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CURSO DE CIENCIAS BIOLOGICAS

Isolamento, caracterização e estrutura primária de uma nova metaloprotease presente na peçonha da serpente *Bothrops moojeni*

Carolina Petri Bernardes

Monografia apresentada à coordenação do Curso de Ciências Biológicas, Universidade Federal de Uberlândia, para obtenção do grau de Bacharel em Ciências Biológicas.

Uberlândia - MG
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Este trabalho apresentado no formato de artigo científico foi enviado à revista *Bioorganic Chemistry* no mês de fevereiro de 2007

Dedico este trabalho aos meus pais, Raquel e Mário e ao meu irmão Lucas, pessoas extraordinárias, que sempre me apoiaram e foram meus modelos, e sem os quais não teria chegado até aqui. Toda a minha admiração, agradecimento e amor sinceros.

Agradecimentos

Ao professor Fábio, pela oportunidade, acolhimento, pela orientação, paciência, carinho, disposição e tempo. Obrigada pela convivência e por ter acreditado em mim. Obrigada pelas conversas e conselhos, saiba que foram fundamentais para o meu crescimento e que são de grande valia para a minha vida.

A minha amiga Rachel que sempre me deu força, e esteve presente em todos os momentos. Foram quatro anos ótimos.

Aos meus amigos de turma, Neydson, Diego, Leo, Gisela, Franciele, Rubens e Samuel (os agregados da turma), por terem tornado através de sua presença e companheirismo esta etapa da minha vida tão especial.

Aos amigos e colegas de trabalho Lara, Fernanda, No. Pessoas importantes, com quem sempre dividi minhas duvidas, acertos, erros e momentos de diversão no laboratório.

Aos colegas de trabalho do laboratório Patrícia de Castilhos, Patrícia Soares, Kelly, Nadia, Carla, Maraísa, Maira, Breno, Philipe, Jamir e Saulo, pela convivência e pela ajuda.

Isolamento, caracterizaçao, e estrutura primaria de uma nova metaloprotease presente na peçonha da serpente *Bothrops moojeni*.

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RESUMO

Uma protease, denominada Bthα, foi purificada da peçonha da serpente *Bothrops moojeni* utilizando as colunas de cromatografia DEAE Sephacel, Sephadex G-75 e Heparina-agarose. A enzima foi purificada como demonstrado pelo perfil de migração na SDS-PAGE, corado com coomassie blue, e possui uma massa molecular aproximadamente de 24,5 kDa. A estrutura primária da Bthα apresenta significante similaridade com outras metaloproteases isoladas de peçonhas de serpentes. A enzima hidrolisa primeiramente a cadeia Aα do fibrinogênio, seguida pela cadeia Bβ, e não apresenta efeito sobre a cadeia γ. A enzima hidrolisa apenas a cadeia β da fibrina, deixando a cadeia α e o dímero γ aparentemente, inalterados. A enzima Bthα não apresentou atividade fosfolipásica A_2, hemorrágica e coagulante. Semelhante a outras enzimas de peçonha de serpente, a enzima Bthα é estável na faixa de pHs entre 4 a 10, e resistente à temperaturas até 70°C por 15 minutos. O efeito de inibidores como o EDTA sobre a atividade fibrino(geno)lítica sugere que a Bthα é uma metaloprotease, e a inibição por β- mercaptoetanol revela a importância

das pontes dissulfeto para a estrutura e sua estabilidade. Aprotinina e benzamidina, inibidores específicos de serino protease, não apresentaram efeitos sobre a atividade enzimática da Bthα. Desde que a enzima Bthα foi descoberta como desfibrinogenante, quando administrada intraperitonealmente em camundongos, é esperado que a ela se torne de interesse médico como agente terapêutico, auxiliando no tratamento e prevenção de trombose arterial.

Palavras-chave: Peçonha de serpente, *Bothrops moojeni,* metaloprotease, fibrinogenase.

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Lista de Abreviaturas

AMBIC: tampão bicarbonato de amônio;

EDTA: Acido etilenodiaminotetracético;

PAGE: Eletroforese em gel de poliacrilamida;

SDS: Dodecil sulfato de Sódio;

SVMP: Metaloprotease de peçonha de serpente;

TEMED: N,N,N`,N`-tetrametiletilenodiamino;

TCA: Ácido tricloroacético

1 Introdução

As peçonhas das serpentes pertencentes às famílias Viperidae e Crotalidae contêm uma grande variedade de proteínas e peptídeos que afetam o sistema homeostático. Essas proteínas podem ser classificadas como coagulantes, anticoagulantes e fibrinolíticas [1] e [2]. O Envenenamento por estas serpentes resulta, geralmente, em sangramento persistente porque a peçonha causa considerável degradação do fibrinogênio e de outros fatores de coagulação, impedindo a formação do coágulo [3]. Os principais componentes da peçonha de serpente responsáveis por estas atividades são as metaloproteases [4], as quais vêm sendo purificadas e caracterizadas de peçonhas do gênero *Bothrops*, incluindo as espécies *Bothrops asper* [5] e[6] *Bothrops atrox* [7] *B. lanceolatus* [8] *B. jararaca* [9] e [10], *B. jararacussu* [11], *B. alternatus.* [12] e *B. neuwiedi* [13] e [14]

Como descrito previamente [15], as metaloproteases fibrino(geno)líticas podem ser classificadas tanto como α ou β fibrinogenases. Até agora não há na literatura nenhum relato de fibrinogenase de peçonha de serpente com especificidade de hidrólise dirigida unicamente à cadeia γ do fibrinogênio. A especificidade pelas cadeias Aα- ou Bβ não é absoluta. A degradação da cadeia Aα é geralmente acompanhada pela degradação da cadeia Bβ. A maioria das enzimas fibrinogenolíticas são metaloprotease com especificidade direcionada às cadeias Aα e menor especificidade às cadeias *Bβ* [16].

O envenenamento por serpentes do gênero *Bothrops* é caracterizado pelos danos locais, incluindo edema, dor, hemorragia, necrose, e por distúrbios sistêmicos tais como coágulopatias, hemorragia sistêmica, e distúrbios renais [17], [18], [19] e [20].

Os acidentes ofídicos com serpentes Bothrópicas ocorrem com freqüência nas regiões: centrais e sudeste do Brasil e por todo o domínio morfoclimático do Cerrado [21],

[22] e [23], e pode causar edema, necrose, infecção, e decréscimo da coagulação e hematoma extradural, às vezes fatal [24].

A principal característica de peçonha da serpente *Bothrops moojeni* é a sua alta atividade proteolítica, responsável pela maioria dos efeitos locais e sistêmicos observados durante o envenenamento por esta espécie de serpente. Neste artigo, descreveremos o isolamento, a caracterização bioquímica e estrutural da Bthα, uma metaloprotease presente na peçonha da serpente *Bothrops moojeni*

2 MATERIAL E MÉTODOS

2.1 Material

A peçonha dessecada a vácuo foi obtida no Serpentarium Bioagents (Batatais - São Paulo, Brasil). Acrilamida, persulfato de amônio, aprotinina, benzamidina, azul de bromofenol, EDTA, fibrinogênio bovino, β-mercaptoetanol, *N,N'*-metileno-bis-acrilamida, padrão de peso molecular para eletroforese, PMSF, dodecil sulfato de sódio (SDS), e *N,N,N',N'*-tetrametiletilenodiamo (TEMED) foram adquiridos da Sigma Chemical Co. (St Louis, MO, USA. Glicina. Tris, e todos os materiais para cromatografia foram adquiridos da Amersham Pharmacia Biotech. Todos produtos químicos restantes eram de grau analítico.

2.2 Isolamento da Bthα

A peçonha de *Bothrops moojeni* (200mg) foi ressuspendida em tampão bicarbonato de amônio 50mM (pH 7,8) e centrifugado a 10.000g por 10min. A solução sobrenadante foi cromatografada em coluna DEAE – Sephacel (1.7 x 15cm) previamente equilibrada com

tampão Bicarbonato de amônio 50mM (pH 7,8) e eluída com um gradiente convexo de concentração (50mM – 0.45M) do mesmo tampão.

Frações de 3mL foram coletadas, e suas absorbâncias a λ = 280nm foram monitoradas. As frações correspondentes ao pico D2 foram reunidas, liofilizadas e dissolvidas no tampão bicarbonato de amônio 50mM, pH 7,8 e aplicada na coluna Sephadex – G75 (1 x 100cm) previamente equilibrada com o mesmo tampão. A fração fibrinogenolítica (pico D2G2) foi liofilizada e aplicada em coluna de Heparina – Agarose (1.0 x 10cm) previamente equilibrada com tampão Tris-HCl (10mM) + $CaCl_2$ (5mM) pH 7,0 e eluida com tampão Tris-HCl (10mM) + NaCl (1M) pH 7,0. O fluxo foi de 40mL por hora e frações de 2mL foram coletadas.

2.3 Estimativa da concentração protéica

A concentração protéica foi déterminada pelo método de [25], utilizando soro albumina bovina como padrão.

2.4 Análise eletroforética

Eletroforese utilizando gel de poliacrilamida a 14% (SDS-PAGE) foi realizada conforme descrito por [26]. A eletroforese foi realizada a 20mA por gel em tampão Tris Glicina pH 8,3 contendo 0,01% SDS. Os padrões de massa moleculares utilizado foram: fosforilare b (97 kDa), soro albumina bovina (66 kDa), ovoalbumina (45 kDa), anidrase carbônica (30 kDa), inibidor de tripsina (20.1 kDa) e α-lactoalbumina (14.4 kDa). Os géis foram corados com coomassie brilhante blue. A massa molecular relativa à enzima purificada foi estimada pelo software de análise de imagem Kodak 1D. A Bthα também foi

submetida a espectrometria de massa utilizando o espectrômetro *Q.TOF MicroTM (Micromass, UK).*

2.5 Déterminação da Estrutura Primária

A proteína em seu estado nativo foi reduzida e alquilada com vinil piridina de acordo com o método descrito por [27]. A amostra foi dissolvida em 1mL de guanidina–HCl 6M em Tris HCl 0,1M, pH 6,8. Nesta solução foi adicionado 30μL de β-mercaptoetanol sob nitrogênio e a amostra foi incubada a 50°C por 4 h. Posteriormente, 40 μL de vinil piridina foi adicionada e as amostras foram incubadas a 37°C durante 2h. A proteína foi desalificada em uma coluna de Vydac C-4; utilizando um gradiente de 0 a 70% de acetonitrila em 0,1% TFA por 90mim com um fluxo de 1mL por minuto. A proteína coletada foi liofilizada e digerida com tripsina. Os peptídeos resultantes foram separados em Vydac C18 Small Pore e submetidos ao seqüenciador automático.As seqüências de aminoácidos obtidas foram comparadas com seqüências de outras proteínas relatadas no banco de dados SWISS-PROT/ TREMBL utilizando os programas FASTA 3 e BLAST.

2.6 Atividades Proteolíticas

2.6.1 Atividade fibrinogenolítica

A atividade fibrinogenolítica foi testada como descrito por [27], com algumas modificações. O fibrinogênio (1,5mg/mL) e a enzima (5μg) foram misturados 1:100 (m/m) e a mistura foi incubada com tampões em diferentes pHs (4.0 – 10.0) a 37°C e por diferentes intervalos de tempo (5, 10, 15, 30, e 60 mim). A reação foi interrompida pela

A dose desfibrinogenante mínima (DDM) foi definida como a quantidade de enzima mínima capaz de tornar o plasma incoagulável.

2.8 Atividade Hemorrágica

A atividade hemorrágica foi testada segundo o método descrito por [29]. Soluções contendo 30ug da enzima Bthα foi injetada na pele dorsal de camundongos subcutâneamente. A pele do camundongo foi retirada 3hs depois, e o diâmetro do halo hemorrágico, da superfície interna da pele, foi medido.

2.9 Estimativa da concentração do fibrinogênio

A estimativa da concentração do fibrinogênio foi determinada pela injeção intraperitonealmente da enzima purificada (2.0μg/g peso corporal) dissolvida em 200μL de salina em grupos de cinco animais. Após uma hora, os animais foram anestesiados com Eter e o sangue foi retirado por punção cardíaca e armazenados em tubos na presença do anticoagulante (EDTA). Os sangues dos animais foram misturados e centrifugados a 3.000g por 10 minutos. O plasma foi removido e a concentração do fibrinogênio foi quantificada pelo aparelho analyzer compact. Camundongos controle receberam 200uL de salina.

2.10 Estabilidade térmica.

Bthα e fibrinogênio dissolvidos em tampão Tris-HCl 50mM pH 8,0, foram incubados por 15 minutos 0 a 70°C. A atividade fibrinogenolítica remanescente foi determinada como descrito anteriormente.

2.11 Inibidores enzimáticos

A inibição da atividade fibrinogenolítica foi determinada incubando Bthα (5μg) em 0,25mL de tampão Tris-HCl 50mM pH 8,0 por 15 minutos a temperatura ambiente (25°) contendo um dos seguintes inibidores: EDTA (5mM), aprotinina (5mM), benzamidina (5mM), e β-mercaptoetanol (5mM). A atividade proteolítica foi determinada como descrito anteriormente.

3 RESULTADOS

3.1 Isolamento e caracterização da Bthα

A cromatografia da peçonha de *Bothrops moojeni* em coluna de DEAE-Sephacel (fig. 1A) resultou na separação de sete frações protéicas (picos D1 – D7). As proteínas presentes na fração D2 apresentaram substancial atividade proteolítica. O segundo passo cromatográfico realizado com a fração protéica D2 em coluna Sephadex–G75 (Fig. 1B) resultou em três picos principais. O pico D2G2 deste fracionamento foi aplicado na coluna heparina-Agarose (Fig.1C). A (Bthα) demonstrou atividade proteolítica sobre a azocaseína. A enzima Bthα apresentou atividade fibrinolitica e fibrinogenolítica, mas não apresentou ações coagulante, fosfolipásica A_2, e hemorrágica.

Um resumo do processo de purificação está mostrado na tabela 1. Considerando a atividade proteolítica sobre a azocaseína, a enzima Bthα foi purificada 1.08 vezes com uma recuperação de 8,7% (m/m). A análise eletroforética (SDS-PAGE) (Fig.2) sob condições desnaturantes e redutoras, indicaram que a enzima Bthα foi altamente purificada (Fig 1C), e possui um massa molecular aparente de aproximadamente 24,5 kDa. A análise no espectrômetro de massa da Bthα indicou a massa molecular de 23.095 Da.

A estrutura primária da Bthα determinada automaticamente pela seqüência de aminoácidos produzidos pela digestão da proteína com tripsina e *S*-pyridyl-ethylated está mostrada na Fig. 3. A seqüência apresenta 72% de similaridade com metaloproteases isoladas de peçonhas de *B. leucurus* e com a hemorragina factor II de *L. Muta;* 77% de similaridade com uma metaloprotease isolada do veneno de *B. jararacussu* [30], e com uma metaloprotease Tipo II da peçonha de *B. asper* (Arce V., Azofeifa G., Flores M., Alape A.; http://ca.expasy.org/uniprot/Q072L5). A enzima Bthα apresentou significante similaridade com várias outras metaloproteases, de acordo com o banco de dados SWISS-PROTEIN analisado pelo programa FASTA 3.

3.2 Atividade proteolítica

A tabela 1 mostra os valores obtidos pela atividade proteolítica sobre a azocaseína em pH 8,0. A Bthα apresenta atividade sobre azocaseína, com uma atividade específica de 192,3 unidades/mg de proteína, comparada com 178,1 unidades/mg de proteína de veneno bruto.

A Bthα se mostrou ativa degradando o fibrinogênio bovino, causando uma rápida hidrólise das cadeias Aα e Bβ em 10 minutos de incubação, como demonstrado pelo perfil eletroforético da atividade (Fig 4A). Enquanto a concentração de proteínas de alto peso molecular decresce com o tempo de reação, a concentração de produtos de baixo peso molecular aumenta substancialmente. A ação da Bthα sobre fibrina mostrou-se ser menos efetiva do que sobre o fibrinogênio. A Bthα degrada a cadeia β, enquanto as cadeias α- e o dímero γ- não são afetados durante o tempo testado (Fig 4B).

3.3 Atividade de Desfibrinogenação

A enzima Bthα causou desfibrinogenação quando administrada intraperitonealmente em camundongos, tornando o plasma incoagulável. Os níveis de fibrinogênio reduziram a zero após uma hora da inoculação (DDM = 2,0μg/g peso corporal); enquanto os animais controle apresentaram 270mg de fibrinogênio por dL de plasma. Os níveis normais de fibrinogênio foram restabelecidos apenas 24 horas depois.

3.4 Efeito dos inibidores de protease

A figura 5 mostra o efeito de diversos inibidores sobre a atividade proteolítica da Bthα. A incubação da enzima purificada por 15 minutos a 37°C com EDTA (5mM), um agente quelante de íons metálicos, inibiu completamente a atividade fibrinogenolítica, indicando que a Bthα é uma protease do tipo metalo. A atividade fibrinogenolítica da enzima foi inibida também pelo β-mercaptoetanol (5mM), entretanto benzamidina e aprotinina (5mM) não apresentaram nenhum efeito de inibição sobre a atividade.

3.5 Estabilidade Térmica

Como mostrado na figura 6, a enzima Bthα exibiu atividade fibrinogenolítica máxima na faixa de temperatura que vai de 20°C a 50°C. Aparentemente, a cadeia Bβ-do fibrinogênio não sofreu proteólise na faixa de temperatura entre 0 a 10°C e 60 a 70°C. Nestas temperaturas a enzima Bthα degradou parcialmente a cadeia Aα-do fibrinogênio. O pH ótimo para a atividade fibrinogenolítica da Bthα é pH 8,0. Entretanto, a enzima é ativa na faixa de pH que vai de 4 a 10 (dados não mostrados).

4 DISCUSSÃO

Proteases de peçonha de serpente são de grande interesse médico, por serem responsáveis por vários efeitos sistêmicos e locais do envenenamento por acidentes ofídicos. A purificação de uma metaloprotease da peçonha de *Bothrops moojeni* (protease A moojeni) foi informada por [31], que demonstrou que esta peçonha possui uma significante quantidade de proteínas básicas, a enzima coagulante batroxobin [32], três serino proteases (MSP1 e MSP2 [33] e MOO3 [34]), e uma metaloprotease (MPB) [35]. BM-PLA2, uma fosfolipase A2 [36] e duas miotoxinas de baixo peso molecular foram isoladas da peçonha da serpente *Bothrops moojeni* [37], [38] e [39].

Neste trabalho, uma nova metaloprotease, Bthα, da peçonha de *Bothrops moojeni* foi purificada por uma combinação de cromatografias de troca iônica (DEAE-Sephacel), gel filtração (Sephadex–G75) e afinidade (Heparina-Agarose). A massa molecular da enzima Bthα analisada em SDS-PAGE (24.5 kDa) e no espectrômetro de massa (23.095 Da) foi similar a de 27 e 22,5kDa relatada por MOO3 e Protease A moojeni, respectivamente [34] e [31], e a metaloproteases de 23, 24, e 28kDa presente em venenos de *B. atrox* [40], *B. asper* [41], e *B. Lanceolatus* [8], respectivamente. Semelhantemente à MSP2, protease A moojeni e MOO3, a enzima Bthα apresentou uma única banda, na presença e ausência de β-mercaptoetanol, como também relatado por fatores hemorrágicos de baixo peso molecular [42].

A estrutura primária da Bthα deduzida, a partir de peptídeos resultantes da digestão com tripsina está demonstrada na Figura 3, onde é exibida similaridade significante com outras metaloproteases purificadas de peçonhas de serpentes. Esses resultados confirmam que a Bthα é também uma metaloprotease. Os peptídeos da hidrólise da Bthα podem ser

alinhados em aproximadamente 80% com as seqüências de outras metaloproteases proveniente de peçonhas de serpente.

A enzima Bthα não causou hemorragia quando aplicada (30μg) subcutaneamente no dorso do camundongo (dados não mostrados). Baseado nos domínios estruturais, as metaloproteases de peçonha de serpente, (SVMPS) são classificadas em quatro classes: P-I a P-IV [43]. As da classe P-I possui somente o domínio metaloproteases; a classe P-II possui também o domínio desintegrina- like em sua estrutura, a classe P-III possui uma terceira característica um domínio rico em cisteína. A classe P IV possui, em adição as características da classe P-III, ligações-dissulfeto, e o domínio lectina-like. A subclasse de proteínas P-1A apresenta intensa atividade hemorrágica, enquanto proteína da subclasse P-1B apresenta pequena, ou nenhuma atividade hemorrágica e incluí Neuwidase da peçonha de *B. neuwiedi* [14] e MOO3 da peçonha de *B. moojeni* [34]. Nossos resultados sugerem que a Bthα pertence a classe P-1B.

Bthα apresentou atividade proteolítica sobre azocaseína, com uma atividade específica de 192,3 unidades/mg de proteína. Considerando esta atividade a enzima Bthα foi purificada 1.08-vezes. A enzima representa 8.7% do total protéico da peçonha (Tabela I). O rendimento apresentado pela enzima Bthα foi maior do que resultados apresentados de outras enzimas fibrinogenolíticas purificadas da mesma peçonha. Os dados apresentados pelas enzimas MSP1, MSP2 e MOO3 foram 0,51; 1,1 e 1,42%, respectivamente. Aparentemente, a Bthα apresentou uma atividade específica baixa quando comparada com a peçonha bruta. Este fato pode ser explicado porque a Bthα não é a única enzima com ação proteolítica presente na peçonha da serpente de *Bothrops moojeni.* De fato, praticamente todas as frações obtidas pela DEAE-Sephacel e Sephadex-G75 foram capazes de hidrolisar a azocaseína (dado não mostrado).

Assim como outras fibrinogenases da peçonha de *Bothrops moojeni* (MSP1 e MSP2 [31] e MPB [32]) a Bthα hidrolisa preferencialmente a cadeia Aα acompanhada pela hidrólise da cadeia Bβ do fibrinogênio. Estas proteases diferem da Bthα porque degradam a cadeia γ, algo que não é muito comum em muitas proteases da peçonha. O coágulo de fibrina é mais resistente à hidrólise pela Bthα do que o fibrinogênio. Apenas a cadeia β foi degradada, enquanto as cadeias α e o dímero γ permaneceram sem alterações (Fig 4B). Estes resultados podem ser explicados por que o fibrinogênio contem uma grande superfície de aminoácidos que são expostos à enzimas proteolíticas. A fibrina, entretanto possui uma estrutura entrelaçada e por isso é menos susceptível à proteólise [44].

A estrutura primária parcial da enzima Bthα e o efeito do agente quelante na atividade proteolítica confirmou que a Bthα é uma metaloprotease. A análise da seqüência indicou que a enzima Bthα possui seis resíduos de cisteína, que podem estar envolvidos na formação de três pontes disulfeto. As pontes disulfeto internas também se mostraram importantes para atividade enzimática e para a integridade estrutural da Bthα. A incubação da enzima Bthα com 5mM de β-mercaptoetanol resultou na inibição da hidrólise do fibrinogênio pela Bthα. Aprotinina e benzamidina não influenciaram na inibição da atividade enzimática. Semelhante a outras proteínas isoladas de peçonha de serpentes [45], [46] e [11] a enzima Bthα não é estável a altas temperaturas (Fig 6) e mostrou um pH ótimo em torno de 4 – 10 (dados não mostrados).

In vivo, a enzima Bthα age como um agente desfibrinogenante, removendo fibrinogênio do plasma. Com minutos após a administração da enzima, ocorreu uma depleção na concentração do fibrinogênio no plasma, e com uma hora os níveis de fibrinogênio foram reduzidos a zero e o plasma permaneceu incoagulável por até 24 horas. Este resultado sugere o uso potencial da Bthα como um agente anticoagulante e

antitrombótico. A sugestão como anticoagulante, com remoção total do fibrinogênio do plasma circulante, por meio de enzimas fibrinogenolítica presentes em peçonhas de serpentes ocorreu pela primeira vez em 1968 [47]. Estudos sobre o mecanismo de ação das enzimas fibrinogenolíticas têm revelado que elas causam uma rápida e eficiente atividade anticoagulante e antitrombótica, em outras palavras, elas desfazem a formação do coágulo de fibrina, quando ocorre disfunção do sistema homeostático. Por esta razão, estas enzimas vêm sendo de grande interesse para a clinica médica, especialmente aquelas com um alto grau de pureza e livre de outros efeitos farmacológicos.

A ação de proteases que podem interferir na coagulação e no sistema fibrino(geno)lítico seguido do envenenamento por picadas de serpentes do gênero *Bothrops* têm sido relatadas [48]. Inúmeras dessas proteínas vêm sendo usadas pela medicina como anticoagulantes ou em pesquisas para investigar seus possíveis potenciais terapêuticos [49], [50] e [51]. Por esta razão, a metaloprotease fibrino(geno)lítica, Bthα, vislumbra-se como uma ferramenta por sua importância enzimática como anticoagulante e antitrombótica, e por ajudar a entender melhor o mecanismo de ação dos venenos das serpentes do gênero *Bothrops*.

Em conclusão, nós purificamos uma metaloprotease, Bthα, com atividade azocaseinolítica, fibrino(geno)lítica e desfibrinogenante da peçonha da serpente *Bothrops moojeni*. As propriedades da Bthα indicam que esta proteína provavelmente pertence à classe das P1-B α SVMPs. Este trabalho sugere que esta toxina pode interferi na cascata de coagulação após o envenenamento.

Legenda das figuras

Figura 1: Purificação da Bthα da peçonha de *Bothrops moojeni:* (A) Separação em resina DEAE-Sephacel – a peçonha bruta (200mg) foi aplicado na coluna (1.7 x 15cm) e eluida a um fluxo de 20 mL por hora com gradiente convexo de tampão Bicarbonato de amônio pH 7,8 de 0,05M a 0,45M. (B): Separação em resina Sephadex-G75; a fração D2 foi aplicada na coluna (1.0 x 100cm) e eluída com tampão bicarbonato de amônio pH 7,8 e coletada a um fluxo de 20mL por hora. (C): Separação em resina Heparina- Agarose: A fração concentrada (D2G2) foi aplicada na coluna (1.0 x 10cm) e a eluição foi realizada a um fluxo de 40mL por hora com um tampão Tris-HCL 0,01M contendo 1.0M de NaCl pH 7.0. Os "pools" foram delimitados por círculos fechados.

Figura 2: Estimação da massa molecular usando gel de poliacrilamida SDS PAGE a 14% (m/v), Tris-glicina pH 8,3 e 20mA. Linha 1- padrão de peso molecular, 2- Peçonha bruta *B moojeni* reduzida, 3- Bthα reduzida. O gel foi corado com coomassie brilhante blue R-250.

Figura 3: Comparação da seqüência da Bthα com outras metaloproteases de peçonha de serpentes. Os resíduos de Cisteína estão em vermelho. MS: Massa Molecular. Mostra o número de seqüências depositadas no banco de dados Swiss-Prot/Trembl.

Figura 4: Hidrólise de (A) fibrinogênio bovino (1,5mg/mL) e (B) fibrina pela enzima Bthα. Linha 1: controle incubado sem a enzima por 60 minutos; 2 –6: controle incubado com a enzima (5 μg) por 5, 10, 15, 30 e 60 minutos, respectivamente.

Figura 5: Hidrólise do fibrinogênio bovino pela enzima Bthα (5 μg) e o efeito de inibidores. Linha 1 : fibrinogênio controle incubado sem a enzima por 60 minutos; 2 – 5: fibrinogênio apos incubação com Bthα e 5mM β-mercaptoetanol, 5 mM EDTA, 5 mM aprotinina e 5 mM benzamidina por 60 minutos respectivamente

Figura 6: Efeito da temperatura na estabilidade da enzima Bthα. Alíquotas de Bthα e fibrinogênio foram incubadas em tampão Tris 0.05M pH 8,0 e atividade fibrinogenolítica foi testada em diferentes temperaturas por 15 minutos, previamente descrita. Linha 1: fibrinogênio controle incubado sem a enzima por 15 minutos; 2- 6 fibrinogênio controle incubado com a enzima por 15 minutos a 0, 10, 20, 30, 40, 50, 60, 70 °C, respectivamente.

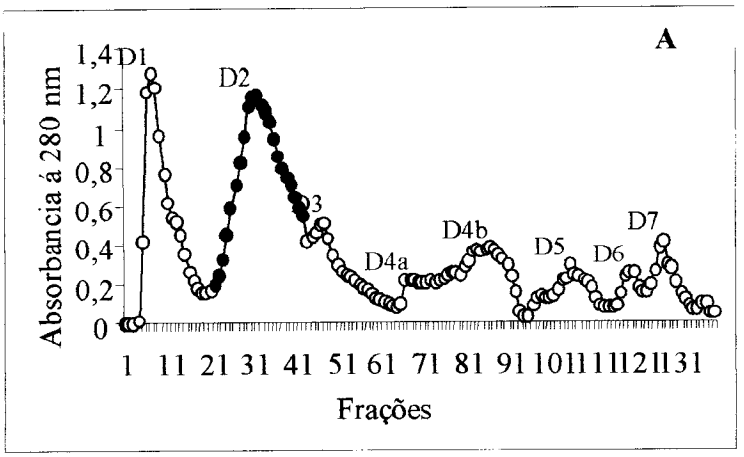
Tabela 1. Resumo dos passos de purificação da enzima Bthα da peçonha de *Bothrops moojeni*

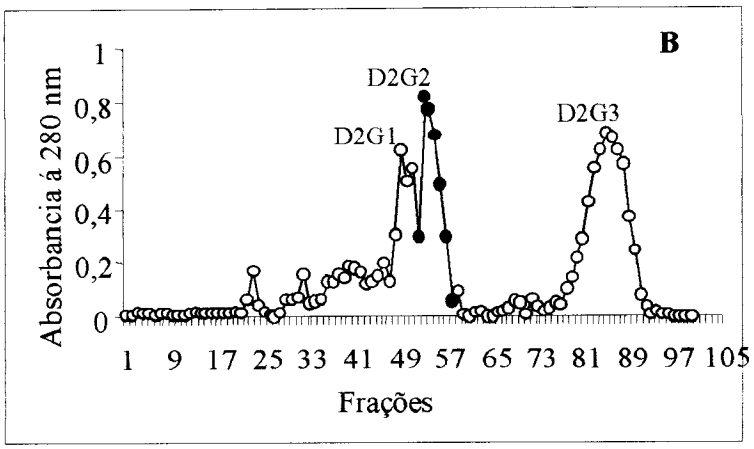
Tabela 1: Resumo dos passos de purificação da enzima Bthα da peçonha de *B. moojeni*:

Amostra	Proteínas totais (mg)	Recuperação (%)	Atividade total (units)*	Atividade específica**	Grau pureza
Peçonha bruta	200.0	100.0	35,620	178.1	1.00
D2	44.70	22.35	8,415	188.2	1.06
D2G2	22.07	11.03	3,244	147.0	0.82
Bthα	17.43	8.71	3,294	192.3	1.08

* A atividade proteolítica foi testada utilizando a azocaseína como substrato.
**Uma unidade de atividade proteolítica foi medida como o aumento de 0.01 unidades de absorbância à 366 nm.

FIGURA 1


A
Absorbancia á 280 nm
1,4
1,2
1
0,8
0,6
0,4
0,2
0
D1
D2
D3
D4a
D4b
D5
D6
D7
1 11 21 31 41 51 61 71 81 91 101 111 121 131
Frações


B
Absorbancia á 280 nm
1
0,8
0,6
0,4
0,2
0
D2G1
D2G2
D2G3
1 9 17 25 33 41 49 57 65 73 81 89 97 105
Frações

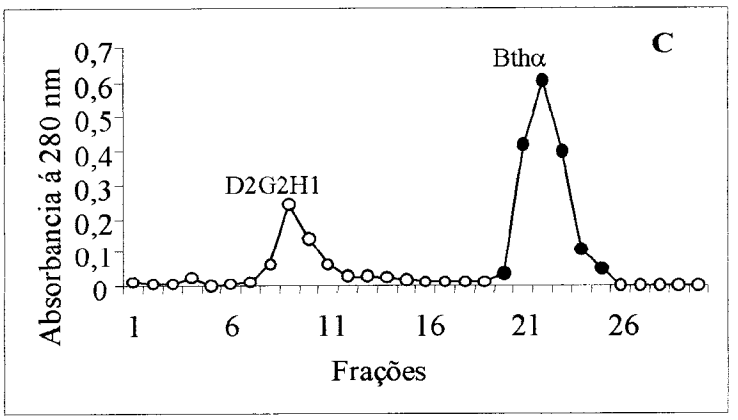

C
Absorbancia á 280 nm
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
D2G2H1
Bthα
1 6 11 16 21 26
Frações

FIGURA 2

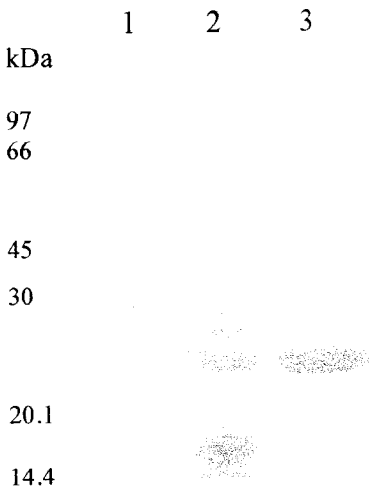
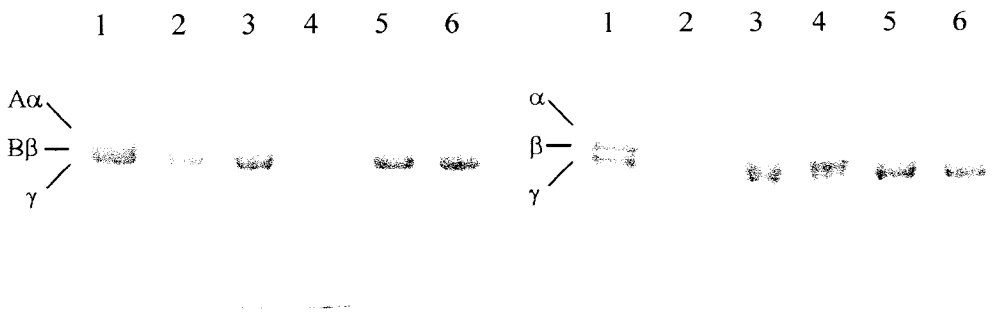


FIGURA 3

Proteína/fonte	Seqüência	MS (Da)	Similaridade (%)	REF
	1 25 50 75			
Btha/*B. moojeni*	FSPRHIELVVVADHGMFKKYNSNLNTIRKWVHEMVNSMNGFYRSVDVTASLANLEVWSKXXXXXXXXXXXXXXXS			
B.jararacussu	KMCGVTETNWESYEPIKKASPSNLTPEQQKFSPRYIEVAVVADHRMFKKYNSNLNTIRKWVHEMVNSMNGVYRSMDVHLSLANLEVWSKKDLINVQKDSRETLKS			
B.asper	QQRFSPRYIELAVVADHGIFTKYNSNLNTIRTRVHEMLNTVNGFYRSVDVHAPLANLEVWSKQDLIKVQKDSSKTLKS			
B. Leucurus	SPRYIELVVVADHGMFKKYNSNLNTIRKWVHEMLNTVNGFYRSMNVDASLVNLEVWSKKDLIKVEKDSSKTLTS			
L. muta	FSQKYIELVVVADHGMFTKYNGNLNTIRTRVHEIVNTLNGFYRSLNILISLTDLEIWSNQDLINVQSAANDTLKT			
	100 125 150 175			
Btha/*B. moojeni*	FGEWRXXDLLPRISHDNAQLLTAIVFDGHVIGRAFTGGMCDPRHSVGVVMDHSPKNLQVAVTMAHELGHNLGMHHDGNQCHCDAASCIMADSLXXXXXXX			
B.jararacussu	FGEWRERDLLPRISHDNAQLLTAVVFDQQTIGRAYIAGMCDPRHSVGVVMDHSKENLQVAVTMAHELGHNLGMEHDENQCHCDAPSCVMASVLSVVLSYE			
B.asper	FGEWRERDLLPRISHDHAQLLTAVVFDGNTIGRAYTGGMCDPRHSVGVVRDHSKNNLWVAVTMAHELGHNLGIXHDTGSCSCGAKSCIMASVLSKVLSYE			
B. Leucurus	FGEWRERDLLPRISHDHAQLLTVIFLDEETIGIAYTAGMCDLSQMVAWGQDY------VAVSMAHELGHNLGMRHDGNQCHCNAPSCIMADTLSKGLSFE			
L. muta	FGEWRERVLLNRISHDNAQLLTAIDLADNTIGIAYTGGMCYPKNSVGIVQDHSPKTLLIAVTMAHELGHNLGMKHDENHCHCSASFCIMPPSISEGPSYE			
	200			
Btha/*B. moojeni*	XXXXXXXXXXXXXXXHNPQCILNEP	23.095		
B.jararacussu	FSDCSQNQYQTYLTKHNPQCILNEPLLTVSGNELLEAGE	41.957	77	UNIPROT Q7T1T4_BOTJR
B.asper	FSDCSQNQYETYLTNHNPQCILNKP	22.857	77	UNIPROT BAP1_BOTAS
B. Leucurus	FSDCSQNQYQTYLTKHNPQCILNKP	22.003	72	UNIPROT LEUCA_BOTLC
L. muta	FSDCSKDYYQMFLTKRKPQCILNKP	22.596	72	UNIPROT Q90391_CROAT

FIGURA 4



A **B**

FIGURA 5

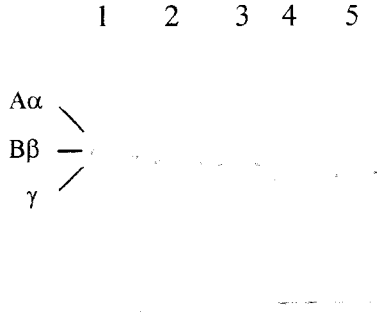
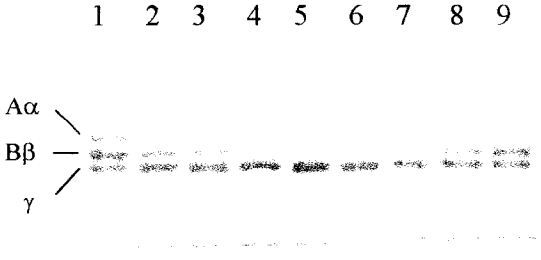


FIGURA 6



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Isolation, properties and partial amino acid sequence of a new metalloproteinase from *Bothrops moojeni* snake venom

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ABSTRACT

A proteinase, here denominated Bthα, was purified by DEAE Sephacel, Sephadex G-75 and Heparin-agarose column chromatography from the venom of *Bothrops moojeni*. The enzyme was purified to homogeneity as judged by its migration profile in SDS-PAGE stained with coomassie blue, and had a molecular mass of about 24.5 kDa. The partial protein sequence of Bthα exhibits significant similarities with other metalloproteinases reported from snake venoms. The enzyme cleaves the Aα-chain of fibrinogen first, followed by the Bβ-chain, and shows no effects on γ-chains. On fibrin, the enzyme hydrolyzed only the β-chain, leaving the α-chain and γ-dimmer apparently untouched. It was devoid of phospholipase A_2, hemorrhagic and thrombin-like activities. Like many venom enzymes, it is stable at pHs between 4 and 10 and resists heating at 70 °C for 15 min. The inhibitory effects of EDTA on the fibrinogenolytic activity suggest that Bthα is a metalloproteinase and inhibition by β-mercaptoethanol revealed the important role of the disulfide bonds in the stabilization of the native structure. Aprotinin and benzamidine, specific serine proteinase inhibitors had no effect on Bthα activity. Since the Bthα enzyme was found to cause defibrinogenation when administered i. p. on mice, it is expected that it may be of medical interest as a therapeutic agent in the treatment and prevention of arterial thrombosis.

Keywords: *Bothrops moojeni*; Metalloproteinase; Snake; Venom.

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1. Introduction

The venoms of *Viperidae* and *Crotalidae* snakes contain a large variety of proteins and peptides affecting the hemostatic system. These proteins may be classified as coagulant, anticoagulant or fibrinolytic factors [1] and [2]. Envenomation by these snakes generally results in persistent bleeding because of the venom causes considerable degradation of fibrinogen and other coagulation factors, preventing clot formation [3]. The main snake venom components responsible for theses activities are metalloproteinases [4], several of which have been purified and characterized from *Bothrops* species, including *B. asper* [5] and [6], *B. atrox* [7], *B. lanceolatus* [8], *B. jararaca* [9] and [10], *B. jararacussu* [11], *B. alternatus* [12] and *B. neuwiedi* [13] and [14].

As indicated previously [15], venom fibrino(geno)lytic metalloproteinases may be classified as being either α- or β-chain fibrin(ogen)ases. Thus far there have been virtually no reports of a fibrino(geno)lytic snake venom enzyme with cleavage specificity directed solely to the γ-chain of fibrin(ogen). Specificity for the α- or β-chains is not absolute since there is substantial degradation of the alternate chain with increasing time. The majority of the fibrino(geno)lytic enzymes are metalloproteinases with specificity directed preferentially towards the Aα-chain and with somewhat lower activity towards the Bβ-chain [16].

Envenoming by *Bothrops* species is characterized by prominent local tissue damage, including edema, pain, hemorrhage and necrosis, and by systemic disturbances such as coagulopathies, systemic hemorrhage and renal failure [17], [18], [19] and [20].

Envenoming by *Bothrops moojeni* is frequent in central and southeastern Brazil, throughout the Cerrado morphoclimatic domain [21], [22] and [23] and may produce

great increment in swelling, necrosis and infection, mild decrement in coagulation action and sometimes fatal extradural haematoma [24].

The main feature of *Bothrops moojeni* venom is its high proteolytic activity, responsible for most of the local and systemic effects observed during envenomation by this snake. In this report, we describe the isolation, biochemical and structural characterization of Bthα, a metalloproteinase from *Bothrops moojeni* snake venom.

2. Materials and methods

2.1. Materials

Desiccated *B. moojeni* venom was purchased from Bioagents Serpentarium (Batatais - São Paulo, Brazil). Acrylamide, ammonium persulfate, aprotinin, benzamidine, bromophenol blue, EDTA (sodium salt), fibrinogen, β-mercaptoethanol, *N,N'*-methylene-bis-acrylamide, molecular weight markers for electrophoresis, PMSF, sodium dodecyl sulphate (SDS) and *N,N,N',N'*-tetramethylethylenediame (TEMED) were from Sigma Chemical Co. (St Louis, MO, USA). Glycine, Tris, molecular mass standards and all chromatographic media were from Amersham Pharmacia Biotech. All other reagents used were of analytical grade.

2.2. Isolation of Bthα

Crude venom of *B. moojeni* (200 mg) was dissolved in 50 mM ammonium bicarbonate buffer (pH 7.8) and clarified by centrifugation at 10,000 g for 10 min. The supernatant solution was chromatographed on DEAE Sephacel column (1.7 x 15cm), previously equilibrated with 50 mM, pH 7.8 ammonium bicarbonate and eluted with a convex concentration gradient (50 mM – 0.45 M) of the same buffer.

Fractions of 3.0 mL/tube were collected, their absorbances at λ = 280 nm were read and those corresponding to peak D2 were pooled, lyophilized, dissolved in 50 mM, pH 7.8 ammonium bicarbonate and applied to 1 x 100 cm Sephadex G-75 column previously equilibrated with the same buffer. The fibrinogenolytic fraction (peak D2G2) was lyophilized and applied to column of Heparin Agarose (1 x 10 cm) previously equilibrated with 10 mM, pH 7 Tris-HCl - 5 mM $CaCl_2$ and eluted with 10 mM, pH 7 Tris-HCl - 1 M NaCl. The flow rate was 40 mL/hr and fractions of 2.0 mL were collected.

2.3. Estimation of protein concentration

Protein concentration was determined by the method of [25], using bovine serum albumin as standard.

2.4. Electrophoretic analysis

Electrophoresis using polyacrilamide gels (SDS-PAGE) was performed as described by [26] using 14% gels. Electrophoresis was carried out at 20 mA/gel in Tris-glycine buffer, pH 8.3, containing 0.01% SDS. The molecular mass standard proteins

used were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa). The slab gels were stained with coomassie blue. The relative molecular mass of the purified enzyme was estimated by Kodak 1D image analysis software. Bthα was also submitted to mass spectrometry using a Q.TOF MicroTM (Micromass, UK) spectrometer.

2.5. Amino acid sequence determination

The sample was dissolved in 1 mL of 6 M guanidine-HCl in 0.1 M tris-HCl, pH 8.6. To this solution was added 30 μL of β-mercaptoethanol under nitrogen and the sample was incubated at 50 °C for 4 h. After this, 40 μL of vinyl pyridine was added and the samples were incubated at 37 °C during 2 h. The protein was desalted on a column of Vydac C-4, using a gradient of 0 to 70% acetonitrile in 0.1% TFA over 90 min at a flow rate of 1mL/min. The collected protein was lyophilized, digested with trypsin. The tryptic peptides were separated on Vydac C18 Small Pore and submitted to automatic protein sequencer and the amino acid sequence of the peptides obtained were compared with the sequences of other related proteins in the SWISS-PROT/ TREMBL data bases using the FASTA 3 and BLAST programs.

2.6. Proteolytic activity

2.6.1. Fibrinogenolytic activity

Fibrinogenolytic activity was assayed as described by [27], with slight modifications. Fibrinogen (5 μg) and enzyme were mixed 1:100 (w/w) and the mixture was incubated in (pH 4.0 – 10.0) buffer at 37 °C for different time intervals (5, 10, 15,

30 and 60 min). The reaction was stopped by the addition of an equal volume of a denaturing buffer containing 2 % sodium dodecyl sulfate (SDS) and 10% β-mercaptoethanol. Reaction products were analyzed by SDS-PAGE.

2.6.2. Fibrinolytic activity

The fibrinolytic activity was assayed as described above. The fibrin was prepared by the addition of 10 U NIH thrombin to 50 μL of fibrinogen solution (1.5 mg/mL in 0.15 M NaCl).

2.6.3. Azocaseinolytic activity

Proteolytic activity was tested on azocasein as substrate. Enzyme (50 μg) was added to 2 mL of azocasein (1 mg/mL) in 0.2 M Tris/HCl, pH 8.0, 4 mM $CaCl_2$ and incubated at 37 °C for 30 min. Five hundred microlites of 5% (m/v) trichloroacetic acid (TCA) was then added to precipitate the undegraded azocasein. After 20 min the solution was filtered and the absorbance of the filtrate measured at 366 nm. One unit of activity was defined as an increase of 0.01 absorbance units at 366 nm.

2.7. Defibrinating activity

Defibrinating activity was tested by the method of [28], with slight modifications. Briefly, four Swiss mice (18 - 20 g) were injected i. p. with increasing dose of the enzyme, dissolved in 200 μL of saline; control animals received 200 μL of saline. After one hour, animals were anesthetized with ether and bled by cardiac puncture. Whole blood was placed in tubes and kept at 25 - 30 °C until clotting

occurred. The minimum defibrinating dose (MDD) was defined as the amount of venom capable of making the plasma incoagulable.

2.8. Hemorrhagic activity

Hemorrhagic activity was determined by the method of [29]. Test solutions (30 μg) were intradermicaly injected into dorsal skin of mice. The skin was removed 3 hr later and the diameters of the hemorrhagic spots were measured on the inside surfaces.

2.9. Estimation of fibrinogen concentration

The estimation of fibrinogen concentration was determined by injecting i.p. of purified enzyme (2.0 μg/g body weight) dissolved in 200 μL of saline in groups of 5 mice. After one hour, the animals were anesthetized with ether and bled by cardiac puncture in the anticoagulant presence (EDTA). The blood of the animals was mixed and centrifuged 3,000 g for 10 min, the plasma was removed and the estimation of fibrinogen was accomplished in analyzer compact instrument. Mice controls received 200 μL of saline.

2.10. Heat stability

Bthα and fibrinogen dissolved in 50 mM Tris-HCl buffer, pH 8.0, were incubated for 15 min at 0 - 70 °C and the remaining fibrinogenolytic activity was determined as previously described.

2.11. Enzyme inhibitors

Inhibition of fibrinogenolytic activity was determined by incubating Bthα (5 μg) in 0.25 mL of 50 mM Tris-HCl, pH 8.0, for 15 min at room temperature (25°C) containing one of the following inhibitors 5 mM EDTA, 5 mM aprotinin, 5 mM benzamidine and 5 mM β-mercaptoetanol. Proteolytic activity on fibrinogen was determined as previously described.

3. Results

3.1. Isolation and characterization of Bthα

Chromatography of the venom from *B. moojeni* on a DEAE Sephacel column (Fig. 1A) resulted in the separation of seven protein fractions (Peaks D1-D7). The proteins present in the D2 fraction showed substantial proteolytic activity. Further chromatography of the D2 protein fractions on a Sephadex G-75 column (Fig. 1B) resulted in the elution of three main peaks of proteins. Peak D2G2 from this fractionation, was applied to a heparin-Agarose Column (Fig. 1C). The adsorbed fractions (denoted Bthα) showed proteolytic activity towards azocasein. Bthα also showed fibrinogenolytic and fibrinolytic activities; no blood-clotting, phospholipase A_2 and hemorrhagic activities were detected

A summary of the purification procedure is shown in table 1. Considering the proteolytic activity on azocasein the Bthα was purified 1.08-fold. Bthα represented 8.7% (w/w) of the initial desiccated venom. Electrophoretic (SDS-PAGE) analysis (Fig. 2) under denaturing and reducing conditions indicated that the Bthα enzyme present in the gel affinity chromatography adsorbed eluate (Fig. 1C) was highly purified, and had

a molecular mass of about 24.5 kDa. Mass spectrometry analysis of Bthα indicated a mass of 23.095 Da (data not shown).

The partial amino acid sequence of Bthα was determined by automated sequencing of peptides produced by digestion of the *S*-pyridyl-ethylated protein with trypsin is shown in Fig. 3. This sequence exhibited 72% of similarities with metalloproteinase isolated from *B. leucurus* and hemorragic factor II of *L. muta* snake venoms; 77% of similarities with the fragment of metalloproteinase isolated from *B. jararacussu* venom [30] and Type II metalloproteinase of venom of *B. asper* (Arce V., Azofeifa G., Flores M., Alape A.; http://ca.expasy.org/uniprot/Q072L5). Furthermore, Bthα shows significant similarities with several others metalloproteinases according to data bank SWISS-PROTEIN Program FASTA 3.

3.2. Proteolytic activity

Table 1 shows values obtained for proteolytic activity upon azocasein at pH 8.0. Bthα showed proteolytic activity towards azocasein, with a specific activity of 192.3 units/mg of protein compared to 178.1 units/mg of protein for the crude venom.

Bthα was active against bovine fibrinogen and caused a rapid degradation of the Aα and Bβ-chain of this substrate within 10 min as demonstrated by the electrophoretic profile of the reaction mixture (Fig. 4A). While the concentration of higher molecular mass protein decreased with increasing digestion time the concentration of lower molecular mass products increased substantially. The action of Bthα on the fibrin showed to be less effective than on the fibrinogen. Bthα digested the β-chain, while the α- and γ-dimer were not affected within the time tested (Fig. 4B).

3.3. Defibrinating activity

Bthα caused defibrinogenation when administered i. p. to mice, making the plasma uncoagulable. The fibrinogen levels reduced to zero after 1 hour of the injection (MDD = 2.0 μg/g body weight), while control animals presented 270 mg of fibrinogen per dL of plasma. The normal fibrinogen levels were only reestablished after 24 hours.

3.4. Effect of protease inhibitors

Figure 5 shows the effects of several inhibitors on the fibrinogenolytic activity of Bthα. Incubation of the purified enzyme for 15 min at 37 °C with EDTA (5 mM), a metal chelating agent, completely abolished its fibrinogenolytic activity, indicating that Bthα is a metallo-type proteinase. The fibrinogenolytic activity of the enzyme was also abolished by β-mercaptoethanol (5 mM), however, benzamidine and aprotinin (5 mM) had little or no effect.

3.5. Heat stability

As shown in Fig. 6, Bthα enzyme exhibited its maximum fibrinogenolytic activity in the temperature range from 20 to 50°C. Apparently, the activity on Bβ-chain of fibrinogen was totally lost at temperature range from 0 to 10 °C and 60 to 70 °C. At those temperatures, Bthα partially digested the Aα-chain of fibrinogen. The optimal pH for the fibrinogenolytic activity of Bthα was 8.0. However, the enzyme was active at the pH ranges of 4-10 (data not shown).

4. Discussion

Proteinases from venomous snakes are of a medical interest since they are responsible for many local and systemic affects of envenomation by snake bites. The isolation of a metalloproteinase from *B. moojeni* (moojeni protease A) was reported by [31], who showed that this venom contained significant amounts of basic proteins, the coagulant enzyme batroxobin [32], three serine proteinases (MSP1 and MSP2 [33] and MOO3 [34]) and one metalloproteinase (MPB) [35]. BM-PLA2, and acid phospholipase A2 [36] and two myotoxins of low mol. weight were isolated from *B. moojeni* [37], [38] and [39].

In this work, a new metalloproteinase, Bthα, from the venom of *B. moojeni* was purified by a combination of gel ion-exchange (DEAE Sephacel), gel filtration (Sephadex G-75) and affinity (Heparin-agarose) chromatographies. The molecular mass of Bthα by SDS-PAGE (24.5 kDa) and mass spectrometry analysis (23.095 kDa) was similar to the 27 and 22.5 kDa reported for MOO3 and protease A moojeni, respectively [34] and [31] and to the 23, 24 and 28 kDa metalloproteinases detected in *B. atrox* [40], *B. asper* [41] and *B. lanceolatus* [8] venoms, respectively. In agreement with MSP2, protease A moojeni and MOO3, Bthα gave a single band in the absence or presence of β-mercaptoethanol, as also reported for other low molecular mass hemorrhagic factors [42].

The partial amino acid sequence of Bthα deduced from tryptic peptides is shown in Fig. 3 where it exhibits significant similarities with other metalloproteinases reported from snake venoms, suggesting the Bthα is a metalloproteinase too. The tryptic peptides of Bthα which were sequenced could be aligned with approximately 80% of the sequences of other snake venom metalloproteinases.

Bthα didn't present hemorrhage on mouse back skin when 30 micrograms of enzyme was intradermicaly injected (data not shown). Based on their domain structure, snake venom metalloproteases (SVMPs) have been classified into four classes, P-I to P-IV [43]. Metalloproteinases of P-I class consist only of the proteinase domain, class P-II consist of proteinase domain and disintegrin-like domain and class P-III have a third, cysteine-rich domain. Class P-IV SVMPs possess, in addition to class P-III domains, disulfide-attached lectin-like domains. Sub-class P-1A proteins display high hemorrhagic activity, while those in class P-1B display little or no activity and include Neuwidase from *B. neuwiedi* venom [14] and MOO3 from *B. moojeni* venom [34]. Our results suggest that Bthα belongs to class PI-B.

Bthα showed proteolytic activity towards azocasein, with a specific activity of 192.3 units/mg of protein. Considering this activity Bthα was purified 1.08-fold. The enzyme represents 8.7% of the total venom proteins. The yield of Bthα was larger than those reported for other fibrinogenolytic enzymes purified by same venom. The yield of MSP1, MSP2 and MOO3 were 0.51, 1.1 and 1.42%, respectively. Apparently, Bthα showed very low specific activity when compared with the crude venom. This fact can be explained because Bthα isn't the only enzyme with proteolytic action from the venom of *B. moojeni*. Indeed, practically all the fractions obtained by DEAE Sephacel (Fig. 1A) and Sephadex G-75 (Fig. 1B) column chromatography were capable of hydrolyzing the azocasein (data not shown)

Like other fibrinogenases from the venoms of *B. moojeni* (MSP1 and MSP2 [33] and MPB [35]) Bthα, preferentially hydrolysis the Aα-chain accompanied by hydrolysis of Bβ-chain of fibrinogen. These proteinases differ from Bthα because they digested the γ-chain, which is not common to many venom proteinases. The fibrin clot was more resistant to hydrolysis by Bthα than fibrinogen. Only the β-chain was

digested, while the α-chain and γ-dimer of fibrin were not affected (Fig. 4B). These results can be explained because fibrinogen contains long stretches of amino acids which are exposed to proteolytic enzymes. Fibrin, however, has a cross-linked structure and is much less susceptible to proteolysis [44].

The partial primary structure of Bthα and the effect of metal chelation (EDTA) on its fibrinogenolytic activity confirmed that Bthα is a metalloproteinase. The sequence analysis indicated that enzyme has six cysteine residues that can be involved in forming three disulfide bonds. Indeed, the internal disulfide bonds were also found to be critical for the activity or structural integrity of Bthα, since incubation with 5 mM β-mercaptoethanol resulted in inhibition of fibrinogen hydrolysis by Bthα. Aprotinin and benzamidine showed no inhibition of enzymatic activity. In common with most proteinases isolated from snake venoms [45], [46] and [11] the enzyme Bthα was not stable at high temperatures (Fig. 6) and it shows an optimum pH around of 4 - 10 (data not shown).

In vivo Bthα acts as a benign defibrinogenating agent to remove fibrinogen from the blood. Within minutes of administration of the enzyme there was a depression in the fibrinogen concentration in plasma and within an hour the fibrinogen levels were reduced to zero and the plasma remain uncoagulable up to 24 hours. This result suggests the potential use of the Bthα as an anticoagulant and antithrombotic agent. The suggestion that anticoagulation could be achieved by totally removing plasma fibrinogen from the circulation by means of a snake venom fibrino(geno)lytic enzymes was first made in 1968 [47]. Studies of the mechanism of action of the fibrino(geno)lytic enzymes has revealed that they cause a fast and efficient anticoagulant and antithrombotic activity, in other words, they undo the formed fibrin clot when there is dysfunction of the haemostatic system. For that reason, these

enzymes have been of great interest to the medical clinic, especially those with a high degree of purity and devoid of other pharmacological effects.

The action of proteinases that may interfere with coagulation and the fibrino(geno)lytic system following envenomation by *Bothrops* snake bites has been reported [48]. A number of these proteins have been used clinically as anticoagulants or in preclinical research to investigate their possible therapeutic potential [49], [50] and [51]. Therefore, the fibrino(geno)lytic metalloproteinase Bthα stands out as a promising tool for its importance as an anticoagulant and thrombolytic enzyme and for a better understanding of the action mechanism of *Bothrops* venoms.

In conclusion, we have purified a metalloproteinase, Bthα, with azocaseinolytic, fibrino(geno)lytic and defibrinating activities, from *B. moojeni* venom. The properties of Bthα indicate that this protein probably belongs to class P1-B of SVMPs. This finding suggests that this toxin could interfere with the coagulation cascade following envenoming.

LEGENDS TO FIGURES

Figure 1. Purification of Bthα from *Bothrops moojeni* venom: **(A)** Separation on DEAE Sephacel: crude venom (200 mg) was applied to column (1.7 x 15 cm) and elution was carried out at a flow rate of 20 mL/hr with convex ammonium bicarbonate gradients buffer, pH 7.8, from 50 mM to 0.45 M. **(B)** Separation on Sephadex G-75: the active fraction (D2) was applied to a column (1.0 x 100 cm) and elution with 50mM ammonium bicarbonate buffer at pH 7,8 was achieved at a flow rate of 20 mL/hr. **(C)** Separation on Heparin-Agarose: the fraction concentrate (D2G2) was applied to a column (1 x 10 cm) and elution was carried out at a flow rate of 40 mL/hr with 10 mM M Tris-HCl buffer containing 1.0 M NaCl at pH 7.0. Fractions pooled are indicated by the closed circle.

Figure 2: Molecular mass estimation using SDS PAGE electrophoresis in 14 % (w/v) polyacrylamide, Tris-glicine buffer, pH 8.3, and 20 mA. Lane 1 - standard proteins, 2 - reduced crude *B. moojeni* venom, 3 - reduced Bthα. The gel was stained with coomassie blue R - 250.

Figure 3: Comparison of the sequences of Bthα from *B. moojeni* venom and other metalloproteinases Cysteine residues are in red. MS: Molecular Masses. REF show the number of the sequence deposited in the data base Swiss-Prot/Trembl.

Figure 4. Proteolysis of **(A)** bovine fibrinogen and **(B)** fibrin by the Bthα enzyme. Lane 1: control incubated without enzyme for 60 min; 2 - 6: control incubated with enzyme for 5, 10, 15, 30 and 60 min, respectively.

Figure 5. Proteolysis of bovine fibrinogen by the Bthα enzyme and effect of inhibitors. Lane 1: control fibrinogen incubated without enzyme for 60 min; 2 - 5: fibrinogen after incubation with Bthα and 5 mM β-mercaptoethanol , 5 mM EDTA, 5 mM aprotinin and 5 mM benzamidine for 60 min, respectively.

Figure 6. Effect of the temperature on the stability of the Bthα enzyme. Lane 1: control fibrinogen incubated without enzyme for 15 min; 2 - 9: control fibrinogen incubated with enzyme for 15 min at 0, 10, 20, 30, 40, 50, 60 and 70 °C, respectively.

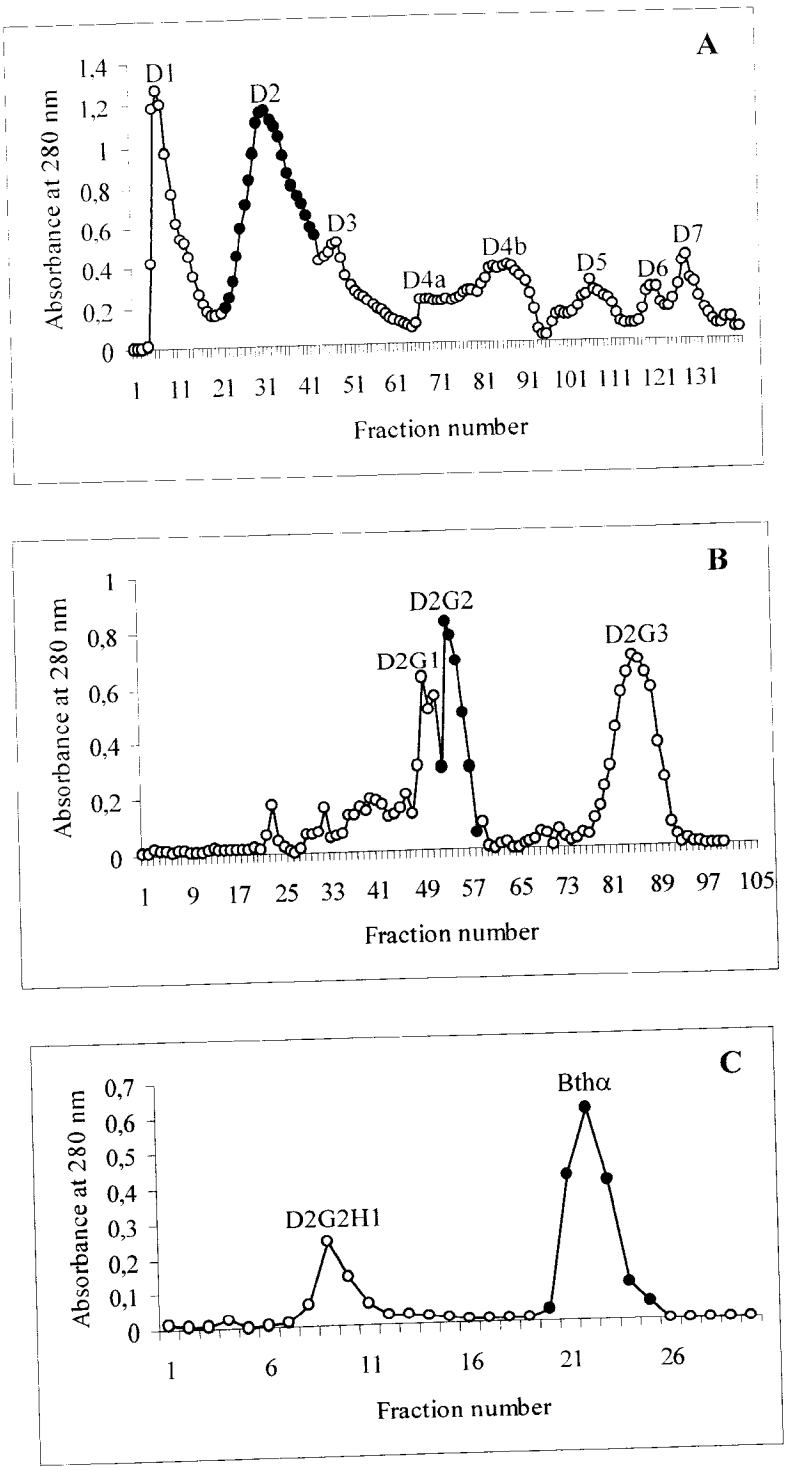
Table1. Summary of the purification steps of the Bthα enzyme from *Bothrops moojeni.*

TABLE 1

Sample	Total protein (mg)	Protein recovery (%)	Total activity (units)*	Specific activity**	Fold purity
Crude Venom	200.0	100.0	35,620	178.1	1.00
D2	44.70	22.35	8,415	188.2	1.06
D2G2	22.07	11.03	3,244	147.0	0.82
Bthα	17.43	8.71	3,294	192.3	1.08

* Proteolytic activity was tested on azocasein as substrate.
**One unit of azocaseinolytic activity was defined as an increase of 0.01 absorbance units at 366 nm.

FIGURE 1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Absorbance at 280 nm
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1,2
1
0,8
0,6
0,4
0,2
0
D1
D2
D3
D4a
D4b
D5
D6
D7
1 11 21 31 41 51 61 71 81 91 101 111 121 131
Fraction number
B
Absorbance at 280 nm
1
0,8
0,6
0,4
0,2
0
D2G2
D2G1
D2G3
1 9 17 25 33 41 49 57 65 73 81 89 97 105
Fraction number
C
Absorbance at 280 nm
0,7
0,6
0,5
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0,3
0,2
0,1
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Bthα
D2G2H1
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Fraction number

FIGURE 2

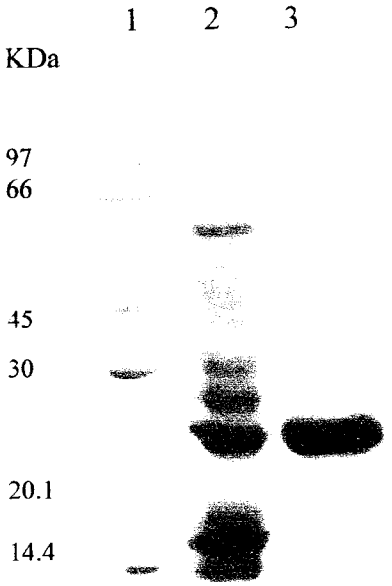
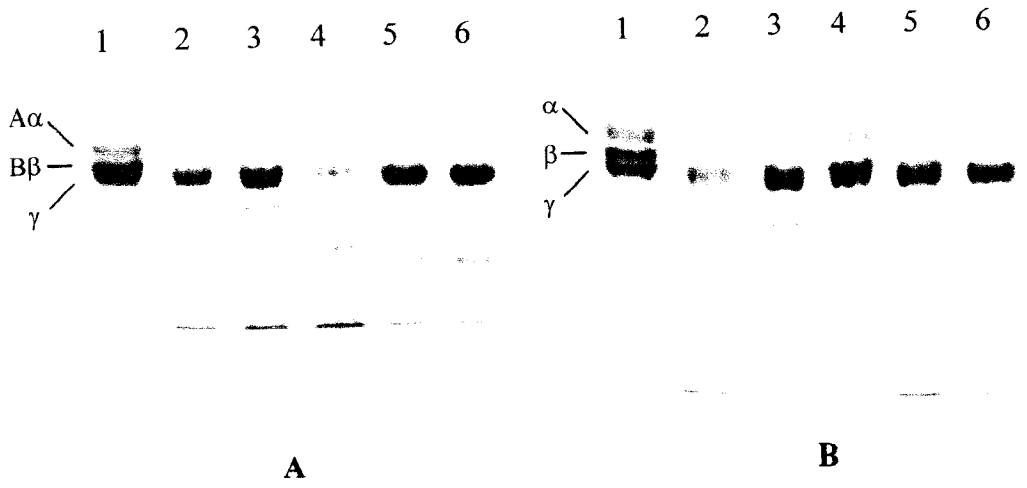


FIGURE 3

Protein/source	Sequence
	1 25 50 75
Bthα/*B. moojeni*	FSPRHIELVVVADHGMFKKYNSNLNTIRKWVHEMVNSMNGFYRSVDVTASLANLEVWSKXXXXXXXXXXXXXXXXS
B.jararacussu	KMCGVTETNWESYEPIKKASPSNLTPEQQKFSPRYIEVAVVADHRMFKKYNSNLNTIRKWVHEMVNSMNGVYRSMDVHLSLANLEVWSKKDLINVQKDSRETLKS
B.asper	QQRFSPRYIELAVVADHGIFTKYNSNLNTIRTRVHEMLNTVNGFYRSVDVHAPLANLEVWSKQDLIKVQKDSSKTLKS
B. Leucurus	SPRYIELVVVADHGMFKKYNSNLNTIRKWVHEMLNTVNGFYRSMNVDASLVNLEVWSKKDLIKVEKDSSKTLTS
L. muta	FSQKYIELVVVADHGMFTKYNGNLNTIRTRVHEIVNTLNGFYRSLNILISLTDLEIWSNQDLINVQSAANDTLKT
	100 125 150 175
Bthα/*B. moojeni*	FGEWRXXDLLPRISHDNAQLLTAIVFDGHVIGRAFTGGMCDPRHSVGVVMDHSPKNLQVAVTMAHELGHNLGMHHDGNQCHCDAASCIMADSLXXXXXXX
B.jararacussu	FGEWRERDLLPRISHDNAQLLTAVVFDQQTIGRAYIAGMCDPRHSVGVVMDHSKENLQVAVTMAHELGHNLGMEHDENQCHCDAPSCVMASVLSVVLSYE
B.asper	FGEWRERDLLPRISHDHAQLLTAVVFDGNTIGRAYTGGMCDPRHSVGVVRDHSKNNLWVAVTMAHELGHNLGIXHDTGSCSCGAKSCIMASVLSKVLSYE
B. Leucurus	FGEWRERDLLPRISHDHAQLLTVIFLDEETIGIAYTAGMCDLSQMVAWGQDY------VAVSMAHELGHNLGMRHDGNQCHCNAPSCIMADTLSKGLSFE
L. muta	FGEWRERVLLNRISHDNAQLLTAIDLADNTIGIAYTGGMCYPKNSVGIVQDHSPKTLLIAVTMAHELGHNLGMKHDENHCHCSASFCIMPPSISEGPSYE

	200	MS (Da)	Similaritie (%)	REF
Bthα/*B. moojeni*	XXXXXXXXXXXXXXXHNPQCILNEP	23.095		
B.jararacussu	FSDCSQNQYQTYLTKHNPQCILNEPLLTVSGNELLEAGE	41.957	77	UNIPROT Q7T1T4_BOTJR
B.asper	FSDCSQNQYETYLTNHNPQCILNKP	22.857	77	UNIPROT BAP1_BOTAS
B. Leucurus	FSDCSQNQYQTYLTKHNPQCILNKP	22.003	72	UNIPROT LEUCA_BOTLC
L. muta	FSDCSKDYYQMFLTKRKPQCILNKP	22.596	72	UNIPROT Q90391_CROAT

FIGURE 4



A B

FIGURE 5

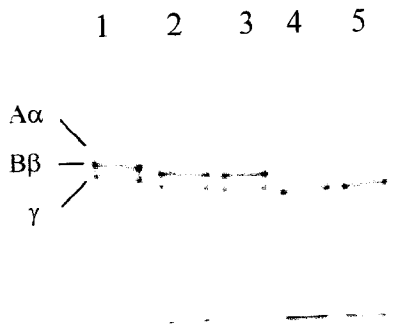
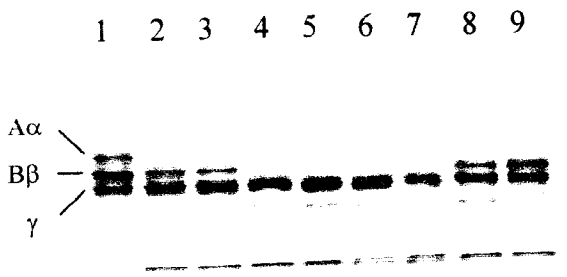


FIGURE 6



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