organismos a partir destes têm aumentado gradualmente durante os anos o que resultou no aumento significativo das publicações na área.

Nattokinase (EC 3.4.21.62) é uma potente enzima fibrinolítica que tem sido descoberta de várias fontes como: Natto japonês (DEEPAK et al., 2008), molho de soja Chungkook jang e doen-jang coreanos (KIM et al., 1996), Douchi chinês YUAN et al., 2012) e pasta fermentada de camarão em toda a Ásia. A Nattokinase tem sido extensivamente investigada por causa da alta atividade fibrinolítica que normalmente apresenta e é conhecida pelo seu mecanismo de ação, pois degrada diretamente a fibrina dos coágulos sanguíneos. Sendo considerada atualmente como um agente promissor para prevenir e tratar doenças cardiovasculares (UNREAN e NGUYEN, 2013). Cheonggukjang (pasta de soja dessalgada fermentada) feito a partir de soja fermentada por espécies de bactérias do gênero *Bacillus*. Estas bactérias secretam diferentes tipos de enzimas fibrinolíticas durante a fermentação do Cheonggukjang. Estas enzimas são normalmente classificadas na classe das subtilisinas (RADNAABAZAR et al., 2011). O Cheonggukjang (CGJ) é similar ao Natto japonês. Para a produção do Cheonggukjang no passado, os grãos da soja eram embebidos e cozidos e então espalhados em palha de arroz e fermentado durante 2-3 dias. O *Bacillus subtilis*, presente na palha, se propagava e criava a mucilagem pegajosa na soja. No CGJ moderno, a soja é inoculada com o *B. subtilis* e reservado para fermentar.

Nas pesquisas sobre a capacidade funcional do CGJ e sua atividade fibrinolítica a maior barreira para o seu uso como fibrinolítico é a instabilidade dessas enzimas nas condições ácidas do estômago e condições de aquecimento durante o processo de cozimento na produção do CGJ (KO et al., 2008).

O Douchi é similar ao Natto japonês em muitos aspectos como a matéria prima, modo de produção e componentes alimentares. Razão no qual tem sido investigado em relação à produção de enzimas fibrinolíticas (ZHANG et al., 2013).

A pasta de camarão fermentada é tradicionalmente produzida utilizando camarão cinza fresco e sal (10-15% m/m). A mistura é triturada e mantida por 2 dias à temperatura ambiente. A mistura parcialmente fermentada é misturada para formar uma pasta e mantida sob o sol por mais 2-30 dias. O produto final é embalado em plástico ou embalagem de madeira, ou ainda em embalagens de vidro. O processo de fermentação é realizado pelas bactérias presentes no

ar, utensílios ou ingredientes que estão presentes na natureza. Desta forma nenhuma cultura comercial é utilizada (WONG; MINE, 2004).

3.3. Agentes fibrinolíticos

A busca por um novo agente fibrinolítico eficaz requer a análise profunda das características dos agentes fibrinolíticos comercialmente disponíveis ou em fase de análise. Requer também o conhecimento acerca do mecanismo de ação desses agentes. Os agentes trombolíticos à base de proteases fibrinolíticas degradam a fibrina, que é o principal componente proteico dos coágulos sanguíneos formados a partir da ativação do fibrinogênio pela trombina (EC 3.4.21.5) (DEEPAK et al, 2010; KIM et al., 2011). Estes fatores sanguíneos são ativados após um dano vascular como propõe a teoria celular (LANE et al, 2005). Em um estado de desequilíbrio, coágulos de fibrina podem não ser hidrolisados resultando em trombose (DUBEY et al, 2011).

O trombo é um coágulo anormal que se desenvolve num vaso sanguíneo. Uma vez formado o coágulo, é provável que o fluxo contínuo de sangue que passa por ele acabe por deslocá-lo e estes coágulos que fluem livremente são conhecidos como êmbolos. Em geral, os êmbolos não param de fluir até atingir um ponto estreitado do sistema circulatório. Por conseguinte, os êmbolos que se originam em artérias de grande calibre ou no lado esquerdo do coração eventualmente bloqueiam artérias sistêmicas de menor calibre ou arteríolas no cérebro, nos rins ou em outros locais. Por outro lado, os êmbolos que têm origem no sistema venoso e no lado direito do coração fluem nos vasos pulmonares, causando embolia arterial pulmonar (GUYTON, 2011).

As causas das condições tromboembólicas são geralmente duplas: em primeiro lugar, qualquer superfície endotelial áspera de um vaso, como a que pode ser causada por arteriosclerose, infecção ou traumatismo, é capaz de iniciar o processo da coagulação. Em segundo lugar, o sangue quase sempre coagula quando flui muito lentamente pelos vasos sanguíneos, devido à formação contínua de pequenas quantidades de trombina e outros pró-coagulantes. Em geral, essas substâncias são removidas do sangue pelo sistema de macrófagos, principalmente pelas células de Kupffer do fígado. Se o sangue estiver fluindo de modo muito lento, as concentrações dos pró-coagulantes em determinados locais elevam-se quase sempre o suficiente para iniciar a coagulação; entretanto, quando o sangue flui rapidamente, esses pró-coagulantes são logo misturados com grandes quantidades de sangue e removidos durante sua passagem pelo fígado (GUYTON, 2011).

Baseado em métodos de avaliação de risco comparáveis, baixa renda, comportamento e fatores de risco relacionados com o estilo de vida são as principais causas de morte e incapacitação no mundo. O principal fator de risco para as mortes e incapacidade é a qualidade da dieta inadequada, que em 2010 levou a 678000 mortes anuais. Os principais fatores que contribuíram para isso foram a ingestão insuficiente de frutas, nozes/sementes, grãos, legumes e frutos do mar, bem como a ingestão em excesso de sódio. Nos EUA, um número estimado de 58000 mortes por DCVS (doenças cardiovasculares) por ano (intervalo de confiança de 95%, 37000-80000) em 2010 foram atribuídas à ingestão de sódio > 2,0g/dia, o que representa 1 em 16 (6,3%) de todas as mortes por DCVS e 1 em 8 (13,1%) mortes por DCVS antes dos 70 anos de idade. Globalmente estima-se 1.65 milhões de mortes por DCVS anualmente (intervalo de confiança de 95%, 1.10-2.22 milhões) foram atribuídas à ingestão de sódio > 2g/ dia, o que representa quase 1 em 10 (9,5%) de todas as mortes por DCVS (MOZAFFARIAN et al., 2014).

Existem mais de vinte enzimas no corpo que contribuem com a coagulação do sangue enquanto que apenas uma, a plasmina, pode lisar e remover o coágulo. A plasmina (EC 3.4.21.7) mantém o fluxo sanguíneo em lesões nos vasos (CHOI et al, 2009; WANG et al, 2011). É uma serino-protease terminal acionada por ativadores de plasminogênio que rompe a rede de fibrina proteoliticamente (DEEPAK et al, 2010; ROVATI et al, 2010).

A Comissão Internacional de enzimas reconhece a fibrolase, uma metaloproteinase, isolada do veneno da cobra *Agkistrodon contortrix contortrix*, como uma enzima fibrinolítica cuja reação é responsável pela hidrólise da ligação -Ala14 -Leu- na insulina B e da ligação -Lys413-Leu na subunidade Aα- do fibrinogênio (NC-IUBMB, 2010). A fibrolase mostrou possuir efetiva atividade trombolítica na desobstrução da artéria carótida em um modelo de trombólise em caninos. Uma versão recombinante da enzima foi criada em levedura pela Amgen, Inc. (Thousand Oaks, CA, USA) e nomeada alfimeprase. A Alfimeprase é idêntica à fibrilase exceto por dois aminoácidos truncados na cadeia amino-terminal e à inserção de um novo aminoácido amino-terminal na proteína truncada. Essas mudanças levaram a uma maior estabilidade da enzima que prolonga o armazenamento (MARKLAND, SWENSON, 2010).

A enzima fibrinolítica microbiana Nattokinase é semelhante à plasmina e foi encontrada pela primeira vez em 1987 em um micro-organismo isolado de um alimento japonês fermentado de soja (natto) e pode reduzir coágulos diretamente por hidrólise da rede

de fibrina, como também através da conversão de plasminogênio em plasmina ativa (RADNAABAZAR et al, 2011).

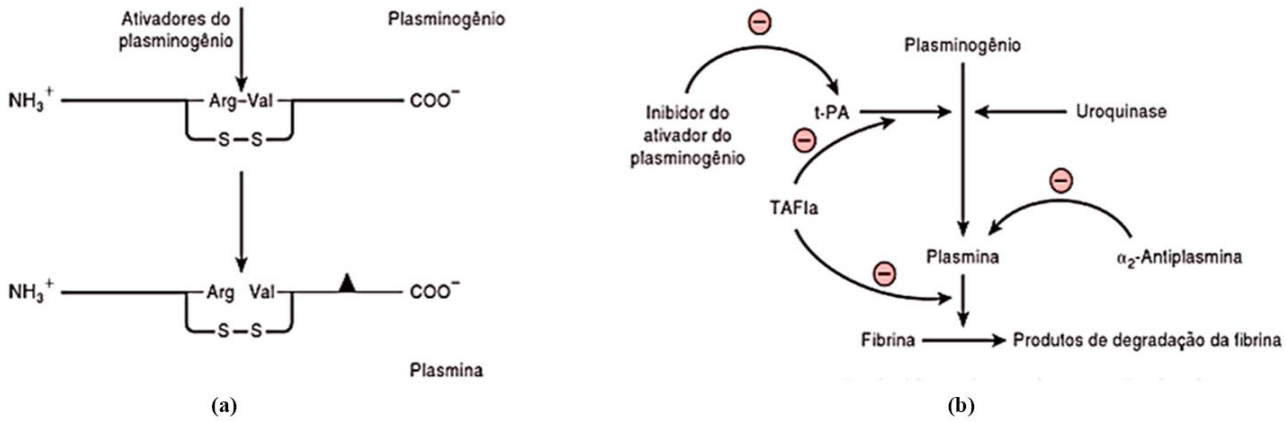
3.3.1. Hemostase e coagulação sanguínea

O termo hemostasia significa prevenção da perda de sangue. Toda vez que um vaso sanguíneo sofre lesão ou ruptura, a hemostasia é mantida por meio de vários mecanismos distintos, que incluem: (1) espasmo vascular, (2) formação de tampão plaquetário, (3) coagulação sanguínea, e (4) crescimento eventual de tecido fibroso no coágulo sanguíneo para obturar o orifício no vaso de forma permanente. A coagulação do sangue no vaso lesado é um dos mecanismos de hemostasia que consiste na formação do coágulo sanguíneo. O destino habitual de um coágulo que se forma num pequeno orifício da parede vascular é ser invadido por fibroblastos: essa invasão começa dentro de poucas horas após a formação do coágulo (promovida, pelo menos em parte, pelo fator de crescimento secretado pelas plaquetas) e prossegue até a organização completa do coágulo em tecido fibroso, dentro de cerca de 1 a 2 semanas. Por outro lado, quando ocorre formação de coágulo sanguíneo maior, como o que ocorre quando o sangue extravasa nos tecidos, substâncias especiais do próprio coágulo tornam-se ativadas e atuam como enzimas para dissolvê-lo (plasmina) (GUYTON, 2011).

A coagulação sanguínea é parte de um sistema de equilíbrio dinâmico no qual de um modo constante se depositam e se dissolvem coágulos de fibrina. O processo de dissolução dos coágulos sanguíneos é denominado fibrinólise. A plasmina, a principal protease responsável pela degradação da fibrina e do fibrinogênio, circula na forma de seu zimogênio inativo, o plasminogênio (90 kDa), e qualquer quantidade pequena de plasmina formada na fase líquida, em condições fisiológicas, é rapidamente inativada pelo inibidor da plasmina de ação rápida, a α2-antiplasmina. O plasminogênio liga-se à fibrina, e assim, incorpora-se aos coágulos à medida que são produzidos; já que a plasmina que é formada quando ligada à fibrina é protegida da ação da α2-antiplasmina, ela permanece ativa. Na maioria dos tecidos do corpo, são encontrados ativadores do plasminogênio de vários tipos, que clivam a mesma ligação Arg-Val no plasminogênio, produzindo a plasmina, uma serino protease de duas cadeias (Figura 1) (MURRAY et al., 2009).

Figura 1(a) Ativação do plasminogênio. A mesma ligação Arg-Val é clivada por todos os ativadores do plasminogênio para dar origem à molécula de plasmina de duas cadeias. O triângulo sólido indica o resíduo de serina do sítio ativo. As duas cadeias de plasmina são mantidas unidas por uma ponte dissulfeto. (b) Iniciação da fibrinólise pela ativação da

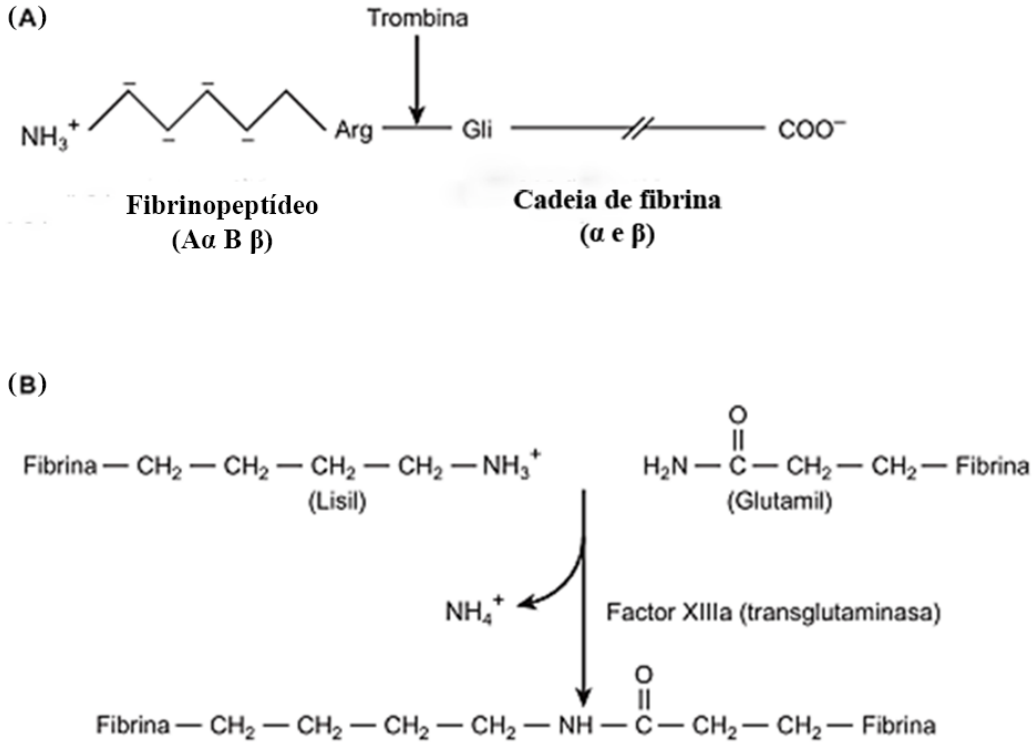
plasmina. Esquema que demonstra os locais de ação do ativador do plasminogênio tecidual (t-PA), da uroquinase, do inibidor do ativador do plasminogênio, da α2-antiplasmina e do inibidor da fibrinólise ativável por trombina (TAFIa) (essas três últimas proteínas exercem ações inibitórias).



Fonte: Murray et al, (2009), p. 588-589

O fibrinogênio é uma glicoproteína plasmática solúvel que possui três pares idênticos de cadeias polipeptídicas (Aα, Bβ, γ) 2 ligadas de maneira covalente por ligações dissulfeto. As cadeias Bβ y γ contém complexos ligados à asparagina. As regiões amino-terminais das seis cadeias se mantém estreitamente próximas mediante várias ligações disulfeto, entretanto as regiões carbóxilo-terminais se separam, o que dá lugar a uma molécula desenrolada, muito assimétrica (Figura 2) (MURRAY et al., 2009).

Figura 2 Formação de um coágulo de fibrina. (A) Quebra, induzida por trombina, das ligações Arg-Gly das cadeias Aα e Bβ do fibrinogênio para produzir fibrinopeptídeos (lado esquerdo) e as cadeias α e β do monômero de fibrina (lado direito). (B) Entrecruzamento de moléculas de fibrina pelo fator XIII ativado (fator XIIIa)



Fonte: Murray et al, (2009), p. 587.

3.4. Potencial biotecnológico de fungos para produção de enzimas

Micro-organismos são a principal fonte de enzimas para aplicação industrial (couros, detergentes e farmacêutica) devido ao rápido crescimento celular e o baixo custo de produção. Os fungos produzem uma variedade de proteases mais ampla do que as bactérias. Além disso, alguns fungos são considerados *GRAS* (sigla do inglês que significa: geralmente reconhecidos como seguros) e eles produzem enzimas extracelulares, que são fáceis de recuperar de caldo fermentado (ZAFERANLOO et al., 2014).

Enzimas microbianas são conhecidas por desenvolverem um papel crucial no metabolismo catalítico, levando ao seu uso em várias indústrias e aplicações. O uso final de

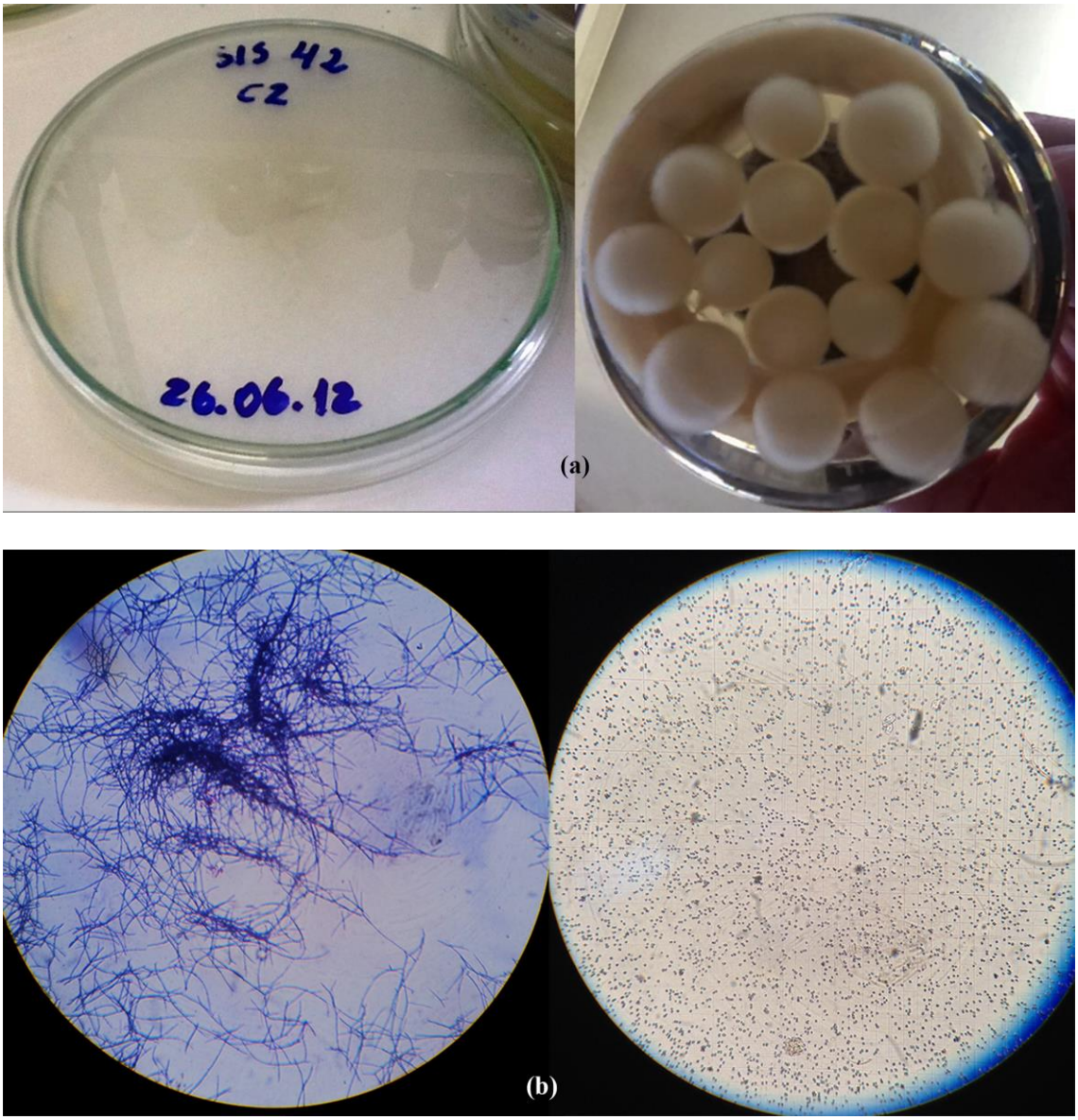
mercado para enzimas industriais é extremamente diversificado com numerosas aplicações comerciais (ADRIO; DEMAIN, 2014). Fungos filamentosos são máquinas celulares versáteis para a produção de enzimas, antibióticos, ácidos orgânicos e outros componentes industrialmente relevantes (POSCH et al., 2012).

Devido a sua diversidade metabólica, alta capacidade de produção, eficiência de secreção e capacidade de realizar modificações pós traducionais os fungos filamentosos são amplamente explorados como fábricas celulares eficientes na produção de metabólitos, substâncias bioativas e proteínas nativas ou heterólogas, respectivamente (WUCHERPFENNIG et al., 2010). Proteases de origem fúngica são secretadas extracelularmente e em alguns casos os metabólitos fúngicos refletem as condições de cultivo. A nutrição e o ambiente físico podem ser um fator dominante para o crescimento fúngico e para os níveis de produção de proteases extracelulares em sistemas de cultura submersa (LU, et al., 2010).

Enzimas fúngicas são importantes para a agricultura, indústria e saúde humana devido à sua estabilidade a altas temperaturas, extremos de pH e especificidade a diversos substratos quando comparadas com enzimas oriundas de plantas e animais (RANI et al., 2012, ZAFERANLOO et al., 2014).

Com cerca de 1,5 milhões de espécies, os fungos possuem o segundo maior número de espécies após os insetos. Com o desenvolvimento da biotecnologia, os fungos têm ganhado importância crucial na produção de uma grande variedade de produtos como alimentos, biocombustíveis, enzimas, antibióticos e químicos. Zygomycota é o mais variado e pouco estudado filo dos fungos. É dividido em dois subfilos, trichomycetos e zygomycetos. Dentro dos zygomicetos, *Mucor* é um gênero com cerca de 3000 membros que são amplamente distribuídos no solo, superfícies de plantas e vegetais em decomposição. Membros do gênero *Mucor* (Figura 3a.b) são conhecidos como deteriorantes em alguns casos mas muito raramente considerados como agentes etiológicos (KARIMI; ZAMANI, 2013).

Figura 3(a) *Mucor subtilissimus* UCP 1262 em 5 dias de crescimento. Cultura em meio Czapek sólido (à esquerda), cultura em meio líquido (à direita); (b) Microscopia óptica da cultura de *M. subtilissimus* UCP 1262 (100x), hifas (à esquerda) e esporos (à direita).



Fonte: Elaborada pelo autor

Espécies do gênero *Mucor* têm sido extensivamente utilizadas em processos biotecnológicos e reconhecidas na literatura como produtoras de proteases (Tabela 1).

Tabela 1 Processos biotecnológicos que utilizam espécies do gênero *Mucor*

Processo biotecnológico	***Espécie***	***Referência***
Biotransformação não-terpenóide	*Mucor* sp.	(SILVA et al., 2014)
Ácidos graxos totais e recuperação de ácido γ- linoleico	*Mucor circinelloides*	(TANG et al., 2014)
Lipase	*Mucor hiemalis* f. corticola	(ULKER; KARAOĞLU, 2012)
Aspartico-protease	*Mucor mucedo*	(YEGIN et al., 2012)
Protease	*Mucor* sp.	(ALVES et al., 2002)
Protease	*Mucor pusillus* Lindt	(SOMKUTI; BABEL, 1967)
Protease	*Mucor miehei*	(ESCOBAR; BARNETT, 1993)
Protease	*Mucor bacilliformis*	(LAHORE et al., 1995)

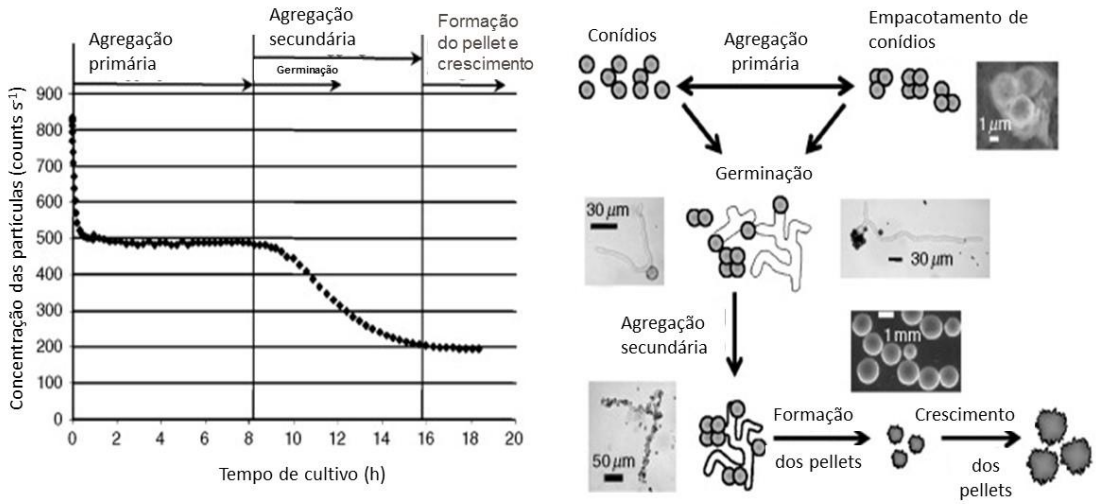
3.5. Produção de proteases fibrinolíticas por fermentação submersa

O custo da produção de enzimas e processo de purificação é o maior obstáculo para a aplicação com sucesso de proteases na indústria e medicina. Para a produção de enzimas fibrinolíticas muitas tentativas têm sido feitas para aumentar a expressão da enzima fibrinolítica, incluindo a seleção de um meio de cultura ideal, otimização das condições ambientais e aumento da expressão de amostras geneticamente modificadas. A seleção dos componentes do meio é normalmente crucial para a produção fermentativa de enzimas fibrinolíticas. Uma vez que diferentes micro-organismos possuem diferentes características fisiológicas, é necessário otimizar os componentes do meio e condições ambientais para o crescimento celular e produção da enzima (KOTB, 2012).

No cultivo submerso, duas formas de crescimento distintas podem ser observadas: a forma micelial e a formação de pellets. Os pellets são caracterizados pelo desenvolvimento

estável de agregados esféricos e podem ser mais ou menos densos, ramificados e redes de hifas parcialmente interligadas (Figura 4).

Figura 4 Modelo de agregação para coagulação de micro-organismos filamentosos

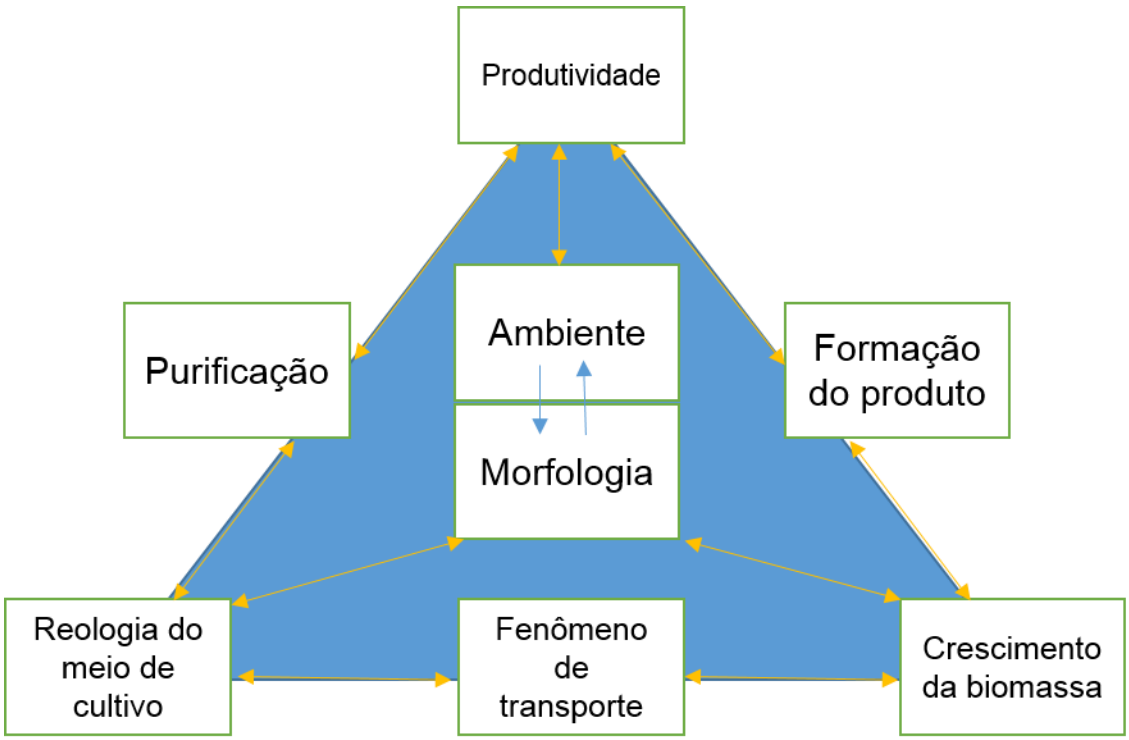


Fonte: GRIMM et al., (2004). p 5.

Algumas vezes a morfologia do tipo pellet é preferida para cultivos industriais e para as etapas de "downstream" por conta das características de menor viscosidade. Nesse tipo de cultivo a transferência de massa, oxigênio e nutrientes é consideravelmente melhor. E a subsequente separação dos pellets do meio de cultivo é mais simples do que em meios de cultivo micelares. Fácil agitação e aeração proporcionam baixos custos de operação por que a carga de energia necessária é muito menor (WUCHERPFENNIG et al., 2010).

Muitas fontes de baixo custo como rejeitos da agricultura têm sido utilizadas e até então essas enzimas fibrinolíticas têm exibido um importante desempenho para a degradação de coágulos de sangue. Portanto, componentes como a glicose, a farinha de soja, o cloreto de cálcio e o sulfato de magnésio também são empregados para aumentar a produção dessas enzimas (Figura 5) (DEEPAK et al, 2010).

Figura 5 Ilustração esquemática do processo biotecnológico para a produção de produtos de origem microbiana, com os principais parâmetros ambiente e morfologia no centro, e a produtividade para ser otimizada no topo do triângulo



Fonte: Adaptado de WUCHERPFENNIG et al., (2010). p. 93.

A Figura 5 ilustra um processo biotecnológico, com a morfologia e o sistema biológico influenciados pelo ambiente. A morfologia e o ambiente são os principais parâmetros do processo e estão desta forma localizados no centro do triângulo, uma vez que eles influenciam todos os parâmetros ambientais. A produtividade está localizada no topo do triângulo, por que é um parâmetro central a ser otimizado. A reologia do meio de cultura e o crescimento da biomassa formam a base, completando o triângulo. Todos os parâmetros ambientais das laterais do triângulo influenciam uns aos outros. A reologia do meio de cultura causa problemas de mistura e influencia o fenômeno de transporte que pode causar problemas ao crescimento celular. A formação do produto é altamente relacionada com o formato de crescimento da biomassa. Otimização do crescimento leva a formação extensiva da formação do produto, bem como alta viscosidade torna complicada a recuperação do produto. Um procedimento de purificação caro e trabalhoso leva a um processo de purificação de alto custo (WUCHERPFENNIG et al, 2010).

Para atender à crescente demanda de enzimas na indústria é necessário melhorar o desempenho do sistema e assim, aumentar o rendimento reduzindo o custo de produção (AGREBI et al, 2010). O custo total da produção de enzimas é um dos maiores desafios para a sua aplicação industrial (MUKHERJEE e RAI, 2011).

3.6. Purificação de enzimas fibrinolíticas

3.6.1. Sistemas de Duas Fases Aquosas (SDFA)

O desenvolvimento da industria farmacêutica tem sido caracterizado por uma tendência de aumento volumétrico da capacidade de produção. Enquanto o esforço tecnológico para o escalonamento dos respectivos processos de cultivo pode ser considerado simples, um considerável esforço tecnológico para a redução dos custos da recuperação dos produtos deve ser enfrentado. Desta forma a redução rápida de volume e concentração do produto tornam-se as principais questões nas etapas iniciais do processo de purificação. Até então, os processos convencionais com base na adsorção e filtração estão prestes a atingir os seus limites em termos de economia, processamento, velocidade e escala. A principal técnica candidata para cumprir com esta tarefa, no entanto pode ser encontrada na utilização dos sistemas de duas fases aquosas (SDFA), que pode combinar um grande número dessas características exigidas (BENSCH et al., 2007).

Sistemas de duas fases aquosas (SDFA) surgem naturalmente da mistura entre diferentes polímeros solúveis em água ou um polímero e um sal específico. Quando dois polímeros específicos são misturados em água dentro de certas concentrações, a mistura é separada em duas camadas (fases) aquosas imiscíveis. No SDFA, cada fase proporciona um ambiente distinto para proteínas e outros solutos. Diferenças nas interações entre solutos e solventes pode levar a uma distribuição desigual que é marcadamente quantificada pelo coeficiente de partição, designado como K, que pode também ser explorado para detectar mudanças na estrutura dos solutos. O coeficiente de partição K de uma proteína é definido como a razão da concentração das proteínas ou da atividade enzimática entre as duas fases (ZASLAVSKY et al., 2013).

Os SDFA têm sido utilizados para recuperação de produtos a partir do extrato bruto da fermentação microbiana ou em sistemas integrados de produção que envolvem a produção da biomolécula durante a fermentação atrelado à separação das moléculas produzidas entre as duas fazes do sistema formado por duas fases aquosas. Como a extração da protease

fibrinolítica em SDFA (PEG/fosfato de sódio) a partir de caldo fermentado de *Streptomyces* sp. DPUA1576 (MEDEIROS E SILVA et al., 2013) e a extração da enzima fibrinolítica a partir do extrato do esporocarpo da *Auricularia polytricha* utilizando SDFA (PEG/fosfato de sódio) (MOHAMED ALI et al., 2014).

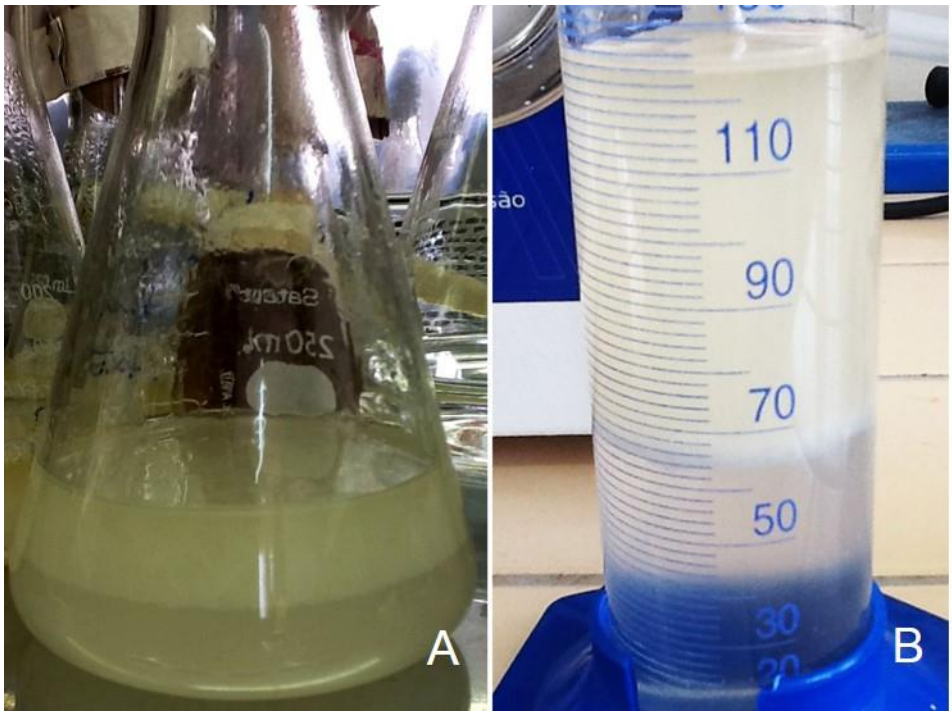
As condições de fermentação para a produção de enzimas fibrinolíticas foram otimizadas pela primeira vez na literatura utilizando *Bacillus subtilis* DC-2 para a produção e extração em sistemas de duas fases aquosas como apresentado no trabalho de Ashipala e He (2008). Este estudo demonstrou que a produção da enzima fibrinolítica foi bastante dependente da concentração do PEG 4000, da concentração do sal Na_2SO_4 e do pH. E a produção da enzima no sistema de produção integrado à separação no SDFA foi maior que em fermentação submersa (homogênea).

3.6.2. Fermentação extrativa utilizando SDFA

Fermentação extrativa utilizando SDFA tem sido descrito como adequado para o crescimento da célula microbiana devido ao alto teor de água e baixa tensão interfacial (PANDEY e BANIK, 2011). O SDFA é formado por mútua incompatibilidade entre dois polímeros ou um polímero e um sal em solução aquosa (ALBERTSSON, 1986).

Processos fermentativos baseados em sistemas de duas fases aquosas oferecem a substituição de separações mecânicas complexas das células por sistemas de particionamento em fases opostas (PANDEY e BANIK, 2011). Um sistema bifásico aquoso é inoculado com as células microbianas (sistema de expressão) que cresce seletivamente em uma fase particular, enquanto o produto de interesse é continuamente particionado para a fase oposta. Este mecanismo tem representado uma tecnologia atrativa para a remoção contínua do produto alvo do meio de fermentação. Por utilizar células microbianas, este sistema supera os baixos rendimentos dos processos fermentativos convencionais, reduzindo a inibição do produto, pois, o produto alvo é recuperado numa fase livre de células, enquanto que as células continuam a produzir na fase oposta como apresentado na Figura 6 (GLYK et al., 2015; SINHA et al., 2000).

Figura 6 Fermentação extrativa utilizando Sistema de Duas Fases Aquosas PEG/sulfato de amônio. (a) Frasco Erlenmeyer contendo meio de cultura adicionado ao SDFA (PEG/sulfato de amônio). (b) separação das duas fases da fermentação extrativa evidenciando a partição das células fúngicas na interfase.



Fonte: Elaborada pelo autor

O SDFA em um processo integrado permite a separação e concentração simultânea da molécula de interesse para a aquisição de um extrato livre de impurezas. Além disso, o ambiente biocompatível dos sistemas bifásicos aquosos facilita a preservação da atividade biológica no extrato enzimático (IANNUCCI et al., 2008). O uso de polímeros e sais no SDFA possui a vantagem de baixa viscosidade e custo econômico se comparado aos sistemas polímero-polímero (SOARES et al., 2015).

Atualmente, as indústrias têm buscado a eficiência econômica e industrial nos processos de separação e purificação de biomoléculas para garantir alto rendimento e pureza. Fermentação extrativa ou recuperação do produto *in situ* tem sido sugerido como solução para

superar a inibição dos produtos e baixa produtividade dos processos biotecnológicos (OOI et al., 2011). Esses sistemas têm sido descritos como ideais para o crescimento das células microbianas devido ao alto teor de água e baixa tensão interfacial (TAN et al., 2015).

3.6.3. Métodos convencionais de purificação de enzimas fibrinolíticas de origem fúngica

Várias enzimas fibrinolíticas têm sido produzidas, purificadas e caracterizadas bioquimicamente a partir de espécies fúngicas. Alguns exemplos estão listados na Tabela 2.

Tabela 2 Métodos de purificação e propriedades bioquímicas de proteases fibrinolíticas produzidas por fungos

Espécie fúngica	Método de purificação	Peso molecular	pH e temperatura ótimos	Classe de protease a que pertence	Referências
***Rhizopus chinensis* 12**	Precipitação com sulfato de amônio, cromatografia de interação hidrofóbica, troca iônica e gel filtração	18,0 – 16,6 kDa	10.5- 45ºC	metaloprotease	(XIAO-LAN et al., 2005)
***Fusarium* sp. CPCC 480097**	Precipitação com sulfato de amônio e troca iônica	28 kDa	8,5 – 45ºC	Quimotripsina serino-metalloprotease	(WU et al., 2009)
Armillaria mellea	Cromatografia de troca iônica e gel filtração	21 kDa	6,0 – 36ºC	Quimotripsina metalloprotease	(LEE et al., 2005c)
Cordyceps militaris	Troca iônica DEAE-Sepharose, Superdex 200 / FPLC/ Superdex 75 colunas	34 kDa	7,0 – 40ºC	Quimotripsina serino metalloprotease	(CHOI et al., 2011)
***Aspergillus oryzae* KSK-3**	Precipitação com sulfato de amônio, Cromatografia de troca iônica e filtração em gel	30 kDa	6,0 – 50ºC	Serino protease	(SHIRASAKA et al., 2012[a])
Perenniporia fraxinea	FPLC	42 kDa	6,0 – (35ºC-40ºC)	Quimotripsina metalloprotease	(KIM et al., 2008)
Schizophyllum commune	Cross-flow filtration and FPLC	21,32 kDa	5,0 - 45ºC	Metalloprotease	(LU, et al., 2010)

3.7. Caracterização estrutural e termodinâmica de biomoléculas

3.7.1. Dicroísmo circular

Um dos grandes desafios para a ciência da química de proteínas e peptídeos é entender o exato mecanismo de dobramento de proteínas. O conhecimento dos mecanismos envolvidos na aquisição da estrutura tridimensional, pode ser empregado para desenhar uma proteína com as propriedades requeridas para aplicação biotecnológica e farmacêutica (SINGH et al., 2014).

A caracterização rápida de novas proteínas é de grande importância para os campos da proteômica e genômica estrutural. Dicroísmo circular (CD) é um método excelente para avaliar rapidamente a estrutura secundária, dobramento e propriedade das ligações das proteínas e peptídeos. Resumidamente, Dicroísmo circular é definido como a absorção desigual da luz circularmente polarizada para a direita e para a esquerda.

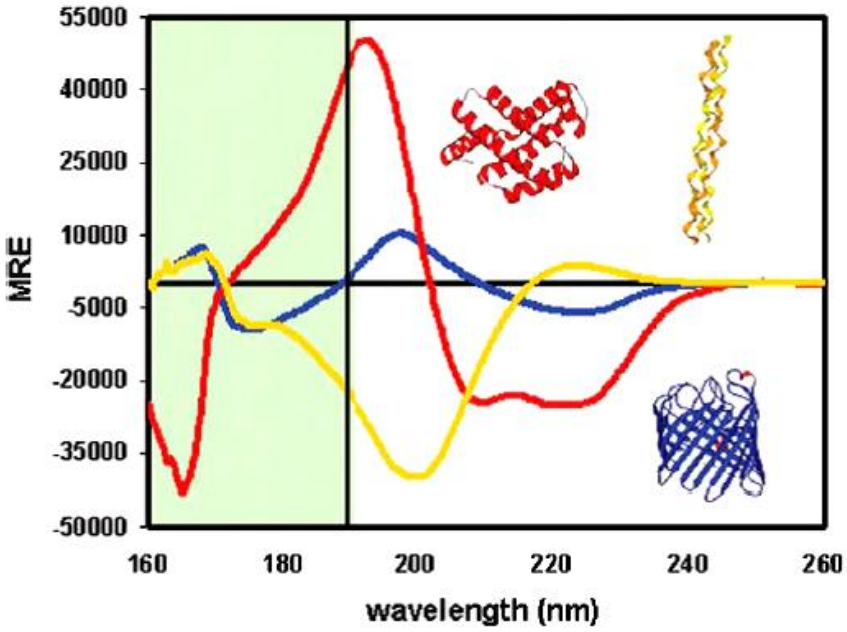
Um feixe de luz possui campos elétricos e magnéticos que são dependentes entre si e oscilam de forma sinusoidal num único plano. Quando visto de frente, a onda sinusoidal pode ser visualizada como a resultante de dois vetores de igual comprimento, que traçam círculos, um para o sentido horário (ER) e o outro que gira para a esquerda (EL). As duas ondas polarizadas circularmente tem existência física. As ondas são 90 graus fora de fase entre si e podem ser separadas utilizando uma variedade de prismas ou de dispositivos eletrônicos que utilizam o efeito de *Pockel*. Quando as moléculas assimétricas interagem com a luz, elas devem absorver a luz circularmente polarizada para a esquerda e para direita em diferentes graus (daí o termo, *dicroísmo circular*) e também possuem diferentes índices de refração para as duas ondas. O resultado é que o plano de onda da luz é rotacionado e que a adição dos vetores ER e EL resulta num vetor que traça uma elipse e a luz é dita por ser elipticamente polarizada (GREENFIELD, 2006).

O perfil das proteínas é dependente da sua conformação, assim o CD pode ser utilizado para estimar a estrutura de proteínas desconhecidas e monitorar as mudanças conformacionais induzidas por temperatura, mutações ou interações de ligação. Entretanto, DC não fornece a estrutura secundária de resíduos específicos, como fazem as determinações estruturais por Cristalografia de raio X e RMN, porém o método tem a vantagem dos dados poderem ser coletados e analisados em poucas horas em soluções de amostras contendo µM de concentração proteica em tampão e em condições fisiológicas. Estruturas secundárias das

proteínas podem também ser estimadas através da Espectroscopia de Transformada de Fourier e Espectroscopia Raman, porém em alguns casos a análise das proteínas devem ser feitas em filmes ou em óxido de deutério (GREENFIELD, 2006).

Análises de CD em região distante ao UV (far UV) (Figura 7), pode estimar quantitativamente a estrutura secundária que pode ser comparada com a gerada através da cristalografia em raio-X ou Ressonância nuclear magnética. O espectro do CD pode ser utilizado para confirmar a integridade da expressão de domínios proteicos de uma proteína multi-domínio. Além disso a perda do sinal do CD pela adição de agentes desnaturantes (como ureia ou cloreto de guanidina) ou pelo aumento da temperatura pode ser usado para estimar a estabilidade do estado nativo das proteínas (KELLY et al., 2005).

Figura 7 Espectro do dicroísmo circular da maioria das proteínas de α-hélice em vermelho, folhas-beta em azul e uma hélice de poliprolina (colágeno) em amarelo. Os dados em baixo comprimento de onda da área em verde para a esquerda da linha preta vertical são normalmente acessíveis apenas utilizando espectroscopia de dicroísmo circular por radiação sincrotron (SRCD). Entretanto, os dados à direita da linha são acessíveis por ambos SRCD e por espectroscopia de dicroísmo circular convencional. MRE significa elipticidade molar residual média.



A espectroscopia de dicroísmo circular (CD) é uma técnica extensivamente utilizada para estimar a estrutura secundária de proteínas e polipeptídeos em solução. Esta técnica pode ser utilizada para se distinguir entre estruturas desordenadas (randômicas) e ordenadas (alfa-hélice ou folhas-beta). O CD detecta diferenças dependentes do comprimento de onda da absorção da luz circularmente polarizada para direita e para a esquerda de peptídeos e proteínas. O espectro do CD de peptídeos desordenados, são usualmente caracterizados por uma banda única abaixo de 200nm enquanto que estruturas em alfa-hélices usualmente apresentam duas bandas negativas a 208 e 222nm juntamente com uma banda positiva a 192nm, estruturas folhas-beta tipicamente apresentam uma banda negativa a 217nm e a uma banda positiva a 195nm (GOPAL et al., 2012).

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CAPÍTULO 1

PRODUCTION OF FIBRINOLYTIC PROTEASE BY *Mucor subtilissimus* UCP 1262 AND BIOCHEMICAL CHARACTERIZATION

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Abstract

Cardiovascular diseases, resulting from the deposit of clots in blood vessels, are the leading cause of death worldwide. These clots can be dissolved by the action of fibrinolytic enzymes. Fibrinolysis (thrombolysis) is a dynamic process in which a fibrin rich thrombus is hydrolyzed into fibrin degradation products by plasmin. Results on the selection of the best fibrinolytic enzyme producer from a group of 36 isolates obtained from Caatinga soil (Serra Talhada, Pernambuco, Brazil) are presented. About 58 % of the isolates were shown to display fibrinolytic activity above 100 U/mL being *Mucor subtilissimus* UCP 1262 the highest producer. Further optimization of the fermentation process, using *M. subtilissimus* UCP 1262 resulted in the production of 1075 U/mL of enzymatic activity, with a specific activity of 16753 U/mg. The maximum activity of the enzyme was observed at pH 5.0 and 28 ºC and the enzyme was shown to be a chymotrypsin-like serine protease. The fibrinolytic enzyme had a capacity of enzymatic degradation of the blood clot of 16.7 % *in vitro*. The fibrinolytic

protease obtained from the culture broth of the *M. subtilissimus* UCP 1262 constitute a new source of fibrinolytic enzyme for future applications.

Keywords: fibrinolytic enzyme, submerged fermentation, blood clots; fibrinolysis; *Mucor;* protease.

1. INTRODUCTION

Fibrin is the major protein component of the blood clots, and is formed from fibrinogen by thrombin action. The insoluble fibrin fibers are hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen activators, such as Tissue type Plasminogen Activator (tPA). In an unbalanced state, the clots are not lysed and thus, thrombosis occurs (XIAO-LAN et al., 2005).

Fibrinolytic agents currently available for treatment of thrombosis are plasminogen activators such as tPA and urokinase-type plasminogen activators and all of these agents exhibit undesirable side effects. Therefore, the search for other fibrinolytic enzymes as therapeutic agents for treatment of thrombotic disorders from diverse sources is needed (PATEL et al., 2012). Several microbial serine proteases possessing fibrinolytic activity have been considered as therapeutic agents for thrombosis and fibrinolytic enzymes have been characterized for the development of superior thrombolytic drugs (MUKHERJEE et al., 2012).

Myocardial infarction, ischemic stroke and many serious cardiovascular diseases, are caused by abnormal clots, or thrombus, that interferes with blood flow, oxygen and nutrient transportation in arteries or veins. In hemostasis, these clots are important in wound healing by termination of bleeding by coagulation. These clots must then be efficiently dissolved via

fibrinolysis, induced by plasmin (LU; CHEN, 2010). In any case, it must be pointed out that these clots are important in wound healing by termination of bleeding by clotting.

During the process of cell growth and enzyme production, numerous nutrient components are required. The application of screening methods is important to find the crucial components and get corresponding proportions. Traditional optimization methods like single-factor methods would greatly increase the work- load, but they could not be completed under normal laboratory conditions. Factorial design is an experimental design method and has been used to accurately estimate the main influence of factors with less experimental time (PORTO et al., 2007). To rapidly screen, it has been applied to critical components from many factors. Submerged fermentation is widely accepted in industry for the production of fungal enzymes (YEGIN et al., 2012).

Therefore, in this work, we present the screening of fibrinolytic enzyme producers isolated from an unexploited environment (Caatinga soil) and preliminary results on the optimization of the culture medium for enzyme production using the highest producing strain *Mucor subtilissimus* UCP 1262 as well as the biochemical characterization of the fungal fibrinolytic enzyme.

2. MATERIAL AND METHODS

2.1. Screening of the fungal strains

The Culture Bank of Microorganisms from Catholic University of Pernambuco (UCP) provided the fungal species that were isolated from Caatinga soil (Northeast region of Brazil). These isolates were allowed to grow at 30°C for 7 days and then transferred to the MS-2 medium described by Porto et al., (1996) a concentration of 10^4 spores/mL. The flasks were incubated at 30°C, pH 7.0 and 120 rpm in orbital shaker. After 96 h of fermentation, cultures

were centrifuged at 10000g, 4°C and for 15 min. The clear supernatant was used for determining enzymatic activity. The optimization of the fermentation process for the production of fibrinolytic protease by *M. subtilissimus* UCP 1262 was made according to a 2^3 factorial design (Table 1).

Table 1 Levels of the independent variables of the full factorial design 2^3 for the production process of the fibrinolytic protease from *M. subtilissimus* UCP 1262

Variable	Level		
	(-1)	(0)	(+1)
NS	S	S+WB	WB
[NS]	1	2	3
[$CaCl_2$]	0	0.5	1

NS: Nitrogen Source, [NS]: Nitrogen Source Concentration, [CaCl2]: Calcium Chloride Concentration, S: Soybean, WB: Wheat Bran, S+WB: Soybean and Wheat bran, (C): Central points.

The independent variables were the nitrogen source: Soybean (S) and Wheat Bran (WB) at different concentrations (%) and Calcium Chloride Concentration (%), using the MS-2 as a base medium, as described by Porto et al., (1996). The response variables were Fibrinolytic Activity (U/mL), Specific Activity (U/mg) and Protease Activity (U/mL). The influences were evaluated by an analysis of variance (ANOVA) with a significance level of 95 %. Statistical analysis of the experimental design was performed using the software Statistic 8.0 (Statsoft Inc., USA).

2.2. Determination of total protein

The protein content was determined by the method described by Bradford (1976) using BSA (bovine serum albumin, working range: 25- 100 mg/mL) as a standard. Each experiment was

performed in triplicate and the average value was then calculated after correction with the corresponding blank.

2.3. Determination of fibrinolytic activity and protease activity

Fibrinolytic activity was measured using a fibrin degradation assay. For this determination, 0.4 mL of 0.072 g fibrinogen/l was placed in a test tube with 0.1 mL of 0.245 M phosphate buffer (pH 7.0) and incubated at 37°C for 5 min. Then, 0.1 mL of a 20 U/mL thrombin solution was added. The solution was incubated at 37°C for 10 min, 0.1 mL of diluted clarified culture medium was added, and incubation continued at 37°C. At 60 min, 0.7 mL of 0.2 M TCA (Trichloroacetic Acid) was added, and mixed.

The reaction mixture was centrifuged at 15000 g for 10 min. After that, 1 mL of the supernatant was collected and the absorbance at 275 nm was measured. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank. In this assay, 1 U (fibrin degradation unit) of enzyme activity is defined as a 0.01 per minute increase in absorbance at 275 nm of the reaction solution (WANG et al., 2011).

Total extracellular protease was assayed at 25°C as described by Ginther (1979) in the culture media previously clarified by centrifugation (12000 g). 0.1 g azocazein/L, in 0.2M Tris-HC1, pH 7.2, containing 0.001M $CaC1_2$, was used as substrate. One unit of activity was defined as the amount of enzyme that produces an increase in the optical density of 0.1 in 1 h at 440 nm.

2.4. Biochemical characterization of the enzyme

The optimum pH, optimum temperature, stability to pH and temperature of the fibrinolytic enzyme in the culture medium clarified by centrifugation, as well as the influence of metal ions, enzyme inhibitors and surfactants on enzyme activity were evaluated. To study the influence of pH and its effect on enzyme stability, the crude extract was mixed with different buffers: sodium acetate (pH 3.0 - 5.0), citrate phosphate (pH 5.0 - 7.0) Tris-HCl (pH 7.0 - 8.5) and glycine-NaOH (pH 8.5 -11.0), and incubated at 37°C for 60 min. The temperature influence in fibrinolytic activity was determined by incubating the crude extract at temperatures ranging between 4°C and 85°C for 30 min. To determine the stability to temperature and pH, aliquots were withdrawn every 15 min for a period of 60 min. These aliquots were submitted to determination of the protease activity.

The enzyme activity of the crude extract was evaluated in the presence of several ions that have been described as inhibitors or activators of protease activity. The influence of ionic solutions was evaluated at a concentration of 0.005 M. The crude extract was exposed to the following ions :zinc sulfate[($ZnSO_4$) .7 H_2O], magnesium sulfate [$MgSO_4$], copper sulfate [$CuSO_4$], ferrous sulfate [$FeSO_4$], calcium chloride [$CaCl_2$], magnesium chloride [($MgCl_2$).$4H_2O$], potassium chloride [KCl], cobalt chloride [($CoCl_2$).$2H_2O$] and incubated at room temperature for 60 min. The ions were dissolved in 0.010 M Tris-HCl pH 7.75 with 0.150 M NaCl.

The influence of surfactants - Tween-20, Tween-80, Triton X-100 and SDS - was studied at surfactant concentrations of 0.5, 0.1 and 1.5 %. The enzyme was incubated with surfactants in 0.010 M Tris-HCl pH 7.75 with 0.150 M NaCl, for 30 min at room temperature and then, the residual protease activity was measured.

For the evaluation of the influence of inhibitors on enzyme activity, the crude extract was exposed to the following inhibitors: PMSF (fluoride methylphenylsulfonyl-$C_7H_7FO_2S$),

mercuric chloride ($HgCl_2$), 2-mercaptoethanol (2-hydroxy-1-ethanethiol-C_2H_6SO) and EDTA (Ethylenediaminetetraacetic acid-$C_{10}H_{16}N_2O_8$) and incubated for 60 min at room temperature. The inhibitors were dissolved in 0.010 M Tris-HCl pH 7.75 with 0.150 M NaCl and the concentration of the solutions was standardized at 0.005 M.

2.5. Substrate specificity

Amidolytic activity was measured spectrophotometrically using the specifically substrates: (S7388 Sigma) N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide – chymotrypsin substrate, (G8148 Sigma) Gly-Arg-*p*-nitroanilide dihydrochloride - urokinase and plasmin substrate. The mixture (0.8 mL) contained 30 µL of crude extract solution, 30 µL chromogenic substrate, and 140 µL of 0.020 M Tris-HCl (pH 7.4). After incubation for 15 min at 37°C, the amount of liberated pNA (*p*-nitroaniline) was calculated by spectrophotometric absorption at 405 nm. One unit of amidolytic activity (AU) was expressed as micro moles of substrate hydrolyzed per minute and per milliliter by the enzyme (KIM et al., 1996).

2.6. Blood clot degradation assay

An *in vitro* blood-clot lysis activity assay was used as described by Prasad et al. (2006). Fresh horse blood was drawn from the jugular vein. 600 µl of blood was transferred to pre-weighted 1.5 mL glass tubes and incubated at 37°C for 2h. After clot formation, serum was completely removed. The tubes containing the clots were then weighted again. The clot weight was determined by subtracting the weight of each tube: 10 % of crude extract with 415 U/mL or various concentrations were added to the tubes containing clots. As can be seen in equation 1:

$$\text{Clot weight} = \text{weight of clot containing tube} - \text{empty tube weight} \quad (1)$$

0.150 M NaCl was also tested as negative control. Treated samples were incubated at 37°C for 24h. Lysed fluid was completely absorbed from each tube with filter paper and the tubes were then re-weighted. Weight differences of each tube before and after incubation were calculated and the percentage of clot lysis in treated and untreated samples was recorded.

3. RESULTS AND DISCUSSION

3.1. Screening of fungal strains

All microorganisms studied showed fibrinolytic activity, demonstrating the biotechnological potential of species isolated from Caatinga Biome soils. Several reports describe the efficient protease biosynthesis by fungi belonging to the genera *Aspergillus* (SHIRASAKA et al., 2012) and *Rhizopus* (XIAO-LAN et al., 2005).

The isolates were screened in MS-2 medium described by Porto et al (1996). Among the 36 isolates studied, 58% showed fibrinolytic activity above 100 U/mL (Figure 1). The microorganism with the highest fibrinolytic activity was *M. subtilissimus* UCP 1262 - 415 U/mL at 96 h of fermentation. Initially, the amount of protein present in the culture media was 0.498 mg/mL and after 72 h of fermentation, it was 0.026 mg/mL, showing the degradation of the protein by the microorganism.

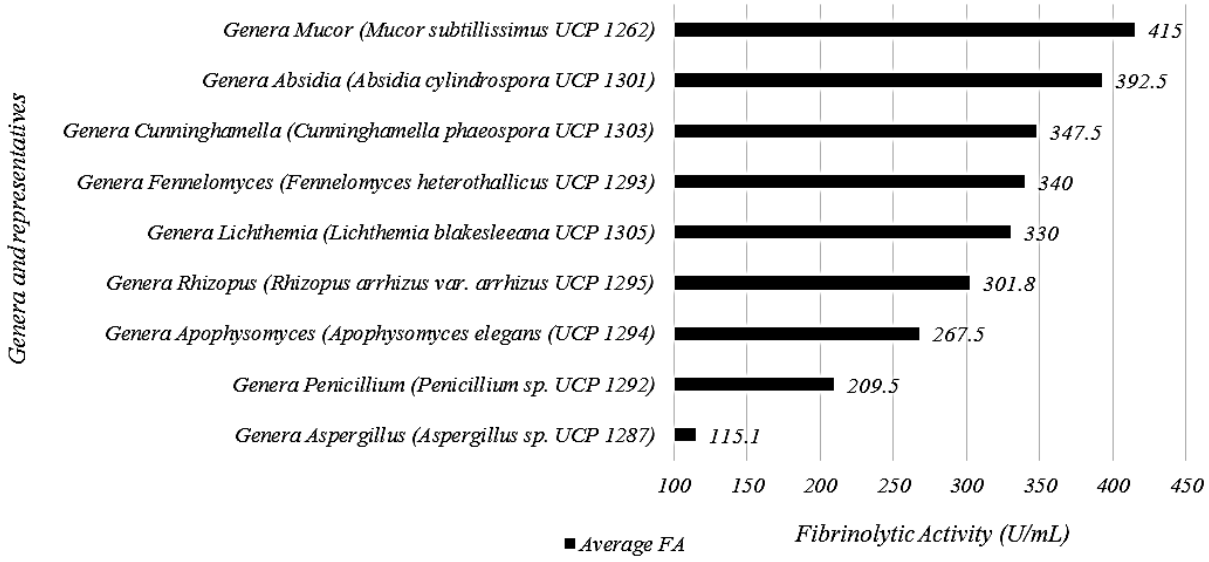


Figure 1 Average of the fibrinolytic activity (FA U/mL) of the fungal species by genus. The representative for the best fibrinolytic activity.

Two closely related species of zygomycetes, *Mucor pusillus* and *Mucor miehei*, secrete aspartate proteases, also known as *Mucor* rennin, into the medium (ANDRADE; SARUBBO, 2002). In the same way, the study was undertaken to explore and assess 37 thermophilic isolated fungal strains in terms of their production of hydrolase and oxide-reductase activities.

Litter, compost and husk proved to be highly interesting sources for laccase-producing microorganisms. This could be attributed to the presence of a relatively high concentration of laccase substrates in these environments (BEN YOUNES et al, 2011).

3.2. Medium composition optimization

Optimization of the medium composition for the production of fibrinolytic by submerged fermentation using the best producer strain was realized through a 2^3 factorial design. The matrix of the design variables and the results for the response variables - Fibrinolytic Activity (U/mL) and specific activity (U/mg) are shown in (Table 2).

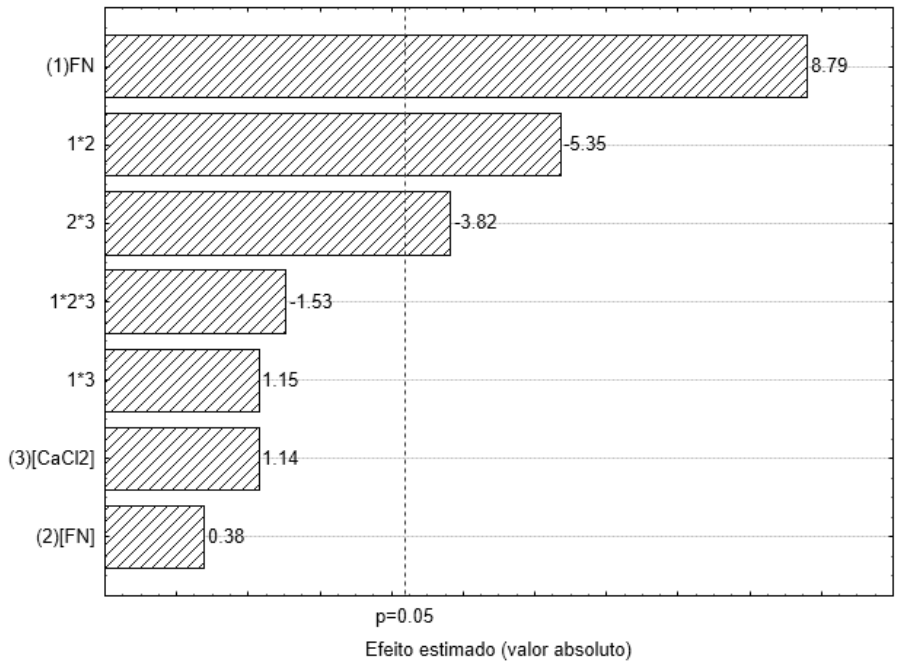
Table 2 Matrix decoded and results of full factorial design 2^3 for the fibrinolytic protease production.

Trial	NS	[NS](%)	[$CaCl_2$](%)	FA(U/mL)	PA(U/mL)
1	S	1	0	950.0	198.0
2	WB	1	0	1025.0	141.0
3	S	3	0	1002.5	274.0
4	WB	3	0	1027.5	192.0
5	S	1	1	965.0	165.0
6	WB	1	1	1075.0	56.0
7	S	3	1	987.5	290.0
8	WB	3	1	1007.5	91.0
9 (C)	S+WB	2	0.5	1035.0	223.0
10 (C)	S+WB	2	0.5	1022.5	190.0
11 (C)	S+WB	2	0.5	1012.5	209.0
12 (C)	S+WB	2	0.5	1025.0	244.0

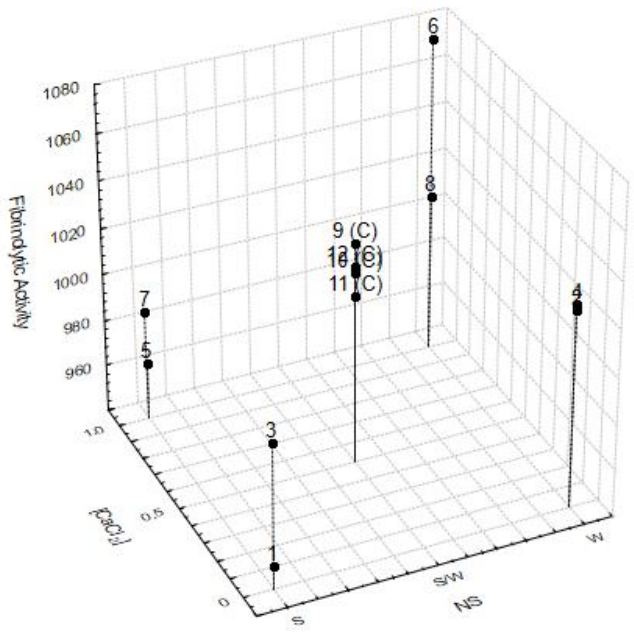
NS: Nitrogen Source, [NS]: Nitrogen Source Concentration, [CaCl2]: Calcium Chloride Concentration, FA: Fibrinolytic Activity, PT: Protease Activity, S: Soybean, WB: Wheat Bran, S+WB: Soybean and Wheat bran, (C): Central points.

The Figure 2 (a), shows the Pareto chart of influences of variables in response Fibrinolytic Activity. The statistical analysis showed that the variable Nitrogen Source (NS) was statistically significant for the response and showed a positive influence on the fibrinolytic activity.

The higher fibrinolytic activity obtained was 1075U/mL, observed at experimental run 6, as shown in Figure 2 (b), using wheat bran – 1 % nitrogen source and 1 % of $CaCl_2$.



(a)



(b)

Figure 2(a) Pareto chart of standardized influences of variables for the fibrinolytic protease production; (b) Simultaneous influences of variables of fibrinolytic protease production

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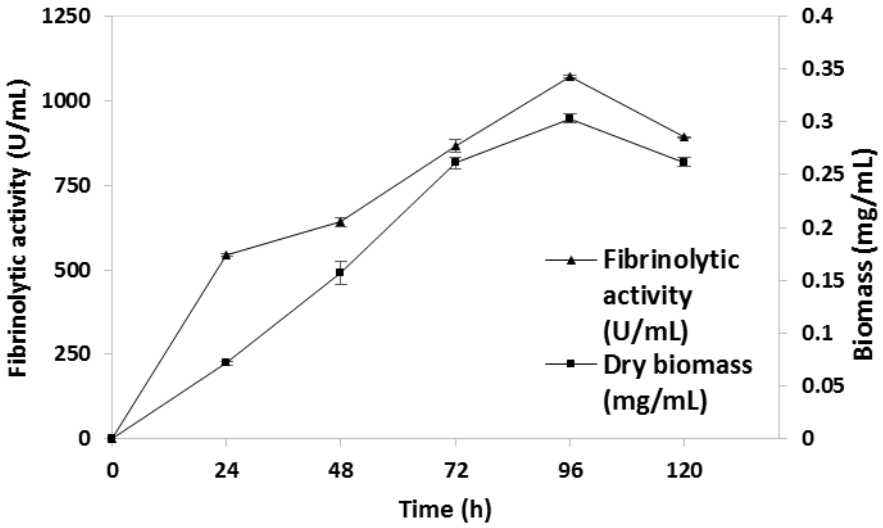
These results confirm the potential of fungi to the production of enzymes into the medium. Among them, representatives of *Mucor* spp. have important biotechnological potential and some of them produce industrial enzymes (ALVES et al., 2002). The fungal biomass was evaluated throughout the fermentation.

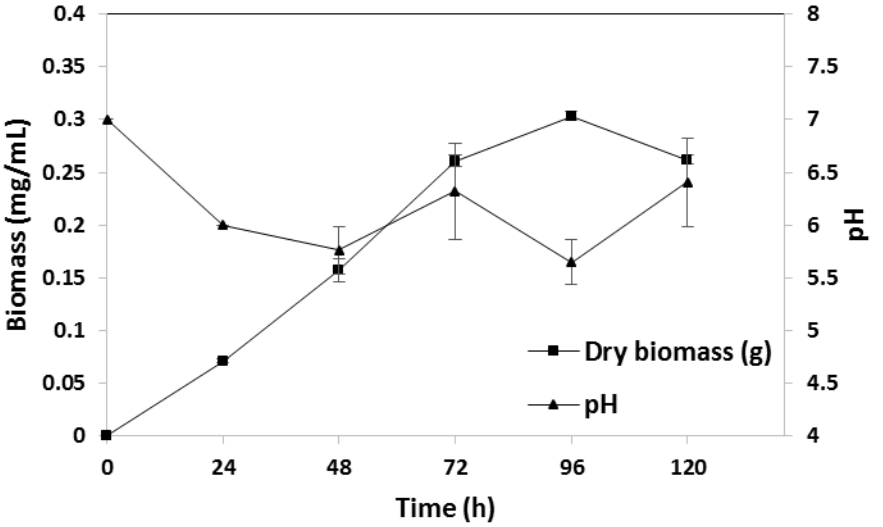
The Figure 3 (a and b) shows the activity levels obtained through the fermentation for a 4 days period. The use of an organic nitrogen source supported the growth of the organism adequately, and the average yield of dry mycelium at 72-120 hours was 0,261 mg/mL.

The apparent inhibition of growth at the final of fermentation was probably caused by the low pH values (pH 5.8 to 6.1) that developed in media. In all trials, mainly in assay 6, the pH drifted towards the acid side, which was probably due to the accumulation of residual anions.

The same characteristics were observed by Somkuti and Babel (1968), for Acid Protease production using *Mucor pusillus* where the pH value at 72 hours of fermentation decreased to acidic levels. The fibrinolytic protease activity reached a maximum level (1075 U/mL) on the fourth day of the fermentation and the biomass development was parallel to enzyme production (Figure 3.a), as it was expected for a primary product of metabolism.



(a)



(b)

Figure 3(a) Biomass from *M. subtilissimus* UCP 1262 and fibrinolytic activity until 120 h of fermentation; (b) pH values from *M. subtilissimus* UCP 1262 fermentation and dry biomass

For milk-clotting protease production by *Mucor mucedo* DSM 809 (YEGIN; FERNÁNDEZ-LAHORE, 2013) the same behavior was observed. At the start of cultivation for the enzyme

production the pH values decreased and was maintained constant up to the end of fermentation (Figure 3.b). Similar results was obtained by Andrade and Sarubbo (2002) using *Mucor circinelloides* for protease production. The optimal initial pH of the medium for proteases production obviously may vary depending on the culture medium and microbial organism under the study. The initial pH of the cultivation media is a parameter impacting on both maximum enzyme production levels and on the properties of the crude extract (YEGIN; FERNÁNDEZ-LAHORE, 2013).

3.3. Biochemical characterization: optimum temperature and pH

The optimum temperature of the protease was at room temperature (28°C ± 3). The crude extract showed 70 % of protease activity at 37°C, 77 % and 10°C, while it decreased to 48 % when subjected to 45°C. Enzymatic activity was completely lost at 75°C. These results differed from those obtained for a fibrinolytic protease from *Aspergillus oryzae* KSK-3, whose optimum temperature was 50°C and was completely inactivated at 60°C (SHIRASAKA et al., 2012). Similarly, the optimum temperature of fibrinolytic enzyme from *Cordyceps militaris* was 25°C (CHOI et al., 2011).

The optimum pH of the obtained fibrinolytic protease was pH 5.0 in the presence of 0.050 M Sodium acetate buffer. The enzyme retained 60 % of its activity at pH 7.0 (0.050 M Tris-HCl buffer) and 30 % at pH 8.5 (0.050 M Tris-HCl buffer). The results show that a considerable lost in activity only occurs at acidic pH (3.0). This is expected as the solubility of a protein will be minimal near this *p*I (Isoelectric point) (GAYLORD; GIBBS, 1962). The enzyme was characterized as acidic-neutral protease since it maintained its activity when tested in the acidic-neutral range (pH 5.0-7.0). Similar results were obtained in the characterization of the fibrinolytic protease produced by *Schizophyllum commune* (PARK et al., 2010).

3.4. Influence of metal ions, protease inhibitors and surfactants

The enzyme activity was stimulated in the presence of the following ions: $ZnSO_4$ (0.005; 0.010 and 0.020 M), $MgSO_4$ (0.005 M), $CuSO_4$ (0.005 M), $FeSO_4$ (0.010 and 0.020 M) and $CoCl_2$ (0.010 and 0.020 M). On the other hand, NaCl (%), KCl (%), MnCl (%), and $CaCl_2$ inhibited enzymatic activity. The $ZnSO_4$ and $CoCl_2$ ions also stimulated the activity of a protease produced by *Schizophyllum commune* whose residual activity was 166 and 154 %, respectively (PARK et al., 2010). The ion $MnCl_2$ promoted protease activity (154 %) (SHIRASAKA et al., 2012), differently from this work. The enzyme present in the crude extract was subjected to the action of protease inhibitors (Table 3). The serino-protease inhibitor, PMSF, induced a significant reduction of the enzyme activity to 43 %. Enzyme activity was also inhibited by Iodoacetic acid (64 %) but was not inhibited in presence of Pepstatin A, β- mercaptoetanol or EDTA.

These results allowed characterizing the obtained fibrinolytic protease as a serinoprotease. Similar results were obtained by Shirasaka et al., (2012), where the enzyme activity was considerably inhibited by serine protease inhibitors PMSF and Pefabloc SC, but not by the chelator agent EDTA.

The enzyme presented a small loss of activity in the presence of the non-ionic surfactant Triton X-100. The anionic surfactant SDS significantly increased fibrinolytic activity in the crude extract (299 %). However, in the presence of other non-ionic surfactants with a longer chain such as Tween 80, the activity was reduced to 37 %. The decrease of activity by Tween 80, was also reported for the alkaline serine protease from the thermophilic fungus *Myceliophthora sp*. which exhibited 20 % of relative activity (ZANPHORLIN et al., 2011).

3.5. Substrate specificities

The hydrolytic activity of the crude extract was measured using several chromogenic substrates. The highest level of fibrinolytic activity was observed with N-Succinyl-Ala-Ala-

Pro-Phe *p*-nitroanilide suggesting that it is a chymotrypsin-like protease. This type of fibrinolytic enzymes has also been reported for *Armillaria mellea* (LEE et al., 2005a) and *Perenniporia fraxinea* mycelia (KIM et al., 2008).

3.6. Blood clot degradation assay

The crude extract produced by *M. subtilissimus* UCP 1262 with a fibrinolytic activity of 415 U/mL showed a capacity of enzymatic degradation of the blood clot of 16.7 %. Similar results were obtained by Mahajan et al., (2012), where *in vitro* assays revealed that the enzyme could catalyze blood clot lysis significantly.

4. CONCLUSION

In the present work, we report the screening, production and characterization of a new fibrinolytic enzyme. About 58 % of the isolates were shown to display fibrinolytic activity above 100 U/mL being *Mucor subtilissimus* UCP 1262 the highest producer. Further optimization of the fermentation process, using *M. subtilissimus* UCP 1262 resulted in the production of 1075 U/mL of enzymatic activity, with a specific activity of 16753 U/mg. The crude extract exhibited maximum activity at pH 5.0 and 28°C. In addition, this enzyme showed high substrate specificity to chymotrypsin and serine proteases. The fibrinolytic enzyme had a capacity of enzymatic degradation of the blood clot *in vitro*. This research has emphasized the importance of the continuous search for novel fungal strains that can support the enzyme production with potential for future applications.

5. ACKNOWLEDGEMENTS

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CAPÍTULO 2

PARTITION OF FIBRINOLYTIC PROTEASE FROM *Mucor subtilissimus* UCP 1262 IN AQUEOUS TWO-PHASE SYSTEM USING PEG/AMMONIUM SULPHATE

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Abstract

Aqueous two-phase partitioning can be used to separate and extract proteins from cell debris or to partition proteins from microbial fermentation broth. Extraction of fibrinolytic protease produced by the fungal specie *Mucor subtilissimus* UCP 1262 at submerged fermentation was carried out using a PEG/ammonium sulphate aqueous two-phase system (ATPS). A 2^3 full factorial design was used to investigate the influence of PEG molar mass, PEG concentration and salt concentration on the responses, namely partition coefficient (K), activity yield (Y) and purification factor (PF). The ATPS was composed of PEG (molar mass 400, 3350 and 8000g/mol) PEG concentrations 15.0, 17.5 and 20.0% (w/w) and ammonium sulphate concentrations 15, 20 and 25% (w/w). The results of the extraction of the fibrinolytic protease from the fermentation broth showed partition coefficient (K=1.5) and in the top phase rich in polymer was obtained (Y) of 79.2 % and (PF) of 12.2. The parameters of this assay were: PEG 8000, 15% and ammonium sulphate concentration 25%. The statistical analysis of the partition coefficient (K) showed that the independent variable ammonium sulphate concentration (%) significantly influenced this response exerted the greatest effect. It was found that increasing the concentration of salt and decreasing the PEG molar mass resulted in an increase of the partition coefficients of the proteins to the top phase. This partitioning effect was greater for the more hydrophobic proteins and particularly in systems having a pH close to the isoelectric point of the protein. The practical application of ATPS has been

demonstrated in a large number of cases including a number of industrial applications with excellent levels of purity and yield. Furthermore, higher values of purification and yield of this enzyme suggests suitable application of fibrinolytic protease from this strain in pharmaceutical industry as a thrombolytic agent.

Keywords: aqueous two-phase system, Fibrinolytic Protease, *Mucor subtillissimus*, PEG, ammonium sulphate

1. INTRODUCTION

Urokinase is a serine protease (EC 3.4.21.73) originally isolated from human urine. It activates plasminogen into plasmin. Streptokinase is produced by beta-hemolytic Streptococcus and also activates plasminogen into plasmin. However, these enzymes have low specificity to fibrin and short half-lives, and are expensive (SUGIMOTO et al., 2007). All these drawbacks provide an opportunity for exploring the effective and safe thrombolytic agent with novel mechanisms of action that can dissolve a thrombus reliably (CHOI et al., 2013). Thrombotic diseases are potentially fatal conditions of multiple origins. Various thrombolytic enzymes, such as streptokinase and urokinase, have been used in the treatment of thrombotic diseases along with recombinant tissue plasminogen activators (JIN et al., 2013).

Thrombosis, the blockage of blood vessels due to blood clot formation, is the proximate cause of these vascular disorders. Fibrinolysis (thrombolysis) is a dynamic process in which a fibrin rich thrombus is hydrolyzed into fibrin degradation products by plasmin. Plasmin is derived from plasminogen by plasminogen activators (PAs) (CHOI et al., 2013).

ATPSs formed by two polymers or by polymer and salt represent traditional systems. Nevertheless, other alternative biphasic systems can be obtained by using surfactants, micellar compounds, or ionic liquids. Due to the high percentage of water present in their composition, ATPSs can provide a gentle environment for the extraction and recovery of sensitive biological materials such as proteins (SILVÉRIO et al., 2012).

Aqueous two-phase systems (ATPS) offer well-known benefits, such as high interfacial contact area of operation, biocompatibility and procedural simplicity. This process is based on the mass transfer between two immiscible liquid phases. Based on physicochemical properties of the target molecule, the separation will occur by addition of two polymers (or a polymer and a salt) and that will cause the formation of two-aqueous immiscible phases, extracting specific groups of molecules to one of phases (COELHO et al., 2013).

The subject of this work is the extraction of the fibrinolytic protease produced by *M. subitilissimus* UCP 1262 using aqueous two phase system (PEG/ ammonium sulphate).

2. MATERIAL AND METHODS

2.1. Production of the crude extract

The process production of fibrinolytic protease was performed using the filamentous fungi *M. subtilissimus* UCP 1262, and using the media MS-2 described by Porto (1996) with minor modifications. The medium composition was: wheat bran filtrate (2%), K_2HPO_4 (0.435%) and 1mL of mineral solution containing: $FeSO_4.7H_2O$ (100 mg), $MnCl_2.4H_2O$ (100 mg), and $ZnSO_4.H_2O$ (100 mg) of distilled water q.s.p 100 mL, NH_4Cl (0.1%), $MgSO_4.7H_2O$ (0.06%), glucose (1%), $CaCl_2$ (1%).

The fungal strain was allowed to grow at 30°C, 6 days, and then transferred to the MS-2 medium at the concentration of 10^4 spores/mL. The flasks were then incubated at 30°C, pH 7.0 and 120 rpm in an orbital shaker. After 96 h of fermentation, cultures were filtrated and centrifuged at 10000×g, 4°C for 15 min. The clear supernatant was used for determining enzyme activity and used for the enzyme extraction.

2.2. Aqueous two-phase system for Fibrinolytic Enzyme (FE) extraction

The influence of phase forming parameters in the FE purification process was performed according to a 2^3 factorial design shown in (Table 1).

Table 1 Levels of the independent variables of the full factorial design 2^3 for the extraction of the fibrinolytic protease from *M. subtilissimus* UCP 1262

Variable	*Level*		
	(-1)	(0)	(+1)
[a] M_{PEG} (g/mol)	400	3350	8000
[b] C_{PEG} (% w/w)	15	17.5	20
[c] C_{SALT} (% w/w)	15	20	25

[a] PEG molar mass; [b] PEG concentration; [c] Ammonium sulphate concentration;

The independent variables PEG molar mass, PEG and Salt $(NH_4)_2SO_4$ concentrations selected according to the binodal curves described by Murari et al. (2015). The response variables were the partition coefficient (K), yield (Y), purification factor (PF) and fibrinolytic activity (FA). The experimental design consisted of 12 trials and 4 replicates in the central point, necessary to calculate the pure error. The effects were evaluated by an analysis of

variance (ANOVA) with a confidence ($p<0.05$) to make estimates of the main effects and interaction effect. Statistical analysis of the experimental design for production and purification of the enzyme was performed using the software Statistic 8.0 (Statsoft Inc, Tulsa, OK, USA).

2.3. **Methodology for analysis of the results**

The partition coefficient of fibrinolytic enzyme (FE) was defined as the ratio of fibrinolytic activity (FA), expressed as U/mL, in the PEG phase (FA_{PEG}) to that in the salt phase (FA_{SALT}) (Eq. 1):

$$K = \frac{FA_{PEG}}{FA_{SALT}} \quad (1)$$

The activity yield was determined as the ratio of total activity in the PEG or salt phase to that in the initial FE solution (FA_i) and expressed as percentage. For this purpose, FA_P was multiplied by V_P ,for each (PEG and salt) phases, and FA_i by the total volume of the initial protease solution (V_i). (Eq. 2):

$$Y_t = \left(\frac{FA_{PEG}.Vp}{FA_i.V_i} \right) \times 100 \quad (2)$$

The purification factor in the PEG phase and salt phase were calculated as the ratio of the respective specific activity for each phase (SA_p) to the specific activity of the initial crude extract (SA_i), all expressed in U/mg (Eq. 3):

$$\mathrm{PF} = \frac{\mathrm{SA}_p}{\mathrm{SA}_\mathrm{i}} \quad (3)$$

2.4. **Substrate specificities**

Amidolytic activity was measured spectrophotometrically using the synthetic substrates: (S7388 Sigma) N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide –chymotripsin substrate, (G8148 Sigma) Gly-Arg-*p*-nitroanilide dihydrochloride - urokinase and plasmin substrate. The mixture (0.8 mL) contained 30 µL of enzyme solution, 30 µL chromogenic substrate, and 140 µL of 20mM TRIS-HCl (pH 7.4). After incubation for 15 min at 37°C, the amount of liberated p-nitroaniline (pNA) was calculated by spectrophotometric absorption at 405 nm. One unit of amidolytic activity (AU) was expressed as nanomoles of substrate hydrolyzed per minute and per millilter by the enzyme (KIM et al., 1996).

3. RESULTS AND DISCUSSION

3.1. Fibrinolytic enzyme purification using ATPS

Conventional techniques such as chromatography, electrophoresis and precipitation have been widely employed for purification of proteolytic enzymes. However, these methods are considerably expensive, providing low yields and not suitable for large scale production. The developments in the field of ATPS can be achieved by combining some downstream processing techniques such as ion-exchange chromatography, gel filtration, precipitation and ultrafiltration for further product purification. One of the most economical downstream processing methods for biomolecule recovery is the aqueous two phase systems (ATPSs) (RATANAPONGLEKA, 2010).

In this work, the crude extract was partitioned and the enzyme was isolated using aqueous two-phase systems (PEG/ $(NH_4)_2SO_4$). The main difference in comparison with conventional systems is the integration of the liquid–liquid extraction technique with fractional precipitation, which can decrease the protein content with no loss of biological activity by removing of contaminant molecules (COELHO et al., 2013).

The fermentation broth was studied to find the best conditions for extraction of fibrinolytic protease using ATPS based on a complete experimental design. The matrix of the design variables and the results of the responses are shown in (Table 2).

The fibrinolytic activity in all the experiments ranged between 135 and 345 U/mL. The fibrinolytic protease partitioned preferentially to the Salt phase (K >1). High recovery values in purification using ATPS can be obtained with high concentration of PEG and salts. Generally, this is attributed to the influence of volume exclusion and the salting out effect, whereby both the interested enzyme and total protein are in the PEG-rich phases. Otherwise in this work, the higher value in fibrinolytic activity (345 U/mL) trial 6 (Fig. 1) was obtained in the Salt phase of the system with the highest M_{PEG} (8000 g/mol), less C_{PEG} (15 % w/w) and highest concentration of salt C_{SALT} (25 % w/w).

Table 2 Matrix of the design variables and the results of the responses of fibrinolytic protease purification

Trial	M_{PEG}[a] (g mol^{-1})	C_{PEG}[b] (%)	C_{SALT}[c] (%)	FA_{PEG}[d] (U/mL)	FA_{SALT}[e] (U/mL)	K[f]	Y_{PEG}[g] (%)	Y_{SALT}[h] (%)	PF_{PEG}[i]	PF_{SALT}[j]
1[k]	400	15	15	-	-	-	-	-	-	-
2	8000	15	15	195.0	152.5	1.27	106.7	94.8	6.6	0.0
3[k]	400	20	15	-	-	-	-	-	-	-
4	8000	20	15	182.5	285.0	0.64	54.4	184.3	11.4	8.2
5	400	15	25	135.0	222.5	0.60	57.0	143.9	5.9	0.0
6	8000	15	25	227.5	345.0	0.65	79.2	253.1	12.2	8.8
7	400	20	25	137.5	162.5	0.84	44.4	121.2	7.9	4.0
8	8000	20	25	152.5	140.0	1.08	60.6	113.1	0.0	3.2
9(C)	3350	17,5	20	150.0	280.0	0.53	69.0	195.0	6.1	7.5
10(C)	3350	17,5	20	177.5	225.0	0.78	79.4	165.1	7.4	5.7
11(C)	3350	17,5	20	150.0	227.5	0.65	63.4	158.4	6.6	6.1
12(C)	3350	17,5	20	175.0	222.5	0.78	78.3	152.2	7.7	6.0

[a] PEG molar mass; [b] PEG concentration; [c] Salt concentration; [d] Fibrinolytic activity PEG phase; [e] Fibrinolytic activity SALT phase; [f] Partition coefficient; [g] Yield PEG phase; [h] Yield SALT

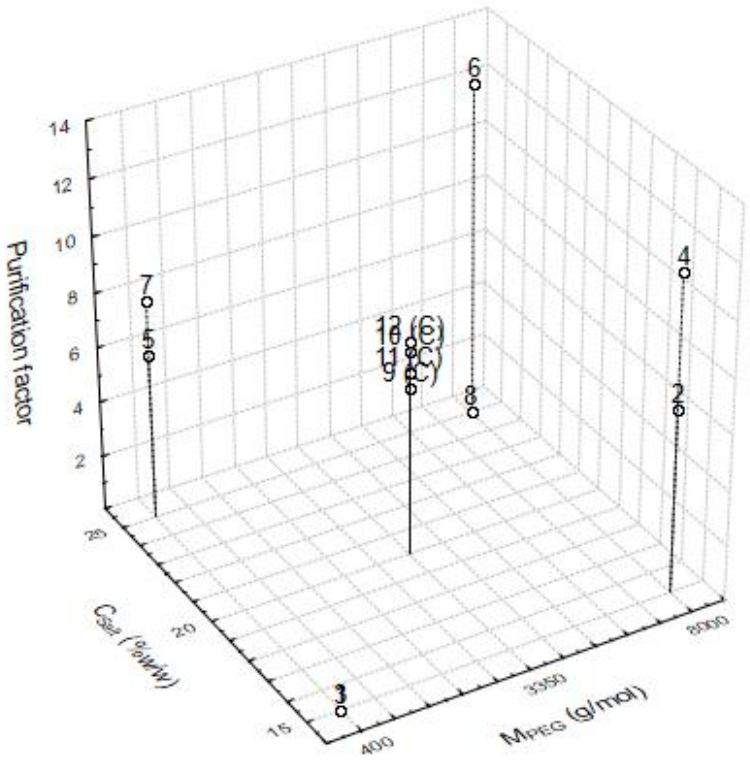

Figure 1 Simultaneous effects of salt concentration (C_{SALT}) and PEG molar mass (M_{PEG}) for fibrinolytic activity in the bottom phase of PEG/ammonium sulphate ATPS.

According to Table 3, an increase of M_{PEG} and C_{SALT} has a positive effect on the partition coefficient. The fibrinolytic enzyme partitioned preferentially to the salt phase and the best fibrinolytic activity was found in the trial 6 (Fig. 1).

In accordance with the work of Chethana et al. (2007) reporting separation of Betalains, the partition coefficient increased when the PEG molar mass increased. In the partition of acid protease from *Mucor bacilliformis* extract in PEG-phosphate systems at the lower PEG molar mass (600 g/mol), all proteins partitioned into the PEG phase, although at the higher PEG molar mass (20000 g/mol), proteins partitioned into the salt phase, but this process is unsuitable for enzyme purification

purposes. On the other hand, utilization of intermediate PEG molar mass (1540, 3350 and 8000 g/mol) was advisable to promote differential partition (LAHORE et al. 1995; MOHAMED ALI et al. 2014).

Table 3 Calculated effect of the responses in the factorial design 2^3 for the extraction of the fibrinolytic protease from *M. subtilissimus* UCP 1262

Effects	**Fibrinolytic Activity**				
	K	**Y_{PEG}**	**Y_{SALT}**	PF_{PEG}	PF_{SALT}
(1) M_{PEG}	6.47*	9.17*	7.09*	7.78*	7.23*
(2) C_{PEG}	0.09	3.83*	-1.36	-2.58	3.00
(3) C_{SALT}	3.75*	3.69*	6.56*	3.82*	3.53*
1x2	-1.31	-2.67	-0.51	-4.46*	-0.60
1x3	4.74*	-5.64	3.31*	-9.38*	-0.14
2x3	3.82*	0.96	4.70*	-7.10*	-4.36*
1x2x3	2.42	2.13	3.85*	-8.98*	-7.97*

* Data statistically significant at 95% confidence ($p < 0.05$)

The hydrophobicity effect in ATPS is related to the components concentration and chemical identity, and this hydrophobicity can vary depending of the constituents. Normally, both phases of the systems are rather hydrophobic, but typically, the PEG phase is more. In this cases the hydrophobic proteins are preferential partitioned to the PEG phase. This effect can be manipulated by changing the polymer concentration or by the addition of salts. Thus, less water is available when the system is more hydrophobic. Normally, this can occur by increasing PEG concentration, but when the PEG molar mass increases, the hydrophilic groups/hydrophobic area ratio decreases, causing a rise in hydrophobicity (Asenjo and Andrews 2011). According to Mohamed ali et al. (2014), when the tie-line (TLL) was increased, the free volume of the Salt phase decreases and

promotes the partition of protein, from the salt phase to the PEG phase or to the interface. Their results also showed that PEG 8000 g/mol was chosen for effective partition of fibrinolytic enzymes while the lowest purification fold was in the trial 1, which was made from a PEG molar mass equivalent to 1000 g/mol.

The high enzyme recovery in the salt rich phase can be explained due to the low molecular weight of the extracted fibrinolytic protease (15 kDa, data not shown), there was not enough force to attract the target enzyme to the PEG phase. As a direct consequence of their small size, proteins typically require high polymer concentrations to create an attraction sufficient to lead to phase separation. Under these conditions, the expressions for the osmotic pressure and the excluded volume theory no longer capture the physics of the polymer solution. In some cases, there is significant evidence that PEG interacts specifically with proteins, and mechanisms other than depletion forces are also likely to be involved. However, these comprise only a small number of cases (DUMETZ et al. 2008).

Most of the crude extract protein migrated to the PEG or to the interphase because of the precipitation effect of the ammonium sulphate. In this work, the C_{PEG} did not show a significant effect over the purification factor and yield, in accordance with Coelho et al. (2013) for the purification of bromelain in ATPS using PEG/ $(NH_4)_2SO_4$ systems. Nevertheless, in the same work higher concentrations of $(NH_4)_2SO_4$ likely leads to bromelain precipitation due the salting-out effect. A decrease in PEG concentration lead to a reduction of the excluded volume effect, allowing part of proteins, including bromelain, to become soluble in the PEG- enriched phase (COELHO et al. 2013).

The variable M_{PEG} showed higher effects to the activity yield for both phases of the system. The results shows high levels of Y at the Salt phase (15 % PEG 8000 and 25 % $(NH_4)_2SO_4$). Adequate results are compared with the literature (Table 4), using the same components but with phase systems using PEG at lower molar mass showing lower values for activity yield (HSU et al., 2009).

The downstream during the purification process from biological samples in industry normally comprises more than three steps. Initially, the microbial cells need to be removed using membrane filtration or centrifugation. The second step is the primary separation or precipitation by ammonium sulphate from the fermentative broth. And, the last step is the final purification but, for traditional chromatographic methods this process is subdivided into 3 or 4 more steps.

Using ATPS (PEG/ ammonium-sulphate), the second and third steps can be realized at the same time. The precipitation of impurities is followed by the isolation of the interest enzyme in a different phase of the system. Both phases can replace a number of steps involved in conventional downstream processing such as extraction, clarification, concentration, and intermediate purification (LOC et al., 2010).

Table 4 Effect of phase composition at various aqueous two-phase systems (PEG/ ammonium sulphate)

Phase composition (%, w/w)	**Origin**	**Biomolecule**	**Yield (%)**	**Reference**
15% PEG 8000/ 25% $(NH_4)_2SO_4$	*Mucor subtilissimus*	Fibrinolytic protease	253.1	This work
18% PEG 900/ 14% $(NH_4)_2SO_4$	Whey	α-lactalbumin and β-lactoglobulin	96.7 for αla and 83.8 for βlg	(RODRIGUES, VENÂNCIO and TEIXEIRA, 2001)
8.5% PEG 6000/ 17.5% $(NH_4)_2SO_4$	Recombinant *Bacillus badius*	Phenylalanine dehydrogenase (PheDH)	95.85	(MOHAMADI and OMIDINIA, 2007)
10.86% PEG 4000/ 36.21% $(NH_4)_2SO_4$	Pineapple (*Ananas comosus* L. Merril)	Bromelain	66.38	(COELHO et al., 2013)

3.2. Substrate specificities

The hydrolytic activity of the purified enzyme was measured using several chromogenic substrates. The highest level of fibrinolytic activity observed with N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide suggests that the enzyme is a chymotrypsin-like protease. This type of fibrinolytic enzymes has also been previously reported (LEE et al. 2005; KIM et al. 2008).

4. CONCLUSION

In the present work, the extraction using ATPS of a new fibrinolytic enzyme from *M. subtilissimus* UCP 1262 was reported. The fibrinolytic protease partitioned preferentially to the Salt phase (K >1). High recovery values in purification using ATPS was obtained and the higher value in fibrinolytic activity (345 U/mL) trial 6 was obtained in the Salt phase of the system with the highest M_{PEG} (8000 g/mol), low concentration of PEG (15 % w/w) and the highest concentration of salt (25 % w/w). This research has emphasized the importance of the continuous search for novel fungal strains that can support the enzyme production with potential for future applications. The purification process facilitated the separation of the enzyme from the fermentation broth reducing the steps of the downstream process.

5. ACKNOWLEDGEMENTS

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CAPÍTULO 3

SIMULTANEOUS PRODUCTION AND RECOVERY *IN SITU* OF FIBRINOLYTIC PROTEASE FROM *Mucor subtilissimus* UCP 1262

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Abstract

Fibrinolytic proteases are enzymes that degrade fibrin and are a promising alternative for thrombolytic therapy. The aim of this study was to evaluate the optimum conditions for the integrated production and purification of fibrinolytic protease from *Mucor subtilissimus* UCP 1262. The integrated process of production and purification was carried out in a culture medium containing wheat bran and by adding polyethylene glycol (PEG) and $(NH_4)_2SO_4$ according to a 2^3 experimental design. In all assays, the enzyme preferentially partitioned to the PEG phase (K>1), with an optimum specific activity of 6419 U/mg in the PEG phase. Fibrinolytic enzyme from *Mucor subitilissimus* UCP 1262 was pre-purified after extractive fermentation in PEG and ammonium sulphate ATPS, in which the fungal strain was able to grown even in high salt concentration, produced and extracted simultaneously to the PEG phase. The results indicate that the use of a low-cost substrate and the integration of fermentation with an aqueous two-phase system extraction may be a promising alternative for the production and extraction of fibrinolytic protease.

Keywords: fibrinolytic protease, *Mucor subitilissimus*, PEG/ammonium sulphate, ATPS, extractive fermentation

1. INTRODUCTION

The cost of enzyme production and downstream processing is a major obstacle to successful application of proteases in industry and medicine. For fibrinolytic enzymes, many attempts have been made to improve expression of the fibrinolytic enzyme, including selection of an ideal culture medium, optimization of environmental conditions, and overexpression by genetically engineered strains. Selection of medium components is usually critical for fermentative production of fibrinolytic enzymes. Since different microorganisms have diverse

physiological characteristics, it is necessary to optimize nutrient components and environmental conditions for cell growth and fibrinolytic enzyme production.

Cardiovascular diseases of both arterial and venous systems are substantial and a rapidly growing problem around the world, affecting people from all socioeconomic backgrounds. Thrombosis plays a major role in diverse circulatory diseases such as myocardial infarction, deep vein thrombosis, and pulmonary embolism. Thrombosis occurs when a thrombus (fibrin clot) is formed inside a blood vessel (CHOI et al., 2014).

Aqueous two-phase systems (ATPS) naturally arise in mixtures of different water-soluble polymers, or a single polymer and a specific salt. When two specific polymers, for example, dextran and ficoll, are mixed in water above certain concentrations, the mixture separates into two immiscible aqueous layers (ZASLAVSKY et al., 2013). ATPS allow process integration on account of simultaneous separation and concentration of the target protein in a clear extract being achieved. Moreover, the biocompatible environment facilitates the preservation of the biological activity of enzyme extracts (IANNUCCI et al., 2008). The use of polymer-salt ATPS has the advantage of low viscosity and economical cost as compared to polymer–polymer (SOARES et al., 2015).

With the integrated process, the aqueous biphasic systems are inoculated with the expression system growing selectively in a particular phase, while the continuously secreted target product is partitioned toward the opposite phase. Process integration results when one single unit operation can achieve the process objectives of two or more discrete processing stages, and consequently, a reduction of the total number of unit operations is possible (CHAVEZ-SANTOSCOY et al., 2010).

This approach represents an attractive technology for the continuous removal of the target product from fermentation broth. As the target molecule is produced by the expression system, thus overcoming low product yields in conventional fermentation processes due to product inhibition and recovery of the target product in one cell-free phase, while the expression system is continuing its production in the opposite phase (GLYK et al., 2015; SINHA et al., 2000).

In this work, we propose a new extractive fermentation process that allows the Polymer (PEG) and Salt $(NH_4)_2SO_4$ of ATPS to perform simultaneously with the *Mucor subtilissimus* UCP 1262 cells to produce and extract the fibrinolytic protease.

2. MATERIALS AND METHODS

The process of production and purification of fibrinolytic protease was performed using the filamentous fungi *Mucor subtilissimus* UCP 1262, and using the media MS-2 described by Porto et al. (1996) with minor modifications. The medium composition was wheat bran filtrate (2%), K_2HPO_4 (0.435%) and 1mL of mineral solution containing: $FeSO_4.7H_2O$ (100 mg), $MnCl_2.4H_2O$ (100 mg), and $ZnSO_4.H_2O$ (100 mg) of distilled water q.s.p 100 mL, NH_4Cl (0.1%), $MgSO_4.7H_2O$ (0.06%), glucose (1%), $CaCl_2$ (1%).

2.1. Medium for extractive fermentation using ATPS

The culture medium for extractive fermentation using ATPS was prepared by mixing PEG and ammonium-sulphate solutions according to the experimental design described in the succeeding section in this study and the culture medium MS-2. After adjusting the pH to 7.2, the system was autoclaved at 121°C for 20 min. Extractive fermentation lasted for 96 h and was performed at 120 rpm agitation and 30°C under different conditions according to a factorial design. At the

end of the process, the flasks were left to settle for 1h and further centrifuged at 12000×g for 15 min to separate both phases (PEG and salt). After that, the phases were subjected to the analytical determinations (protein content and fibrinolytic activity).

2.2. Factorial Design 2^3 for Aqueous two-phase extractive fermentation

The influence of phase-forming parameters in the fibrinolytic protease extractive fermentation process was performed according to a 2^3 factorial design shown in Table 1. The independent variables PEG molar mass and PEG and salt (ammonium sulphate) concentrations were made according to the previous studies (unpublished data). The response variables were the partition coefficient (K), PEG (FA_{PEG}) and salt (FA_{SALT}) fibrinolytic activities and Activity Yield (Y) of the PEG phase. The experimental design consisted of 12 trials and 4 replicates in the central point, necessary to calculate the pure error.

Table 1 Levels of the independent variables of the full factorial design 2^3 for the extractive fermentation process of the fibrinolytic protease from *Mucor subtilissimus* UCP 1262

Variables	***Levels***		
	(-1)	(0)	(+1)
PEG molar mass (g/mol)	400	3350	8000
PEG Concentration (% w/w)	15	17.5	20
Ammonium sulphate Concentration (% w/w)	15	20	25

2.3. Methodology for analysis of the results

The partition coefficient of FP was defined as the ratio of fibrinolytic activity (FA), expressed as U/mL, in the PEG phase (FA_{PEG}) to that in the salt phase (FA_{SALT}), Eq. (1):

$$K = \frac{FA_{PEG}}{FA_{SALT}} \quad (1)$$

In order to assess the recovery operation, the fibrinolytic activity yield was calculated using Eq. (2):

$$Y_{PEG} = \frac{100}{1+\left(\frac{1}{V_R}\right)*\left(\frac{1}{K}\right)} \quad (2)$$

where V_R is the volume ratio of the top phase to the bottom phase.

In order to analyze the purification performance of the operation, the CF (concentration factor) was described as the ratio of the fibrinolytic specific activity (SA_{PEG}) in the top phase to the initial specific activity (SA_{CE}) in the crude extract (homogeneous fermentation) (OOI et al., 2011), Eq. (3):

$$PF = \frac{SA_{PEG}}{SA_{CE}} \quad (3)$$

2.4. Determination of total protein

The protein content was determined by the method described by Bradford (1976) using BSA (bovine serum albumin, working range: 25- 100 mg/mL) as a standard. Each experiment was performed in triplicate and the average value was then calculated after correction with the corresponding blank.

2.5. Determination of fibrinolytic activity

Fibrinolytic activity was measured using a fibrin degradation assay. For this determination, 0.4 mL of 0.072 g/l fibrinogen was placed in a test tube with 0.1 mL of 0.245 M phosphate buffer (pH 7.0) and incubated at 37°C for 5 min. Then, 0.1 mL of a 20 U/mL thrombin solution was added. The solution was incubated at 37°C for 10 min, 0.1 mL of diluted enzyme solution was added, and incubation continued at 37°C. At 60 min, 0.7 mL of 0.2 M TCA (trichloroacetic Acid) was added, and mixed. The reaction mixture was centrifuged at 15000 xg for 10 min. After that, 1 mL of the supernatant was collected and the absorbance at 275 nm was measured. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank. In this assay, 1U (fibrin degradation unit) of enzyme activity is defined as a 0.01 per minute increase in absorbance at 275 nm of the reaction solution (WANG et al., 2011).

3. RESULTS AND DISCUSSION

The extractive fermentation or recovery of the fibrinolytic protease from *Mucor subtilissimus* UCP 1262 *in situ* was carried out using an aqueous two phase system composed of Polyethylene glycol and ammonium sulphate as a polymer/salt system composition. The main difference with this system composition, in comparison with other conventional systems, is the integration of the liquid–liquid extraction technique with fractional precipitation (COELHO et al., 2013).

Based on previous studies for extractive fermentation of fibrinolytic enzymes, we suggest a mechanism for simultaneous precipitation/separation using ammonium sulfate as a Salt phase at high concentrations. The Salt and PEG concentrations used were selected according to the factorial design 2^3 (Table 2), and the best fibrinolytic activity was found in the PEG phase 23.15 U/mL in the trial 7 containing PEG 400 (20%) and Salt (25%) (Figure 1).

Table 2 Matrix of the design variables and the responses of fibrinolytic protease integrated process of production and purification

Trial	M_{PEG}[a] (g/mol)	C_{PEG}[b] (%)	C_{SALT}[c] (%)	FA_{PEG}[d] (U/mL)	FA_{SALT}[e] (U/mL)	K[f]	Y[g] (%)
1	400	15	15	16.12	4.98	3.23	70.80
2	8000	15	15	10.98	4.61	2.38	48.78
3	400	20	15	15.97	5.10	3.13	75.79
4	8000	20	15	11.08	5.86	1.89	48.61
5	400	15	25	25.30	4.98	5.07	71.73
6	8000	15	25	23.18	6.40	3.62	76.52
7	400	20	25	23.15	4.12	5.62	78.94
8	8000	20	25	1.94	4.23	0.45	18.08
9 (C)	3350	17.5	20	16.04	5.31	3.02	50.18
10 (C)	3350	17.5	20	16.78	5.75	2.91	49.30
11 (C)	3350	17.5	20	11.46	4.28	2.67	47.13
12 (C)	3350	17.5	20	9.28	4.75	1.95	39.42

a PEG molar mass; **b** PEG concentration; **c** Salt concentration; **d** Fibrinolytic activity PEG phase; **e** Fibrinolytic activity SALT phase; **f** Partition coefficient; **g** Activity Yield.

The Fibrinolytic enzyme partitioned preferentially to the PEG phase of the system in most of the trials, being the higher partition coefficient (K: 5.62), obtained in trial 7 of the factorial design composed by PEG 400 (20%) and Ammonium sulphate (25%) (Table 2). Microbial cells were retained at the interface. Statistical analysis showed that the variables PEG molar mass

(M_{PEG}) and the interaction between PEG molar mass (M_{PEG}) and concentration of ammonium sulfate (C_{SALT}) were statistically significant for the partition coefficient (K) response (Figure. 2)

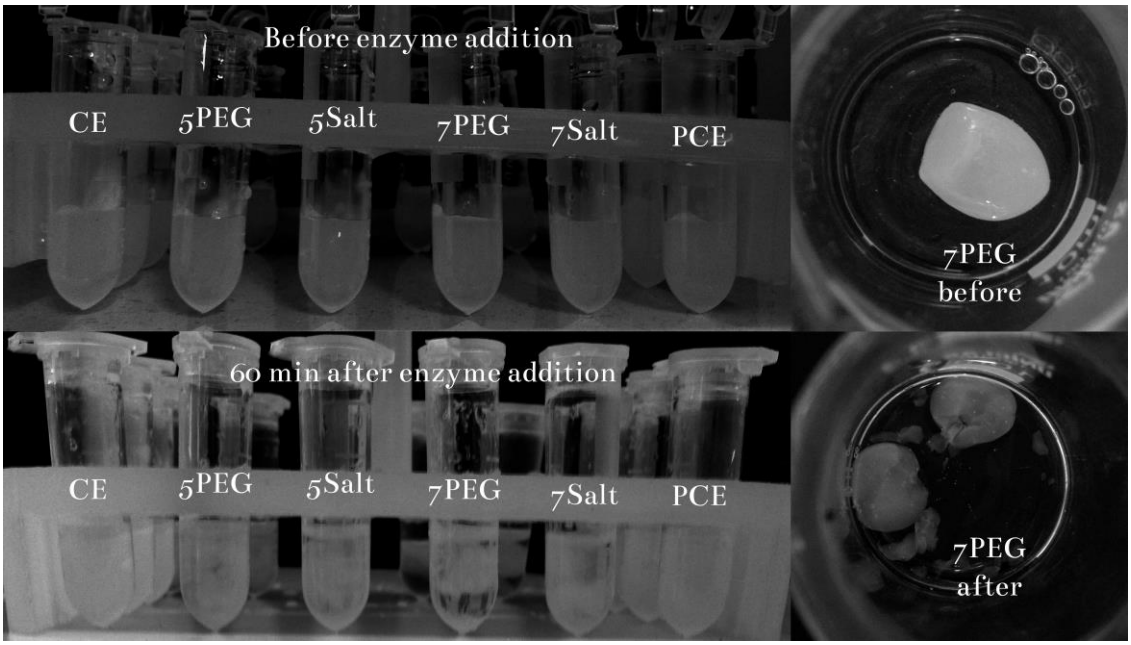

Figure 1 Fibrin clot liquefaction by Fibrinolytic Enzyme from *Mucor subtilissimus* UCP 1262. CE- Crude extract (homogeneous fermentation); 5PEG- trial 5 (PEG phase) of extractive fermentation; 5Salt- trial 5 (Salt phase) of extractive fermentation; 7PEG- trial 7 (PEG phase) of extractive fermentation; 7Salt- trial 7 (Salt phase) of extractive fermentation; PCE- acetone-precipitated crude extract (homogeneous fermentation).

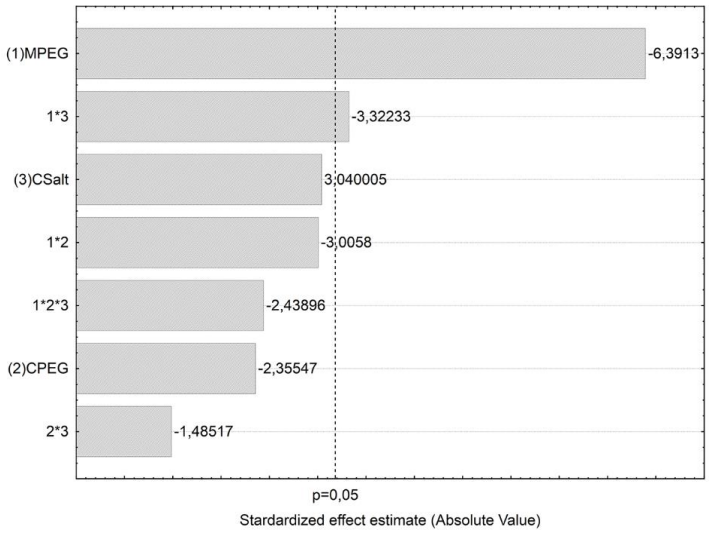

Figure 2 Pareto chart for the standardized effects of the variables: (1) PEG molar mass –M_{PEG}, (2) PEG concentration – C_{PEG}, (3) ammonium sulphate concentration – C_{SALT} and on partition coefficient of Fibrinolytic protease. The extension of bars across the vertical dotted line (p=0.05) represents the dimensions of significance.

The M_{PEG} can also affect the phase formation, where polymers with higher molar mass are more capable to induce ATPS formation (MURARI et al., 2015). Comparing the results from homogeneous fermentation with the extractive fermentation using ATPS it is possible to measure the purification of the process.

The fibrinolytic Specific Activity in the PEG phase (SA_{PEG}) of 135.51 U/mg was greater than the crude extract 19.27 times-fold, since the Specific Activity of the crude extract of the homogeneous fermentation (SA_{CE}) was 7.032 U/mg. The advantages of using extractive fermentation to obtain a more concentrated target enzyme resulting in process selectivity and reduced product inhibition, was also described by Sales et al. (2013).

According to Hotha and Banik (1997) for alkaline protease extractive fermentation, the partition coefficient, K, was increased when the PEG molar mass 9000 decreased to 4000 (g/mol). The total activity yield compared with the control (homogeneous fermentation) was

2.80 times higher when the ATPS was made entirely with the fermentation, and 2.26 times higher when the ATPS was added after 45h of fermentation. The enzyme production was higher when using PEG molar mass 4000 then using PEG 6000 or 9000 (g/mol).

Evaluating the partition and recovery of 6-pentyl-α-pyrone (6PP), Rito-Palomares et al. (2001), selected the best system composition as PEG 1450 (7.4%) sodium sulphate (16.2%) as the first ATPS for *in situ* recovery study. However, the *Trichoderma harzianum* cells were completely inhibited due to the high ionic strength of the Salt phase. The authors adopted a different strategy carrying on a biomass production cultivation (without ATPS) for a duration of 53 h followed by the recovery of mycelium and resuspension in the ATPS.

Even then, the fungal cells were inhibited and the production was not detected. The performance of salts in promoting phase separation is reflected in the lyotropic series (a classification of ions based upon salting-out or salting-in ability). Salts with multi-charged anions are most effective. The Hofmeister series determines the increase in surface tension of water by salts. Hence, as an approximation, those salts that favor salting-out raise the surface tension of water the most. As $(NH_4)_2SO_4$ has much a higher solubility than phosphate salts, it is the reagent of choice for salting-out (KELLY et al 2005).

The addition of components for separation systems ATPS (PEG and Salts) after the homogeneous fermentation, when the microorganism is already growing, is a strategy to avoid growth inhibition. Normally, this strategy is used when the ATPS is composed of high salt concentrations with high ionic strength of the bottom phase, causing that the fungal spores never germinate.

In these cases where the cultures are carried out in which the ATPS were loaded at the beginning, the growth is completely inhibited and no production of the target product was detected (RITO-PALOMARES et al., 2001). However, our findings are not in keeping with this

previous work, showing that the salt concentrations used changed the dynamics of the aeration and agitation due to viscosity, but did not inhibit the fungal growth and production of the enzyme.

The effect of the variables on the enzyme production and extraction in *M. subtilissimus* UCP 1262 for partition coefficient is shown in Figure 2. Most of the variables markedly increased the fibrinolytic enzyme content in the PEG phase trials of the factorial design. As shown in Pareto chart of standardized effects of yield variable in Extractive fermentation (Figure 3), the reduction of M_{PEG} and C_{PEG} increased the activity yield, and the maximum yield (78.94%) was obtained when grown on MPEG 400 (g/mol) and CPEG 20% (w/w).

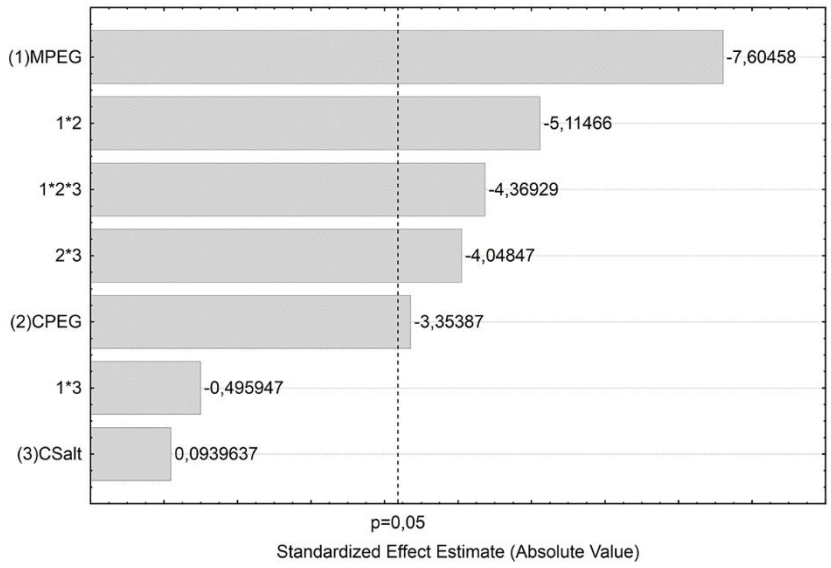

Figure 3 Pareto chart for the standardized effects of the variables: (1) PEG molar mass –M_{PEG}, (2) PEG concentration – C_{PEG}, (3) ammonium sulphate concentration – C_{SALT} and on activity yield. The extension of bars across the vertical dotted line (p=0.05) represents the dimensions of significance.

According to Ashipala and He (2008), the decrease of PEG 4000 and Na_2SO_4 concentration levels, increased the fibrinolytic activity in PEG phase, and was attributed this increase of enzyme activity to a higher secretion of the enzyme in presence of PEG.

High PEG concentration can may be a limiting factor. Using a high viscous media, the agitation greatly impacts on the productivity and bioprocess yield, influencing the distribution of air bubbles, homogenization and transfer of oxygen in the culture medium as reported by Viana Marques et al. (2010). Many reports of integrated process using ATPS contain PEG as a polymer with different molar mass and different salts are described (Table 3).

Table 3 Several examples of extractive fermentations using ATPS

ATPS	Microorganism	Target product	Reference
PEG 4000/ phosphate	*B. thuringiensis*	Alkaline protease	Hotha and Banik (1997)
PEG 4000/sodium sulfate	*Bacillus subtilis* DC2	Fibrinolytic protease	Ashipala and He (2008)
PEG 8000/ phosphate	*Streptomyces* sp. DAUFPE 3060	Clavulanic acid	Viana Marques et al. (2010)
PEG 4000/ dextran T500	*Bacillus licheniformis* MTCC 1483	*Phosphatase*	Pandey and Banik (2011)
PEG 8000/ sodium sulfate	*Bacillus sp.* UFPEDA 485	Fibrinolytic protease	Sales et al. (2013)
PEG 4000/ phosphate	*Aspergillus tamarii* URM 4634	Xylanase	Da Silva et al (2014)

Interestingly, the range between PEG 2000 to 8000 (g/mol) is optimum for this type of process. Polymers with intermediate molar mass are more preferable than the systems containing higher molar mass because of their lower viscosity. The high viscosity may limit the oxygen mass transfer, decrease the growth of cells and reduce enzyme production (SHOW et al., 2012). In this work, we showed that variable M_{PEG} exercised statistically significant influence for all the responses, being the crucial variable for the partitioning and recovery of the enzyme (Figure 2 and 3).

The extractive fermentation technique for enzyme production has been extensively applied and reported in the literature (VIANA MARQUES et al., 2010; WANG; DAI, 2010; OOI et al., 2011; SHOW et al., 2012; GUTIÉRREZ et al., 2013; SALES et al., 2013). The formation of two-phase systems for simultaneous cultivation and downstream process provides a fast, efficient and low cost mechanism for enzyme recovery.

4. CONCLUSION

Fibrinolytic enzyme from *Mucor subitilissimus* UCP 1262 was pre-purified after extractive fermentation in PEG and ammonium sulphate ATPS, in which the fungal strain was able to grown even in high salt concentration, produced and extracted simultaneously to the PEG phase. The fibrinolytic enzyme produced and extracted liquefied the fibrin clot *in vitro*. These results demonstrated that extractive fermentation using PEG –ammonium sulphate ATPS has the potential to improve fibrinolytic protease recovery *in situ*.

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CAPÍTULO 4

PURIFICATION AND STRUCTURAL CHARACTERIZATION OF A NOVEL PROTEASE WITH FIBRINOLYTIC ACTIVITY FROM *Mucor subtilissimus* UCP 1262

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Abstract

A novel protease with fibrinolytic activity was purified from *Mucor subtilissimus* UCP 1262 using a two-step purification protocol. The enzyme was pre-purified using cetonic precipitation and adsorbed by ion exchange chromatography on DEAE-sephadex G50. Compared to the crude enzyme extract, the specific activity of the enzyme increased 5.30 fold with a recovery of 36.31%. The two-dimensional electrophoresis system (2DE) coupled with SDS-PAGE showed a single protein band of approximately 15.3 kDa and isoeletric focusing point of 3.9, exhibiting a nature as an acidic enzyme. Fibrinolytic activity was exhibited, which was stronger than plasmin activity and also had a higher affinity for the N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (*SAApNA*) and azocasein substrates, suggesting to be a chymotrypsin-like protease. Protein unfolding induced by pH and temperature are common approaches to study proteins *in vitro*. The far UV circular dichroism (CD) of the fibrinolytic protease showed the secondary structure with most content of α-helix. These results demonstrate an viable enzyme purification protocol. The highly purified fibrinolytic enzyme may represent a new source of therapeutic agents to treat thrombosis.

1. INTRODUCTION

Proteases constitute a large group of hydrolytic enzymes that cleave the peptide bond of proteins and degrade them into smaller peptides and amino acids. Among the different industrial enzymes, proteases have applications mainly in the food industry, in creating detergents and use with processing leather products (ANITHA AND PALANIVELU, 2013).

Microbial enzyme production requires investigation of the parameters which may affect enzyme yield, for optimization of production and effective downstream processing techniques. Various natural-active enzymes purified from microbial sources have been used extensively in several

fields. Of these, fibrinolytic enzymes have received attention for their potential medicinal use for thrombotic disease. Cardiovascular disease is a leading cause of morbidity and mortality worldwide, and most of the fibrinolytic agents available currently for clinical treatment are barely satisfactory (BI et al, 2013).

The microbial diversity may facilitate targeting novel fibrinolytic proteases with desired therapeutic attributes. In recent years, thrombolytic agents found in non-animal sources have received considerable attention because of their security (KUMAR et al, 2013). Fibrin is formed from fibrinogen by the action of thrombin. Fibrin is lysed by plasmin, which is activated from plasminogen by tissue plasminogen activator. Hemostasis is a complex process that maintains the balance between fibrin formation and fibrinolysis. However, when fibrin hydrolysis is not complete because of a balance disorder, thrombosis can occur (SHIRASAKA et al., 2012).

The abnormal clot obstructs the flow of blood and nutrients to vital tissues within the vascular system (LU et al, 2010). Due to prevalence, these cardiovascular diseases are expected to impose an ever-increasing impact on our society socially and financially (KIM et al., 2011). The question of how some proteins are able to achieve extreme thermal stability is important both because of its industrial potential and practical applications and because of the fundamental physical and chemical processes involved. Probing the conformational behavior of a single-chain polypeptide at high temperatures provides insight into the different forces involved in the stabilization of a protein (HAQ; KHAN, 2005). Therefore, in this study we attempted to identify a thrombolytic agent as a protease with fibrinolytic activity from *Mucor subtilissimus* UCP 1262. The purification and structural characterization of this protease are also described.

2. MATERIAL AND METHODS

2.1. Culture conditions for enzyme production

The culture conditions of *Mucor subtilissimus* UCP 1262 for enzyme production were preliminarily optimized (30°C, 120 rpm). A 6-day culture of *Mucor subtilissimus* UCP 1262 in 7 mL Czapek Agar medium was used. The spores suspended at 10^4 of concentration was inoculated into 100 mL medium liquid described by Porto et al (1996) with minor modifications so that wheat was added as a nitrogen source.

The medium consisted of wheat bran filtrate (1%), K_2HPO_4 (0.435%), and mineral solution [$FeSO_4.7H_2O$ (100 mg), $MnCl_2.4H_2O$ (100 mg), and $ZnSO_4.H_2O$ (100 mg)], NH_4Cl (0.1%), $MgSO_4.7H_2O$ (0.06 %), and $CaCl_2$ (1%) plus glucose (1%) dissolved in 100 mL of distilled water. The culture medium was added in a shaking flask (250 mL), which was incubated at 30°C on a shaker (120 rpm) for 4 days. The culture broth was obtained by filtration and used for enzyme purification.

2.2. Purification of protease with fibrinolytic activity

The fermentation broth (300 mL) was submitted to precipitation using 70% acetone. The acetone was added to the supernatant and stirred for 15min at 4◦C, and then centrifuged for 40 min at 12,000 × g. The resulting precipitate was collected through centrifugation at 15000xg, 4°C, for 30 min. The pellet was dissolved in 100mM sodium acetate buffer, pH 7.5, and the solution was dialyzed against the same buffer overnight at 4°C. The desalted proteins (about 1 mg) was concentrated by lyophilization and subsequently loaded into a DEAE-Sephadex chromatography process, using a column (8.0 x 1.0 x 1.0 cm) equilibrated with sodium acetate buffer (100 mM, pH 7.5). The adsorbent sample was then eluted with 0.5 M potassium chloride in the same buffer in 1mL fractions The protein-containing fraction was pooled and the enzyme solution was

concentrated for further analysis. Then absorbance at 280nm was monitored throughout the whole process.

2.3. Determination of total protein

The protein content was determined according to Bradford (1976) method using bovine serum albumin (working range: 25- 100 mg/mL) to obtain a standard curve. Each experiment was performed in triplicate.

2.4. Determination of Fibrinolytic Activity

Fibrinolytic activity was measured using the fibrin degradation assay (WANG et al, 2011). Initially, 0.4 mL of 0.72 % fibrinogen was placed in a test tube with 0.1 mL of 245 mM phosphate buffer (pH 7.0) and incubated at 37°C for 5 min. Then, 0.1 mL of a 20-U mL^{-1} thrombin was added. The solution was incubated at 37°C for 10 min, 0.1 mL of diluted enzyme solution was added, and incubation continued at 37°C. This preparation was mixed after 20 - 40 min. At 60 min, 0.7 mL of 0.2 M trichloroacetic acid was added and mixed. The reaction mixture was centrifuged at 15000x g for 10 min. After that, 1 mL of the supernatant was collected and the absorbance at 275 nm was measured. Each experiment was performed in triplicate and the average value was then calculated after correction of the corresponding blank. In this assay, 1 U (fibrin degradation unit) of enzyme activity was defined as a 0.01/min increase in absorbance at 275 nm of the reaction solution.

2.5. Substrate specificity

Amidolytic activity was measured spectrophotometrically using the synthetic substrates: (S7388 Sigma) N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide –Chymotrypsin substrate, (G8148 Sigma) Gly-Arg-*p*-nitroanilide dihydrochloride - urokinase and plasmin substrate. The mixture (0.8 mL)

contained 30 µL of enzyme solution, 30 µL chromogenic substrate, and 140 µL of 20mM TRIS-HCl (pH 7.4). After incubation for 15 min at 37°C, the amount of liberated *p*-nitroaniline (pNA) was calculated by spectrophotometric absorption at 405 nm. One unit of amidolytic activity (AU) was expressed as micromoles of substrate hydrolyzed per minute and per milliliter by the enzyme. Each value is the mean of three determinations (KIM et al., 1996).

2.6. Determination of molecular mass and isoelectric focusing point (IEF)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12.5% polyacrylamide running gel according to (LAEMMLI, 1970).

The molecular mass was approximately calculated using a molecular mass marker (Novex Sharp Pre-Stained Protein Standard (10ul) as a standard analyzed on a NuPAGE Novex 4-12% Bis-Tris Gel with 1% MES running buffer. Protein bands were detected by staining with Coomassie brilliant blue R-250 and Silver Stain.

The isoelectric focusing point (pI) of the enzyme was determined by two-dimensional electrophoresis (2-DE) according to the manufacturer's procedure. Isoelectric focusing (IEF) was carried out using 13-cm Immobiline DryStrip gels containing a preformed pH gradient immobilized in homogeneous polyacrylamide gels with a pH range of 3–10 (GE Healthcare).

The purified enzyme was loaded by in-gel rehydration with a reselling solution containing 8 M of urea, 0.3% DTT and 0.2% (v/v) pH 3–10 IPG buffer. IEF was carried out at 20°C in a Multiphor II Electrophoresis System (GE Healthcare), wherein the voltage was linearly increased from 300 to 3500 V at 4 V/min and kept constant for a further 3 h. After IEF, the strip was equilibrated for 15 min in buffer containing 8 M urea, 20% glycerol, 2% SDS, 2% DTT and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of DTT. After equilibration, the strip was

loaded on a 12.5% SDS-PAGE gel for second-dimensional separation. The gel was stained with Coomassie Brilliant Blue R250.

2.7. Effect of inhibitors in protease activity

To evaluate the effect of inhibitors, the purified sample was exposed to the following inhibitors, the concentration of the solutions was standardized at 5 mM: PMSF (fluoride methylphenylsulfonyl-$C_7H_7FO_2S$), mercuric chloride ($HgCl_2$), 2-mercaptoethanol (2-hydroxy-1-ethanethiol-C_2H_6SO) and EDTA (Ethylenediaminetetraacetic acid acetic - $C_{10}H_{16}N_2O_8$) and incubated for 60 min at 37°C. The inhibitors were dissolved in 0.1 M Tris-HCl, pH 7.75, with 0.150 M NaCl.

2.8. Circular dichroism (CD)

Far-UV CD (195–260 nm) spectra of proteins were measured using a JASCO J-815 spectropolarimeter at room temperature. A solution of protein (110 μL, 1 mg/mL) was placed into a 0.2 mm pathlength cell, and the CD spectra were acquired with 20 nm/min scan speed at 0.2 nm step size and 1.0 nm bandwidth under constant purging with nitrogen. Three spectra were accumulated and averaged for each sample.

3. RESULTS AND DISCUSSION

3.1. Purification, determination of molecular mass and isoelectric point (IEF)

The fibrinolytic enzyme was purified using the steps described in Table 1, including acetone precipitation and ion exchange chromatography with DEAE-Sephadex column. The specific activity of the enzyme after purification, in comparison with the crude extract, was increased 5.30-fold, with a recovery of 36.31%. The yield of the purified enzyme after DEAE-Sephadex ionic

exchange chromatography was about 36.3 % of the relative fibrinolytic activity of the culture supernatant and the enzyme had a specific activity of 47.28 U/mg of protein.

Purification of fibrinolytic protease using acetone at different volume concentrations is reported: 5 volumes, 3 volumes, and 2.5 volumes, as a primary precipitation agent for the recovery of proteases. Precipitation was also reported with acetone at different concentrations: 80% (v/v), 66% (v/v); or 44, 66, and 83% (v/v), followed by centrifugation and/or drying. Precipitation of enzymes can also be achieved by the use of water-soluble, neutral polymers such as polyethylene glycol (KUMAR AND TAKAGI, 1999).

A single-step chromatographic procedure, anion-exchange chromatography, was performed for purification of the fibrinolytic enzyme. Due to the residues of the culture supernatant, it was directly subjected to the column after clarification pre-treatment (Figure 1).

This was an advantage when the purification strategy used in this work was compared to the purification of proteolytic enzymes produced under solid state fermentation conditions, where a number of steps need to be applied to remove excess salt (YEGIN et al, 2012). Anion-exchange chromatography was a particularly critical step to the success of Neanthes japonica (Iznka) fibrinolytic enzyme (NJF) purification (WANG et al, 2011) and fibrinolytic enzyme NJF purified from the second active fraction (DENG et al, 2010).

The fibrinolytic enzyme purified from mycelium of *Perenniporia fraxinea* showed 14.2-fold with a yield of 0.8% (KIM et al, 2008). The purified protease with fibrinolytic activity appeared as a single protein band on SDS-PAGE (Figure 2), and had a molecular weight of 15 kDa and isoeletric point of 3.9 (Figure 3). The purification steps of the protease have been summarized in Table 1.

The fibrinolytic activity (FA) was eluted as two peaks as showed in Figure 1 the second peak with maximum fibrinolytic activity was collected, dialyzed and concentrated by lyophilization, and used as the purified enzyme preparation. Here, the fibrinolytic protease appeared as a single band in

SDS-PAGE as shown in Figure 2 and the protein spot in 2-DE as showed in Figure 3 and the purity of the enzyme was thus confirmed.

Table 1 Purification steps of the protease with fibrinolytic activity from *Mucor subtilissimus* UCP 1262

Step	FA* (U/mL)	FA* (U)	PT* (mg/mL)	V* (mL)	PT* (mg)	SA* (U/mg)	Fold	Y*(%)
Crude Extract	5.35	1605	0.006	300	1.8	891.66	1.00	100
Cetonic precipitate	5.41	162.51	0.0104	30	0.31	520.86	5.84	101.25
DEAE-Sephadex	1.96	5.90	0.0416	3	0.12	47.28	5.30	36.31

*FA: Fibrinolytic activity, PT: total protein, V: Volume, SA: Specific activity, Y: Yield.

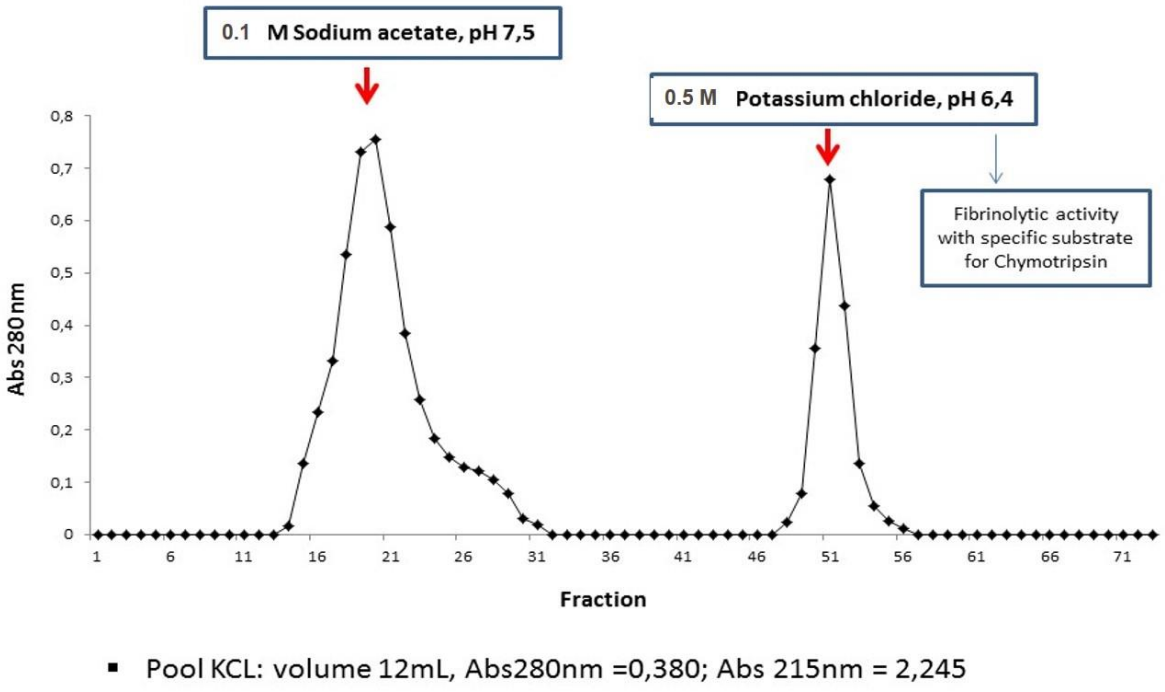


Figure 1 Purification of fibrinolytic protease produced by *Mucor subtilissimus* UCP 1262. Anion-exchange chromatography on a DEAE-Sephadex column (8.0x1.0 cm) equilibrated with 0.1M Sodium acetate (pH 7.5), then eluted at a flow rate of 1.2 mL min $^{-1}$ with linear gradient of 0.5M Potassium chloride (pH 6.4).

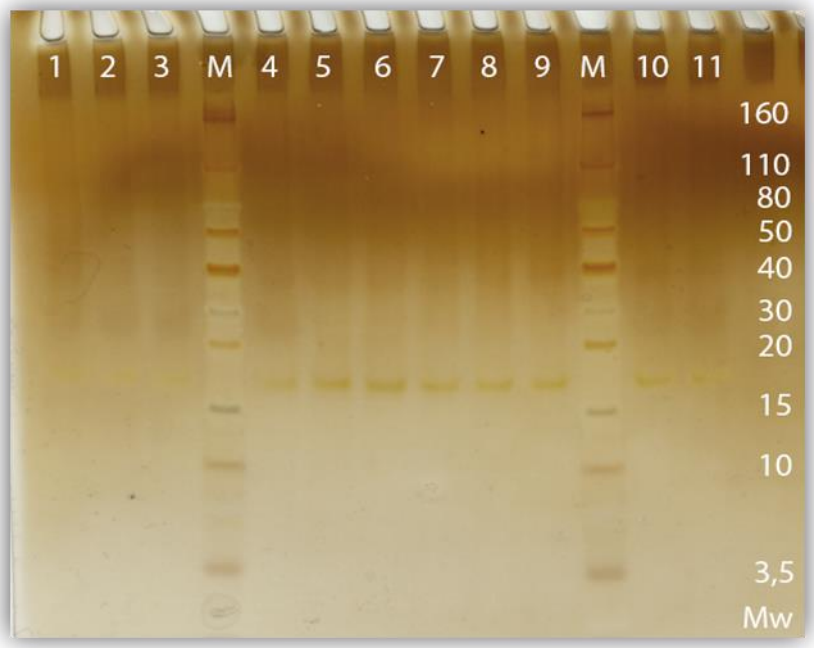


Figure 2 SDS PAGE of the purified fibrinolytic protease. M: Marker; Mw: Molecular weight; Columns 1 to 11: pH values.

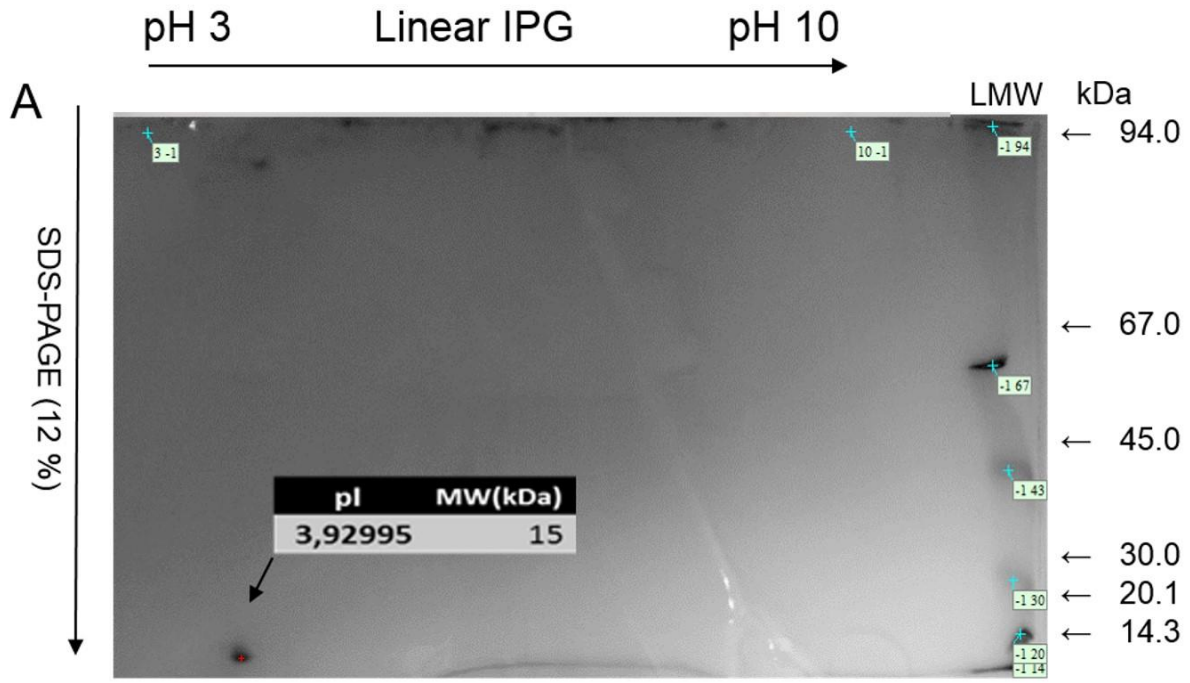


Figure 3 2-DE of the purified fibrinolytic protease. The first dimension was completed on immobilized pH 3-10 linear IPG, obtained using Image Master softwere Platinum (GE Biosciences) LMW: Low weight marker. pI: Isoelectric point. MW (kDa): Molecular weight.

The purified fibrinolytic enzyme from *Mucor subtilissimus* UCP 1262 with 15 kDa is as a similar molar mass as the fibrinolytic enzyme purified from *Paecilomyces tenuipes* (14 kDa). These enzymes have a molar mass lower than other fibrinolytic enzymes from fungi: *Paecilomyces tenuipes* (KIM et al, 2011), *Fusarium* sp. CPCC 480097 (WU et al, 2009), *Armillaria mellea* (LEE et al, 2005), *Aspergillus oryzae* KSK-3 (SHIRASAKA et al, 2012) and *Perenniporia fraxinea* (KIM et al, 2008). Comparative studies of thrombolytic enzymes from fungus with other emblematic thrombolytic agents revealed that these are low molar mass proteases, which may be an evidence for less immunogenicity character. Therefore, administration of these proteases in thrombolytic therapy, should not elicit the immune response against it. The therapeutic perspective of some of these agents is being evaluated in clinical trials (ARBIND and JAGDEEP, 2011).

The isoelectric focusing point (pI) of enzyme was determined by two-dimensional electrophoresis (2-DE) and showed a pI of 3.9 (Figure 3). The isoelectric point for fibrinolytic protease is not close to neutral pH, thus the effect on solubility is not a concern for future formulation.

The hydrolytic activity of the purified enzyme was measured using several chromogenic substrates. The highest level of fibrinolytic activity was observed with N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide suggesting that it is a chymotrypsin-like protease. This type of fibrinolytic enzymes has also been reported from *Armillaria mellea* (LEE et al, 2005), *Perenniporia fraxinea* mycelia (KIM et al, 2008) and *Fomitella fraxinea* (LEE et al, 2006). The purified sample with fibrinolytic activity was subjected to the protease inhibitors. For the serinoprotease inhibitor, PMSF, the fibrinolytic enzyme showed a residual activity of 36.5%. It has also been inhibited by iodoacetic acid (54.5%) but was not inhibited in presence of pepstatin A, β-mercaptoethanol and EDTA with residual activity of 93.9, 98.6 and 132% respectively. These results allowed characterizing fibrinolytic protease as a serinoprotease. Similar results were obtained by Shirasaka et al, (2012), when the

enzyme activity was considerably inhibited by serine protease inhibitors PMSF, but not by chelator agent EDTA.

3.2. Circular dichroism (CD) spectroscopy of the fibrinolytic protease

The CD analysis was used to investigate the structural stability of the fibrinolytic protease through pH and thermal unfolding. The secondary structure of the fibrinolytic protein was shown to contain a significant amount of α-helix (Fig 4.a). The far-UV CD spectrum of the fibrinolytic protease as a function of pH and temperature is shown in Fig. 4 and 5.

3.3. The pH induced denaturation of fibrinolytic protease

Far UV CD spectra was obtained at series of pH values from pH 2.0 to 10.0. The far UV CD spectra, was stable in the pH range of 3-7. At the extreme acidic pH (2.0) reach a random coil structure (Fig 4.b), at the same way at the alkaline pH (8-10). The decrease in α-helix content and increase in β-sheet and unordered structure suggest different modes of unfolding in thermal and unfolding by pH (acid-induced denaturation).

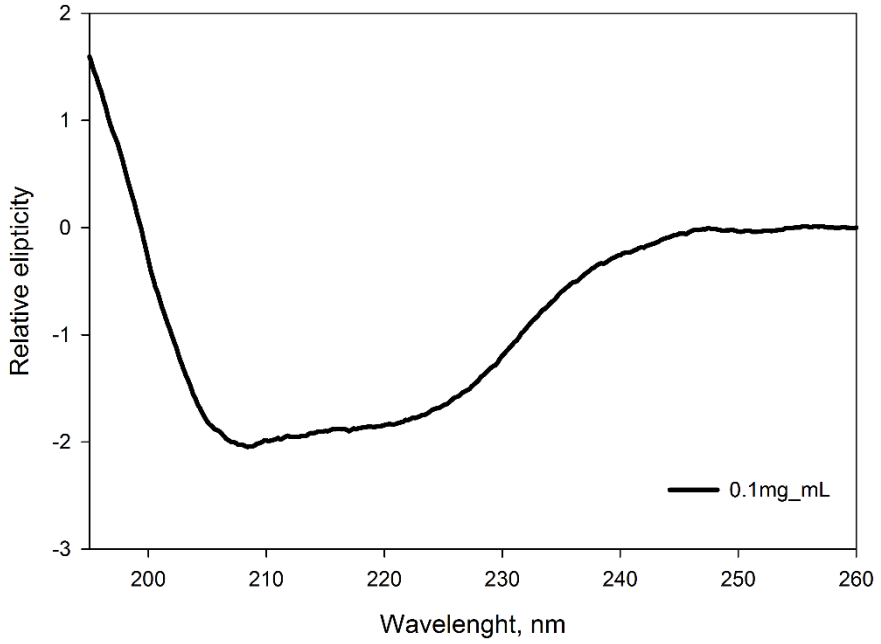
The far UV spectra of fibrolase (PRETZER et al., 1991) displayed essentially no change in band shape or position over the pH range 5 to 9. The spectral changes at pH 2 and 3 were consistent with loss of α-helical structure. SDS-PAGE electrophoresis gel results (Fig. 2), correlated well with the pH effect on the secondary structure. The gel showed no change in the location of the fibrinolytic protease band over the entire pH range 2 to 10. Band intensity corresponded qualitatively to protein concentration results and sample at pH 4 to 9 showed no variation of the fibrinolytic protease band. However, at acidic pH values, no fibrolase band was clearly seen. This destabilization while changing pH could be a result of unfavorable changes in the electrostactic network of the protein

and, probably due to loss of electrostatic interactions necessary in maintaining the structure (HAQ AND KHAN, 2005).

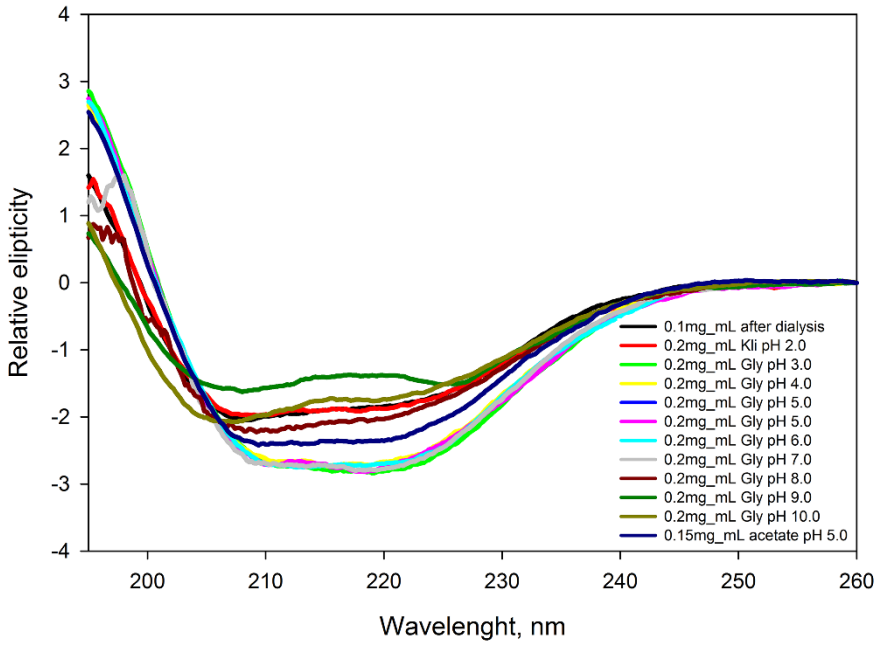
3.4. Thermal Denaturation of fibrinolytic protease

The fibrinolytic protease lost the secondary content at 60°C, indicating unfolding of the protein (Fig 5.a). Monitoring the secondary structure during thermal denaturation by far UV CD spectroscopy showed a partial decrease in the ellipticity when heated to 60°C. The complete loss of the structure was observed at and above 65°C. The thermal denaturation thermodynamic parameters were generated as follows. The initial temperature was 30 °C and the thermal denaturation assay finished at 76.67 °C. The *Tm* (denaturation temperature) detected was 58.14 °C, the denaturation enthalpy (is defined as thermal dynamic state (dH) that consist of the internal energy of the system used to desaturate the molecule. This parameter was 85.14 Kcal/mol. The (dS) Denaturation entropy is defined as a measure of molecular disorder within a macroscopic system. The fibrinolytic enzyme showed a 0.2570 Kcal/mol/K.

The thermal denaturation curve (Fig 5 b) with change in ellipticity at 222 nm, indicated *Tm* of the protein to be 58.14°C (Table 2). The spectra above 65°C visibly show the transformation of the structure into a randon coil. The effect of temperature and pH on the activity and conformation of the thrombolytic protein fibrolase was examined (PRETZER et al., 1991). Although, upon heating above 37°C, fibrolase unfolded irreversibly. The *Tm* was 50°C in pH 8.0 and 43°C in pH 5.0

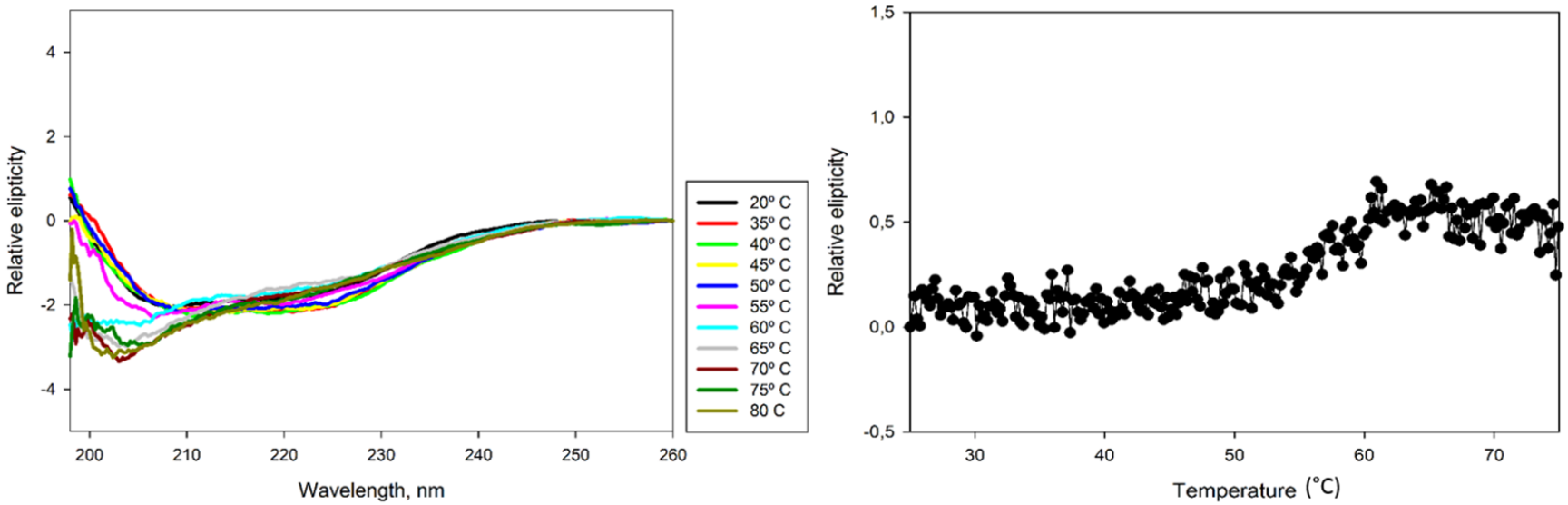


(a)



(b)

Figure 4 (a) Far UV circular dichroism spectra of fibrinolytic protease analyzed in 10 mM acetate buffer pH 5.0 at 25°C. (b) Effect of different pH on secondary structure of fibrinolytic protease monitored by far UV circular dichroism. A spectrum of the purified protein after dialysis is in black.



(b) **(b)**

Figure 5 (a) Thermal denaturation pattern of 0.2 mg/mL fibrinolytic protease in 10mM Tris buffer pH 7.5. (b) Circular dichroism signal at 222nm of the protein sample was monitored in at temperature gradient of 1°C/min from 25 to 80°C.

4. CONCLUSIONS

A chymotrypsin-like serine-protease with fibrinolytic activity from *Mucor subtilissumus* UCP 1262 was purified. The 15kDa molecular weight fibrinolytic protease is one of the lowest among so far reported from fungal enzymes. The structure of the fibrinolytic protease was shown to contain a significant amount of α-helix. Protein unfolding induced by pH and temperature were applied to study the protein conformational changes and showed from the thermal denaturation curve, change in ellipticity at 222 nm, indicated *Tm* of the protein to be 58.14°C. The purified fibrinolytic protease may represent a potential source of new therapeutic agents to treat thrombosis.

5. ACKNOWLEDGEMENTS

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CONSIDERAÇÕES FINAIS

A seleção dos componentes do meio e do micro-organismo é normalmente crucial para a produção fermentativa de enzimas fibrinolíticas. Uma vez que diferentes micro-organismos possuem diferentes características fisiológicas, é necessário selecionar a melhor composição do meio e condições ambientais para o crescimento celular e produção da enzima. O desenvolvimento de um processo de produção e purificação da enzima fibrinolítica foi realizado e organizado em etapas visando a redução de etapas e rapidez no processo de produção e purificação da enzima além de caracterização da protease fibrinolítica purificada.

- Inicialmente no capítulo 1, foi selecionada, dentre espécies fúngicas isoladas de amostra de solo da Caatinga, a espécie fúngica *Mucor subtilissimus* UCP 1262 de acordo com a melhor atividade fibrinolítica. Este micro-organismo foi utilizado para selecionar a melhor condição de cultivo para a produção da enzima utilizando-se um substrado de baixo custo (farelo de trigo) visando-se reduzir os custos do processo inicial de produção da enzima. A enzima foi caracterizada no extrato bruto quanto ao pH e à temperatura, apresentou afinidade ao substrato sintético para a Quimiotripsina e degradação de coágulo sanguíneo *in vitro*.

- Posteriormente, no capítulo 2, o extrato bruto enzimático livre de células foi submetido à extração e pré-purificação da enzima utilizando o Sistema de duas fases aquosas (PEG/ sulfato de amônio), sendo este sal do sistema escolhido por ser extensivamente utilizado nas etapas de pré-purificação de extrato bruto enzimático. Por ser um método biocompatível o SDFA utilizado, foi capaz de extrair a enzima fibrinolítica, mantendo a atividade enzimática e recuperando a enzima para a fase sal do sistema. Este processo de purificação facilitou a separação da enzima a partir do caldo fermentado reduzindo os passos iniciais do processo de purificação.

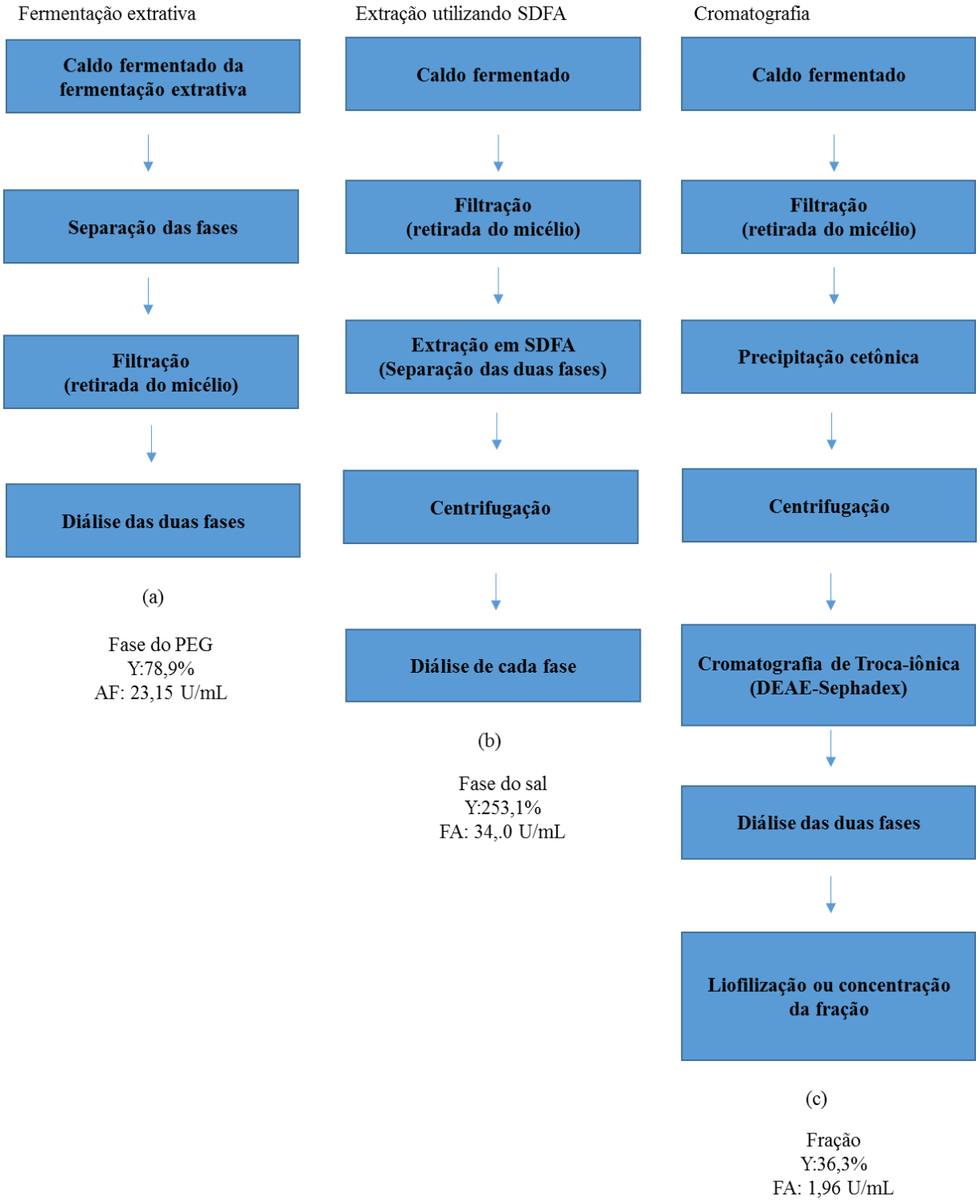
- No capítulo 3, baseado nos resultados do capítulo 2 (extração da protease fibrinolítica em SDFA), foi sugerido um mecanismo para a precipitação/separação utilizando sulfato de amônio como a fase do sal em altas concentrações. A protease fibrinolítica produzida por *Mucor subtilissimus* UCP 1262 foi pré-purificada utilizado fermentação extrativa em SDFA (PEG/sulfato de amônio), no qual a amostra fúngica manteve-se viável para o crescimento mesmo em altas concentrações de sal, onde a enzima foi extraída simultaneamente ao crescimento microbiano para a fase do PEG. Os resultados indicaram que o uso de um substrato de baixo custo (farelo de trigo) e a integração da fermentação com um

sistema de extração pode ser uma alternativa promissora para a produção e extração de proteases fibrinolíticas.

- No capítulo 4, o extrato bruto inicial foi submetido ao método cromatográfico de purificação com o intuito de se caracterizar molecular e estruturalmente a enzima. A enzima apresentou 15 kDa de peso molecular, um dos mais baixos já publicados de enzimas fibrinolíticas produzidas por fungos. E ponto isoelétrico no pH 3,9, que por ser distante do pH neutro não provoca maiores questionamentos em relação a formulações futuras de um agente terapêutico, pois a enzima é solúvel a pH neutro. A estrutura secundária da protease fibrinolítica mostrou conter maior teor em α-hélice e se mostrou estável entre o pH 5 e 9, não demonstrando nenhuma modificação no perfil do UV distante. O experimento de desnaturação térmica apresentou a temperatura de desnaturação da enzima *Tm*: 58,14°C e total mudança conformacional apenas a partir de 65°C. A enzima purificada e caracterizada pode representar uma fonte potencial de novos agentes fibrinolíticos para o tratamento de doenças cardiovasculares.

As etapas de purificação da enzima através de Sistema de duas fases aquosas convencional, fermentação extrativa utilizando SDFA e cromatografia líquida estão esquematizados na Figura 1 desta seção de considerações finais.

Figura 1. Organograma com os passos do processo de produção e comparação entre os métodos de purificação propostos neste trabalho. Comparação da Fermentação extrativa (a); extração em Sistema de duas fases aquosas (b); Cromatografia de troca Iônica (c) da protease fibrinolítica produzida por *Mucor subtilissimus* UCP 1262



ANEXOS

Effects of small changes in polymer hydrophobicity on their interaction with different protein conformations

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Abstract. We have previously shown that increasing the hydrophobicity of PEG by adding a methyl group to every other monomer unit allowed the resulting polymer to alter protein folding and inhibit protein aggregation to amyloid fibrils. Here we examined the effects of this substitution on proteins capable of adopting multiple conformations (folded, partially folded or a molten globule) depending on conditions. We have selected several proteins (α-lactalbumin, apomyoglobin, carbonic anhydrase, staphylococcal nuclease and cytochrome C,) and examined them in different conditions (pH, temperature, salt concentrations, presence of cofactors) where they are present in different conformations. We were especially interested is relative sensitivity of folded and molten globule conformations of these proteins to the presence of polymers. We used far UV CD to test the changes in the protein secondary structure, near UV CD for changes in tertiary structure and quenching of intrinsic protein fluorescence by acrylamide to evaluate changes in solvent accessibility of aromatic residues.

Keywords: macromolecular crowding, molten globule, hydrophobicity, protein folding

1. Introduction

Protein folding is a complex process that proceeds via a stochastic search of the most stable conformation [1]. For some proteins it is a highly cooperative two-state process. In many other cases a natively folded state doesn't exist or is never reached and a protein remains fully or partially unfolded. In addition, a variety of folding intermediates can exist differing by the degree of compactness and preservation of the native secondary and tertiary structure. For example, a typical molten globule is characterized by native-like secondary structure and lack of close side chain packing [2-4]. Other folding intermediates may have a partially disrupted secondary structure (e.g. a pre-molten globule) [5, 6] or a more compact tertiary structure (e.g. a dry molten globule) [3]. These intermediate states can be kinetically or even thermodynamically stable in mildly denaturing conditions such as low pH or elevated temperature. Molten globules and other partially unfolded states play an important role in biology as they are involved in protein-chaperone interactions [7-9], protein misfolding and aggregation [10-12] and other biological processes.

Protein structure is highly dependent on the environment. One of the important factors influencing it *in vivo* is presence of other macromolecules creating a crowded environment [13]. Macromolecular crowding is primarily driven by excluded volume effect that decreases the effective volume available for the proteins in the cell and thus increases the effective protein concentration and promotes more compact protein conformations. In addition, there is evidence for weak, non-specific interactions between proteins and other biopolymers [14-18]. Molten globules are especially sensitive to macromolecular crowding and other protein-polymer interactions due to their metastability. Depending on the protein and conditions, crowding can either stabilize a molten globule state or facilitate its conversion to a folded state [19, 20]. We have previously examined the effect of increasing non-specific protein-polymer interactions on protein structure by examining interactions of proteins with two polymers with similar structures and slightly different hydrophobicities. We used PEG (MW 4400) and its derivative UCON (MW 5400). UCON is a 1:1 copolymer of ethylene glycol and 2-propylene glycol. The structures of these polymers are very similar except for the presence of an additional methyl group on every other monomer unit of UCON making it more hydrophobic. We found that intrinsically disordered proteins and proteins capable of forming molten globules were especially sensitive to the presence of these polymers. Here we

expanded this study to the molten globules in order to examine the effects of macromolecular crowding and other protein-polymer interactions on these conformational states of proteins.

2. Materials and methods

2.1. Materials

Staphyloccocal nuclease and apomyoglobin were the gifts from Dr. Munishkina (University of California Santa Cruz). UCON 50-HB-1500 (MW 5.5 kDa) was from Dow Corning, PEG (MW 4 kDa) and dextran (MW 6-8 kDa) were from Alfa Aesar. All other proteins and chemicals were from Sigma, Fisher Scientific or VWR Scientific.

2.2. Methods

2.2.1. Circular dichroism

Far-UV CD (195–260 nm) spectra of proteins were measured using a JASCO J-815 spectropolarimeter at 25°C. A solution of protein (110 µl, 1 mg/mL) was placed into a 0.2 mm pathlength cell, and the CD spectra were acquired with 20 nm/min scan speed at 0.2 nm step size and 1.0 nm bandwidth under constant purging with nitrogen. Three spectra were accumulated and averaged for each sample. Near UV CD (260-320 nm) spectra were acquired using the same parameters in a 10 mm pathlength cell with 2.5 mL of a 0.4 mg/mL protein solution. The same buffer was used for CD, fluorescence and UV-vis measurements.

2.2.2. Fluorescence quenching

Protein solution (13.3 µl, 3 mg/mL, 0.05 mg/mL final concentration) was mixed with buffer (final volume 400 µl) containing different concentrations of acrylamide (0 – 0.5 M). The solution was incubated for 1 h at 25°C, and then intrinsic protein fluorescence was measured. Excitation wavelength was 280 nm and emission spectrum was recorded in 295-350 nm range. Excitation and emission slits were at either 2.5 or 5 nm. Emission spectra were recorded in triplicate and averaged. The measurements were performed in duplicate for each sample. The data resulting from the quenching of the intrinsic fluorescence were analyzed using the Stern-Volmer equation (Eq.1),

$$\frac{F_0}{F} = 1 + K_{sv}[\mathrm{Q}] \qquad \text{Eq. 1}$$

where F_0 is the fluorescence emission in the absence of acrylamide, F is the emission at a specific concentration of acrylamide, K_{sv} is the dynamic quenching constant (Stern-Vollmer constant), and Q is the concentration of acrylamide.

2.2.3. UV-visible spectroscopy

UV and visible spectra of proteins were measured using a JASCO VP-630 spectrophotometer at 25°C. Protein solution (800 μl, 0.3 mg/mL) was placed into a 10 mm pathlength cell, and the UV-vis spectra were acquired in 240-490 nm range with 50 nm/min scan speed at 0.2 nm step size. Three spectra were accumulated and averaged for each sample. Appropriate buffer was used as a blank.

4. Results and discussion

For our studies we selected five folded proteins known to form molten globule folding intermediates: α-lactalbumin, cytochrome C, apomyoglobin, carbonic anhydrase and staphylococcal nuclease (SNAse). We employed far UV CD spectroscopy to examine the effects of polymers on protein secondary structure, near UV CD spectroscopy to examine their effect on tertiary structure, quenching of intrinsic protein fluorescence by acrylamide in the presence of polymers to assess the accessibility of aromatic residues and hydrophobicity of their environments and absorbance in the visible region to test the effect of polymers on the heme environment of hemoglobin. We primarily used two polymers: PEG (MW 4.5 kDa) and UCON (MW 5.5 kDa), a PEG derivative containing methyl groups in every other monomer unit. In some cases we also used dextran (MW 6-8 kDa) as an example of a compact, highly hydrophilic polymer often used to create the excluded volume effect.

3.1. α-Lactalbumin.

α-Lactalbumin, a regulatory subunit of lactose synthase, is a primarily α-helical calcium-binding protein containing four disulfide bonds (MW 14 kDa) [21, 22]. It has been previously shown to convert to partially unfolded states either at low pH or upon removal of calcium at pH 7.5 and 37°C [23-27]. We used the holo form of this protein both in the presence of calcium (pH 8.5, 1 mM $CaCl_2$) and in the absence of either calcium or EGTA (pH 7.5) as a control. We have examined several conditions where protein structure is partially unfolded: 1) pH 7.5, 25°C, 1 mM EGTA; 2) pH 7.5, 37°C, 1 mM EGTA; 3) pH 2.0, 25°C; 4) pH 2.0,

37°C. Condition 1 represents a folded state of apo α-lactalbumin, condition 2 has been described as a temperature-induced molten globule state [23, 26] while conditions 3 and 4 represent well-characterized acid-induced molten globule states of this protein differing by the extent of the remaining secondary structure [21, 26]. Far UV CD spectra of α-lactalbumin at pH 7.5 both in the absence and in the presence of EGTA were similar to that of the holo enzyme indicating the protein secondary structure is preserved upon removal of calcium and even conversion to a molten globule state at 37°C. However, near UV CD spectra showed some loss of intensity for the apo enzyme and almost completely disappear for the apo enzyme at 37°C confirming its conversion to a molten globule. At low pH far UV CD spectra corresponding to a significantly more disordered structure are observed and near UV CD spectra are virtually absent indicating that acid-induced molten globules of α-lactalbumin (conditions 3, 4) are a lot more disordered than the temperature-induced one at neutral pH (condition 2).

Far UV CD data showed that addition of polymers had no effect on the secondary structure of holo α-lactalbumin at pH 8.5 in the presence of calcium. For the holo protein at pH 7.5 no changes were observed in the presence of PEG while in the presence of UCON an increase in the intensity of the peak at 205 nm consistent with more disordered structure was observed. Polymers didn't affect the far UV CD spectra of the apo protein at 25°C but some increase in intensity of the peak at 205 nm was observed at 37°C in the presence of UCON. As for the acid-induced unfolded states of the protein, addition of polymers had no effect on the protein secondary structure at pH 2, 25°C while at pH 2, 37°C a small red shift in the CD spectrum was observed in the presence of PEG and a small blue shift – in the presence of UCON. These results indicate partial loss of secondary structure in the presence of UCON for α-lactalbumin in some conditions (pH 7.5; pH 7.5, 1 mM EGTA, 37°C; pH 2.0, 37°C) and a possible partial gain of secondary structure in the presence of PEG at pH 2.0, 37°C.

Near UV CD spectrum of holo α-lactalbumin in the presence of $CaCl_2$ was not affected by the addition of PEG and slightly increased in intensity in the presence of UCON. Both PEG and UCON significantly decreased the intensity of the near UV CD spectrum of holo α-lactalbumin in the physiological conditions while only UCON had this effect on the spectrum of apo-α-lactalbumin at 25°C. Near UV CD spectra of the molten globule states of the protein at pH 2 were extremaly weak and appeared not to be affected by the presence of polymers.

Fluorescence quenching data showed that acrylamide accessibility of aromatic residues of holo-α-lactalbumin both in the presence and in the absence of calcium was increased by the

addition of PEG and increased even farther in the presence of UCON. For the apo enzyme at 25°C accessibility of aromatic residues decreased in the presence of PEG and further decreased in the presence of UCON indicating increased protein folding in the presence of polymers. In the molten globule states (both at pH 7.5, 37°C and at pH 2) effect of polymers on the aromatic residue accessibility was quite small.

Overall, effect of the polymers on the protein structure strongly depended on variable being observed. Secondary structure was not significantly affected by polymers although some loss of it was observed in the presence of UCON for holo protein at pH 7.5 and for molten globule states at 37°C. Significant loss of tertiary structure in the presence of UCON was observed for both holo and apo protein at pH 7.5, 25°C. For holo protein this loss was also observed in the presence of PEG. Significantly increased accessibility of aromatic residues was observed in the presence of UCON for holo protein and for the acid-induced molten globule at 37°C while for apo protein at pH 7.5, 25°C accessibility of the aromatic residues was decreased in the presence of polymers. When the effect of the polymers was observed, UCON consistently contributed more to the loss of protein structure compared to PEG, similar to earlier observations.

3.2. Apomyoglobin.

Myoglobin is a small, primarily α-helical, heme-containing protein involved in oxygen transfer in the muscle [28, 29]. Apo form of this protein (lacking a heme) is known to form molten globule-like structures at acidic pH [30-32]. NMR studies showed that at neutral pH apomyoglobin is folded and has a similar conformation to a holo protein [30]. Absence of the heme results in highly plastic structures of both the native state of apomyoglobin and its molten globule states at low pH [33, 34]. We examined apomyoglobin at neutral pH (pH 7.5) and at pH 2.0 in the presence of different salt concentrations (no salt; 0.75 M NaCl; 1.1 M NaCl) as it has been previously shown that addition of salt to apomyoglobin at low pH induces its partial folding [35]. Optimal salt concentrations for these experiments were determined by examining changes in the far UV CD spectra of apomyoglobin at pH 2.0 in the presence of increasing concentrations of NaCl (0 – 1.5 M). We found that a spectrum of apomyoglobin at pH 2.0 in the absence of salt corresponds to a partially unfolded α-helical protein (minima at 204 and 222 nm). The spectrum changes with the addition of NaCl with the minimum at 206 nm shifting to 210 nm and decreasing in intensity. This change is likely due to further protein folding. The transition occurs at 0.4 – 1.1 M NaCl and thus we picked

two salt concentrations (0.75 M and 1.1 M) corresponding, respectively, to the midpoint and the endpoint of this transition.

Examination of far UV CD spectra of apomyoglobin in the presence of polymers showed no effect of polymers at pH 7.5. At pH 2 in the absence of salt polymers had a significant effect on the structure of apomyoglobin with the peak in far UV CD shifting from 204 to 206 nm and an intensity of the shoulder at 225 nm increasing significantly in the presence of both polymers although PEG appeared more effective. At higher salt concentrations at pH 2 the effect of polymers was much smaller and the spectra didn't change significantly. Protein precipitation was observed at pH 2.0, 1.1 M NaCl in the presence of 10% PEG. Near UV CD spectra of apomyoglobin were essentially non-existent in all four conditions and thus no effect of polymers on them could be observed (data not shown).

Fluorescence quenching experiments showed that at most conditions additions of PEG lead to decreased accessibility of aromatic residues while addition of UCON lead to significantly increased accessibility of these residues. The effects of polymers were especially large for at pH 2 and 0.75 M NaCl where addition of both PEG and UCON increased Stern-Vollmer constants with UCON increasing it 4-fold. High sensitivity of this state to polymers could be due to its nature as an intermediate in the transition from a highly unfolded state to a molten globule.

Overall, we observed that secondary structure of a primarily unfolded state of apomyoglobin (pH 2, no salt) was especially sensitive to the addition of polymers (especially PEG). On the other hand, solvent accessibility of aromatic residues of the intermediate state (pH 2, 0.75 M NaCl) was altered the most by the presence of the same polymers (especially UCON).

3.2. Carbonic anhydrase.

Carbonic anhydrase (CA) is a 30 kDa enzyme with a β-sheet rich structure [36]. It has been extensively used as a model for a variety of biophysical studies [37] because it is a stable, monomeric, well characterized and widely available protein. CA is known to convert to molten globule states in a variety of conditions including low pH [38-42]. Here we have examined the effects of polymers on bovine CA at pH 7.5 and at pH values 3.2-4.0 where it forms a variety of partially disordered states [38].

3.2. Cytochrome C.

Cytochrome C is a folded, primarily α-helical protein with the heme cofactor. We have examined this protein in three conditions: A- pH 7.5; B - pH 2.0 and C - pH 2.0, 0.5 M NaCl. We found that addition of polymers didn't have a significant effect on far UV CD spectra of this protein at pH 7.5. However, at pH 2 addition of UCON resulted to a significant spectral shift (a peak at 208 nm increased in intensity and shifted to 204 nm while the peak at 222 nm significantly decreased in intensity) consistent with protein unfolding. Addition of PEG in the same conditions didn't lead to observable spectral changes although protein precipitation was observed at the highest PEG concentration (15%).

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Figures

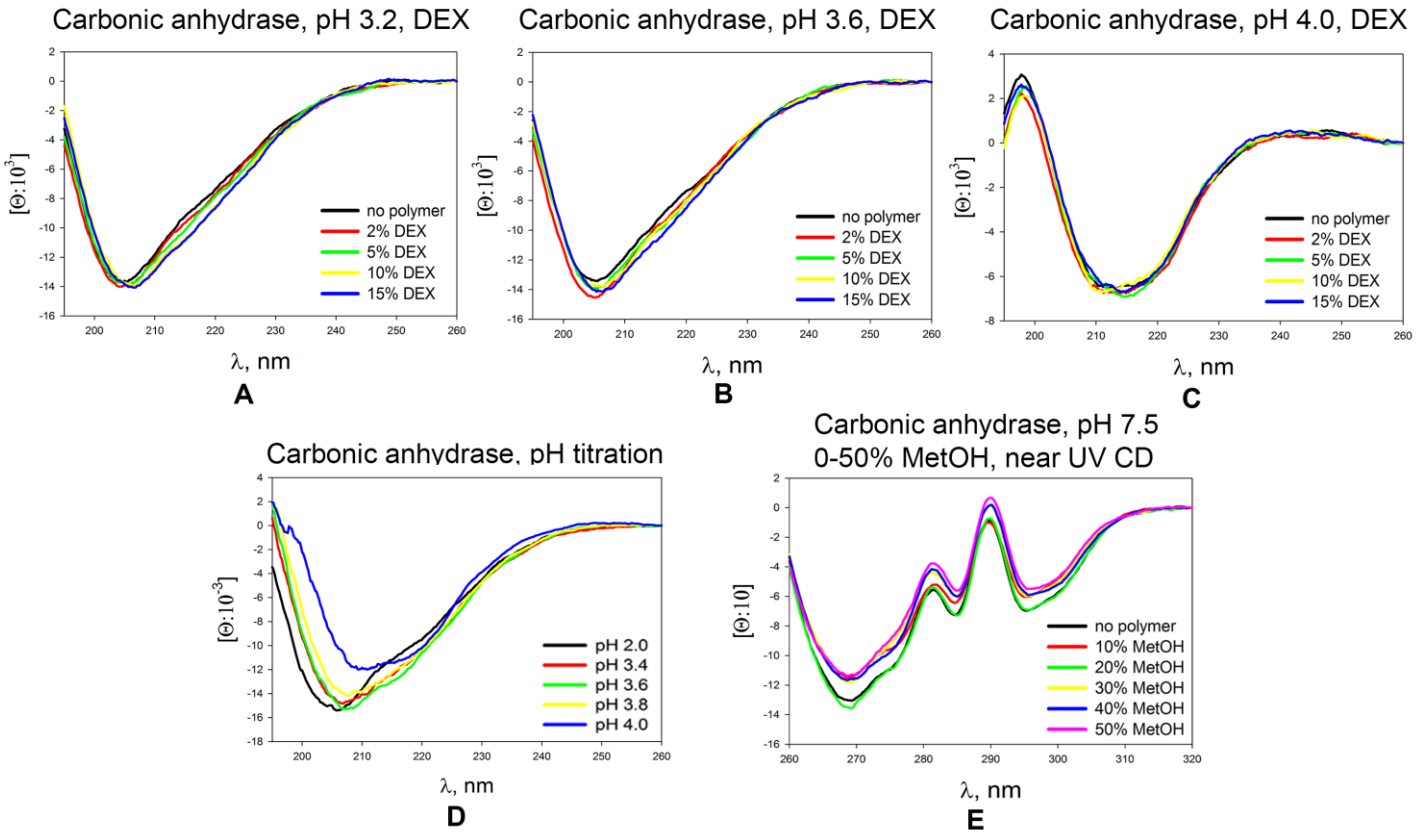

Fig 1 Effect of Dextran and pH on the secondary structure of CA in the crowded environment monitored by far UV circular dichroism. A spectrum of the protein with no additives is in black, with 2% DEX – red, with 5% DEX – light green, with 10% DEX- light yellow and 15% DEX – blue. Spectra are normalized at 217 nm.

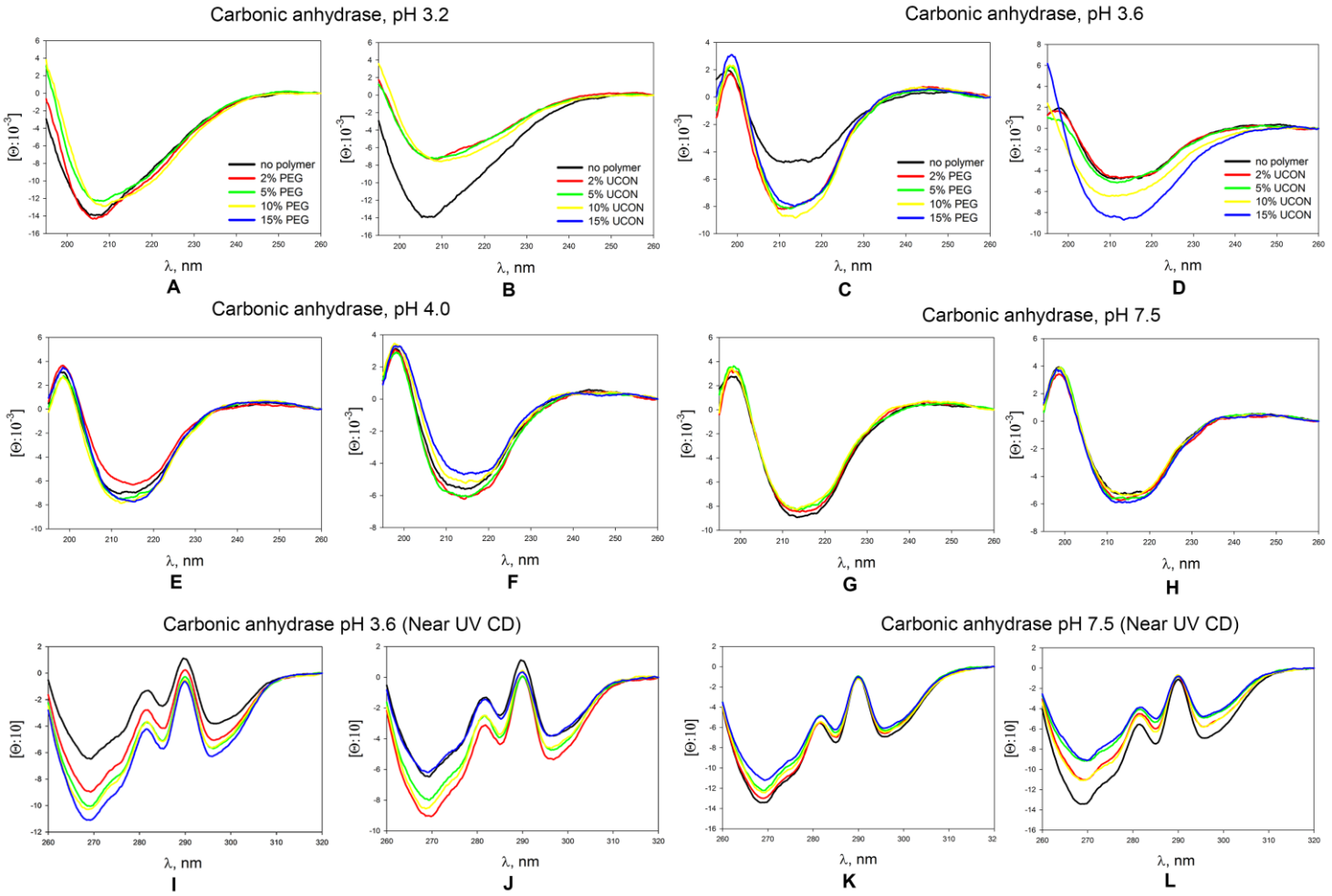

Fig 2 Effect of PEG and UCON on the secondary structure of CA in the crowded environment monitored by far UV circular dichroism. A spectrum of the protein with no additives is in black, with 2% PEG and UCON – red, with 5% PEG and UCON – light green, with 10% PEG and UCON - light yellow and 15% PEG and UCON – blue. Spectra are normalized at 217 nm.

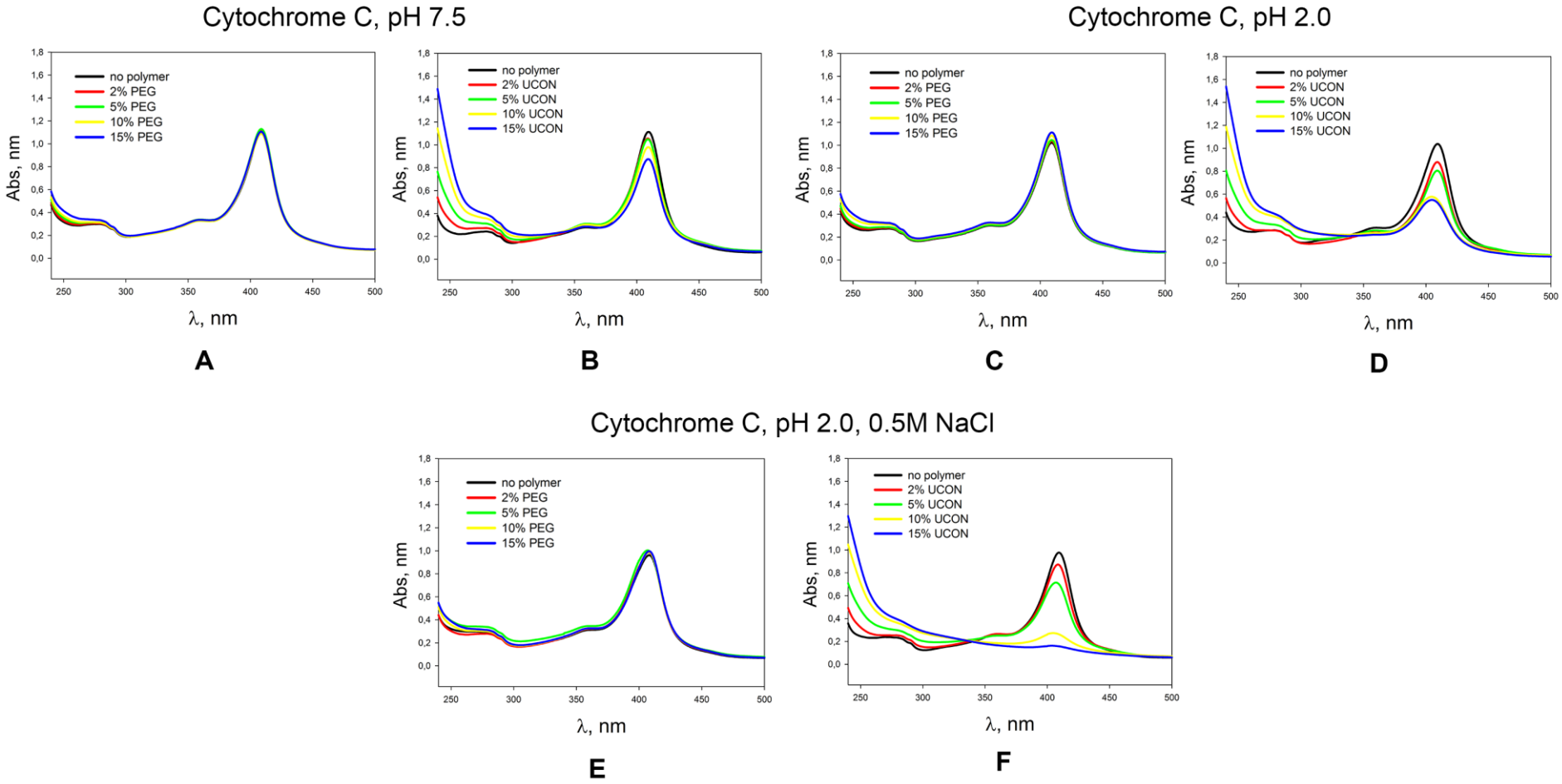

Fig 3 Effect of PEG and UCON on the secondary structure of Cyt C in the crowded environment monitored by far UV circular dichroism. A spectrum of the protein with no additives is in black, with 2% PEG and UCON – red, with 5% PEG and UCON – light green, with 10% PEG and UCON - light yellow and 15% PEG and UCON – blue. Spectra are normalized at 217 nm.

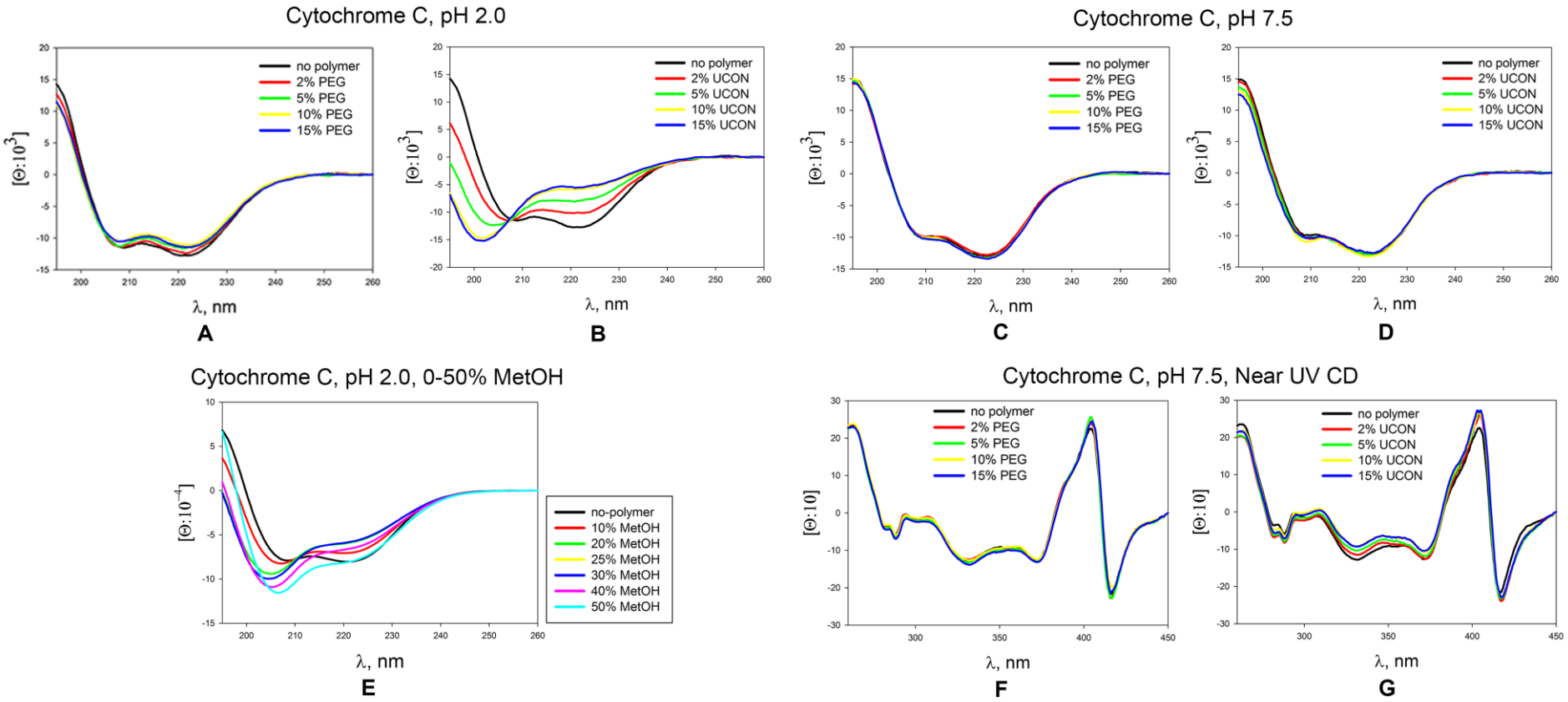

Fig 4 Effect of PEG and UCON on the secondary structure of SNase in the crowded environment monitored by far UV circular dichroism. A spectrum of the protein with no additives is in black, with 2% PEG and UCON – red, with 5% PEG and UCON – light green, with 10% PEG and UCON - light yellow and 15% PEG and UCON – blue. Spectra are normalized at 217 nm.

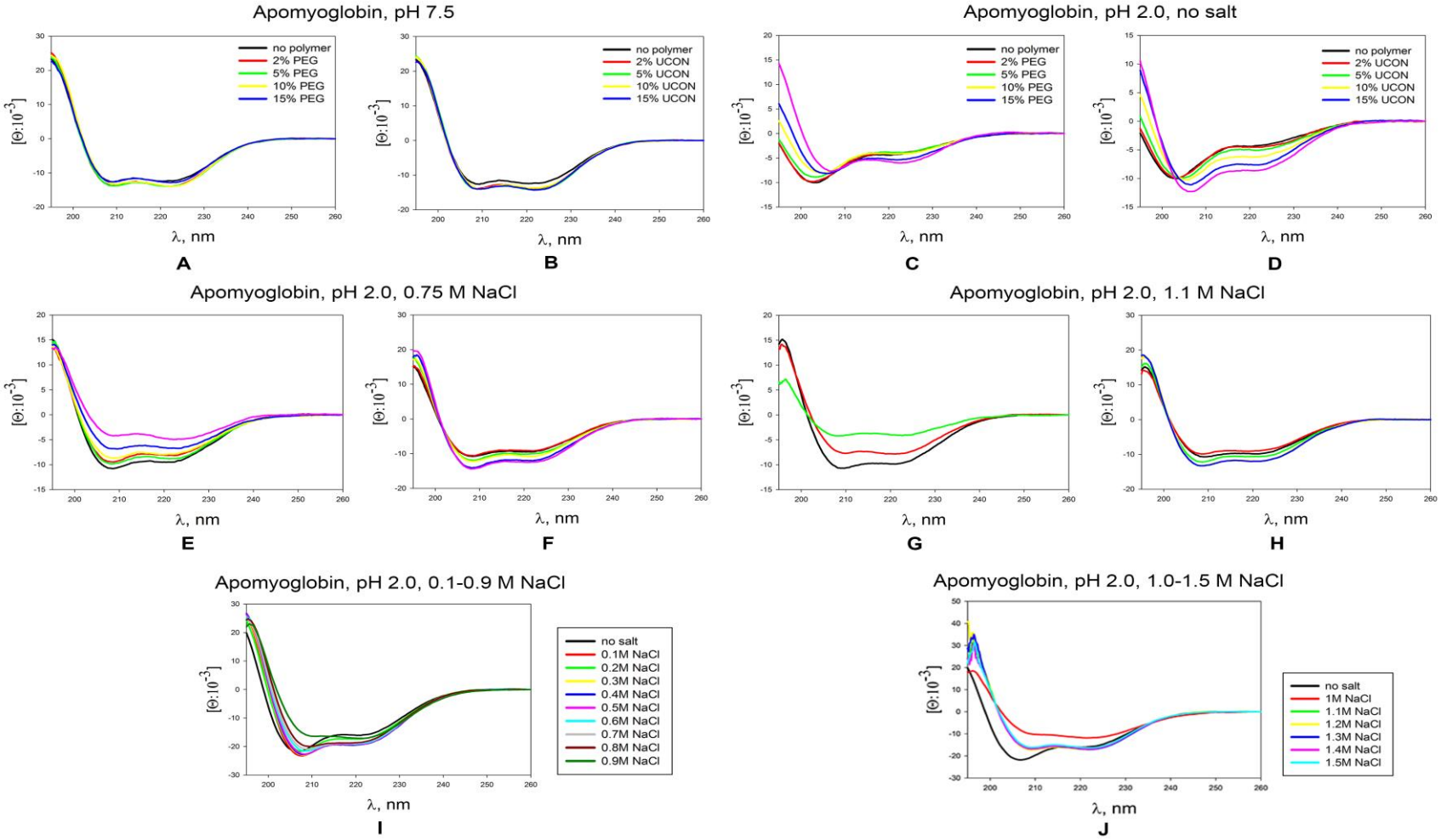

Fig 5 Effect of PEG and UCON on the secondary structure of ApoM in the crowded environment monitored by far UV circular dichroism. A spectrum of the protein with no additives is in black, with 2% PEG and UCON – red, with 5% PEG and UCON – light green, with 10% PEG and UCON - light yellow and 15% PEG and UCON – blue. Spectra are normalized at 217 nm.

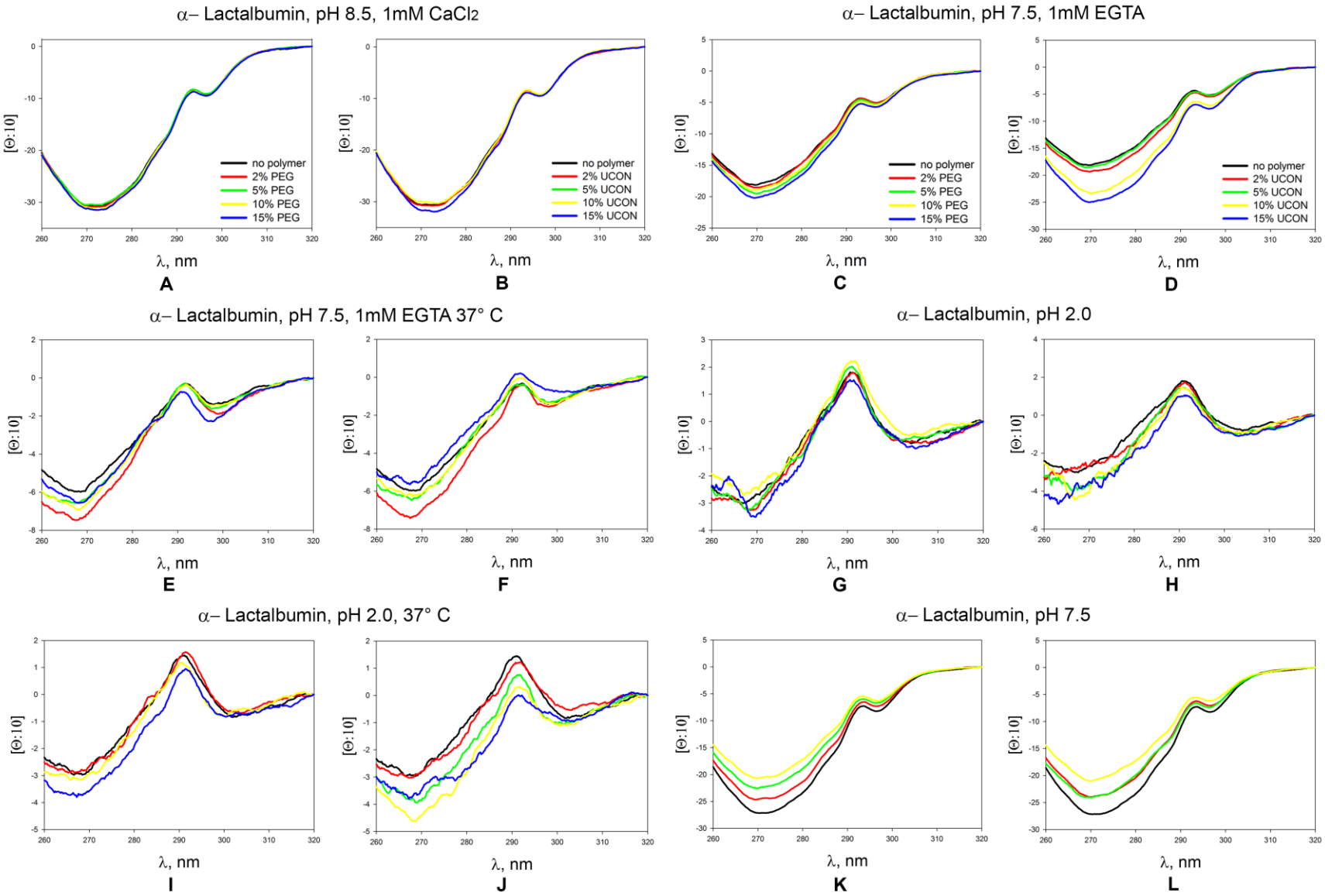
α– Lactalbumin, pH 8.5, 1mM CaCl2
α– Lactalbumin, pH 7.5, 1mM EGTA
α– Lactalbumin, pH 7.5, 1mM EGTA 37° C
α– Lactalbumin, pH 2.0
α– Lactalbumin, pH 2.0, 37° C
α– Lactalbumin, pH 7.5
[Θ:10]
λ, nm
no polymer
2% PEG
5% PEG
10% PEG
15% PEG
2% UCON
5% UCON
10% UCON
15% UCON
A
B
C
D
E
F
G
H
I
J
K
L

Fig 6 Effect of PEG and UCON on the secondary structure of a-LA in the crowded environment monitored by Near UV circular dichroism. A spectrum of the protein with no additives is in black, with 2% PEG and UCON – red, with 5% PEG and UCON – light green, with 10% PEG and UCON - light yellow and 15% PEG and UCON – blue. Spectra are normalized at 217 nm.

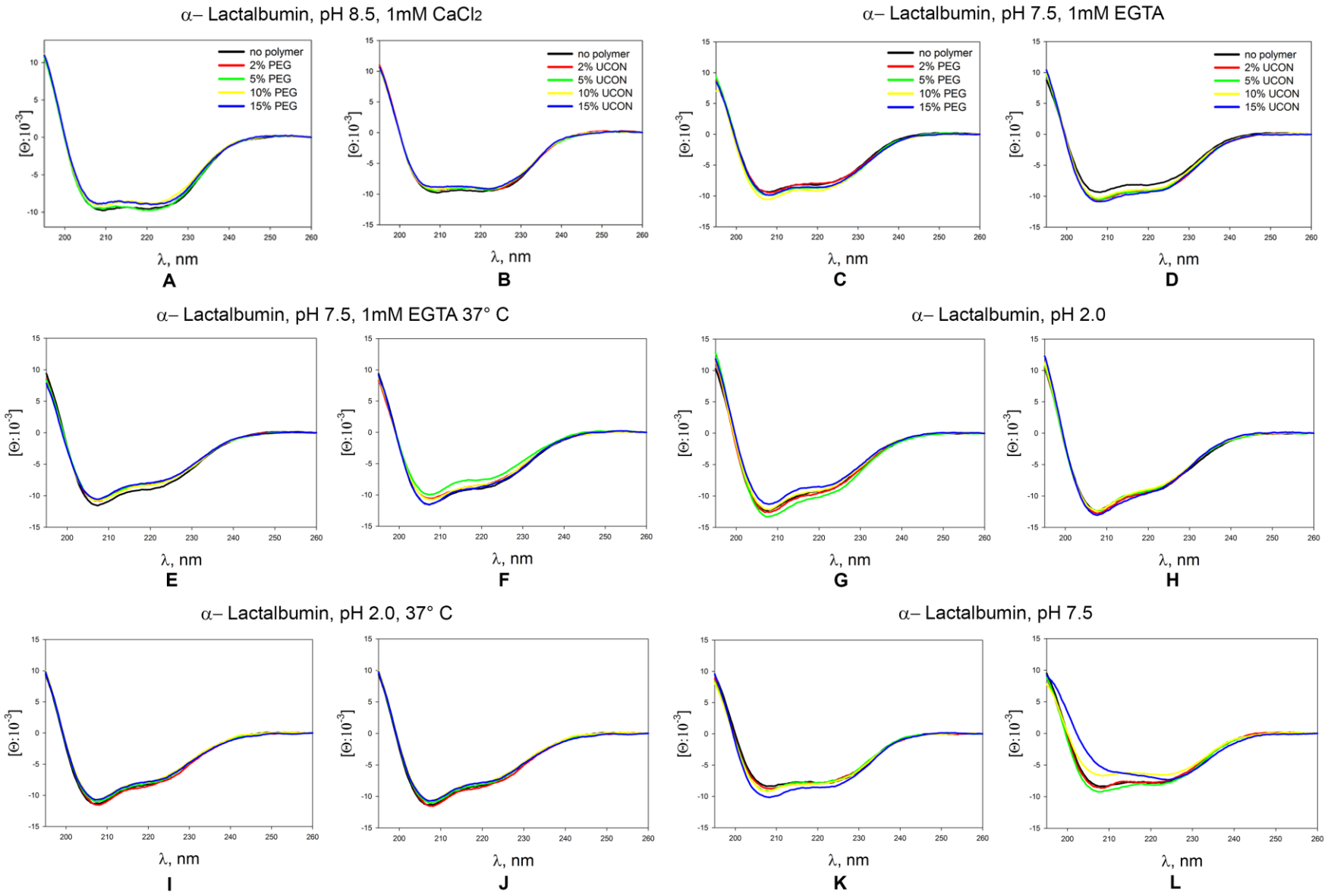
α– Lactalbumin, pH 8.5, 1mM CaCl2
α– Lactalbumin, pH 7.5, 1mM EGTA
α– Lactalbumin, pH 7.5, 1mM EGTA 37° C
α– Lactalbumin, pH 2.0
α– Lactalbumin, pH 2.0, 37° C
α– Lactalbumin, pH 7.5
no polymer
2% PEG
5% PEG
10% PEG
15% PEG
2% UCON
5% UCON
10% UCON
15% UCON
[Θ:10^-3]
λ, nm
A
B
C
D
E
F
G
H
I
J
K
L

Fig 7 Effect of PEG and UCON on the secondary structure of a-LA in the crowded environment monitored by far UV circular dichroism. A spectrum of the protein with no additives is in black, with 2% PEG and UCON – red, with 5% PEG and UCON – light green, with 10% PEG and UCON - light yellow and 15% PEG and UCON – blue. Spectra are normalized at 217 nm.

Table 1. Stern-Vollmer constants (K_{SV}, M^{-1}) for acrylamide quenching of intrinsic protein fluorescence in the presence and absence of polymers.

Protein	Conditions	K_{SV}, no polymers	K_{SV}, 10% PEG	K_{SV}, 10% UCON
α-Lactalbumin	pH 8.5, 1 mM $CaCl_2$	8.6±0.1	9.3±0.9	15.1±0.3
	pH 7.5	7.4±0.9	7.1±1.3	8.6±1.5
	pH 7.5, 25°C, 1 mM EGTA	11.9±0.7	11.0±1.3	9.7±0.6
	pH 7.5, 37°C, 1 mM EGTA	19.7±0.5	17.7±0.7	19.4±0.2
	pH 2.0, 25°C	13.7±1.4	13.3±1.2	17.7±0.1
	pH 2.0, 37°C	14.2±1.5	14.0±0.1	18.3±0.7
Apomyoglobin	pH 2.0	22.1±2.3	19.3±0.4	32.7±0.2
	pH 2.0, 0.75 M NaCl	9.4±1.0	14.4±1.5	20.2±0.7
	pH 2.0, 1.1. M NaCl	16.0±2.0	9.7±0.6	17.4±2.1
	pH 7.5	11.3±0.6	8.5±0.3	15.0±0.3
Carbonic anhydrase	pH 3.2	10.5±0.8	9.7±0.9	11.2±0.7
	pH 3.6	9.3±1.7	9.4±0.8	9.8±1.7
	pH 4.0	11.2±1.5	9.5±0.7	11.8±0.5
	pH 7.5	14.1±0.5	9.5±0.5	10.4±0.4
Staphyloccocal nuclease	pH 2.5	12.6±0.7	13.1±1.9	20.7±1.0
	pH 3.0	13.2±3.0	10.8±1.3	20.6±0.5
	pH 7.5	20.0±1.2	10.5±1.1	14.7±2.7
	pH 2.0	4.1±1.0	7.2±0.5	24.9±0.1

Cytochrome C	pH 2.0, 0.5 M NaCl	4.8±1.3	8.4±0.1	26.0±3.0
	pH 7.5	3.0±0.1	10.4±3.1	35.4±1.1