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DAYANNE PINHO ROSA

DETERMINAÇÃO DE PARÂMETROS ESTRUTURAIS E TERMODINÂMICOS DA ISOFORMA α-TRIPSINA BOVINA EM SOLVENTES AQUO-ORGÂNICOS

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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica e Farmacologia da Universidade Federal do Espírito Santo como requisito para obtenção do título de Mestre em Bioquímica e Farmacologia.
Área de Concentração: Bioquímica.
Orientador: Prof. Dr. Alexandre Martins Costa Santos
Co-orientador: Prof. Dra. Juliana Barbosa Coitinho Gonçalves

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RESUMO

Os solventes orgânicos são comuns em processos industriais que utilizam enzimas, mas, ao mesmo tempo, sabe-se que alteram suas propriedades. Assim, os efeitos do solvente orgânico aquoso (etanol) em diferentes concentrações na estrutura α-tripsina foram investigadas por técnicas espectroscópicas e análise de dados termodinâmicos. A espectroscopia de absorção UV-Vis, a fluorescência intrínseca do triptofano e o espalhamento dinâmico da luz (DLS) sugerem a formação de estados parcialmente dobrados, em vez de estados agregados, em alta concentração de etanol (> 60% v/v de etanol/tampão), exibindo pouca perda de estrutura secundária, mas alterações significativas na estrutura terciária. Os dados termodinâmicos (T_m e ΔH) sugerem um afrouxamento de interações intramoleculares fracas, o que se reflete em um aumento de flexibilidade de tal forma que a capacidade catalítica pode ser aumentada ou diminuída de acordo com a concentração de etanol no sistema. Os resultados globais sugerem que na faixa de 0-60% v/v de etanol/tampão, a α-tripsina sofre fenômenos de multimerização reversíveis, mantendo a sua atividade catalítica. No entanto a partir de 60% v/v de etanol/tampão, a população de estados parcialmente enovelados com menor atividade catalítica é predominante.

Palavras chaves: tripsina, isoformas, enzimologia, solventes orgânicos, estrutura, termodinâmica.

ABSTRACT

Organic solvents are common in industrial processes that use enzymes but, at the same time, it is known that they change the properties thereof, thus the effects of aqueous-organic solvent (ethanol) in different concentration on the α-trypsin structure have been investigated by spectroscopic techniques and thermodynamic data analysis. The results from spectroscopic measurements, including far-UV Circular Dichroism, UV-Vis absorption spectroscopy, intrinsic tryptophan fluorescence and dynamic light scattering (DLS) suggest the formation of partially folded states, instead of aggregates states, at high ethanol concentration (> 60% v/v ethanol), with little loss of secondary structure, but with significant tertiary structure changes. The thermodynamic data (T_m and ΔH) suggest a loosen of intramolecular weak interactions, which reflects in a flexibility increase such that the catalytic capacity can be increased or decreased according to the ethanol concentration into the system. Overall results we suggest that in range of 0-60% v/v ethanol/buffer, α-trypsin undergoes reversible multimerization phenomena maintaining its catalytic activity. However from 60% v/v ethanol/buffer, population of folded partially states with less catalytic activity are predominant.

Key words: trypsin; isoforms; enzymology; organic solvent; structure; thermodynamic.

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PARTE I

1 REVISÃO LITERÁRIA

1.1 Enzimas em meios aquo-orgânicos

Embora a água seja considerada como o solvente da vida, é um solvente bastante fraco para a maioria das reações sintéticas. Os solventes orgânicos são normalmente necessários para aumentar a solubilidade dos substratos hidrofóbicos, deslocar o equilíbrio termodinâmico para favorecer a síntese por hidrólise e suprimir reações secundárias aquo-dependentes. No entanto, a maioria das enzimas naturais exibe uma menor eficiência catalítica em solventes orgânicos em comparação com soluções aquosas nativas (STEPANKOVA et al., 2013). A presença de água é essencial para o funcionamento de várias biomoléculas (LEVY; ONUCHIC, 2006). Ao interagir com as moléculas presentes no meio, a água forma uma camada de hidratação, que influencia as propriedades estruturais e dinâmicas das biomoléculas (BALL, 2008). Porém, o mínimo nível de hidratação necessário para o correto funcionamento de uma proteína ainda está em definição e provavelmente leva em conta quais propriedades desta devem ser consideradas, como por exemplo as diferenças estruturais e termodinâmicas de cada proteína (FOGARTY; POTESTIO; KREMER, 2015).

As enzimas possuem várias qualidades desejáveis para uma ampla gama de aplicações, desde a síntese de intermediários farmacêuticos até a produção em larga escala de biocombustíveis a partir de fontes renováveis (ILLANES et al., 2012). Isto é principalmente devido à sua capacidade de converter um amplo espectro de substratos; sua alta estabilidade em temperaturas extremas; sua alta quimio-, regio- e enantiosseletividade. No entanto, as vantagens dos biocatalisadores, como sua biodegradabilidade, alta especificidade e atividade em condições suaves, refletem o fato de que as enzimas evoluíram para trabalhar em ambientes celulares e, portanto, são geralmente intolerantes às duras condições do processo industrial (IYER; ANANTHANARAYAN, 2008; SCHMID et al., 2001).

Existem duas soluções possíveis para lidar com a baixa tolerância das enzimas em relação aos solventes orgânicos: (I) otimização das condições do processo para o

biocatalisador disponível e (II) preparação de biocatalisadores que podem funcionar sob condições ideais do processo (LUETZ; GIVER; LALONDE, 2008). Enquanto no passado as condições do processo costumavam ser projetadas em torno das limitações da enzima, hoje as enzimas são geralmente projetadas para atender às rigorosas especificações do processo (CARREA; RIVA, 2000). Durante as últimas três décadas, inúmeros estudos têm lançado luz sobre os efeitos dos solventes orgânicos na estrutura e função enzimática. O aumento da compreensão das mudanças moleculares na estrutura enzimática e no mecanismo catalítico em meios de reação não-convencionais levou ao desenvolvimento de muitos métodos complementares, desde a adição de simples agentes estabilizadores até abordagens de engenharia de proteínas altamente sofisticadas, para aumentar a estabilidade enzimática em relação a solventes orgânicos. Curiosamente, tem sido observado que os biocatalisadores podem ser manipulados a um nível tal que funcionam mesmo em solventes orgânicos puros (STEPANKOVA et al., 2013).

Muitos trabalhos têm sido realizados para se avaliar a atividade enzimática em meio aquo-orgânico. Em um deles, Zaks e Klibanov (1988a) utilizaram as enzimas Polifenoloxidase, Álcool Desidrogenase e Álcool Oxidase em solventes com hidrofobicidades e concentrações de água diferentes. Eles verificaram que a concentração de água nos solventes orgânicos não interferia na atividade enzimática e que esta atividade estava relacionada à quantidade de água ligada à proteína, provavelmente porque as moléculas de água na interface da enzima formam um “cluster” em torno de regiões polares e carregadas da enzima.

Outros estudos mostram também que a quantidade de água necessária para catálise depende das características de cada enzima. Lipases são altamente ativas quando poucas moléculas de água estão associadas com a molécula proteica (VALIVETY; HALLING; MACRAE, 1992a, 1992b). Álcool oxidase, álcool desidrogenase e polifenoloxidase são ativas somente quando um elevado número de moléculas de água está ligado com a molécula de enzima, formando uma monocamada (ZAKS; KLIBANOV, 1988a). A α-quimotripsina, entretanto, necessita de quantidade inferior a 50 moléculas de água por molécula de enzima para exibir sua atividade mínima (ZAKS; KLIBANOV, 1988b).

Ensaio utilizando a α-quimotripsina em acetato de etila com diferentes concentrações de água verificaram que há um aumento da velocidade de reação

com o incremento do grau de hidratação da enzima até atingir uma condição ótima (WEHTJE; ADLERCREUTZ; MATTIASSON, 1993). Isso, provavelmente, se deve ao aumento da flexibilidade interna da molécula da enzima. Um aumento acima do ponto ótimo de hidratação resultou na diminuição da atividade enzimática. Nesse caso, a água atuaria como um substrato na reação enzimática especialmente em reações hidrolíticas, produzindo reações secundárias e diminuindo o rendimento do processo químico (LIMA; ANGNES, 1999). A atividade das enzimas, quando em solventes miscíveis que não apresentam limite de solubilidade, aumenta até uma determinada concentração ("ponto ótimo"). Se a concentração destes solventes for maior do que este ponto, a enzima começa ser inativada (KHMELNITSKY et al., 1991b; MOZHAEV et al., 1989).

Experimentos realizados por Gorman e Dordick (1992) mostraram que os solventes podem alterar a atividade enzimática por retirarem a água da camada de hidratação da enzima. Isto ocorre principalmente com o uso de solventes muito polares e o resultado é a perda das atividades catalíticas por desnaturação ou inativação. Segundo os mesmos autores, o uso de solventes orgânicos causa redução na flexibilidade da molécula, se comparado ao meio aquoso. Isso ocorre por causa da influência direta da constante dielétrica. Quanto menor o valor dessa constante, maior a facilidade de interação entre os resíduos de aminoácidos, aumentando a rigidez da enzima e a interação proteína-proteina. Estudo realizado por Klibanov (1989) mostra que moléculas mais rígidas apresentam maior termoestabilidade, provavelmente pela retenção da "impressão" do sítio ativo onde, anteriormente, havia ligantes. Portanto, a escolha do solvente e o controle do meio de hidratação influenciam diretamente o grau de flexibilidade da enzima.

A influência da polaridade de solventes sobre as moléculas de água presentes nas enzimas também foi avaliada por Khmelnitsky et al. (1991), que aumentaram a hidrofilicidade na superfície da α-quimotripsina usando di-anidridopirometílico, e observaram que esta modificação ajudou a manter as moléculas de água no microambiente enzimático. Porém algumas substâncias, como certos carboidratos, conseguem reduzir a atividade da água ao redor da enzima (SANCHEZ-MONTERO et al., 1991). Isto faz com que a enzima tenha sua vida útil aumentada.

Melhorias contínuas e uma melhor compreensão da biocatálise em meios de reação não convencionais podem não apenas aumentar o potencial econômico dos

processos enzimáticos existentes, mas também permitir que novas áreas, até então impedidas pela instabilidade intrínseca das enzimas, sejam exploradas. A estabilidade proteica relaciona-se intimamente com as questões contemporâneas da ciência proteica, interações proteína-solvente e dobramento de proteínas. A engenharia da estabilidade enzimática é, portanto, de interesse comercial e científico (STEPANKOVA et al., 2013).

A partir dos dados na literatura que relatam a influência de solventes orgânicos sobre a estrutura e a atividade das enzimas de forma geral, e conhecendo a importância das serino-proteases em processos biológicos e industriais (BARRETT, 1970; DAVIE; FUJIKAWA; KISIEL, 1991; STOYTCHEVA, 2013), o nosso grupo resolveu investigar a influência do solvente em diferentes concentrações de solventes orgânicos sobre a tripsina, que é uma serino-protease.

1.2 Serino-Proteases

Serino-proteases são enzimas que fazem a hidrólise da ligação peptídica de proteínas, sendo que um dos aminoácidos responsáveis pela atividade catalítica é uma serina muito reativa (VOET; VOET, 2011).

1.2.1 Características das serino-proteases

As serino-proteases constituem a família das enzimas mais estudadas até os dias atuais, sendo amplamente pesquisada por mais de 60 anos. Os membros mais bem caracterizados são a quimotripsina, a tripsina e a elastase (VOET; VOET, 2011), que fazem parte da superfamília das quimotripsinas (Fig. 1). Estas pertencem à família S1, caracterizada por um sistema de tríade catalítica formada por resíduos de aspartato, histidina e serina. Estes resíduos trabalham juntos para controlar a nucleofilicidade do resíduo de serina durante a catálise (KUMAR; VENKATESU, 2012). As serino-proteases apresentam dois domínios similares, provavelmente originados por modificações durante duplicação gênica, e entre esses domínios encontra-se o sítio ativo contendo a tríade catalítica (LESK; FORDHAM, 1996; VOET; VOET, 2011).

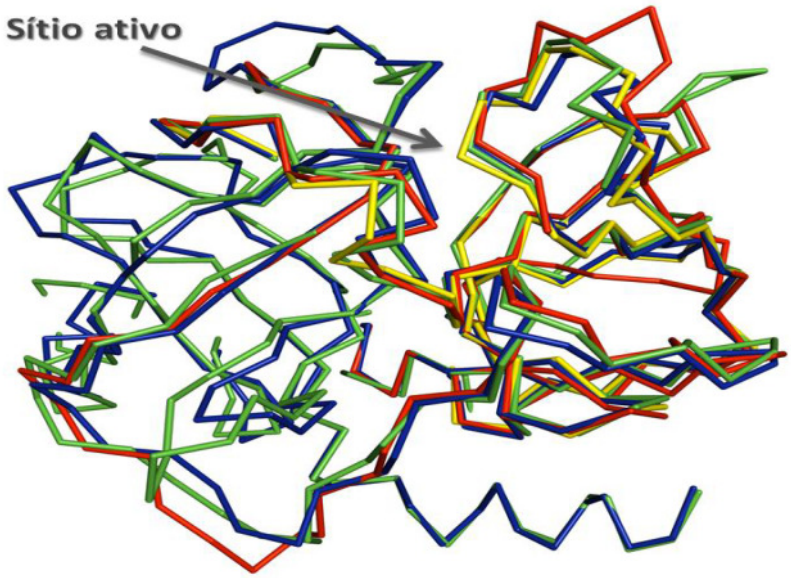

Figura 1: Superposição da estrutura terciária de quatro serino-proteases e seus centros ativos. Note que o enovelamento é semelhante. Enzimas: tripsina bovina - 5PTP (azul), quimotripsina bovina - 2CHA (vermelho), elastase porcina - 3EST (verde) e calicreína porcina - 2PKA (amarelo).

1.2.2 Formação das isoformas da tripsina

A tripsina foi isolada de diversos vertebrados e invertebrados, como porcos, ovelhas, bois, cachorro, homem, entre outros (SIMPSON; HAARD, 1984). De acordo com Keil (1971), as tripsinas são uma família de enzimas de peso molecular variando de 20 a 25 kDa que catalisam preferencialmente a hidrólise de ésteres e ligações peptídicas envolvendo o grupo carboxílico de aminoácidos básicos e na qual um resíduo de histidina e um de serina participam da catálise. A tripsina bovina é uma enzima proteolítica ativa, obtida pela clivagem do tripsinogênio (seu zimogênio), que tem atividade proteolítica restrita (Fig. 2). O tripsinogênio é produzido pelo pâncreas e secretado no intestino, onde sofre a ação de enteropetidases (enzimas presentes na mucosa do duodeno), formando a β-tripsina, que apresenta atividade proteolítica e auto-ativadora (BODE; FEHLHAMMER; HUBER, 1976; BODE; SCHWAGER; HUBER, 1978). A clivagem da ligação peptídica do tripsinogênio ocorre entre os resíduos de aminoácidos Lys6 e Ile7 do lado carboxílico da cadeia (contagem feita na sequência do tripsinogênio). Com isso, ocorre a saída do hexa-peptídeo N-terminal “Val-Asp-Asp-Asp-Asp-Lys” (DAVIE; NEURATH, 1954).

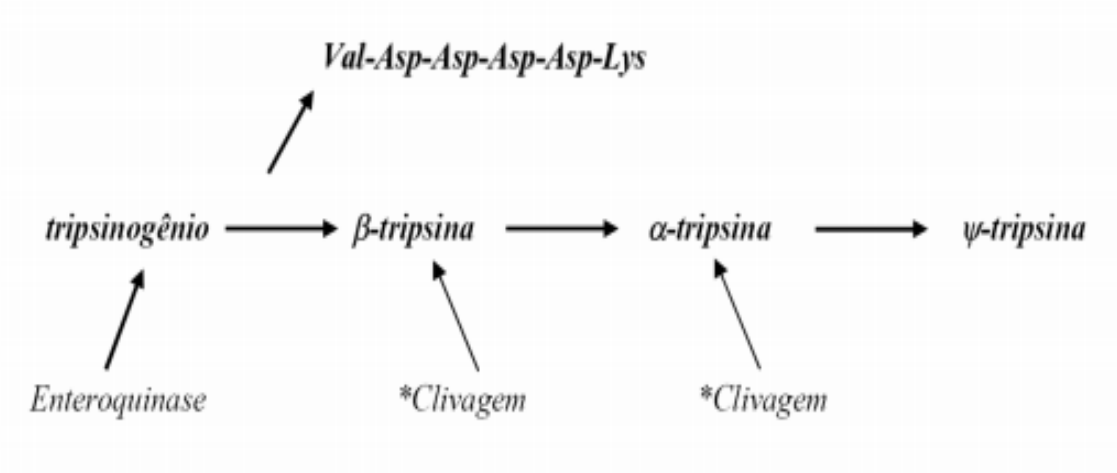

Figura 2: Esquema de formação das isoformas de tripsina.

O grupo α-amino da Ile7 forma uma ponte salina com o resíduo Asp194 (PERKINS; WUTHRICH, 1980), levando a alterações estruturais na β-tripsina recém-formada. Essa nova conformação torna a β-tripsina duzentas vezes mais ativa que o tripsinogênio (ROBINSON; NEURATH; WALSH, 1973).

O processo de autólise continua com a clivagem da ligação entre os resíduos Lys131 e Ser132 na β-tripsina, gerando uma outra forma ativa conhecida como α-tripsina (Fig. 3) (SCHROEDER; SHAW, 1968).

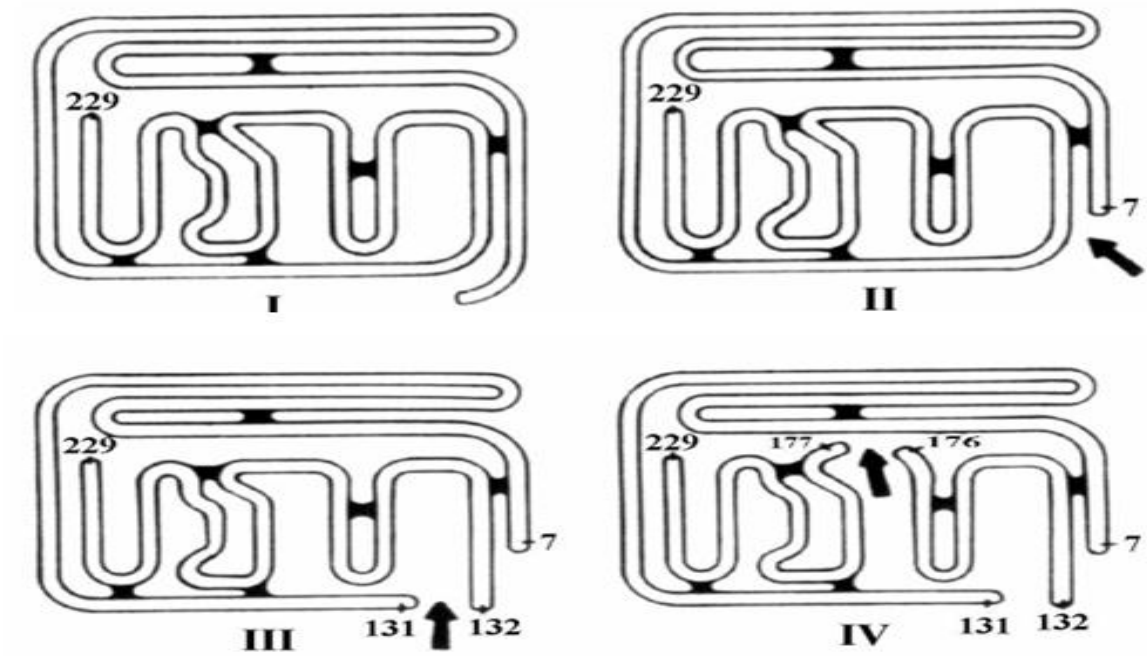

Figura 3: Pontos de clivagem do tripsinogênio e formação das isoformas ativas da tripsina. As setas mostram os pontos de clivagem. I- tripsinogênio, II- β-tripsina, III- α-tripsina, IV- Ψ-tripsina. Fonte: KEIL, 1971.

A α-tripsina formada apresenta estrutura terciária similar a da isoforma β-tripsina, porém apresenta menor quantidade de folha beta (FOUCAULT et al., 1974). A α-tripsina, quando comparada à isoforma beta, possui menor atividade para substratos amidásicos, mas nenhuma diferença significativa é encontrada quando se utiliza substratos do tipo éster (FOUCAULT et al., 1974).

1.2.3 Estrutura secundária e terciária

Quanto à estrutura tridimensional, o trabalho de Bode e cols (BODE; CHEN; BARTELS, 1983; BODE; SCHWAGER, 1975; BODE; SCHWAGER; HUBER, 1978), realizado com métodos cristalográficos, mostrou que a β-tripsina apresenta um arranjo globular com dois domínios estruturalmente semelhantes. O primeiro domínio é formado pela sequência compreendida entre o 1º e o 105º resíduo de aminoácido, e o segundo domínio é formado pela sequência de aminoácidos entre as posições 106 a 223. Esses domínios estão unidos assimetricamente e entre eles está o sítio ativo.

O tripsinogênio e as isoformas de tripsina (β, α e ψ) são constituídos, respectivamente, por uma, duas e três cadeias polipeptídicas. Estas cadeias estão interligadas por seis pontes dissulfeto (Fig. 4). Uma ponte dissulfeto (13-143) interliga os dois domínios, outra ponte dissulfeto (Cys (31)- Cys (47)) estabiliza o primeiro domínio e as outras quatro pontes estão no segundo domínio (MIKES et al., 1966; WALSH; NEURATH, 1964).

As moléculas de α- e ψ-tripsina, até a presente data, não possuem suas estruturas tridimensionais determinadas; assim todos os estudos sobre as isoformas de tripsina bovina se baseiam na estrutura de sua molécula precursora, a β-tripsina. Embora algumas características cinéticas da α- e ψ-tripsina já tenham sido descritas (FOUCAULT et al., 1974; FOUCAULT; SEYDOUX; YON, 1974), pouco se sabe da sua caracterização termodinâmica.

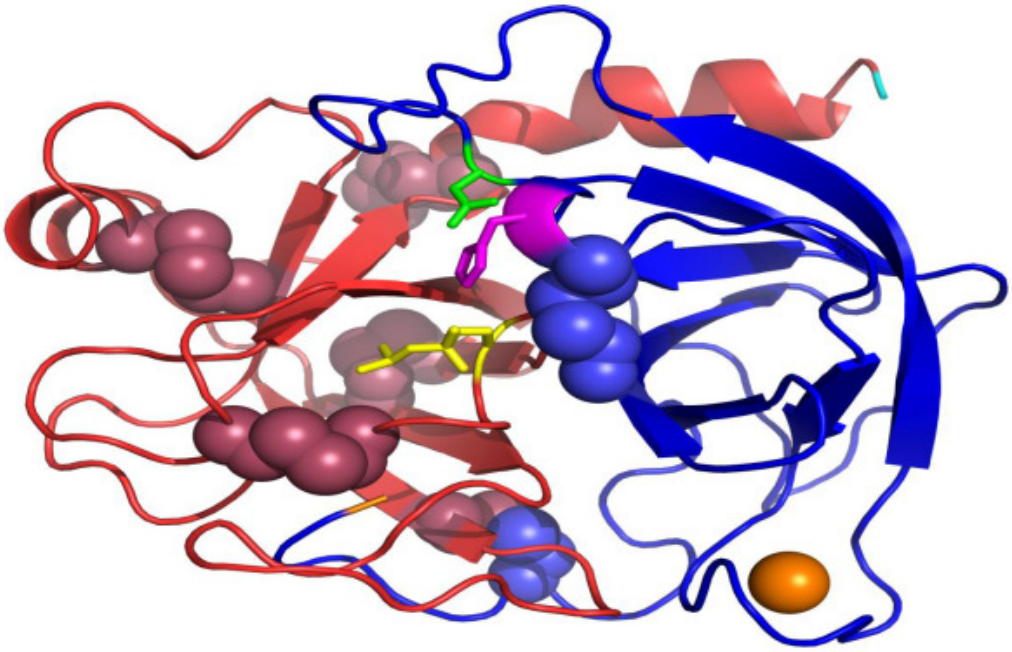

Figura 4: Estrutura tridimensional de β-tripsina bovina. O primeiro domínio está realçado em cor azul (1-105) e o segundo domínio realçado em cor vermelha (106-223). O resíduo de aminoácido N-terminal está representado em cor laranja e o C-terminal em cor ciano. Os resíduos de aminoácidos que compõe a tríade catalítica estão representados em modo de bastão Ser195 (amarelo), His57 (lilás) e Asp102 (verde). As pontes dissulfeto estão representadas pelas esferas azuis no primeiro domínio, vermelhas no segundo domínio e azul-vermelha no interdomínio (ligação entre os dois domínios). O íon metálico Ca2+ que está em seu sítio de ligação é representado por uma esfera de cor laranja acondicionada em uma alça da molécula de proteína (5ptp.pdB).

1.2.4 Tripsina e solventes orgânicos

Existem alguns estudos com a tripsina em solventes orgânicos, porém são poucos os trabalhos realizados com a isoforma purificada. Trabalhos com a tripsina comercial mostram que o solvente orgânico exerce influência sobre a estrutura desta enzima em graus variados, dependendo do tipo e da concentração do solvente. A adição de solventes orgânicos miscíveis em água provoca pequenas alterações no sítio catalítico, o que se reflete na alteração da eficiência da atividade catalítica da tripsina (GUINN; BLANCH; CLARK, 1991). Em elevadas concentrações, a alteração de estrutura é suficiente para causar a perda da atividade catalítica (GUINN; BLANCH; CLARK, 1991).

Estudos utilizando a α-quimotripsina sugerem que a inativação desta enzima ocorre em uma concentração de solvente crítica, que varia conforme o tipo de solvente utilizado, e está atrelada a mudanças conformacionais reversíveis na enzima (MOZHAEV et al., 1989). Porém o trabalho de Simon et cols (2001) registrou que a tripsina, em meio contendo 95% v/v de etanol, apresenta uma atividade catalítica similar quando comparado ao meio puramente aquoso. Apesar das semelhanças na estabilidade e atividade, pequenas alterações conformacionais foram observadas. A alteração conformacional sugerida está relacionada às características das folhas-β presente nestas duas soluções (SIMON et al., 2001), pois a quantidade de folhas antiparalelas aumentou quando o solvente orgânico foi adicionado ao meio, levando a um possivelmente aumento da rigidez da estrutura. A capacidade de catalisar substratos em meios orgânicos parece ser devido à rigidez estrutural da enzima que resulta em elevada barreira cinética, impedindo a desnaturação da proteína (ZAKS; KLIBANOV, 1988b).

Quase não há relato sobre estudos envolvendo a isoforma α-tripsina e solventes orgânicos. Trabalho não publicado realizado por nosso grupo (PEREIRA, 2015) avaliando a influência do tipo e concentração do solvente orgânico na atividade e na estabilidade termodinâmica da enzima observou que a adição de solvente orgânico a α-tripsina não causou processos de agregação significativo na mesma, porém alterou significativamente os parâmetros cinéticos da enzima em presença do solvente orgânico. Trabalhos utilizando agentes desnaturantes ou calor para avaliação de modificações estruturais e conformacionais da tripsina sugerem que a modificação da estrutura ocorre em mais de uma etapa. Trabalhos com a isoforma purificada α-tripsina mostram uma possível formação de estados intermediários em processos de desnaturação induzidos por agentes químicos (MARTINS et al., 2003). Zajizek et al (1981) utilizaram a técnica de desnaturação térmica para acompanhar modificações estruturais na β-tripsina e sugeriram em seu trabalho que o processo de desnaturação ocorre de forma sequencial.

1.3 Aplicações da Tripsina

A tripsina é uma enzima digestiva cuja função bioquímica pode ser explorada em diversas áreas, como a farmacêutica, a biotecnológica industrial e a bioquímica. Algumas dessas aplicações serão descritas a seguir.

1.3.1 Aplicação Farmacêutica

A tripsina tem sido estudada há vários anos, principalmente para o emprego no tratamento de inflamações. Vários estudos demonstram sua eficácia no tratamento de doenças inflamatórias e o aumento da eficácia de analgésicos e antibióticos, quando usado em associação com estes últimos.

A tripsina, por exemplo, tem sido administrada por via intramuscular no tratamento de tromboflebites (INNERFIELD; ANGRIST; SCHWARZ, 1953). O uso de tripsina isolada ou associada a substâncias analgésicas e antibióticas se mostrou eficaz no tratamento de desordem gastrointestinal, lise de coleções protéticas, adjuvante no tratamento tromboembólico e no tratamento de inflamações (SHERRY; FLETCHER, 1960). Pacientes portadores lesões ulcerativas, tromboflebites e arteriosclerose nos membros inferiores e cujo uso de esteróides estava contra-indicado foram tratados com Parenzyme® (tripsina + alfa-quimotripsina) (HAZAN, 1960). Ugolini fez uso tópico de tripsina para digestão de tecidos necróticos (UGOLINI, 1963). Pereira e Padovan comprovaram que a associação de tripsina e quimotripsina ao paracetamol é mais eficiente do que o tratamento com as enzimas ou paracetamol isolados (PEREIRA; PADOVAN, 1968). Barros, em sua tese, estudou a influencia do Parenzyme® (tripsina + alfa-quimotripsina) e Parenzyme® analgésico (tripsina + alfa-quimotripsina +paracetamol) no desenvolvimento de tecido de granulação e observou que a ação das enzimas associadas ao paracetamol é melhor do que o uso de um dos dois isoladamente (BARROS, 1989). Outra associação terapêutica estudada e utilizada em outros países é o Phlogenzym® (tripsina + bromelina + rutosídeo), com ação anti-inflamatória e com pronunciada diminuição de dor em casos de osteoartrite (KLEIN et al., 2006; MANHART et al., 2002).

Para a indústria farmacêutica, é frequentemente problemática a produção de medicamentos a base de proteínas, já que elas possuem características próprias,

não podendo a característica de uma ser extendida a outra (VELLARD, 2003). A tripsina, por exemplo, é produzida na forma sólida e, nesta forma farmacêutica, encontra-se desnaturada. Quando em contato com o conteúdo gástrico, apenas uma pequena parte volta a seu estado nativo, o que resulta em baixa atividade. A indústria farmacêutica costuma, então, colocar grandes quantidades de princípio ativo para compensar as perdas por agregação e não-renaturação da enzima. Porém, o aumento da concentração pode causar um efeito reverso: mais proteínas agregam, pois o processo cinético de conversão das enzimas em produtos insolúveis ocorre mais rápido do que o processo termodinâmico de enovelamento e desenovelamento das proteínas. Assim, estudos sobre a termodinâmica e a estabilidade da tripsina e suas isoformas, bem como a atividade enzimática de cada isoforma isolada, são importantes para possibilitar a produção de formulações farmacêuticas mais estáveis, para definir parâmetros mais claros na realização do controle de qualidade dessas formulações e para fornecer à população um medicamento mais estável (maior durabilidade) e mais confiável em seu efeito.

1.3.2 Aplicação industrial

A biocatálise industrial é reconhecida como um dos principais motores da indústria química (KAUL; ASANO, 2012). A tripsina, por ser uma enzima proteolítica com elevada atividade e relativa facilidade de extração, é amplamente explorada nas indústrias de alimentos e bebidas para, por exemplo, aumentar a qualidade de massas e a maciez de carnes, na produção de hidrolisados proteicos e durante a estabilização fria de cerveja, entre outros (SHAHIDI; JANAK KAMIL, 2001; STOYTCHEVA et al., 2013). Apesar de a enzima atuar de maneira eficiente nestes sistemas, as condições não são favoráveis a catálise, pois em muitas situações os substratos são pouco solúveis na condição de atuação da enzima.

Outros exemplos interessantes do uso da tripsina que geram produtos com alto valor agregado podem ser enumerados. O quanin, que é um pigmento prata esbranquiçado usado na fabricação de pérolas, é extraído das escamas de peixes usando enzimas proteolíticas como a tripsina (WINDSOR; BARLOW, 1981). Outro exemplo interessante é o tratamento do leite com tripsina para prevenir a oxidação do leite, provavelmente devido à melhora na eficiência da ligação do cobre à proteína do leite modificada pela enzima (SHAHIDI; JANAK KAMIL, 2001). Outro

exemplo é o uso da tripsina reduzindo o tempo de fermentação de molhos a base de peixe, sem causar alteração em suas características sensoriais (CHAVEESUK; SMITH; SIMPSON, 1993).

1.3.3 Aplicação em laboratórios de pesquisa

Na pesquisa, a tripsina é utilizada em protocolos de cultura de células e tecidos, agindo por meio da reação de dissociação enzimática, que leva ao rompimento da matriz extracelular para obtenção de células individualizadas, com a finalidade de transferir as culturas para um novo substrato (BANUMATHI et al., 2009; SOLEIMANI; NADRI, 2009; YANG et al., 2009). Também utilizada na identificação de proteínas por meio de técnicas de sequenciamento de peptídeos (SCHUCHERT-SHI; HAUSER, 2009).

2 JUSTIFICATIVA

A α-tripsina é uma enzima que já está parcialmente caracterizada e possui comprovada atividade esterásica e amidásica em aproximadamente 50% cada. O emprego em meio aquoso é muito comum em processos catalíticos, porém seu uso é limitado devido ao fato de poucos substratos serem solúveis em água. O uso de solventes orgânicos ou mistura aquo-orgânica é uma forma direta de aumentar a solubilidade de substratos apolares, tornando a reação possível. No entanto, os solventes orgânicos afetam a atividade enzimática. Desta forma, o estudo dos parâmetros cinéticos, estruturais e termodinâmicos de enzima em sistemas aquo-orgânicos pode fornecer informações importantes para o entendimento da atividade da enzima em meio aquo-orgânico e possibilitar possíveis aplicações em reações de biocatálise. Os parâmetros cinéticos e termodinâmicos em alguns solventes aquo-orgânicos já foram determinados em trabalho anterior, e foi observado que alguns desses parâmetros se alteram com a adição de solventes orgânicos à α-tripsina. Assim, pretende-se entender quais alterações estruturais ocorreram nesta proteína que refletem as alterações termodinâmicas vistas no trabalho de Pereira, E.V. (2015).

3 OBJETIVOS

3.1 Objetivo geral

Trabalhos anteriores com a enzima indicam os tipos e concentrações de solventes orgânicos que conferem à enzima atividade catalítica ótima (PEREIRA, E.V., 2015). A partir destas determinações, o presente trabalho busca fazer a determinação dos parâmetros estruturais da enzima alfa tripsina em solvente orgânico.

3.2 Objetivos específicos

Determinar os parâmetros estruturais da isoforma α-tripsina em meio aquo-orgânico por:

- Espalhamento de luz dinâmico (DLS) monitorando os estados supramacromoleculares da enzima;
- Dicroísmo circular (CD) monitorando a mudança da estrutura secundária em função da concentração de solventes orgânicos;
- Fluorescência (FL) monitorando a mudança da estrutura terciária indiretamente em função da concentração de solventes orgânicos;
- Absorção no ultravioleta (ABS-UV) monitorando a exposição de resíduos aromáticos na superfície da enzima em função da concentração de solventes orgânicos.

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PARTE II

DELINEAMENTO EXPERIMENTAL

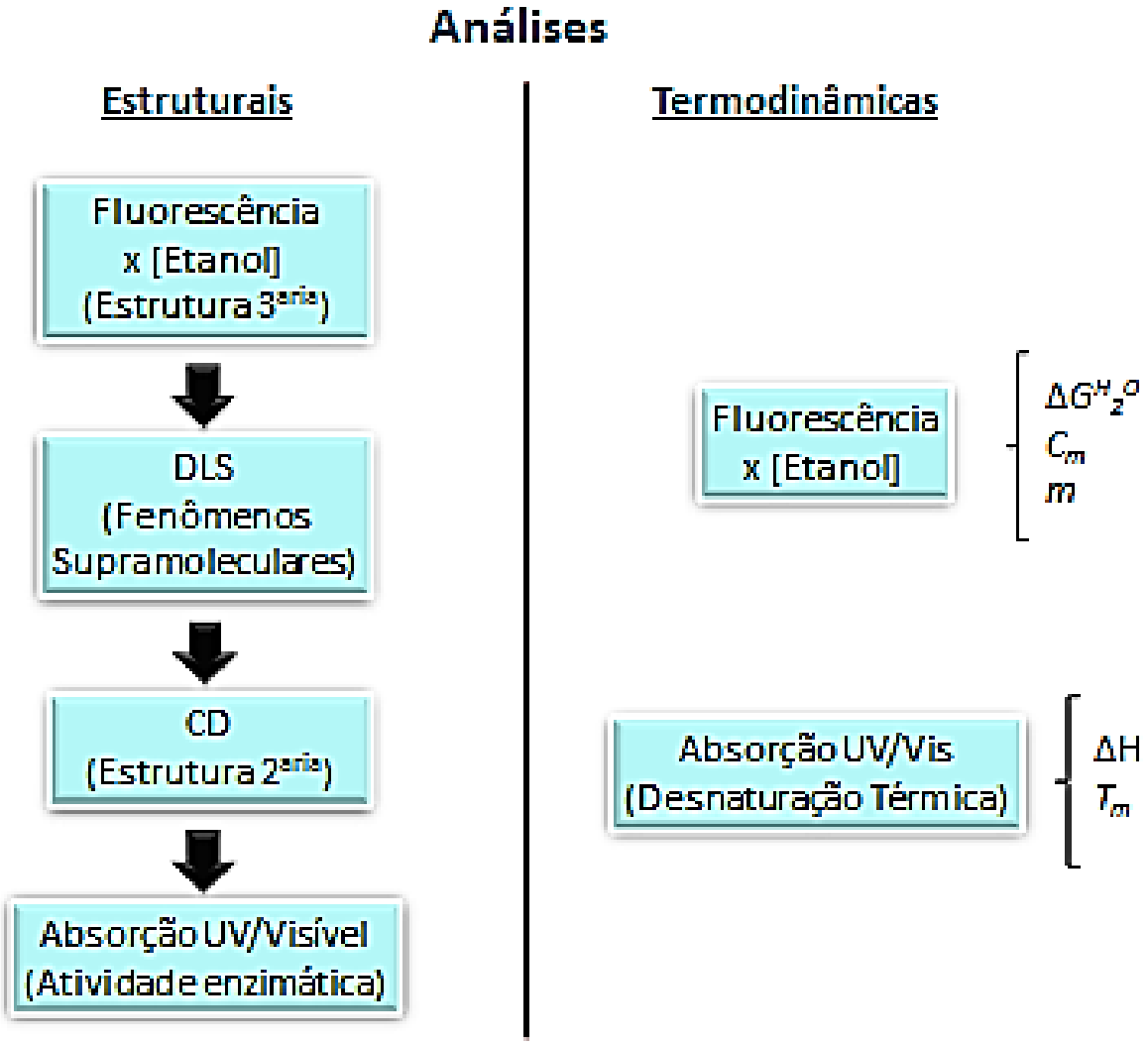

Figura 5: Delineamento experimental. Os ensaios estruturais e termodinâmicos foram realizados concomitantemente. As análises estruturais buscaram avaliar perfil de rearrajo das unidades da α-tripsina. As análises termodinâmicas forneceram os dados de estabilidade estrutural desta isoforma.

PARTE III

ARTIGO

Determination of Structural and Thermodynamic Parameters of bovine α-trypsin isoform in Aqueous-Organic Media

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1 INTRODUCTION

It is increasing the number of industrial processes that use enzymes as catalyzer, such as both animal and human health food, textile and cellulose areas [1] being organic solvent commonly found in all these processes. The use of organic solvent as a reaction medium has dramatically increased the diversity of enzymatic catalysis [2]. However, the addition of organic solvent to protein solutions can lead a widespread degeneration route, and understanding its mechanism is essential for process improvement. The organic solvents are widely applied in industrial processes, allowing a more efficient catalysis of nonpolar substrates. However, in elevated concentration, the protein molecules can aggregate, leading to loss of enzyme activity [3–5]. This fact may cause financial loss, as decay in production yield, need to use larger amounts of enzymes to achieve the desired yield and/or low post-process recovery rate. Perhaps, the biggest difficulty for the industries is to find enzymes that can withstand some industrial conditions as both temperature and pH changes [6] as well as organic solvent. Therefore, the study of inductor factor of protein aggregation is of great interest for industries, particularly for both pharmaceutical and biotechnology industries. Currently, molecular enzymology studies are performed using model protein whose physical and chemical properties (molecular weight, secondary/tertiary structure, catalytic activity) are well known.

Trypsin (EC 3.4.21.4) is the most studied enzyme and has a lot of physical and chemical data determined [7–13]. It is a serine protease family member, playing an important role in the digestive process with proteolytic function and activation of zymogens secreted into the intestinal tract[13,14], fertilization [15], blood coagulation[16,17] and others. Despite many studies have been conducted with commercial or extracted from animal pancreas trypsin, the trypsin isoforms began to be discovered from the 60's [7]. Many studies have shown that, although catalytically similar, there are differences between the isoforms, especially in respect of their specific activity (amidasic or esterasic) and structural properties [6,10]. The α-trypsin is an isoform derived of β-trypsin isoform, which in turn is derived from pancreatic trypsinogen, being a typical globular protein (223 amino acid residues and about 24 kDa) [6]. Commercial preparations of trypsin are isoforms mixtures containing substantial quantities of active forms as ψ-, β- and α-trypsin and others until not studied [18].

There are some studies about trypsin isoforms mixed [19,20], obtained through commercial preparation, in aqueous-organic systems. However, few works have been performed with pure isoforms [21]. As structural and thermodynamic informations cannot be deduced one from the other [19], the objective of this study was the understanding the structural and thermodynamic behavior of α-trypsin in aqueous-organic media. It will allow the optimization of industrial process that uses trypsin isoforms as catalyzer.

2 MATERIALS AND METHODS

2.1 Materials

Commercial bovine trypsin (EC 3.4.21.4) type I (Lot 8003), Tris-(hydroxymethyl) aminomethane, Nα-benzoyl-Dl-arginine-p-nitroanilide (BAPNA) and sinapinic acid were purchased from Sigma (St. Louis, MO, USA). Calcium chloride was purchased from Casa da Química (Diadema, SP, Brazil). Potassium chloride and dimethyl sulfoxide (DMSO) were purchased from Vetec (Rio de Janeiro, RJ, Brazil). Acetic acid, hydrochloride acid and ethanol were purchased from Synth (Diadema, SP, Brazil). Glycine was purchased from Dinâmica (Diadema, SP, Brazil). SP-SEPHADEX-C50 matrix cationic exchanger was purchased from Pharmacia Fine Chemicals INC (Uppsala, Sweden). Type I water was obtained with a ThermoScientific^{TM} Barnstead^{TM} Easypure^{TM} II ultrapure water system (Thermo Scientific, Ohio, USA).

2.2 Protein purification and purity of trypsin isoforms

2.2.1 Purification of α-trypsin isoform

The purification of α-trypsin isoform was performed using the conditions described by Lacerda e cols [18]. The α-trypsin fractions were pooled and dialyzed against 1 mmol.L^{-1} HCl at 4ºC by a dilution factor of 10^6 times sample volume for 24 h and then lyophilized, aliquoted, and stored at 2 – 8 ºC.

2.2.2 Purity of α-trypsin isoform by mass spectrometry

The α-trypsin purity was determined by mass spectrometry (MS) [22,23], using Matrix assisted Laser Desorption/Ionisation-Time of Flight (MALDI-ToF) technique. For MS experiments, an aliquot of 0.05 $mg.mL^{-1}$ α-trypsin was dissolved in a sinapinic acid matrix solution, whose proportion was 1:3 (v:v) sample:matrix. This mixture was applied in triplicate into a MALDI target plate (MTP Anchorchip 384 x 600) and dried at room temperature for 15 min. The average molecular mass was obtained in an AUTOFLEX III MALDI-Lift Tof-Tof (Bruker TM) in linear positive mode. The Flex Analysis Software (BrukerDaltonics) was used to interpret mass spectra. The raw data had its baseline subtracted and, soon after, the curves received a smoothing treatment. Then, the protein has had its molecular mass determined.

2.3 Hydrodynamic radius

Hydrodynamic radius was measured by Dynamic Light Scattering (DLS) technology using a NPA152 Zetatrac® (Microtrac Instruments, Inc, USA) instrument equipped with a solid state diode laser (780 nm, 5 mW) light source. The equipment is localized at Federal Institute of Education, Science and technology of Espírito Santo, Aracruz, ES, Brazil. The acquisitions were performed using 70% of laser power with 3s for each acquisition, and a total time of 2 minutes/acquisition. Since the purpose of this assay was verify the hydrodynamic radius variation as a function of ethanol concentration increasing, two systems were tested: the first one was composed of only protein/buffer and the second of ethanol/buffer from 20% to 80% (v/v). The final concentration of α-trypsin (0.5 $mg.mL^{-1}$) and buffer (50 $mmol.L^{-1}$ of Glycine buffer pH 3.0 with $CaCl_2$ 20 $mmol.L^{-1}$) were maintained in both systems. The temperature in all tests was 25 ºC. The data were obtained through the mean ± SD of the acquisitions in each sample and Microtrac FLEX^{TM} software was used in the analysis of raw data.

2.4 Fluorescence spectroscopy

The fluorescence spectroscopy was used for investigation of structural behavior of trypsin at aqueous-organic media. Fluorescence intensity was measured using a Hitachi F-2000 Spectrofluorimeter (Hitachi Ltd., Tokyo, Japan) linked to a thermal bath and a 1 cm path-length quartz cuvette. The equipment was located to Laboratory of Cardiovascular Biochemistry at UFES. Measures were performed at

0.01 mg.mL^{-1} of α-trypsin in 100 mmol.L^{-1} of glycine buffer pH 3.0 with 20 mmol.L^{-1} of $CaCl_2$ at 25 ºC varying the ethanol concentration from 10% to 95% (v/v). The samples were excited at 280 nm (Tyr and Trp amino acids residues) and emission measures were collected from 310 to 370 nm range, with 1 nm interval. The photomultiplier was adjusted to 400V and the scan rate was 600 nm.min^{-1}. Origin Pro 8® was used in the analysis of raw data. The results were expressed as mean ± S.D. (n = 3).

2.5 Circular Dichroism (CD) spectroscopy

The CD technique was used to measure the secondary structure percentage of α-trypsin at aqueous-organic media. A Jasco J-810 spectropolarimeter (Jasco Co., Tokyo, Japan) equipped with a thermoelectric sample temperature controller (Peltier system) was used to record CD measurements, using a 1 mm path-length cell. For each test, the buffer spectrum was subtracted from the corresponding protein spectrum. Each spectrum represented the average of 10 accumulations recorded from 190 to 260 nm range at 25 ºC over nitrogen atmosphere, with a band pitch of 0.5 nm, with a response time of 4 s and high sensitivity and scan speed of 100 nm.min^{-1} [18,23]. The enzyme was diluted directly in 30 mmol.L^{-1} potassium chloride solution (pH 3.0), obtaining a final concentration of 0.5 mg.mL^{-1}. Additionally, it was prepared more four solutions keeping the same concentration of α-trypsin and buffer, and only varying the ethanol concentration (20%, 40%, 60% and 80% v/v). The CD intensities were expressed as ellipticity (mdeg). Percentages of the different secondary structures, α-helix, β-sheet and random coil were estimated using the CDSSTR® algorithms and databases accessed through DICHROWEB [18,24,25].

2.6 Thermal denaturation monitored by UV-spectroscopy

In order to monitor the α-trypsin thermal denaturation in aqueous-organic media, the method of absorption variation between unfold and fold states was applied [8] monitoring two wavelengths (279 and 285 nm). An Evolution™ 300 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc, USA) equipped with a thermoelectric sample temperature controller (Peltier system) was used to record the absorption measures. The temperature range was from 298.15K up to 353.25K with

scan rate of 1 $K.min^{-1}$ and acquisition interval of 30s. Measures were performed with solutions at 0.5 $mg.mL^{-1}$ of α-trypsin in 50 $mmol.L^{-1}$ of glycine buffer pH 3.0 with 20 $mmol.L^{-1}$ of $CaCl_2$. Additionally, it was prepared other four solutions keeping the same concentration of α-trypsin and buffer, but adding 20%, 40%, 60% and 80% (v/v) of ethanol/buffer. Origin Pro 8® was used in the analysis of raw data. The results were expressed as mean ± S.D. (n = 3).

2.7 Amidasic activity assay

The enzymatic activity (amidasic) was performed according to Lacerda and cols [18], using the chromogenic synthetic substrate Nα-benzoyl-dL-arginine-*p*-nitroanilide hydrochloride (BA*p*NA). For each assay, 100 µL of α-trypsin solution at 2.5 $mg.mL^{-1}$ (stock solution at pH 3.0) was diluted in 395 µL of 100 $mmol.L^{-1}$ of Tris-HCl buffer pH 8.0 with 20 $mmol.L^{-1}$ of $CaCl_2$. The same solution was prepared, but adding ethanol at 20%, 40%, 60% and 80% (v/v). These samples solutions were centrifuged at 12,396 *g* during 1 min at 25 ºC and the supposed pellet and the supernatant were separated in different tubes. So the volume of the pellet and supernatant was completed to the initial volume (495 µL). Protein concentration was determined in the both supernatant and supposed pellet by absorbance at 280 nm. All these samples were incubated at 37 ºC during 10 min and then was added 5 µL of substrate solution (BA*p*NA) at 90 $mmol.L^{-1}$ (solubilized in DMSO); thus, the final protein concentration was set to 0.5 $mg.mL^{-1}$. After 15 min, 125 µL of aqueous solution of acetic acid 60% v/v was added and then the chromogenic product (*p*-nitroaniline) absorbance was measured at 410 nm using NanoDrop®2000 Thermo UV-Vis Spectrophotometer (Thermo Fisher Scientific, Inc, USA). The results were expressed as mean ± S.D. (n = 3).

2.7.1 Influence of ethanol on α-trypsin molar extinction coefficient ($\varepsilon_{trypsin}$)

In order to ensure whether the ethanol may influence on the α-trypsin apparent concentration, the measure of activity at organic media was performed using α-trypsin at 2.5 $mg.mL^{-1}$ in HCl aqueous stock solution at pH 3.0 . Starting this stock protein solution, it was done five serial dilutions from 0.5 up to 0.1 $mg.mL^{-1}$. These dilutions were done in 50 $mmol.L^{-1}$ of Glycine buffer pH 3.0 with 20 $mmol.L^{-1}$ of

$CaCl_2$. It was also prepared other four solutions keeping the same concentration of α-trypsin and buffer, but adding 20%, 40%, 60% and 80% v/v of ethanol. A Evolution™ 300 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc, USA) equipped with a thermoelectric sample temperature controller (Peltier system) was used to record the absorption measures. So it was performed scanning from 250 to 310 nm range at 25ºC with scan rate of 600 $nm.min^{-1}$ and 1 nm interval and 10 acquisitions per measure. Maximum absorbance of each curve was collected for each sample and $\varepsilon_{trypsin}$ was calculated by linear regression. GraphPad Prism® was used in the analysis of raw data.

2.7.2 Influence of organic solvent on molar extinction coefficient of p-nitroaniline ($\varepsilon_{p\text{-}NA}$)

In order to ensure whether the ethanol may influence on the chromophore apparent concentration, it was performed absorbance measures adding ethanol to the product formed in the enzymatic reaction (*p*-NA). A stock solution of *p*-NA at 90 $mmol.L^{-1}$ solubilized in DMSO was diluted from $1.5x10^{-5}$ $mmol.L^{-1}$ up to $9.0x10^{-5}$ $mmol.L^{-1}$ in 100 $mmol.L^{-1}$ of Tris-HCl buffer pH 8.0 with 20 $mmol.L^{-1}$ of $CaCl_2$, in order to maintain the same solution condition found at the item 2.7. Other four solutions were prepared keeping the same concentration of *p*-NA and buffer, but adding 20%, 40%, 60% and 80% (v/v) of ethanol. A Evolution™ 300 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc, USA) equipped with a thermoelectric sample temperature controller (Peltier system) was utilized to record the absorption measures. The absorption measurement was performed at 410 nm [26] at 25 ºC. GraphPadPrism® was used in the analysis of raw data and the $\varepsilon_{p\text{-}NA}$ values were obtained through Absorbance *versus p*-NA Concentration graphic. The results were expressed as mean ± S.D. (n = 3).

2.8 Thermodynamic parameters

The graphics generated by the raw data of both fluorescence and thermal denaturation assays were submitted to nonlinear regression models. Thus, it was provided thermodynamic parameters in some concentrations of ethanol as well as in its absence.

2.8.1 Calculation of T_m

The raw data of thermal denaturation curves obtained for systems containing only buffer and containing 40%, 60% and 80% v/v ethanol were submitted a nonlinear regression fit (Boltzman model) using Origin 8.0® software for obtaining the x0 parameter which is equivalent the T_m values.

2.8.2 Calculation of ΔH_{vH} (van't Hoff enthalpy).

The ΔH_{vH} of α-trypsin for some ethanol concentration were calculated from thermal denaturation profile from UV spectroscopy (285 nm) and fitted by nonlinear regression using Eq. 1 according to Cohen and Pielak [27], assuming a two-state transition [28].

$$y_{obs} = \frac{(y_n + m_n T) + (y_d + m_d T)\left\{ e^{\left[\frac{\Delta H_{vH}}{R\left(\left(\frac{1}{Tm}\right)\left(\frac{1}{T}\right)\right)} \right]} \right\}}{1 + e^{\frac{\Delta H_{vH}}{R\left(\frac{\frac{1}{Tm1}}{T}\right)}}} \quad (1)$$

In Eq. (1), y_{obs} is an experimental observable value, T is the temperature in Kelvin, R is the gas constant, T_m is the midpoint transition temperature and ΔH_{vH} is the enthalpy of unfolding at T_m. The parameters y_n and y_d refer to the y intercepts of the native and denatured baselines, respectively, while m_n and m_d are the slopes of the baselines in native and denatured state, respectively. All fits were performed using Origin 8.0® software.

2.8.3 Calculation of ΔG^{H_2O} of a-trypsin by chemical denaturation

For calculation of ΔG^{H_2O}, that is the free energy of unfolding at zero denaturant concentration, it was used the fluorescence *versus* ethanol concentration raw data. Using the methodology described by Shaw e cols [29], ΔG^{H_2O} was determined by linear extrapolation of ΔG *versus* ethanol concentration graphic. The C_m parameter, that is the solvent concentration wherein 50% of the protein population is in the

folding conformation, was determined using Origin 8.0® software for obtaining a fitted sigmoid curve (Boltzman model).

2.8.3.1 Calculation of denaturant m value (m)

The *m* value is the dependence of free energy of unfolding on denaturant concentration. The raw data of fluorescence versus ethanol concentration assay (item 2.4) were used for obtaining a ΔG versus ethanol concentration graphic (Fig. 1B). The straight line equation was generated and corresponds to the Eq. (2):

$$\Delta G = \Delta G^{H_2O} - m[\text{denaturant}] \quad (2)$$

Where ΔG^{H_2O} is the free energy of unfolding at zero denaturant concentration (the x axis intercept) and [denaturant] is the denaturant concentration [30]. In this work, the ethanol was considered the denaturant agent.

3 RESULTS AND DISCUSSION

3.1 Purification and purity of α-trypsin isoform

A cationic exchange benchtop chromatography was performed according to Santos and cols [22] in order to obtain the isolated α-trypsin isoform. The chromatographic result was similar to that obtained by Lacerda and cols [18]. The α-trypsin isoform was eluted at sixth peak in elution order (results not showed), according profile presented by Lacerda and cols [18] and Santos and cols [22]. Results of mass spectrometry showed that the selected fraction has just one molecule and that the experimental average molecular mass of α-trypsin isoform is 23.312 KDa, being similar to the theoretical molecular mass. Thus, both previous results demonstrate the α-trypsin purity.

3.2 Fluorescence Spectroscopy

The conformational change, which may occur with the use of organic solvent in different concentrations, was indirectly monitored through the amino acids residues

movement [31–34] by fluorescence spectroscopy. Only Tyr and Trp residues were monitored since they exhibit intrinsic fluorescence and have enough quantum yield to provide a good fluorescence signal [31–35].

3.2.1 Thermodynamic stability of α-trypsin by solvent denaturation

The determination of protein thermodynamic stability is an important initial data to characterize a biomolecule. The free energy variation in absence of denaturant influence (ΔG^{H_2O}) is a parameter used to ascertain the protein stability. The higher its value, the more stable is the native state of a protein. Using the fluorescence spectroscopy technique, it was possible to obtain some thermodynamic parameters that reflect the α-trypsin isoform stability.

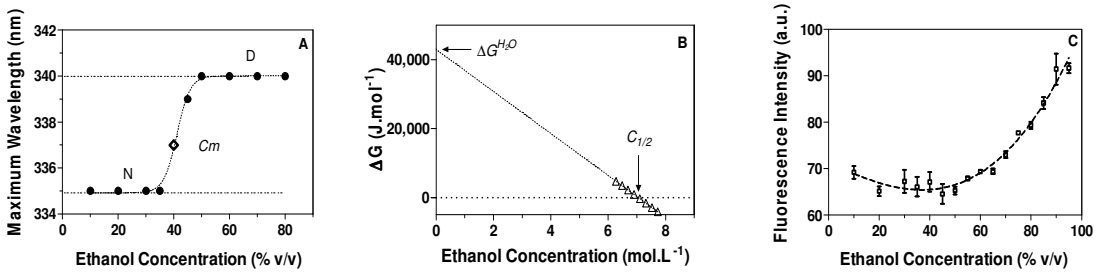

Fig. 1: Fluorescence spectroscopy of α-trypsin at function of ethanol concentration. Intrinsic tryptophan emission spectra were measured with excitation at 280 nm for 0.01 mg.mL^{-1} α-trypsin in 100 mM glycine buffer at pH 3.0 and 20 mM $CaCl_2$ at 25 ºC. α-Trypsin denaturation profile (figure A), the free energy variation (figure B), and emission intensity (figure C) are plotted against ethanol concentration. The dotted line in figure B is a linear extrapolation in order to determine the energy free variation. The dotted line in B and C figures are just to guide the eye in graphic. C_m is the organic solvent concentration wherein 50% of the protein population is in the folding conformation.

The graphic of maximum wavelength shift versus ethanol concentration (Fig. 1A) showed a sigmoidal model that is typical of a two-state process [36] monitored by spectroscopic techniques [37] and also with a single symmetric transition with 95% of reversibility. This reversibility is generally valid for chemical denaturation [38]. The results of linear extrapolation [29,39] obtained by denaturation curves are showed in Table 1 and Fig. 1B. The value obtained for α-trypsin isoform is in good agreement with a range of theoretical values (5 – 17 kcal.mol^{-1} or 20.92 – 71.128 kJ.mol^{-1}) [40,41] obtained by other groups and experimental values previously obtained by our group [6,42]. The result of our work demonstrates that α-trypsin remained stable

during the purification and storage process. In addition, the stability of our purified enzyme is higher than that found in other works with α-trypsin and other isoforms.

Table 1: Stability properties of α-trypsin monitored by fluorescence at different ethanol concentration at pH 3.0.

ΔG_{H2O} (KJ.mol^{-1}) [a]	*Cm* (% v/v) [b]	*m* (KJ.mol^{-1}.M^{-1}) [c]
42.997	41	6.095

[a] Free energy variation in absence of denaturant.
[b] The organic solvent concentration wherein 50% of the protein population is in the folding conformation.
[c] The dependence of the free energy of unfolding on denaturant concentration

Specific m [30] and Cm [43] were also obtained for α-trypsin in ethanol. The midpoint transition Cm, where the polypeptide was partially unfolded, is equal to 41% v/v of ethanol. These thermodynamic parameters for this isoform will be important for future comparisons since they have never been determined.

Thus, the set of thermodynamic results suggests that the α-trypsin isoform was isolated in predominantly stable state and didn't undergo any kind of irreversible process during separation and purification process. Also, small conformational changes may have occurred but without causing alteration in the biological activity of the trypsin molecule.

3.2.2 Fluorescence emission profile of α-trypsin induced by solvent denaturation

The raw data (λ_{max}) from emission curves of intrinsic fluorescence of α-trypsin were fitted using a sigmoidal model (Boltzman) and the result was used to obtain the denaturation curve as a function of solvent concentration (Fig. 1A). As can be seen in Fig. 1A, there was a significant red shift of the maximum wavelength (λ_{max}) between the native and denatured emission plateaus ($\Delta\lambda$ = 5nm). In general, the wavelength of maximum emission suffers a bathochromic shift to lower values as the polarity of the environment or the fluorophore decreases [44]. This result indicates that the solvent induces protein denaturation as the organic solvent concentration increases [45]. One possibility for this shift is an interaction of the indole ring from tryptophan amino acid residue with the polar solvent [31]. The Guinn's work [44] have suggested

that the red shift may be caused by increasing in the tryptophan residues exposure to the solvent or by movement of polar groups within the protein closer to the tryptophan side chain [44]. Moreover, this energy shift may be occurred due to both hydrogen bonding interactions with the imino group and the general effects of solvent polarity [46,47].

The results presented in Fig. 1C were plotted from the maximum fluorescence intensity obtained from the emission scan curves as a function of ethanol concentration. In Fig. 1C, an increase in fluorescence intensity signal was observed as ethanol concentration increased. This increasing becomes significant from the solution containing 70% v/v of ethanol. This result is in excellent agreement with Guinn and cols [44], who have suggested that structural changes in commercial trypsin begins to occur between 60% and 70% organic solvent. The fluorescence emission increasing versus ethanol concentration was unusual since several papers show that the process of protein denaturation is followed by a decrease in the fluorescence signal as the denaturant is added [31–33,45,48]. Evaluating these result, it was hypothesized that there was probably the formation of an aggregate [49] or an intermediate state [50], but it is hard to draw conclusions based only on fluorescence intensity since many factors may influence these intensity changes, such as solvent polarity and concentration of quenching species [44]. Then, in order to elucidate the formation of an aggregate or an intermediate state other spectroscopic tests were performed.

3.3 Hydrodynamic radius

In order to understand what have happened when organic solvent was added to α-trypsin aqueous solution, it was performed measures by DLS [51]. Fig. 2 shows the particle size distribution as a function of ethanol concentration. In both the buffer and 80% v/v ethanol solutions, there is only a single particle size, that is, a monotonic profile. However a polytonic distribution was observed in the system containing 20% v/v ethanol, being the population composed of three particle sizes, with the main population presenting radius of 1,400 nm. Analyzing the particle size variation in the protein system, it can be observed that the particle average size increased 1,308 times when ethanol was added to the system. In turn, the difference between systems containing different organic solvent concentrations has resulted in an

increase of only 3 times between the lowest and the highest concentration of ethanol used in this work.

The insert of Fig. 2 shows a considerable sharp increase in the particle diameter when α-trypsin is solubilized in aqueous-organic systems containing 20% v/v of ethanol, suggesting a supramolecular process. As the difference in particle size between α-trypsin states in presence of 20% v/v and 80% v/v ethanol was low, it was not necessary to perform tests at other concentration ranges. Work of Martins and cols [52] showed that the variation of hydrodynamic radius between native and denatured states for α-trypsin was only 0.97 nm. Considering the results of Martins and cols [52], our results could not be considered as a simple dimerization or loss of native structure, as such processes would not be able to cause such a diameter change observed in this work. However, the DLS results (Fig. 2) don't allow us to distinguish whether the change seen is related to an aggregate or multimeric state of α-trypsin [53].

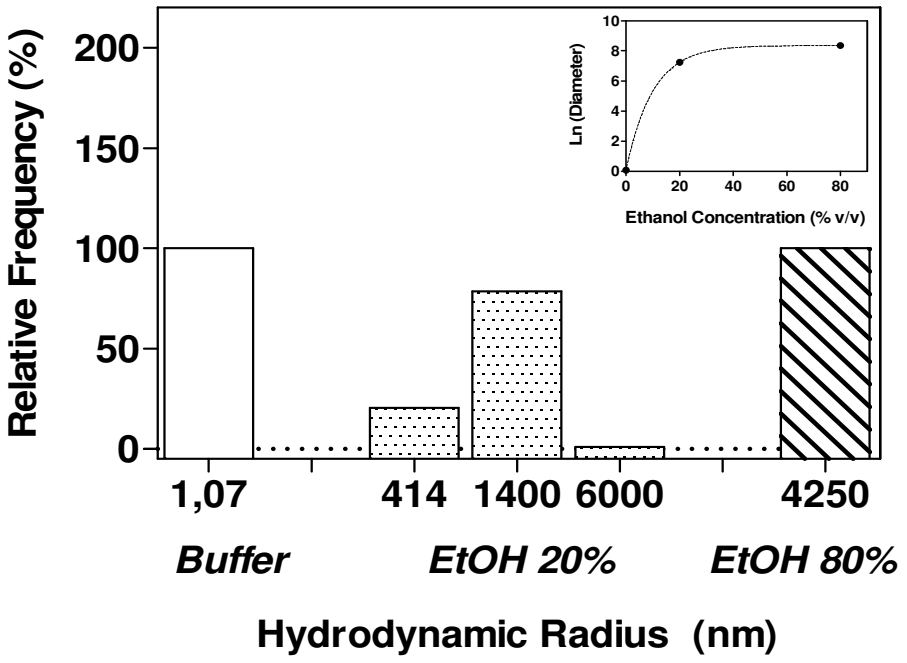

Fig. 2: DLS of α-trypsin isoform in different organic solvent concentration. *Main figure*: Distribution profile of supramolecular states versus ethanol concentration (% v/v). The value of percent intensity is shown along with the hydrodynamic radius obtained by DLS data. DLS measurements performed at 25 ºC with c = 0.5 mg.mL-1 of α-trypsin. *Insert*: Evolution of α-trypsin hydrodynamic radius versus ethanol concentration (% v/v). The particle size is expressed in neperian logarithm (ln).

Thus, based on our results, it was raised two hypotheses: α-trypsin formed insoluble aggregates molecules, which are characterized by irreversible denaturation and loss of enzymatic activity; or the formation of soluble agglomerates of α-trypsin monomers without impair to their enzymatic activity. Studies involving α-chymotrypsinogen, which is also a serine-protease, show the formation of aggregates through chain polymerization mechanism when both pH and ionic strength present low values [54]. For other proteins under these same conditions (low pH and ionic strength value), Fink and cols found a formation of intermediates that form multimeric soluble aggregates and have a high content of defined secondary structure, a condition known as “aggregation of partially folded intermediates” [55]. Comparing the result of Bittar and cols [42] to the work conditions performed by our group, there wasn’t aggregation for trypsin isoform at protein concentration range tested.

3.4 Secondary structures percentage of α-trypsin in aqueous-organic media

Circular Dichroism spectroscopy technique was used to monitor changes of secondary structure content in order to find out aggregation signature [38,56] in α-trypsin as ethanol concentration increases. Our CD results for α-trypsin in aqueous system are in good agreement with other works for the same isoform [18]. Foucault et cols [10] worked with the isolated isoform and observed values different from ours, but the pH system (pH 2.0) was more acidic than we have used.

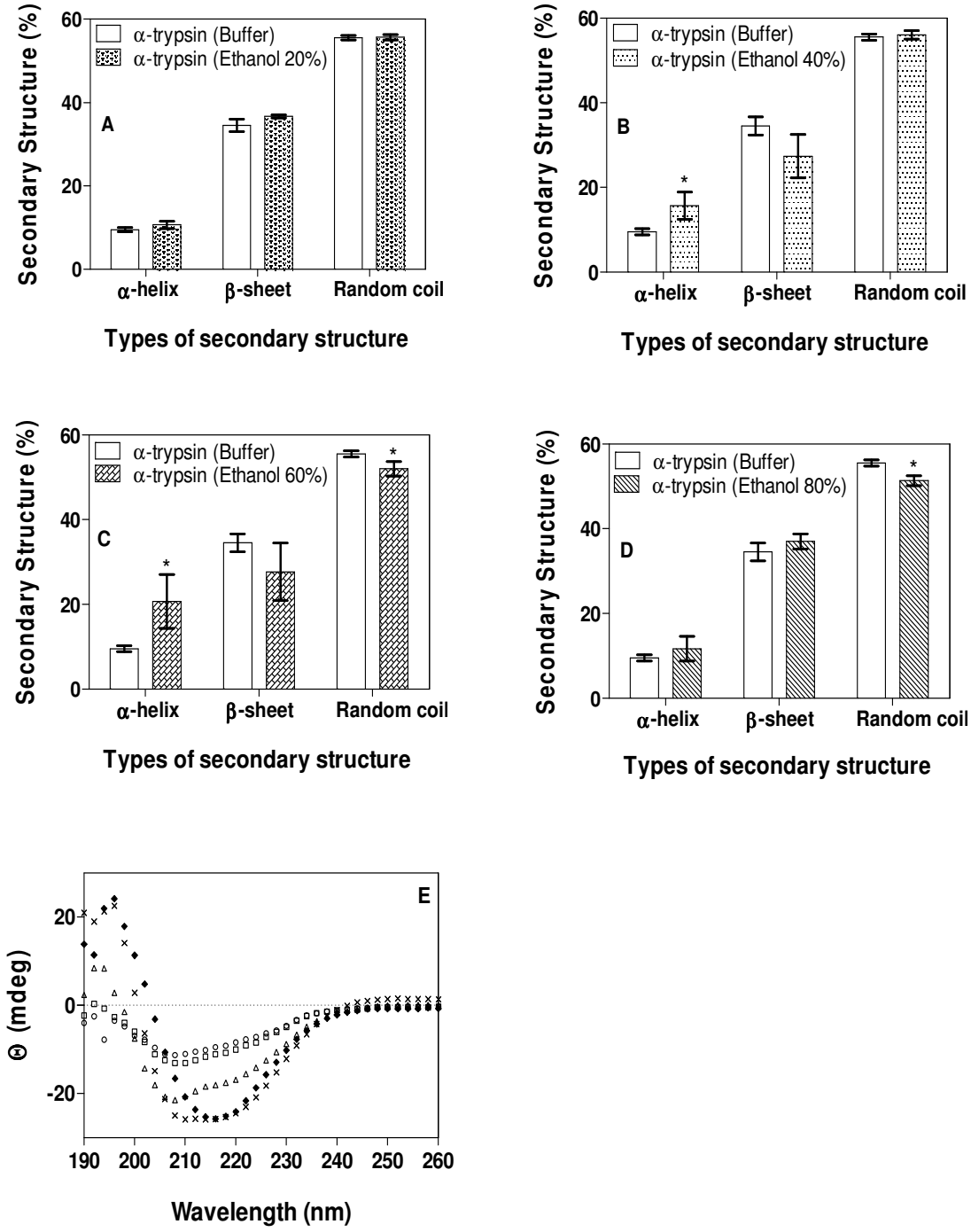

Fig. 3: Secondary structure distributions of α-trypsin secondary at function ethanol concentrations monitored by far-UV Circular Dichroism are represented in figures A-D. Figure E shows CD spectra of α-trypsin at only buffer system (open circle) and at different ethanol concentrations: 20% v/v (open square), 40% v/v (open up triangle), 60% v/v (x) and 80% v/v (solid lozenge). CD measurements were performed at 25 ºC with c = 0.5 mg.mL^{-1} of α-trypsin in 30 mmol.L^{-1} of KCl pH 3.0. Each bar represents the mean ± S.D. (n = 3). The graphic symbols denote the significance levels when compared with control groups. Significantly different from control: *P<0.05.

The Fig. 3 shows the CD results for α-trypsin in buffer system and in systems containing 20% v/v, 40% v/v, 60% v/v and 80% v/v ethanol/buffer. In each figure, there is a comparison of the percentage changes of secondary structure for systems with organic solvent and systems containing only buffer. α-trypsin in 20% (v/v) ethanol/buffer (Fig. 3A) does not present significant alteration in the percentages of secondary structure when compared with buffer system. On the other hand, in 40% and 80% (v/v) ethanol/buffer (Fig. 3B and 3D), α-trypsin showed significant percentage changes in α-helix and random coil contents, respectively. At 60% (v/v) ethanol/buffer (Fig. 3C) α-trypsin presented significant percentage changes in both α-helix and random coil structure. The analysis of systems containing 40% v/v and 60% v/v ethanol/buffer suggests that the addition of organic solvent to the medium induces destabilizing changes of these systems. This affirmation is based on the increase of percentage of α-helix secondary structure seen in Fig. 3B and Fig. 3C compared to the system with buffer, and is confirmed by analysis of raw spectrum (Fig. 3E), that shows a broadening of the maximum negative [20]. According to Griebenow & Klibanov [57], the α-helix content increases because the fraction of the dissolved (and more denatured) protein decreases. However, the percentages in the system containing 80% v/v ethanol/buffer are similar to those of the systems without organic solvent.

This similarity of secondary structure between purely aqueous environment and high ethanol concentration environment was also seen by Simon and cols [20], for both commercial trypsin and α-chymotrypsin. In the Fig. 3E, it can be observed that did not occurred a decrease in both negative and positive maxima, which is a classical signature for intermolecular β-sheet type structures [58,59], although the type of β-sheet can differ, as can be seen by different shapes of the CD spectra [20]. Thus, our results does not demonstrate significant signature of protein aggregation, i.e., an expressive increase in the β-sheet percentage [60,61] and/or no decrease of broad of negative maximum in range of 0 to 60% v/v ethanol/buffer. The general evaluation of the secondary structure percentage results (Fig. 3) points to small conformational changes when organic solvent is added to the system, which is in good agreement with Fink [49] and Mares-Guia [62]. Also, our results are similar to Khurana and cols [50] and to Barnett and cols [54] whose conclusions were that protein unfolding in the

absence of aggregation results in minimal secondary structure changes but significant tertiary structure changes (Fig. 1A).

3.5 Catalytic activity assay in presence of ethanol

The results obtained by CD spectroscopic assay points to formation of partially folded structures instead of aggregation processes when ethanol is added into the medium as seen in previously studies [49,50,54,55]. However, other tests were necessary in order to support this idea and discard totally the aggregation hypothesis. The DLS assay indicates the formation of agglomerated or aggregated structure, but this result does not allow to affirm which of these kind of process has occurred. Based on the knowledge that protein aggregation is generally an irreversible phenomenon [63,64] and that may lead to loss of catalytic capacity, the influence of organic solvent on the aggregate formation was tested by measurements of the activities in the system phase correspondent to the supposed aggregate and supernatant. It is already well known that organic solvent can affect protein structure and stability which may cause, for example, increase or decrease in enzymatic activity [4,65–67], but the causes of this occurrence are multifactorial (Log P [68], dielectric constant and pH [54,55]).

The influence of organic solvent on the $\varepsilon_{p\text{-}NA}$ (product of BA*p*NA catalysis) and $\varepsilon_{\alpha\text{-}trypsin}$ were also taken into account, as in other works [69]. The data analysis of this possible influence showed that the increase of organic solvent concentration in the system affects only the $\varepsilon_{p\text{-}NA}$. Thus, in order to obtain the product (*p*-NA) concentration, it was used the $\varepsilon_{p\text{-}NA}$ obtained experimentally for each organic system. In addition, for eliminating the influence of protein concentration on activity in the supposed pellet and supernatant, the amount of product released was normalized by the protein concentration in each phase.

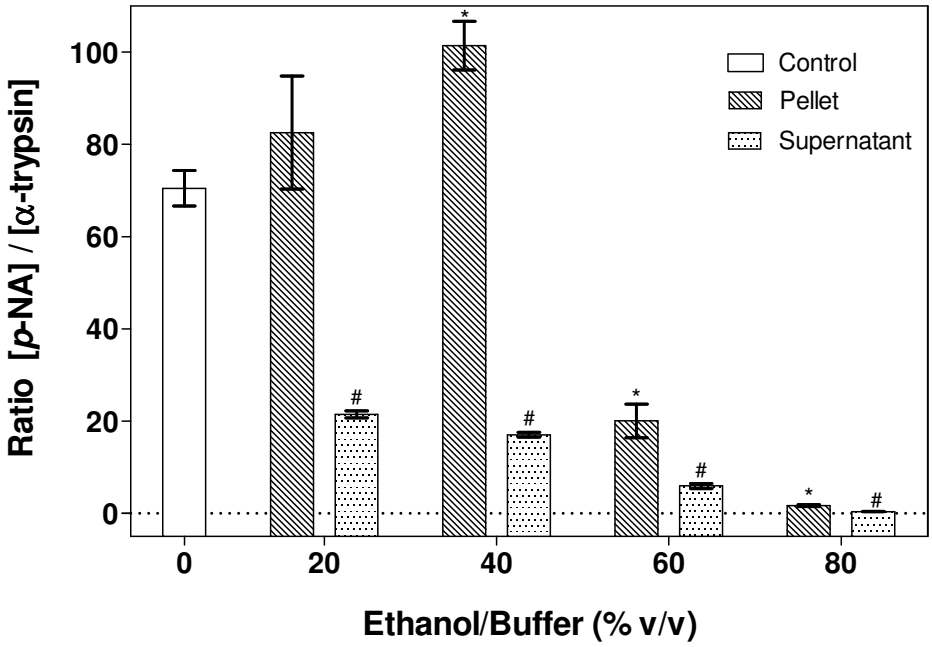

Fig. 4: Catalytic activity of α-trypsin versus ethanol concentration in supernatant and supposed precipitate. Protein concentration was experimentally determined in supernatant and in supposed precipitate. Amidasic product results were normalized for individual concentration found for each ethanol concentration. The control refers to solution with protein but with no ethanol. Each bar represents the mean ± S.D. for triplicate. The graphic symbols denote the significance levels when compared with control groups. Significantly different from control: *P<0.05 for pellet and #P<0.05 for supernatant.

The result shown in Fig. 4 demonstrates the existence of enzymatic activity in the supposed pellet with addition of ethanol/buffer concentration up to 60% v/v. However, two trends about the influence of this organic solvent on the enzymatic activity can be observed: the first one, which ranges from 0 to 40% v/v ethanol/buffer, an increase in enzymatic activity is observed in the supposed pellet; the second one, for values from 60% v/v ethanol/buffer, a decrease in catalytic activity is seen. Kudryashova and cols [70] have studied the ethanol effects on α-chymotrypsin activity and found a profile in excellent agreement with our results showed in FIg. 4. The discrepancy in the catalytic activity of α-trypsin in purely aqueous environments and in those with a high organic solvent concentration (80% v/v) can be explained, from the structural point of view, by the different types of β-sheet [20]. Although the results for CD measurements show an overall similar β-sheet (parallel + antiparallel) content on systems at 0 and 80% v/v ethanol (Fig. 3D), the characteristic of this secondary structure changes according to the medium, what can be seen by the different forms of the CD spectrum (Fig. 3E), and is in agreement with previous work [20,57].

De Diego and cols [64] have also observed a drastic reduction of α-chymotrypsin enzymatic activity as a function of solvent organic addition and they have concluded that the enzyme conformation had probably been modified from the native state to an inactive state with a rigid refolded conformation. Guinn and cols [44] have seen no major changes in the overall structure up to approximately 70% organic solvent, but at higher concentration, they saw a more motional freedom consistent with unfolding of some fraction of surrounding protein, including the active site unfolding. So, the change in the enzymatic activity of α-trypsin could have occurred due a conformational change of amino acids located in the active site or, according to Gorman & Dordick, it may be a result of a physical disruption of the enzyme-bound water which leads the enzyme to deactivation [71].

In addition to the observations made for the supposed pellet, we can also observe that the reduced activity in the supernatant phase can be support by our DLS results (Fig. 2), which indicated the occurrence of an agglomeration process in solution containing only 20% v/v ethanol/buffer. This is directly reflected in the number of units available to perform the catalytic activity: the more clusters are, the fewer units will be available for this purpose. Therefore, the analysis of structural and enzymatic data supports the hypothesis of inexistence of irreversible process in systems containing up to 60% v/v ethanol/buffer, but reinforces the idea of a possible formation of supramolecular structures of α-trypsin isoform which is capable of maintaining high enzymatic activity up to 60% v/v. Above this ethanol concentration, our CD results support the hypothesis of occurrence of process that induce irreversible changes in α-trypsin without aggregate formation.

3.6 Thermodynamic analysis

The thermodynamic stability is directly related to the stability of native protein conformation [72,73] and the thermodynamic parameters were used to understand α-trypsin behavior in organic media system. The thermal protein unfolding profile of α-trypsin in organic solvent (20-80% v/v ethanol/buffer) showed a sigmoidal curve (data not shown), typical of a two-state process denaturation monitored by spectroscopic techniques [37]. The thermal denaturation reversibility was estimated to be >95% for α-trypsin.

Thus, the thermodynamic parameters obtained by of mathematical analysis performed according to methodology presented by Cohen e Pielak [27] are shown in Table 2. *Tm* and *ΔH* parameters vary in a non-linear way when organic solvent is added. Besides that, from 0 to 60% v/v (ethanol/buffer), the values of *Tm* and *ΔH* decreased when compared to the control system, but the values of these parameters increased when the percentage of ethanol raised to 80% v/v.

Table 2: Thermal denaturation of α-trypsin at pH 3.0 obtained by UV-Vis absorption spectroscopy at 280 nm.

Ethanol / Buffer (% v/v)	***Tm*** **(K)** [a]	ΔH_{Tm} **(KJ.mol^{-1})**[b]
0	329.3 ± 0.8	235.39 ± 11.41
40	311.0 ± 1.5	151.75 ± 8.69
60	327.0 ± 1.0	114.06 ± 11.99
80	338.1 ± 0.2	253.07 ± 10.05

[a] *Tm* is the temperature wherein 50% of the protein population is in the folding conformation.
[b] Transition enthalpy at *Tm*. This parameter reflects the denaturation curve shape.

Based on the information that *Tm* is an intensive property of material and that *ΔH* is an extensive one, the analysis of the parameter *Tm* suggests that there was a destabilizing conformational change in the α-trypsin with addition of organic solvent from 0 to 60% v/v, which would justify the Tm value reduction. Furthermore, these data suggest that the buffer-ethanol mixture affected the flexibility of α-trypsin structure, probably due to a weakening of intramolecular interactions or due to a decreasing of solvation layer stability of protein that takes place when enzymes are placed in polar organic solvents [71].

Comparing the ΔH values between organic solvent (up to 60% v/v ethanol/buffer) and buffer systems, it can be observed that there was a significant decrease that may be caused by a loosening of α-trypsin structure, which requires a lower amount of energy for α-trypsin denaturation. In addition to the previous spectroscopic observations, we can also observe that the possibility of enzyme aggregate formation was excluded for the 0-60% v/v ethanol/ethanol systems, since our data did not show the signature of thermodynamic parameters that would describe a protein aggregation, that is, similar Tm values and lower ΔH values were observed when 40% v/v and 60% v/v ethanol/buffer systems are compared to the protein-buffer

system. Comparing 0% and 80% v/v ethanol systems, it can be seen in Table 2 that the Tm and ΔH values for aqueous-organic systems increase significantly in comparison with buffer system, which may be related to increase of strength of weak intramolecular interactions, leading an induction of system stabilization by organic solvent, so that the protein component undergoes a stabilizing conformational change.

Thus, the analysis of the thermodynamic parameters presented in Table 2 suggests that may occur changes in the strength of intramolecular interactions in protein or the remodeling of solvation layer type and concentration-dependent. In addition, the hypothesis of aggregate protein induced by organic solvent can be discard.

4 CONCLUSION

Our studies have shown that addition of ethanol induces modifications in inter- and intramolecular interactions of α-trypsin molecules at pH 3.0. In the intermolecular field, the addition of low concentration of ethanol (up to 50% v/v) is able to modify the biomolecules organization in the system, causing them to become energetically stable in agglomerate shape. The agglomerate showed catalytic activity in system up to 60% v/v ethanol/buffer. In the intramolecular field, the data showed a reorganization of defined secondary structure and a significant change in tertiary structure, suggesting a possible formation of partially folding states of α-trypsin in organic system from 60% v/v ethanol/buffer. The lowering of catalytic activity as a function of ethanol concentration may have occurred due to change in tertiary structure and this may possibly modify the position of catalytic site amino acid residues from 60% v/v ethanol/buffer. Still, the thermodynamic data suggest a loosening of tertiary structure as progressive amounts of ethanol are added to system, causing an increase in the distance between the amino acids residues, which results in a reduction of intramolecular interactions (up to 60% v/v ethanol/buffer). This structure relaxation facilitates the position change of some amino acid residues, including those of the catalytic site. From 60% v/v ethanol/buffer, the α-trypsin structure presents a trend of structure tight, leading to the loss catalytic activity. In this way, the enzymatic activity is compromised in systems with predominant amount of ethanol (from 60% v/v ethanol/buffer). Finally,

the characterization of the α-trypsin pure isoform generated more exact (structural and thermodynamic) data on its properties in aqueous organic media.

Thus, results obtained in this work will enable to use the α-trypsin isoform instead of trypsin commercial preparations in industrial processes making them more efficient and less expensive. Beside provide data for application of trypsin in industrial biotechnological processes which are performed in aquo-organic media.

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PARTE IV

PERSPECTIVA

Após a realização deste trabalho, outras dúvidas surgiram e ficarão como sugestões para futuras investigações:

- Realização de ensaios de FTIR e SAXS para determinar o nível de alteração da estrutura secundária e o formato das partículas aglomeradas por influência do etanol;
- Determinação do tempo de vida ou “Shelf life” da enzima em sistema contendo até 60% v/v de etanol/tampão;
- Determinação completa dos parâmetros termodinâmicos da isoforma α-tripsina em várias concentrações de etanol por calorimetria diferencial de varredura (DSC);
- Estudo da ação de outros solventes orgânicos comumente utilizados em processos industriais sobre os parâmetros estruturais e termodinâmicos da isoforma α-tripsina.