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TRIPSINA DO PEIXE AMAZÔNICO TAMBAQUI, *Colossoma macropomum* (Cuvier, 1818): ESTRUTURA, FUNÇÃO E APLICAÇÃO

Recife
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Tese apresentada para o cumprimento das exigências para obtenção do título de Doutor em Ciências Biológicas pela Universidade Federal de Pernambuco.

Orientador: Prof. Dr. Ranilson de Souza Bezerra (UFPE)
Co-orientadores: Prof. Dra. Márcia Vanusa da Silva (UFPE)
Prof. Dr. Vitor Marcelo S.B.B. de Oliveira (UNIFESP)

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“A dupla-hélice é uma estrutura sucinta, mas sua mensagem não poderia ser mais prosaica: a vida é uma simples questão de química.”

James D. Watson

RESUMO

A presente tese reporta a estrutura, função e aplicação como aditivo pré lavagem de uma tripsina do peixe amazônico tambaqui (*Colossoma macropomum*). O capítulo um traz a aplicação de tripsinas purificada dos peixes tambaqui e tilapia do Nilo (*Oreochromis niloticus*) como componentes para soluções de pré-lavagem de roupas, comparando-as com tripsina de porco, subtilisina bacteriana e quimotripsina bovina. No capítulo dois, o efeito do cálcio sobre a tripsina do tambaqui e do porco foi comparado através de ensaios bioquímicos, eletroforéticos, de espectrometria de massas e de fluorescência intrínseca. Já o capítulo três abordou o uso de dinâmica molecular e dicroísmo circular na análise da estrutura da tripsina do tambaqui, porco e salmão submetidas a variações de temperatura, bem como a criação de um banco de dados com sequências aniônicas e catiônicas de tripsinas de peixes e animais homeotérmicos. Com os resultados apresentados na presente tese, pode-se afirmar que a tripsina do tambaqui apresenta uma estrutura mais flexível que a do porco, e que consegue suportar um leque maior de temperaturas e agentes denaturantes, principalmente por conta de sua baixa propensão à autodigestão. Assim, considerando que a tripsina do tambaqui é mais ativa e robusta do que as tripsinas de mamíferos, pode-se dizer que ela apresenta potencial para aplicações industriais, principalmente aquelas que se dão em presença de agentes denaturantes e em temperaturas entre 25 e 60 °C.

Palavras-chave: Autólise; Cálcio; Proteases de peixe; Aditivo pré-lavagem; Dinâmica molecular; Estrutura de proteínas.

ABSTRACT

The present work reports on the functionality, structure of a trypsin from the Amazonian fish, tambaqui (*Colossoma macropomum*) and its application as prewash additive. Chapter one shows the application of trypsins from tambaqui and Nile tilapia (*Oreochromis niloticus*) asa pre-wash components for detergent, in comparison to porcine trypsin, bacterial subtilisin and bovine chymotrypsin. In chapter two, the effect of calcium on tambaqui and porcine trypsin was compared through biochemical, eletrophoretical, mass spectrometry and intrinsic fluorescence analysis. Finally, chapter three shows the use of molecular dynamics and circular dichroism to analyses the structure of tambaqui, porcine, and salmon trypsin subjected to temperature variations, as well as the assemble of a sequences data bank with anionic and cationic trypsins from fish and homeothermic animals. In the light of the results reported in the present thesis, it can be stated that tambaqui trypsin is an enzyme with a flexible structure, with high activity, that can also endure a greater range of temperatures and denaturing agents than porcine trypsin, mainly due to its low propensity for auto digestion. Therefore, considering that tambaqui trypsin is more active and robust than the mammal homologues, it has a great potential for industrial applications, especially in the presence of denaturing agents at mildly high temperatures.

Keywords: Autolysis; Calcium; Fish proteases; Molecular dynamics; Pre-wash additives; Protein structure.

Lista de Ilustrações

Revisão de Literatura

Capítulo 1

Capítulo 2

Capítulo 3

Lista de tabelas

Revisão de Literatura

Capítulo 1

Capítulo 2

Capítulo 3

Lista de abreviaturas e siglas

Abz	Orto-aminobenzoil
AFT	Anionic Fish Trypsin
AHT	Anionic Homeotherm Trypsin
ANOVA	Análise de Variância
BapNa	N-α-Benzoil-L-Arginina *p*-Nitroanilida
BLAST	Basic Local Alignment Search Tool
BSA	Albumina do Soro Bovino
CD ou DC	Dicroísmo Circular
CFT	Cationic Fish Trypsin
CHT	Cationic Homeotherm Trypsin
DMSO	Di-Metil-Sulfoxido
DM ou MD	Dinâmica Molecular
EC	Comitê Enzimático
EDTA	Ácido Etilenodiaminotetracético
FAO	Food and Agriculture Organization of the United Nations
GRAVY	Grand Average of Hydropathy
HPLC	Cromatografia Liquida de Alta Eficiência
LABENZ	Laboatório de Enzimologia
MALDI	Matrix-assisted laser desorption/ionization
MPA	Ministério da Pesca e Aquicultura
NCBI	National Center for Biotechnology Information
PDB	RCSB Protein Data Bank
PMSF	Fluoreto de Fenil-Metil-Sulfonil
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
SDS	Dodecil Sulfato de Sódio
SDS - PAGE	Eletroforese em Gel de Poliacrilamida com SDS
TLCK	Tosil - Lisina Clorometil Cetona
Tris-HCl	Hidrocloridrato de 2-Amino-2-(Hidroximetil)-1,3-Propanodiol
UV	Ultravioleta
z-FR-MCA	Carbobenzoxi-Phe-Arg-7-amido-4-metilcoumarina

Lista de símbolos

Aminoácidos

Ala (A)	Alanina
Arg (R)	Arginina
Asn (N)	Asparagina
Asp (D)	Ácido aspártico
Cys (C)	Cisteína
Glu (E)	Glutamina
Gln (Q)	Ácido glutâmico
Gly (G)	Glicina
His (H)	Histidina
Ile (I)	Isoleucina
Leu (L)	Leucina
Lys (K)	Lisina
Met (M)	Metionina
Phe (F)	Fenilalanina
Pro (P)	Prolína
Ser (S)	Serina
Thr (T)	Treonina
Trp (W)	Triptofano
Tyr (Y)	Tirosina
Val (V)	Valina
C-18	Resina cromatográfica com grupamentos acoplados contendo 18 átomos de carbono cada
Dnp	2,4-di-nitro-fenil
k_{cat}	Constante catalítica
kDa	Quilo Daltons
K_m	Constante de Michaelis e Menten
λ_{max}	Comprimento de onda com emissão máxima
M.M.	Massa molecular
V_{max}	Velocidade máxima

SUMÁRIO

1 Introdução

O tambaqui (*Colossoma macropomum*) é um peixe amazônico que atualmente figura como o segundo mais produzido no Brasil (MPA, 2013). Seu interesse para a aquicultura se deve ao fato dele suportar bem as condições adversas e estresses causados pelo cultivo, apresentando bom crescimento e alimentação (VAL e HONCZARYK, 1995). Além disso, o tambaqui também demonstra boas perspectivas de penetrabilidade no mercado nacional e internacional, por conta da sua carne de qualidade e sabor único (LIMA e GOULDING, 1998). Porém, para se alcançar o sucesso em cultivos, como o tambaqui vem fazendo, é preciso que várias informações sejam reunidas acerca dos aspectos ecológicos, etológicos e fisiológicos do animal. Neste âmbito, o estudo das enzimas digestórias também contribui para o melhor entendimento de sua fisiologia digestória, auxiliando no desenvolvimento de rações mais eficientes.

Com o objetivo de preencher esta lacuna de conhecimento sobre as enzimas digestórias do tambaqui, o laboratório de Enzimologia da UFPE iniciou em 1996 a caracterização das proteases ácidas e alcalinas deste animal (BEZERRA et al, 2000). No decorrer do desenvolvimento destes primeiros trabalhos foi observado que as proteases alcalinas deste peixe eram estáveis a temperaturas de até 55°C, bem como apresentavam uma ampla faixa de atuação de temperatura e pH (BEZERRA et al, 2000). Enzimas com alta atividade e estabilidade são de interesse comercial, e por isso, as aplicabilidades biotecnológicas potenciais das proteases alcalinas do tambaqui foram investigadas. Considerando-se que o maior mercado consumidor de proteases alcalinas, tanto em termos de valor como volume, é o da indústria de detergentes (KIRK et al, 2002), esta foi a primeira área escolhida para se testar a aplicação destas enzimas do tambaqui. As proteases avaliadas demonstraram ser compatíveis com detergentes comerciais, tenso-ativos e oxidantes, indicando assim o alto potencial das enzimas do tambaqui para esta aplicação (ESPÓSITO et al, 2009).

A tripsina é a principal enzima responsável pela atividade proteolítica nos cecos pilóricos e intestino dos peixes, e por isso, um investimento foi feito no sentido de sua purificação e caracterização no tambaqui (BEZERRA et al, 2001). Posteriormente, o estudo desta enzima foi aprofundado com o sequenciamento de 20 dos seus aminoácidos N-terminais, bem como a avaliação de sua especificidade de hidrólise frente a substratos peptídicos sintéticos fluorescentes (MARCUSCHI et al, 2010, Apêndice A). Este conjunto de resultados publicados até o momento aponta para a versatilidade da tripsina do tambaqui, e por esta razão, a presente tese propôs ampliar esta linha de pesquisa, através de um estudo estrutural, funcional e aplicado desta enzima.

Na presente tese a compatibilidade da tripsina do tambaqui e da tilápia do Nilo frente a agentes tenso-ativos e detergentes foi estudada e melhor compreendida. Adicionalmente, outros aspectos estruturais e bioquímicos da enzima nativa também foram avaliados, por técnicas de dicroísmo circular, fluorimetria, eletroforese e atividade enzimática. O gene que codifica a tripsina do tambaqui também foi sequenciado (GenBank: JQ437817.1), e utilizado em ensaios de bioinformática, que envolveram a análise da estrutura tridimensional da proteína através de modelagem computacional por homologia, bem como a execução de dinâmicas moleculares (DM).

Os resultados obtidos apontam para a existência de uma tripsina no trato digestório do tambaqui que possui uma estrutura flexível, porém pouco propensa à autólise, o que faz com que a enzima seja mais ativa e robusta do que os homólogos de mamíferos. Esta combinação de características, mais comuns em peixes adaptados aos ambientes tropicais, é procurada nas enzimas para aplicações industriais. Adicionalmente, a presente tese traz a reunião e análise de um banco de dados de 227 sequências de tripsinas, com o intuito de contribuir para uma melhor compreensão das diferenças entre as tripsinas dos animais homeotérmicos e pecilotérmico provenientes de diversas condições ambientais.

2 Revisão bibliográfica

2.1 Peixes tropicais: anatomia e fisiologia

Os peixes são animais pecilotérmicos e por isso estão sujeitos às temperaturas do ambiente no qual se acham inseridos. A depender da zona climática, essas temperaturas podem variar de 0 a 40 °C (Tabela 1). Na presente tese, será dada ênfase à zona climática tropical que, de acordo com o modelo climático clássico de Köppen (1936), adequado por Peel et al (2007) para as condições de precipitação e clima atuais do planeta, ocupa cerca de 40% da superfície da Terra e se localiza entre os trópicos de Câncer e Capricórnio (Figura 1). Por isso, os peixes que vivem nas regiões tropicais precisam se adaptar a altas temperaturas e umidade constante (VAL et al, 2005). Estas condições são tipicamente encontradas, por exemplo, nos rios e lagos amazônicos, cujas temperaturas podem variar de 25 a 34 °C (DAIRIKI e SILVA, 2011).

Tabela 1 – Limites de temperatura tolerados por peixes de águas geladas, frias, mornas e tropicais.

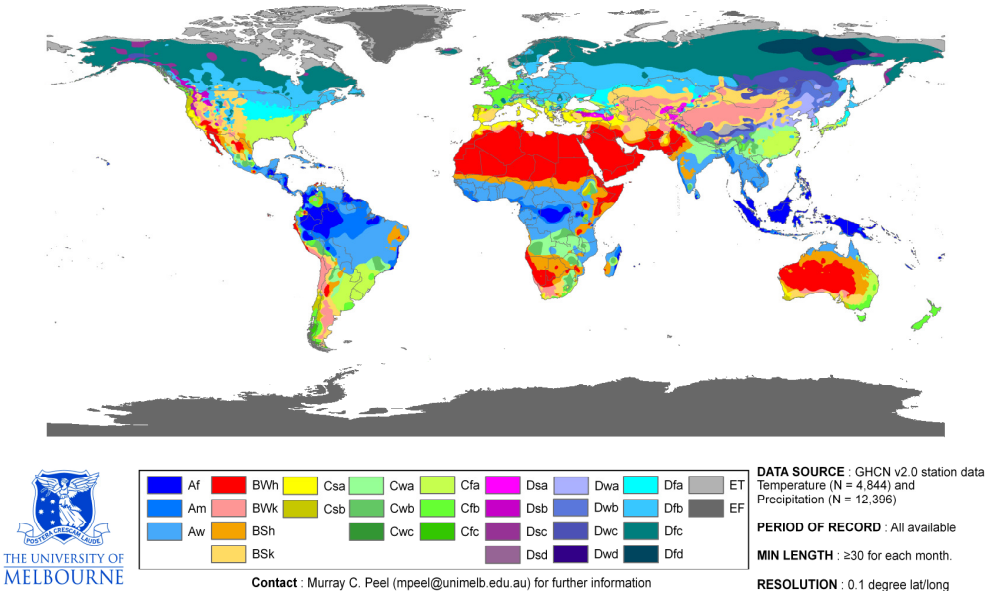
Tipo de água	Espécie	TILMin-TILMax (°C)	Variação de temperatura (°C)
Gelada	Salvelino ártico (*Salvelinus alpinus*)	0-19.7	6-15
	Salmão do atlântico (*Salmo salar*)	-0.5-25	13-17
Fria	Walleye (*Stizostedion vitreum*)	0-30	20-23
	Robalo riscado (*Morone saxatilis*)	2-32	13-24
Morna	Enguia européia (*Anguilla anguilla*)	0-39	22-23
	Bagre de canal (*Ictalurus punctatus*)	0-40	20-25
Morna/tropical	Carpa comum (*Cyprinus carpio*)	0-35.7	26.7-29.4
Tropical	Tilápia de barriga vermelha (*Tilapia zillii*)	7-42	28.8-31.4
	Tilápia da Guiné (*Tilapia guineensis*)	14-34	18-32

Legenda: TILMin - Temperatura Inicial Letal Mínima (indica a temperatura mínima na qual o peixe pode sobreviver); TILMax - Temperatura Inicial Letal Máxima (indica a temperatura máxima na qual o peixe pode sobreviver). **Fonte:** Ficke et al (2007)[1].

[1] Disponível em: <http://link.springer.com/article/10.1007/s11160-007-9059-5> Acesso em 14 jan 2014.

Apesar do impacto que a temperatura e a composição química da água (grau de oxigenação, pH, presença de sais, concentração de compostos orgânicos, coloração) têm sobre o metabolismo dos peixes, eles ainda apresentam as mesmas vias metabólicas clássicas descritas para os animais terrestres. Além disso, o ritmo metabólico dos peixes é mantido próximo ao dos animais homeotermos, e não diminui tanto quanto se esperaria, levando-se em consideração suas temperaturas corporais mais baixas (HALVER e HARDY, 2002). A explicação para esse fenômeno pode ser encontrada na adaptação[2] da "maquinaria bioquímica" à temperatura e a outros fatores abióticos (SOMERO, 2004).

Figura 1 - Mapa com a classificação climática de Köppen-Geider.



Legenda: A primeira letra das siglas indica os 5 grandes grupos climáticos e as letras acompanhantes definem os sub-climas. (A) Tropical: (Af) floresta tropical/equatorial, (Am) monção, (Aw) savana; (B) Árido: (Bw) deserto, (BSh) estepe quente, (BSk) estepe frio; (C) Temperado: (Cs) verão seco, (Cw) inverno seco, (Cfa) sem temporada seca e verão quente, (Cfb) sem temporada seca e verão morno, (Cfc) sem temporada seca e verão frio; (D) Frio: (Ds) verão seco, (Dw) inverno seco, (Dfa) sem temporada seca e verão quente, (Dfb) sem temporada seca e verão morno, (Dfc) sem temporada seca e verão frio, (Dfd) sem temporada seca e verão muito frio; (E) Polar: (Et) tundra, (Ef) congelado. **Fonte:** Peel et al (2007)[3].

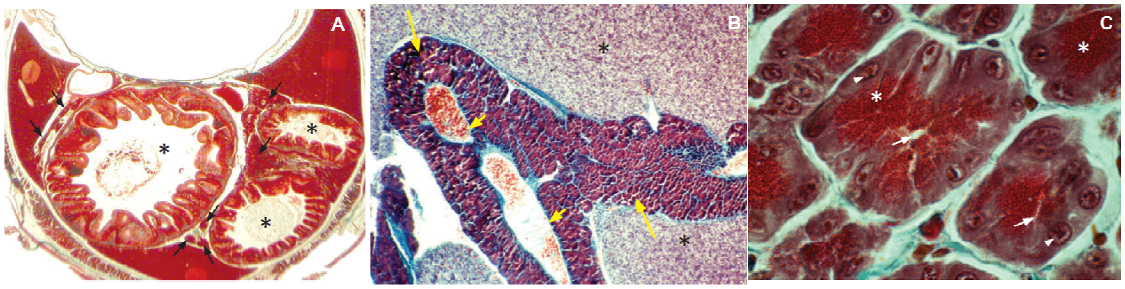
Tal como o metabolismo, a anatomia gastrointestinal dos peixes segue o mesmo princípio básico dos outros vertebrados (WILSON e CASTRO, 2011). Assim, apesar de apresentar algumas variações, a depender de filogenia, alimentação, ontogenia e habitat, o trato digestório dos peixes pode ser dividido, de maneira geral, em quatro grandes partes: (1) boca e faringe, onde ocorre a captura e processamento mecânico dos alimentos; (2) esôfago e

[2] Vide o tópico 2.4.4 para um discussão mais aprofundada
[3] disponível em: <http://www.hydrol-earth-syst-sci.net/11/1633/2007/ hess-11-1633-2007-supplement.zip>. Acesso em: 04 nov 2013.

estômago, cujo pH ácido e enzimas iniciam a transformação química do alimento, liberando nutrientes solúveis; (3) intestino, que é responsável pela continuação da transformação química dos alimentos e absorção dos nutrientes, íons e água oriundos da dieta; e (4) reto, o qual executa a função final de absorção e excreção (RUST, 2002; WILSON e CASTRO, 2011).

Além dos órgãos principais, os peixes também apresentam glândulas e órgãos acessórios, que auxiliam na digestão dos alimentos, como os cecos pilóricos, o fígado e o pâncreas. Os cecos pilóricos são tubos de fundo cego, presentes na maioria dos osteichthyes e em alguns chondrichthyes, sendo responsáveis por aumentar a área de digestão e absorção dos alimentos (WILSON e CASTRO, 2011). O fígado dos peixes tem funções semelhantes às apresentadas pelos mamíferos, como a assimilação de nutrientes; produção de bile; detoxificação do organismo; e manutenção da homeostase. Já o pâncreas é normalmente espalhado de forma difusa na gordura e no mesentério (Figura 2A) que circundam o intestino, estômago, fígado (hepatopâncreas, Figura 2B) e vesícula biliar, sendo que um pâncreas discreto também pode ser encontrado em chondrichthyes (Figura 2C) (GENTEN et al, 2009).

Figura 2 – Cortes histológicos indicando a presença de células pancreáticas em peixes.



Legenda: Em todas as lâminas foi usada a coloração tricrômica de Masson. (2A) Corte transversal na altura da nadadeira ventral do peixe *Danio rerio* (paulistinha), com aumento de 8-40x. As setas apontam as massas de pâncreas difuso no mesentério ao redor do (*) intestino; (2B) Corte histológico longitudinal do hepatopâncreas de *Cyprinus carpio*, com aumento de 100-250x. A imagem mostra o tecido (*) hepático, com setas curtas apontando a veia porta aferente (eritrócitos em laranja) e o setas longas apontando o pâncreas exócrino;. (2C) Corte transversal do pâncreas de *Scyliorhinus canicula*, com aumento de 400-640x. As setas apontam o lúmen do intestino, cercado de células (*) acinares (grânulos de zimogênio corados de vermelho), e as pontas de seta apontam os núcleos celulares. **Fonte:** Genten et al (2009)[4].

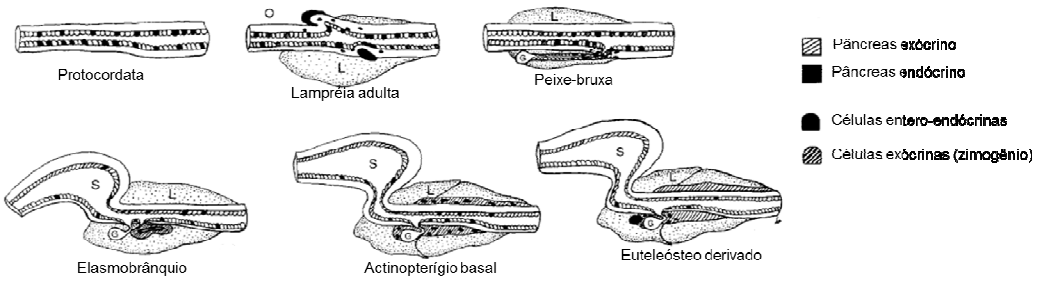
No que se refere ao pâncreas, suas funções podem ser divididas em duas: exócrino e endócrino. O pâncreas exócrino dos peixes, seja ele difuso ou discreto, é composto de um conjunto de células piramidais, organizadas em ácinos, que produzem e guardam as enzimas digestórias na sua forma inativa (zimogênios) (Figura 2C). Essas enzimas são então

[4] Disponível em : <http://onlinelibrary.wiley.com/doi/10.1111/j.1095-8649.2009.02318_3.x>. Acesso em: 03 set 2013.

transportadas através de dutos até os cecos pilóricos, regiões proximais do intestino, ou vesícula biliar (BAKKE et al 2011). Somento ao chegar aos órgãos de destino, as enzimas são ativadas e então são capases de executar suas funções digestórias.

O pâncreas endócrino é composto por estruturas de origem endodérmica formadas por ilhotas de Langerhans. Essas ilhotas consistem em um agregado que secreta hormônios no sistema sanguíneo ou linfático, que são posteriormente transportados aos órgãos de destino (GENTEN et al, 2009). Comparando-se diferentes grupos de peixes (Figura 3), pode-se observar uma tendência de migração das ilhotas para fora do epitélio do trato digestório, levando à formação de um agregado celular independente (o corpúsculo de Brockmann). Esta migração possibilita uma melhor atuação das células endócrinas, uma vez que elas se tornam menos influenciáveis pelas condições locais de hiperglicemia e hiperaminoacidemia do intestino, atuando assim de forma mais sistêmica (TAKEI e LORETZ, 2011).

Figura 3 - Diagrama representado a distribuição do tecido pancreático em diferentes peixes.



Legenda: (O) esôfago; (S) estômago; (G) vesícula biliar; O intestino é o tubo alongado, sem marcação. **Fonte:** Adaptado de Youson e Al-Mahrouki (1999) [5].

Dentre as responsabilidades metabólicas do pâncreas endócrino, está a regulação do metabolismo da glicose, na qual a insulina se destaca como um eficiente efetor hipoglicêmico. Nos mamíferos, a insulina, aliada a outros hormônios, executa um controle rígido da normoglicemia, mantendo a concentração de glicose no corpo em 7 mM. Já nos peixes não existe uma faixa glicêmica padrão, a qual pode variar consideravelmente de uma espécie para outra, principalmente entre os onívoros e carnívoros (POLAKOF et al, 2011). Esta característica metabólica dos peixes fez com que muitos pesquisadores considerassem que estes animais eram intolerantes à glicose, aparentando sofrer de uma diabetes tipo 2 não tratada (MOON et al, 2001). Porém, de acordo com Polakof et al (2011), estudos mais recentes têm demonstrado que os peixes também produzem insulina e possuem

[5] Disponível em: < https://tspace.library.utoronto.ca/bitstream/1807/696/2/Ontogentic_and_phylogenetic_development.pdf>. Acesso em: 23 set 2013.

glicoreceptores, mas apresentam uma resposta à variação de glicemia mais lenta do que a dos mamíferos. Por isso, os peixes tendem a permanecer em estado hiper ou hipoglicêmico por mais tempo do que os mamíferos.

A glicemia e outros aspectos da fisiologia dos peixes ainda precisam ser melhor compreendidos pela comunidade científica. Assim, esperamos que os resultados apresentados na presente tese possam contribuir para o desenvolvimento desta área de conhecimento.

2.2 Objeto de estudo: Tambaqui (*Colossoma macropomum*)

O tambaqui (Figura 4) ou cachama, *C. macropomum* (Cuvier, 1818), é um peixe teleósteo da ordem characiformes, família serrasalmidae, subfamília serrasalminae e gênero *Colossoma.* O termo "*Colossoma*" (corpo sem chifres) deriva do latim e se refere ao fato dos tambaquis, não possuírem um espinho pré-dorsal, característica que os diferenciam morfologicamente do grupo dos pacus (LIMA e GOULDING, 1998). Adicionalmente, o tambaqui adulto apresenta um corpo alongado, que pode atingir até um metro de comprimento e 30 kg, sendo reconhecido como o segundo maior peixe de escamas do mundo (GOULDING e CARVALHO, 1982). O corpo grosso e robusto do tambaqui também apresenta cavidades internas, que lhe permitem uma reserva de gordura visceral que pode chegar a representar 10% de sua massa corpórea. Esta gordura é uma fonte de energia importante para os períodos em que o alimento é escasso e o peixe precisa de energia para migrar.

Figura 4 – Espécime jovem de tambaqui.

Fonte: Acervo pessoal. Fotografia de Marina Marcuschi (2009).

A subfamília serrasalminae é nativa da América do Sul e formas selvagens de tambaqui são encontradas nas bacias Amazônica, Orinocó e do Prata (Figura 5) (GOULDING e CARVALHO, 1982). Os rios, lagos e Igarapés destas bacias são bastante heterogêneos, sendo formados por águas brancas (pH 6,2-7,2), negras (pH 3,8-4,9) ou claras (pH 4,5-7,8). O tambaqui habita todos estes tipos de água, mas tem preferência pelos ambientes mais ácidos (ARIDE et al, 2007). Outro aspecto comum aos ambientes nos quais o tambaqui é encontrado é a hipóxia, causada pela baixa solubilidade e alta demanda do oxigênio em águas tropicais (VAL et al, 2005). O tambaqui é muito bem adaptado à hipóxia, sendo capaz de realizar

respiração aquática de superfície, para a qual ele incha o seu lábio inferior (Figura 6B) e forma uma protuberância que direciona a água mais oxigenada da superfície para dentro da sua boca (ARIDE et al, 2007).

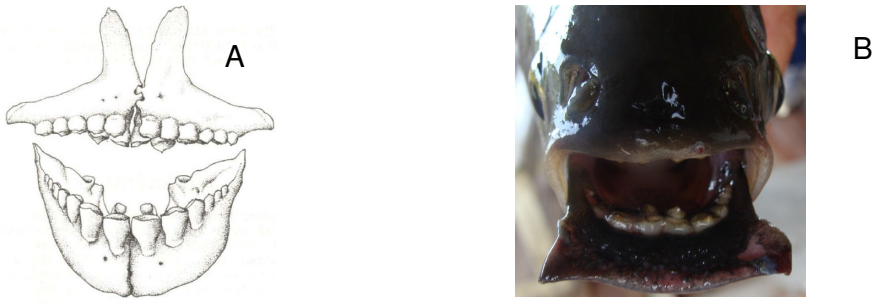
Figura 5- Bacias hidrográficas da América do Sul

Legenda: Em negrito as bacias nas quais o tambaqui selvagem é encontrado. (1) Costa caribenha; (2) Bacia Magdalena; **(3) Bacia Orinocó;** (4) Costa do atlântico norte; **(5) Bacia amazônica**; (6) Bacia do Tocantins; (7) Nordeste do Atlântico oeste; (8) Bacia do Parnaíba; (9) Nordeste da costa do Atlântico leste; (10) Bacia do São Francisco; (11) Costa do Atlântico leste; **(12) Bacia de La Plata**; (13) Costa do Atlântico sudeste; (14) Bacia do Colorado; (15) Bacia do Rio Negro; (16) Costa do Atlântico Sul; (17) Patagônia Central; (18) Costa do Pacífico – Colômbia/Equador; (19) Costa do Pacífico – Peru; (20) Costa do Pacífico – Norte do Chile; (21) Costa do Pacífico – Sul do Chile; (22) Região de La Puna; (23) Bacia das Salinas Grandes; (24) Bacia do Mar chiquita; (25) Região dos Pampas. **Fonte:** FAO, 2009[6].

Outro aspecto que afeta a morfologia do tambaqui é seu hábito alimentar. Este peixe consome sementes e frutos nos períodos de cheia e zooplâncton durante a seca (LIMA e GOULDING, 1998; GOULDING e CARVALHO, 1982). Por conta disso, a dentição do tambaqui é própria para o consumo de alimentos duros (Figura 6A), formada por duas fileiras de dentes molariformes fortes, separadas por um espaço triangular. Já os seus rastros branquiais são bem desenvolvidos, o que corrobora seus comportamento como filtrador (LIMA e GOULDING, 1998; GOULDING e CARVALHO, 1982).

[6]Disponível em: <http://www.fao.org/geonetwork/srv/en/metadata.show?id=37174>. Acesso em 10 Jan 2014.

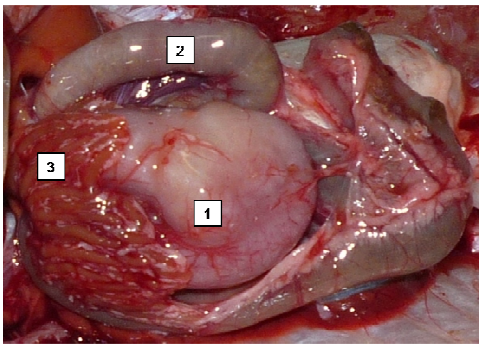
Figura 6 - Região bucal do tambaqui



Fonte: (A) Goulding e Carvalho (1982); (B) Yoshioka[7] (2010).

Os hábitos alimentares do tambaqui também têm efeito sobre sua anatomia digestória (Figura 7). O tambaqui possui um estômago elástico e bem definido, um intestino de 5 a 5,5 vezes maior que o seu corpo, bem como cecos pilóricos desenvolvidos e provavelmente voltados para a digestão de alimentos de origem vegetal. Estas características são tipicamente encontradas em animais onívoros, herbívoros e detritívoros, como é o tambaqui (LIMA e GOULDING, 1998).

Figura 7 – Trato digestório do tambaqui.



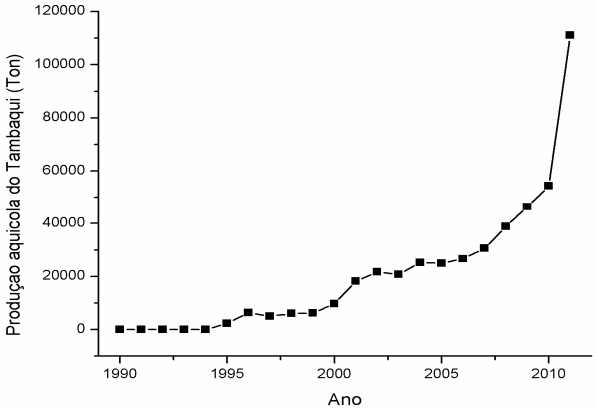
Legenda: (1) estômago, (2) intestino, (3) cecos pilóricos. **Fonte:** Acervo pessoal, fotografia de Marina Marcuschi (2009).

Dado que o tambaqui tem alto teor de gordura, a preparação culinária deste peixe consiste preferencialmente em assar ou fritar suas postas. Antes do século XX, o tambaqui selvagem era capturado e consumido pelas populações que viviam próximas aos rios amazônicos. Mas isso mudou a partir da década de 30 do século passado, quando se iniciou no Brasil o interesse em cultivar organismos aquáticos. Para o tambaqui, este cultivo só deslanchou efetivamente nos anos 90 (Figura 8) e, desde então, vem crescendo. Em 2011, o tambaqui foi o segundo peixe mais cultivado no Brasil, com 111.084 toneladas produzidas

[7]Disponível em: http://www.cpafap.embrapa.br/aquicultura/download/boas_praticas_elianeyoshioka.pdf

(MPA, 2013). Além da América do Sul, é possível se encontrar tambaqui em outras partes do mundo, como América Central, Filipinas e Havaí.

Figura 8. Produção aquicultura do tambaqui (*C. macropomum*), no Brasil de 1990 a 2011



Fonte: Elaboração própria com base em dados da FAO (2014)[8].

[8]Disponível em: <http://www.fao.org/fishery/statistics/global-aquaculture-production/query/en>. Acesso em: 08 jan 2014.

2.3 Tripsina

2.3.1 Histórico

O conhecimento empírico acerca das "modificações espontâneas das soluções orgânicas", ou fermentação, existe desde o período em que as antigas civilizações aprenderam a transformar pastas de amido, frutas e cereais em pães, álcoois e ácidos (DUBOS, 1950). Desde a Antiguidade, a fermentação e seus efeitos aguçaram a curiosidade dos pensadores, alquimistas e filósofos. Mas, foi apenas em 1789, que Lavoisier, em sua publicação *Traité élémentaire de chimie* (Tratado elementar de química), relatou a primeira reação química associada à fermentação: a decomposição do açúcar em dióxido de carbono e etanol (BUCHNER, 1907). Em 1835, Jöns Berzelius afirmou que deveria existir algo que aumentava a eficiência da fermentação dos açúcares, denominando este efeito de "força catalítica das substâncias". A respeito, declarou o pesquisador:

> "É uma nova força, capaz de produzir atividade química, tanto de natureza inorgânica como orgânica [...], (a qual) não é uma capacidade independente das propriedades eletroquímicas das substâncias" (BERZELIUS, 1835 apud TROFAST, 2006, p.31, Tradução nossa).

Em 1875, Wilhelm Kühne proferiu uma palestra, na qual propôs designar-se como 'enzima' os fermentos amorfos e desorganizados que vinham sendo estudados e cuja ação pode ocorrer fora do organismo de origem (KÜHNE, 1876a). Mais especificamente, este autor propôs que se denominasse como tripsina a enzima do pâncreas responsável por digerir proteínas em soluções alcalinas, neutras ou levemente ácidas (KÜHNE, 1876a). Ainda na mesma conferência, Kühne citou o trabalho de Heidenhain que cunhou o termo zimogênio, ao afirmar que nas células pancreáticas a tripsina não é encontrada em sua forma final, mas sim em sua conformação inativa (KÜHNE, 1876b).

Em 1907, o pesquisador Eduard Buchner ganhou o prêmio Nobel por sua pesquisa e descoberta da fermentação fora das células vivas. Os trabalhos de Buchner demonstraram que o agente reponsável pelas modificações químicas da fermentação vem dos seres vivos, mas permanece ativo mesmo fora dos organismos de origem (BUCHNER, 1907). Estas evidências derrubaram a teoria do vitalismo defendida por Pasteur e outros pesquisadores da época, e abriram as portas para o desenvolvimento da disciplina da enzimologia.

Assim, o estudo das tripsinas prosseguiu com John H. Northrop, para quem as propriedades químicas e físicas só poderiam ser propriamente investigadas nas enzimas puras. Com esta ideia, Northrop revolucionou o ramo de purificação de proteínas, tema que lhe rendeu um prêmio Nobel em 1946 (NORTHROP, 1946). Ainda em 1932, Northrop e Kunitz publicaram um trabalho apresentando a primeira purificação, por cristalização, de uma enzima

proteolítica do pâncreas bovino. Em consideração a Kühne, eles denominaram esta enzima de tripsina. Nos sete anos que se seguiram, Northrop e seus colegas do *Rockefeller Institute for Medical Research* purificaram várias outras enzimas pancreáticas, seus zimogênios e inibidores, descrevendo seus mecanismos de ação e inibição (NEURATH e SCHWERT, 1950). Estes pesquisadores conseguiram demonstrar e consolidar a noção de que as enzimas são moléculas de natureza proteica e que a atividade enzimática depende da integridade molecular dessas proteínas (NORTHROP e KUNITZ, 1932).

No que se refere aos estudos do mecanismo de ação das tripsinas, ainda em 1937 Bergmann et al propuseram que a tripsina purificada por Northrop hidrolisava ligações peptídicas na porção carboxiterminal do aminoácido arginina; já em 1939 Hofmann e Bergmann demonstraram que a tripsina também é capaz de hidrolisar na porção carboxiterminal do aminoácido lisina. Já os trabalhos de Schaffer, Dixon e colaboradores (DIXON et al, 1958a; SCHAFFER et al, 1953, 1958) introduziram a ideia de que a atividade proteolítica da tripsina, da quimotripsina e de outras enzimas semelhantes se dava pela ação de uma serina reativa em seus sítios ativos. Adicionalmente, estes pesquisadores observaram que os resíduo de histidina e ácido aspártico também estavam envolvidos na atividade destas serino proteases (DIXON et al, 1958b).

Os passos seguintes das pesquisas com tripsinas envolveram a obtenção de sequências primárias (HARTLEY e KAUFFMAN; 1966; WALSH e NEURATH, 1964), bem como a resolução da suas estruturas terciárias (STROUD et al, 1974; HUBER et al, 1974). Desta forma, vários aspectos da estrutura, mecanismo de ação, interação com substratos e inibidores da tripsina foram comprovados e/ou desvendados. Já nos anos 90, com o avanço dos estudos de mutação sítio dirigida, passou-se a destrinchar a importância real de resíduos de aminoácido específicos para a atividade, reconhecimento de substrato e autólise da tripsina e de outras serino proteases. Neste âmbito, uma das descobertas médicas mais instigantes foi a de que a troca de uma arginina na posição 117 por uma histidina teria relação com o desenvolvimento da pancreatite hereditária humana (VÁRALLYAY et al, 1998; WHITCOMB et al, 1996).

Atualmente, os estudos acerca das tripsinas envolvem o efeito de inibidores como agentes terapêuticos, aplicação laboratorial em proteômica, expressão heteróloga e efeito de reagentes químicos diversos sobre a estrutura destas enzimas. De acordo com o Merops[9] (o banco de dado de peptidases e seus inibidores) a tripsina (EC 3.4.21.4) é classificada como

[9] Disponível on-line em <http://merops.sanger.ac.uk/>. Acesso em: 25/08/2013

uma serino endoprotease do clã PA, família S1, que é capaz de clivar ligações peptídicas na porção carboxiterminal dos aminoácidos arginina e lisina.

2.3.2 Estrutura e mecanismo de ação

Em 1964, Walsh e Neurath compararam a estrutura primária do quimotripsinogênio com o tripsinogênio bovino e constataram que estas duas enzimas se assemelham em muitos aspectos: são serino proteases; têm peso molecular e pontos isoelétricos semelhantes; e têm composição geral de aminoácidos e sequência primária similares (Figura 9). Em 1966, a sequência de quimotripsinogênio publicada por Walsh e Neurath (1964) foi revisada e corrigida por Hartley e Kauffman. A partir de então, esta última sequência tornou-se o modelo padrão para a classe das enzimas quimotripsinas-símile, da qual fazem parte todas as enzimas com a tríade catalítica His-Asp-Ser, inclusive a tripsina.

Figura 9. Alinhamento do quimotripsinogênio com o tripsinogênio bovino.

THE STRUCTURAL SIMILARITY OF TRYPSINOGEN AND CHYMOTRYPSINOGEN

```
                          1   2   3   4   5   6   7   8   9  10  11  12  13  14  15  16  17  18
Chymotrypsinogen     cys-gly-val-pro-ala-ile-gln-pro-val-leu-ser-gly-leu-ser-arg-ILE-VAL-GLY-
Trypsinogen                                                  val-asp-asp-asp-asp-lys-ILE-VAL-GLY-
                                                              1   2   3   4   5   6   7   8   9

 19  20  21  22  23  24  25  26  27  28  29  30  31  32  33  34  35  36  37  38  39  40  41
asp-glu-glu-ala-val-pro-gly-ser-trp-PRO-trp-GLN-VAL-SER-LEU-gln-asp-lys-thr-GLY-phe-HIS-PHE-
gly-tyr-thr-cys-gly-ala-asn-thr-val-PRO-tyr-GLN-VAL-SER-LEU-asn-       -ser-GLY-tyr-HIS-PHE-
 10  11  12  13  14  15  16  17  18  19  20  21  22  23  24  25          26  27  28  29  30

 42  43  44  45  46  47  48  49  50  51  52  53  54  55  56  57  58  59  60  61  62  63  64
CYS-GLY-GLY-SER-LEU-ILE-ASN-glu-asn-TRP-VAL-VAL-thr-ALA-ALA-HIS-CYS-gly-val-thr-thr-ser-asp-
CYS-GLY-GLY-SER-LEU-ILE-ASN-ser-gln-TRP-VAL-VAL-ser-ALA-ALA-HIS-CYS-tyr-lys-ser-gly-ile-gln-
 31  32  33  34  35  36  37  38  39  40  41  42  43  44  45  46  47  48  49  50  51  52  53

 65  66  67  68  69  70  71  72  73  74  75  76  77  78  79  80  81  82  83  84  85  86  87
VAL-val-val-ala-gly-glu-phe-asp-gln-gly-ser-ser-ser-glu-lys-ile-gln-lys-leu-lys-ile-ala-lys-
VAL-arg-leu-gly-glu-asp-asn-ile-asn-val-val-glu-gly-asp-glu-gln-phe-ile-ser-ala-ser-lys-ser-
 54  55  56  57  58  59  60  61  62  63  64  65  66  67  68  69  70  71  72  73  74  75  76

 88  89  90  91  92  93  94  95  96  97  98  99 100 101 102 103 104 105 106 107 108 109 110
val-phe-lys-asn-SER-lys-TYR-ASN-ser-leu-thr-ile-ASN-ASN-asn-ILE-thr-LEU-leu-LYS-LEU-ser-thr-
ile-val-his-pro-SER-   -TYR-ASN(pro,leu,thr,asn)ASN-ASN-asp-ILE-met-LEU-ile-LYS-LEU-lys-ser-
 77  78  79  80  81      82  83  84  85  86  87  88  89  90  91  92  93  94  95  96  97  98

111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133
ALA-ALA-SER-phe-ser-gln-thr-VAL-ser-ala-val-cys-LEU-PRO-ser-ala-ser-asp-asp-phe-ala-ALA-GLY-
ALA-ALA-SER-leu-asn-ser-arg-VAL-ala-ser-ile-ser-LEU-PRO-thr-ser-cys-       -gla-ser-ALA-GLY-
 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115         116 117 118 119

134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156
THR-thr-CYS-val-thr-thr-GLY-TRP-GLY-leu-THR-arg-tyr-thr-asn-ala-asn-thr-PRO-ASP-arg-LEU-gln-
THR-gln-CYS-leu-ile-ser-GLY-TRP-GLY-asn-THR-lys-ser-ser-gly-thr-ser-tyr-PRO-ASP-val-LEU-lys-
120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142

157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179
gln-ala-ser-leu-PRO-leu-LEU-SER-asn-thr-asn-CYS-LYS-lys-tyr-trp-gly-thr-lys-ILE-lys-asp-ala-
cys-leu-lys-ala-PRO-ile-LEU-SER-asp-ser-ser-CYS-LYS-ser-ala-tyr-pro-gly-gln-ILE-thr-ser-asn-
143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165

180 181 182 183 184 185 186 187 188 189         190 191 192 193 194 195 196 197 198 199 200
MET-ile-CYS-ALA-GLY-ala-ser-gly-val-ser-      -SER-CYS-met-GLY-ASP-SER-GLY-GLY-PRO-leu-VAL-
MET-phe-CYS-ALA-GLY-tyr-leu-glu-gly-gly-lys-asn-SER-CYS-gln-GLY-ASP-SER-GLY-GLY-PRO-val-VAL-
166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188

201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223
CYS-lys-lys-asn-gly-ala-trp-thr-leu-val-GLY-ILE-VAL-ser-SER-TRP-GLY-SER-ser-thr-cys-ser-thr-
CYS-ser-gly-lys-leu-gln-               -GLY-ILE-VAL-   -SER-TRP-GLY-SER-gly-cys-ala-gln-lys-
189 190 191 192 193 194                 195 196 197     198 199 200 201 202 203 204 205 206

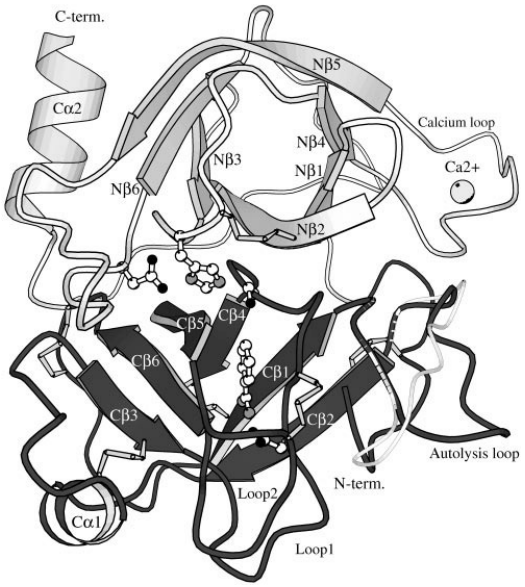
224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246
ser-thr-PRO-GLY-VAL-TYR-ala-arg-VAL-thr-ala-leu-VAL-asn-TRP-val-gln-GLN-THR-leu-ALA-ala-ASN
asn-lys-PRO-GLY-VAL-TYR-thr-lys-VAL-cys-asn-tyr-VAL-ser-TRP-ile-lys-GLN-THR-ile-ALA-ser-ASN
207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229
```

Fonte: Walsh e Neurath (1964).

As quimotripsinas maduras têm 229 aminoácidos, enquanto as tripsinas têm entre 222 e 224 aminoácidos. Esta diferença faz com que alguns aminoácidos-chave (principalmente os da tríade catalítica) apresentem numerações diferentes nas duas enzimas (Figura 9). Assim, para manter uma padronização numérica entre as sequências, convencionou-se alterar a numeração das tripsinas para que ela esteja igual à numeração da quimotripsina bovina publicada por Hartley e Kauffman (1966). Por isso, é comum se encontrar na literatura notações de tripsinas sem os resíduos de número 35, 36, 68, 126, 131, 205, 206, 207, 208, 218 e com resíduos com numeração 184b, 188b e 221b (SMALÅS et al, 1994; LEIROS et al, 1999). Essas modificações, contudo, não implicam a ocorrência de deleções ou inserções nas sequências. Trata-se, tão somente, de uma padronização.

Da mesma forma que a sequência primária, a estrutura tridimensional das tripsinas também é semelhante à das quimotripsinas. Ambas são organizadas em dois barris-β compostos de 6 fitas cada (Figura 10). Entre estes barris, encontra-se o sítio ativo, formado pela tríade catalítica (His57/Asp102/Ser195) e pelo sítio de reconhecimento do substrato, sendo este específico para cada enzima (Asp189/Gly216/Gly226, nas tripsina). Além disso, as tripsinas apresentam um loop de ligação ao cálcio (Glu70/Asn72/Val75/Glu77/Glu80) e outro de autólise (resíduos 141 a155).

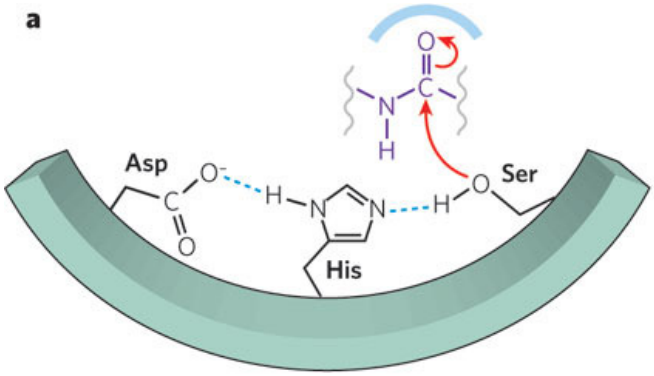
Figura 10 – Estrutura geral de uma tripsina aniônica de salmão (2TBS) sobreposta com uma tripsina aniônica bovina (3PTB).



Legenda: (C-temr) porção carboxiterminal; (Cβ) β-folha do domínio C; (Cα) α-hélice do domínio C; (Nβ) β-folha do domínio N. **Fonte:** Leiros et a 2000.

Os aminoácidos da tríade catalítica interagem entre si através de pontes de hidrogênio, que se formam entre os átomos Nδ1-H da His57 e Oδ1 do Asp102 e entre os átomos Nε2 da His57 e OH da Ser195 (Figura 11). Estas pontes de hidrogênio são essenciais para a atividade proteolítica das serino proteases, tanto que mudanças no pH podem afetar esta interação. Quando o pH do meio se encontra inferior a 6 (pK_R da Histidina), o átomo Nε2 da His57 é protonado, impedindo a formação da ponte de hidrogênio e tornando a enzima inativa (HEDSTROM, 2002).

Figura 11. Aminoácidos que compõem a tríade catalítica do sítio ativo das serino proteases.

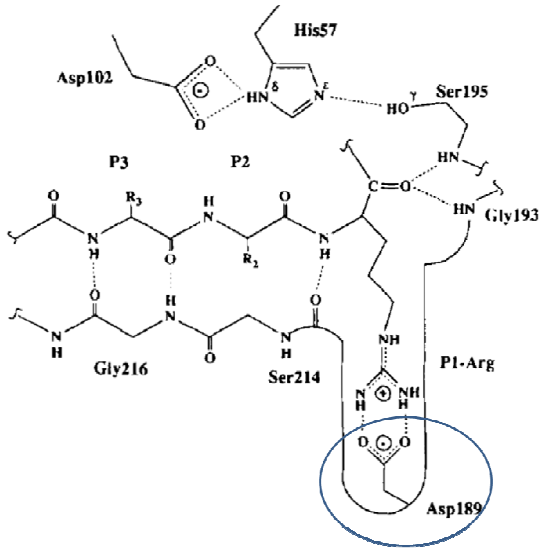


Fonte: Erez et al (2009)[10].

Próximo ao sítio catalítico encontra-se o sítio de reconhecimento do substrato (Asp189/Ser214/Gly216), região que não participa ativamente da hidrólise, mas confere especificidade de substrato à enzima (PERONA e CRAIK, 1995). A configuração na qual a Asp189 (Figura 12) se encontra faz com que a tripsina tenha preferência por aminoácidos com cadeias laterais longas e carregadas positivamente na posição P1, como arginina e lisina (OLSEN et al, 2004).

[10] Disponível em: < http://www.nature.com/nature/journal/v459/n7245/box/nature08146_BX1.html>. Acesso em : 21 jan 2014.

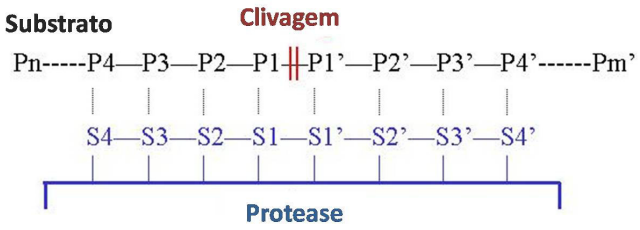
Figura 12 - Representação esquemática da interação de um peptídeo com o sítio ativo da tripsina.



Fonte: PERONA e CRAIK (1995).

A denominação "P1" acima indicada vem da padronização de nomenclatura da ligação protease-substrato publicada por Schechter e Berger (1968), apresentada na Figura 13. Neste modelo, os resíduos de aminoácido da enzima são numerados, a partir do sítio catalítico, de S1 a Sn (sendo Sn o aminoácido N-terminal) e de S1' a Sm' (sendo Sm' o aminoácido C-terminal). Já os resíduos de aminoácido do substrato para esta mesma enzima são numerados, a partir do sítio de clivagem de P1 a Pn (sendo Pn o aminoácido N-terminal) e de P1' a Pm' (sendo Pm' o aminoácido C-terminal).

Figura 13. Representação esquemática do complexo enzima-substrato.



Fonte: Expasy[11], 2005

Outra estrutura bem conservada nas tripsinas dos vertebrados é o loop de cálcio (PAPALEO et al, 2005). Apesar da tripsina não ser uma metaloprotease, vários ensaios

[11] Disponível em: <http://web.expasy.org/peptide_cutter/peptidecutter_enzymes.html>. Acesso em 10 jan 2014.

bioquímicos já demonstraram que a presença de cálcio no meio contribui para o aumento da estabilidade e atividade desta enzima (SIPOS e MERKEL, 1970). Bulaj e Otlewski (1994) avaliaram a estrutura do tripsinogênio bovino com e sem cálcio. Eles observaram que a ligação deste íon não gera modificações estruturais importantes no tripsinogênio, mas tem um efeito amplo sobre a molécula, aumentando a sua entalpia e temperatura de denaturação. Mesmo assim, ainda se sabe pouco sobre como a ligação do cálcio efetivamente contribui para a estabilidade das tripsinas.

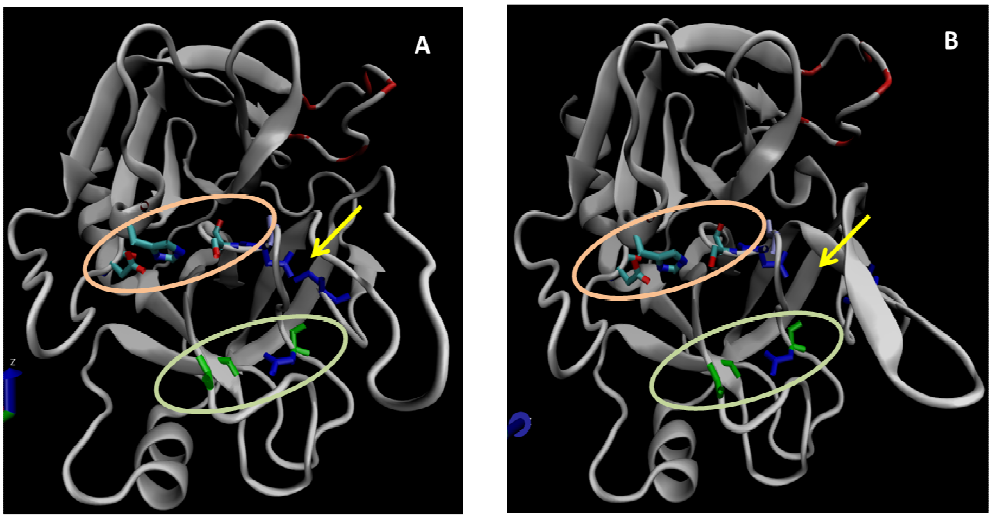
Mais uma estrutura comum às tripsinas é o loop de autólise. Este loop é assim chamado por conter um ponto de clivagem (K145), que leva à conversão da β-tripsina em α-tripsina, uma forma menos ativa da tripsina (SCHROEDER e SHAW 1968; FEHLHAMMER et al, 1977). Este ponto de autólise, porém, não está presente em todas as tripsinas, não sendo encontrado na tripsina de rato, por exemplo, (VARALLYAY et al, 1998) e nem nas tripsinas de peixe. Mas, K145 não é o único ponto de autólise existente nas tripsinas, já que qualquer ligação peptídica que contenha os resíduos arginina ou lisina terá potencial para ser hidrolisada por outra tripsina. Uma tripsina de rato, por exemplo, possui 13 Lys e Arg em sua sequência, porém nem todas estão susceptíveis a autólise na proteína nativa. (VARALLYAY et al, 1998).

Em 1996, Whitcomb et al apresentaram um aminoácido-chave para a autólise da tripsina, o R117. A mutação R117H está associada à pancreatite hereditária humana e postula-se que a alta resistência à autólise derivada desta mutação seja a causa para o desenvolvimento da pancreatite (VARALLYAY et al, 1998). Para testar esta hipótese, Archer et al (2006) induziram esta mutação em pâncreas de camundongos transgênicos. Estes autores observaram que os camundongos com a tripsina mutada apresentaram maior resposta inflamatória e lesão do tecido pancreático do que os modelos controle. Mesmo assim, é importante que se tenha cautela ao afirmar que esta mutação é a causa da pancreatite, pois o pâncreas possui mecanismos de defesa, como por exemplo, inibidores, contra os efeitos deletérios da ativação prematura das proteases (GRAF e SZILÁGYI, 2003). Ademais, vários trabalhos têm demonstrado que existem outras mutações, proteases e moléculas envolvidas no desenvolvimento da pancreatite em mamíferos (GRÁF e SZILÁGYI, 2003).

Assim, o principal mecanismo de defesa contra a autólise das células pancreáticas é a produção das enzimas proteolíticas em suas formas inativas, os zimogênios (CHEN et al, 2003). No caso do tripsinogênio, a presença de um peptídeo sinal na porção N-terminal é o suficiente para inpedir sua atividade. Uma vez no intestino, a enzima é ativada pelas enteroquinases e por outras tripsinas através da clivagem da ligação entre a Lys15 e a Ile16

(Figura 14). O aminoácido Isoleucina é apolar, mas a liberação da sua porção N-terminal permite que uma ponte salina com a Asp194 seja formada. Com esta ligação Ile16-Asp194 é gerada a "cavidade do oxiânion", a qual tem um papel fundamental no mecanismo de ação da enzima (HEDSTROM, 2002).

Figura 14 - Comparação entre a estrutura de uma tripsina bovina nativa (2PTN) e seu tripsinogênio (1TGS).



Legenda: (A) Tripsina bivina ativa; (B) Tripsinogênio bovino. A seta amarela aponta a ponte salina formada entre a Ile16 e a Asp194, ausente no tripsinogênio. O circulo em laranja aponta os resíduos da tríade catalítica e o círculo em verde os resíduos de reconhecimento do substrato **Fonte:** Desenhado com o programa Visual Molecular Dynamics (VMD).[12]

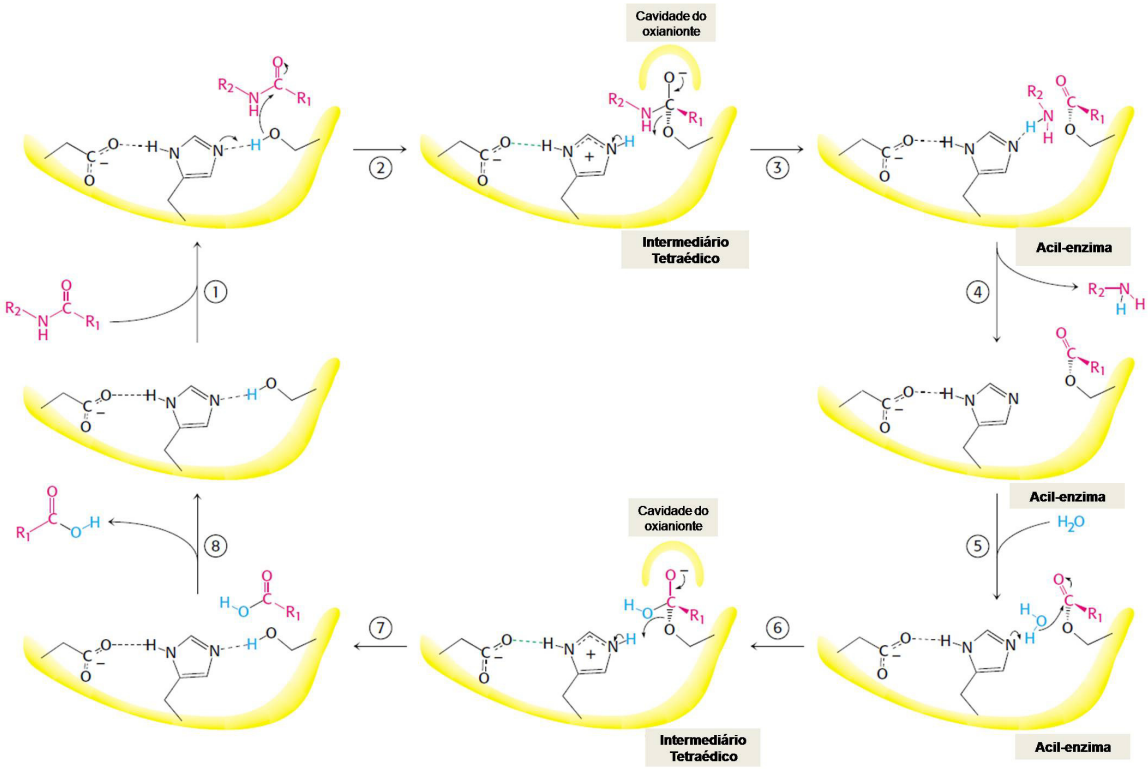
O mecanismo de ação das proteases varia de acordo com o aminoácido que lhe confere a especificidade catalítica. Mas, em linhas gerais, a hidrólise de uma ligação peptídica se inicia com a perturbação da ressonância entre a amida e a carboxila da ligação peptídica alvo, terminando com a adição de uma molécula de água para que o produto final seja liberado (HEDSTROM, 2002; POLGÁR, 2005).

A Figura 15 ilustra o mecanismo de ação das tripsinas, o qual consiste de 8 etapas. (1) Acoplamento do substrato (peptídeo) ao sítio ativo; e formação do complexo enzima-substrato com Ser195; (2) Ataque nucleofílico da Ser195 à carbonila da ligação peptídica a ser quebrada, com formação de intermediário oxiânion, o qual é estabilizado pelas cargas positivas da cavidade do oxiânion; (3) Colapso do intermediário tetraédrico e formação de um complexo acil-enzima; (4) dissociação do primeiro produto de hidrólise, o componente amínico do peptídeo clivado; (5) Entrada de uma molécula de água no sítio ativo; (6) Realização do segundo ataque nucleofílico pela molécula de água; (7) Colapso do

[12] Disponível para download em: <http://www.ks.uiuc.edu/Research/vmd/>

intermediário tetraédrico; (8) dissociação do segundo produto de hidrólise, o componente carboxílico do peptídeo clivado (KASSERRA e LAIDLER, 1969; HEDSTROM, 2002; PERONA e CRAIK, 1995; POLGÁR, 2005; BERG et al, 2008). A reação catalisada pela enzima é 10^9 vezes mais rápida do que seria se ocorresse naturalmente (PERONA e CRAIK, 1995). Através deste mecanismo a tripsina também pode hidrolisar outros compostos orgânicos, como amidas, anilidas, ésteres e tioésteres (HEDSTROM, 2002).

Figura 15 – Mecanismo de ação das tripsinas



Fonte: Berg et al (2008).

2.3.3 Aplicabilidade comercial da tripsina e de outras proteases

Uma das primeiras aplicações industriais das enzimas proteolíticas foi na fabricação de queijo, inicialmente com o uso de sumo de plantas e posteriormente de extratos de estômago de animais jovens (RAWLINGS et al, 2007). As proteases estão entre as principais enzimas comercializadas no mundo, representando cerca de 60% do contingente deste mercado (RAO et al, 1998). As principais aplicações industriais das proteases são: produção de laticínios, pães, derivados de soja, produtos têxtil e de depilação de couro e pele, processamento de carnes, e detergentes.

Dentre as aplicações descritas acima, a formulação de detergentes é um dos focos da presente tese. O emprego de enzimas neste ramo se iniciou em 1913, com o uso de extratos de pâncreas suíno (WOLFGANG, 2004). Esta prática, porém, só foi se intensificar a partir da década de 1960, com a introdução da Alcalase® (Novozymes), uma subtilisina extraída da bactéria *Bacillus licheniformis* (MAURER, 2004; WOLFGANG, 2004). Assim como a Alcalase®, outras enzimas da família das subtilisinas são utilizadas hoje em dia na composição de detergentes (ANWAR e SALEEMUDDIN, 1998; RAWLINGS et al, 2007; MAURER, 2004).

As enzimas provenientes de microrganismos continuam sendo a principal fonte de enzimas comerciais, mas há uma procura por novas fontes de moléculas bioativas, e os organismos aquáticos têm potencial para suprir esta demanda (BOUGATEF, 2013). As proteases desses animais podem ser utilizadas por indústrias de alimentos, detergentes, produtos farmacêuticos e couro, bem como em processos de biorremediação (SHAHIDI e KAMIL, 2001; ALI et al, 2009; ESPÓSITO et al, 2009a; 2009b; 2010; NASRI et al, 2012; KTARI et al, 2012). Dentre as enzimas alcalinas dos animais aquáticos, a mais abundante e melhor estudada é a tripsina. Esta enzima pode ser aplicada, por exemplo, no sequenciamento de outras proteínas, na remoção de resíduos proteicos aderidos a vidros, tecidos ou plásticos, bem como na dissociação de culturas de células. De acordo com Jónsdóttir et al. (2004), tripsinas extraídas do bacalhau podem ser aplicadas na elaboração de remédios para inflamações, fungos, acne e outros problemas dermatológicos. Estes autores também citam um medicamento denominado *Penzim*, o qual contém esta enzima em sua formulação e que vem sendo comercializado para o tratamento de psoríases e eczemas.

Além de suas utilizações na forma solúvel, a tripsina pode ser imobilizada em suportes insolúveis e assim ser reaproveitada várias vezes. A enzima imobilizada pode ser aplicada na remoção de fatores antinutricionais presentes em componentes de rações e alimentos (ex: soja), na produção de hidrolisados proteicos para rações e suplementos alimentares, bem como na purificação de inibidores específicos (AMARAL et al., 2006).

2.3.4 Comparação entre tripsina de peixes e outros animais

Como já foi discutido previamente, os peixes podem ser encontrados em ambientes muito variados no que se refere à temperatura, à composição da água e às condições físico-químicas do meio (VAL et al, 2005). Mesmo assim os peixes apresentam as mesmas rotas metabólicas que os animais homeotermos terrestres, além de um metabolismo tão eficiente quanto (HALVER e HARDY, 2002). Para que isso seja possível, estes animais usam de

estratégias adaptativas, adequando sua fisiologia e morfologia às exigências dos estresses do ambiente. Uma forma de fazer esta adequação é através de pequenas mutações em suas enzimas, principalmente em organismos que vivem em temperaturas extremas.

Os trabalhos com enzimas de peixes adaptadas ao frio se iniciaram no final de 1960, com estudos sobre a Lactato Desidrogenase (LDH) de peixes de diversos ambiente. Nestes estudos os autores observaram que as adaptações bioquímicas frente às baixas temperaturas podem se dar por três vias: (1) modificações na concentração das enzimas no organismo; (2) variação genética ligada à alteração de propriedades cinéticas, como o k_{cat} ou (3) modulação da atividade de enzimas pré-existentes por alterações covalentes nas enzimas ou pelo uso de composto moduladores de baixa massa molecular (SOMERO, 2004). No que se refere ao aumento da atividade catalítica, ela ocorre principalmente por conta da maior flexibilidade estrutural da proteína (FELLER et al, 1996). Como consequência, tem-se um aumento na constante de ligação ao substrato (Km), bem como uma desordenação maior na estrutura, em comparação aos hortólogos mesofílicos (SOMERO, 2004).

A questão da flexibilidade da estrutura está muito ligada à adaptação à temperatura, de forma que enzimas termofílicas tendem a ter uma estrutura mais rígida, enquanto que as psicrofílicas tendem a ser mais flexíveis (FELLER et al, 1997). Tendo em vista que as proteínas são macromoléculas naturalmente instáveis, a simples adição de algumas ligações intramoleculares (por exemplo, pontes de hidrogênio, interações hidrofóbicas, pontes salinas etc.) pode tornar as suas estruturas mais estáveis (FELLER et al, 1996). Neste âmbito, Feller et al (1996) listaram alguns aspectos relacionados ao aumento da flexibilidade nas proteínas: (1) Diminuição no número de resíduos de prolína; (2) aumento do número de resíduos de glicina; (3) aumento do número de aminoácidos de caráter hidrofílico; e (4) diminuição dos aminoácidos de caráter hidrofóbico.

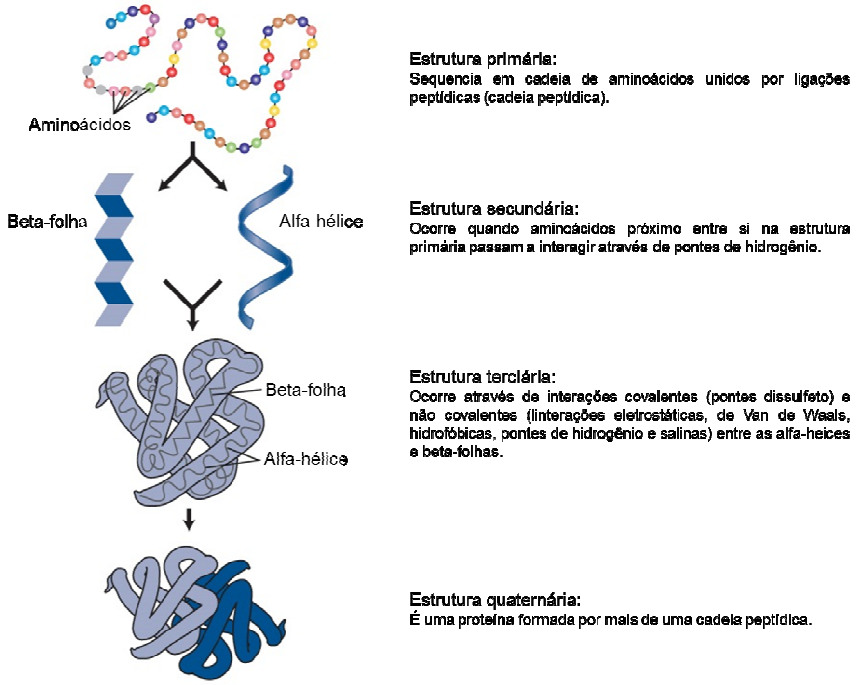
Tratando-se das adaptações à temperatura encontradas nas tripsinas, Leiros et al (1999) descreveram as seguintes para tripsinas adaptadas ao frio: Hidrofobicidade reduzida, com baixa razão de (Ile + Leu)/(Ile+Leu+Val); (2) Estabilidade reduzida na porção C-terminal; Mutação na prolína da posição 152 e nos aminoácidos Pro28-Tyr29, bem como a deleção da tirosina na posição 151. Estes autores sugerem que estas modificações melhoram o acesso do substrato ao sítio ativo e aumentam a flexibilidade da proteína.

2.4 Estudo estrutural e bioquímico das proteínas

2.4.1 Noções gerais de estrutura das proteínas

Ao ler um livro de bioquímica, básica como Lehninger et al (2006), Berg et al (2008) ou Voet et al (2000), nos deparamos com os conceitos clássicos de organização da estrutura proteica em quatro níveis. (primário, secundário, terciário e quatenário, Figura 16).

Figura 16 – Níveis de organização das proteínas.



Fonte: National Human Genome Research[13] (Tradução nossa).

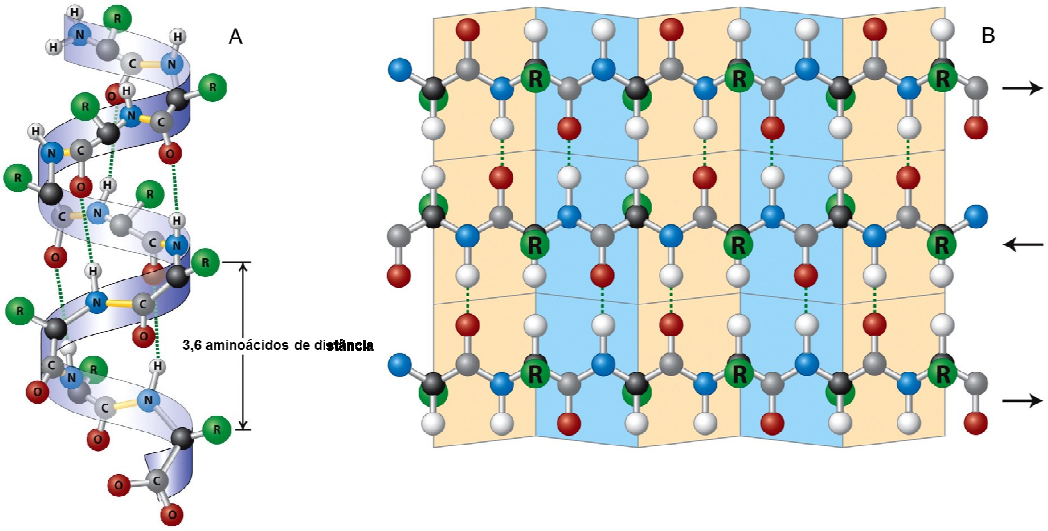
Estes conceitos estruturais, que hoje consideramos "básicos", começaram a ser desenvolvidos há apenas 63 anos. Em dois artigos publicados em 1951, 7 anos antes da publicação da primeira estrutura de uma proteína, Linus Pauling, Robert Corey e H. Branson propuseram duas possíveis configuração para as cadeias polipeptídicas das proteínas fibrosas e globulares: α-hélices(Pauling et al, 1951a) e β-folhas (Pauling et al, 1951b).

A α-hélice (Figura 17A) descrita por Pauling, Corey e Branson, é formada pela interação entre o grupo carbonila de um resíduo com o grupo amida de outro. Cada volta da hélice apresenta 3,7 resíduos de aminoácidos, com os resíduos adjacentes apresentando um deslocamento de 1,5 Å. Já as β-folhas são formadas por duas ou mais cadeias polipeptídicas,

[13] Disponível em: <http://www.genome.gov/Glossary/index.cfm?p=viewimage&id=169>. Acesso em 21 jan 2014.

quase que completamente distendidas, dispostas em conformações antiparalelas (Figura 17B) ou paralelas. Por conseguinte, as pontes de hidrogênio são formadas entre os grupos carbonila e amida de aminoácidos paralelamente disposto. Nesta conformação, os aminoácidos adjacentes têm um deslocamento de 3,3 Å. Além das α-hélices e β-folhas, existe uma estrutura secundária de conexão, conhecida como β-volta, a qual não apresenta estrutura periódica regular, mas é rígidas e bem definidas (Berg et al, 2008).

Figura 17 – Esquematização da estrutura secundária das proteínas.

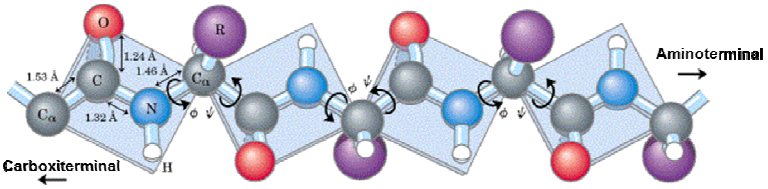


Legenda: (A) Estrutura da α-Hélice; (B) Estrutura da β-folha. Fonte: Alberts et al (2008)[14].

Com os trabalhos desenvolvidos por Linus Pauling e seus contemporâneos, passou-se a compreender as formas que as cadeias peptídicas podem tomar. Mas, o que determina se um aminoácido de uma proteína vai formar uma α-hélice, uma β-folha ou participar de um loop? A resposta está na própria composição dos aminoácidos que formam a estrutura primária das proteínas. Os átomos que participam da ligação peptídica (O carbono da carbonila e o Nitrogênio da amina) estão no mesmo plano espacial. Já as ligações destes átomos com os carbonos alfa (Cα) de seus respectivos aminoácidos encontram-se em planos diferentes (Figura 18), e estas ligações podem assim rotacionar. Os ângulos de torção em torno do carbono alfa são denominados φ (entre o Cα e o N) e ψ (entre o Cα e a carbonila) e cada aminoácido vai se comportar de uma forma, a depender dos resíduos vizinhos.

[14] Disponível em: (A) <http://www.bio.miami.edu/tom/courses/protected/MCB6/ch03/3-04.jpg>; (B) <http://www.bio.miami.edu/tom/courses/protected/MCB6/ch03/3-05a.jpg>. Acesso em 21 jan 214

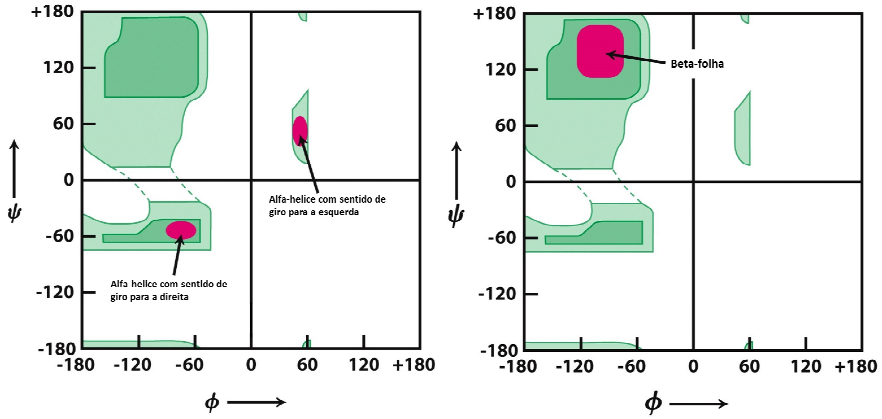
Figura 18 – Configuração espacial padrão de resíduos de aminoácidos em uma cadeia peptídica.



Fonte: IBM-Jena, on-line.[15]

Neste âmbito, Ramachandran et al (1963) desenvolveu um *plot* hoje muito utilizado em trabalhos de estrutura de proteína. Com o uso deste *plot* é possível determinar se a posição teórica tomada por um aminoácido em uma estrutura é fisicamente possível ou não, utilizando-se como base os ângulos de rotação φ e ψ (Figura 19).

Figura 19 – Plot de Rachamandran



Fonte: Berg et al, 2008.

Hoje em dia o estudo da estrutura secundária das proteínas está bem mais avançado do que contemporâneos de Pauling imaginavam. Para se ter uma ideia, em 1950, Neurath e Schwert publicaram um artigo de revisão no qual afirmaram o seguinte:

> "Apesar dos rápidos progressos recentes, a analise dos aminoácidos das proteínas ainda não progrediu o suficiente para render uma estrutura estereoquímica completa de qualquer proteína em termos de sua sequência de resíduos de aminoácidos. A perspectiva de se determinar a natureza dos centros catalíticos das proteinases através de procedimentos analítico diretos, portanto, parece remota." (NEURATH e SCHWERT, 1950, tradução nossa).

Mas, ainda em 1958 Max Perutz e John Kendrew conseguiram resolver a primeira estrutura da uma proteína, usando cristalografia de raio-x. Este feito lhes rendeu um Nobel em química em 1962, e abriu os caminhos para o campo de estudo das estruturas de proteínas.

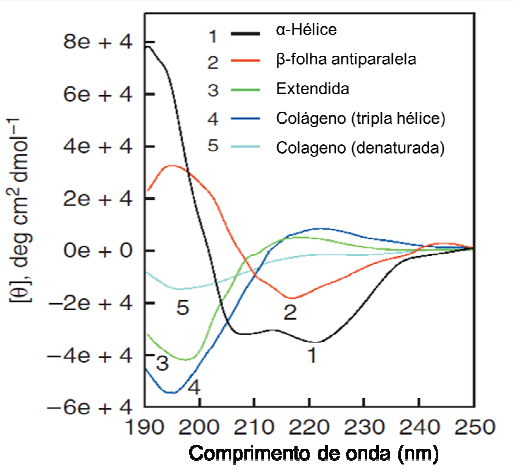
[15] Disponível em: <http://www.imb-jena.de/~rake/Bioinformatics_WEB/basics_peptide_bond.html>. Acesso em 21 Jan 2014.

2.4.2 Técnicas aplicadas ao estudo estrutural das proteínas

Uma das técnicas mais comumente aplicadas no estudo estrutural das proteínas é o Dicroísmo Circular (DC), o qual usado para se avaliar a interação de proteína com ligantes, bem como para determinar se uma proteína esta enovelada corretamente ou se uma mutação afeta tua conformação ou estabilidade. O DC pode ser definido como a absorção diferenciada da luz circular polarizada para a esquerda e para a direita. Este fenômeno é possível porque os aminoácidos, excetuando-se a glicina, são moléculas assimétricas e, portanto, eles irão absorver a luz polarizada para a direita e para a esquerda em intensidades diferentes. Esta diferença na absorbância gera um vetor com uma trajetória elíptica. O grau de "elipticidade" de uma molécula, ou seja, o ângulo da tangente entre o menor e o maior eixo da elipse formada, é a unidade utilizada na análise do dicroísmo circular (DOUGLAS, 2013; GREENFIELD, 2006).

A análise da estrutura secundária das proteínas é normalmente realizada na luz ultravioleta distante do visível (180 a 260 nm). Já no espectro de luz ultravioleta próxima do visível (250 a 320nm), pode-se utilizar o conjunto de aminoácidos aromáticos da proteína para estimar o "fingerprinting" da sua estrutura terciária (KELLY et al, 2005). Os espectros gerados pela análise de dicroísmo circular vão variar de acordo com a composição de estrutura secundária da proteína (Figura 20) e podem ser posteriormente analisados por softwares on-line, para determinar a concentração percentual de cada estrutura da proteína.

Figura 20. Espectros de DC em UV-distante de vários tipos de estrutura secundária.



Fonte: Greenfield (2007).

É importante se ter em mente, porém, que a técnica do dicroísmo é muito limitada para aplicações em estudos de estrutura terciária. Para tanto, outras metodologias deverão ser

utilizadas, como cristalografia com difração de raio-x e espectroscopia de ressonância nuclear magnética (RMN). Ambas as metodologias fornecem detalhes atômicos da estrutura de macromoléculas cristalizadas (raio-x) ou em solução (RMN) (BERG et al, 2008). Contudo, a obtenção de amostras em quantidade e qualidade ótimas para a realização desses ensaios envolve experimentos laboriosos, dispendiosos e que necessitam de uma grande quantidade de proteína pura (SHEN e CHOU, 2009). Portanto, uma alternativa aos experimentos de bancada é a elucidação das estruturas secundária e terciária da proteína através de sua sequência de aminoácidos, *in silico*.

A estrutura primária de uma proteína pode ser obtida através do sequenciamento por técnicas como degradação de Edman, espectrometria de massa ou MALDI (Matrix-assisted laser desorption/ionization). A degradação de Edman foi desenvolvida em 1950 por Pehr Edman, e consiste remoção seriada dos aminoácidos da extremidade N-terminal de um peptídeo. No caso de sequências com mais de 50 aminoácidos, faz-se necessária a realização de uma digestão da proteína, para se gerar peptídeos que possam ser analisados (EDMAN, 1950; BERG et al, 2008). Já a espectrometria de massa usa uma relação ionização/tempo de vôo de peptídeos oriundos da digestão de uma proteína para se inferir a sequência da proteína estudada. Adicionalmente, o estudo da sequência primária de uma proteína pode ser feito pela clonagem e sequenciamento do gene que codifica a proteína.

Com a sequencia primária em mãos, a modelagem teórica da conformação terciária da proteína pode ser feita *ab initio* (do zero, deduzindo-se matematicamente a conformação) ou por homologia (por comparação com estruturas conhecidas de proteínas semelhantes) (AHSAN et al., 2001). O lado positivo da técnica de modelagem por homologia para uma proteína como a tripsina, é que já existem várias estruturas desta enzima disponíveis na literatura. Ao desenhar um modelo, os programas utilizados levam em consideração a energia envolvida na deformação das ligações covalentes, bem como as torções e os ângulos que os átomos das proteínas podem formar (FORSTER, 2003). Contudo, é importante que uma vez que o modelo esteja pronto, se faça uma avaliação de sua estrutura, para ter certeza de que a mesma é fisicamente possível. Para tanto, pode-se utilizar ferramentas on-line de validação de modelos, disponíveis em sites como o Swiss-modell (ARNOLD et al, 2006).

Uma vez obtida a estrutura, seja por cristalografia, RMN ou modelagem, pode-se estimar *in silico*, como a estrutura desta proteína se comporta em diversas situações teóricas, como mudança de temperatura e interação com ligantes. Para tanto, pode-se utilizar programas como o Gromacs (VAN DER SPOEL et al, 2005) ou o Charmm (BROOKS et al, 2009). Estes programas simulam as equações Newtonianas de movimento em um sistema

com centenas de milhões de partículas, possibilitando que se analise como cada átomos de uma molécula se comporta (APOL et al, 2010).

Finalmente, mais uma forma de se estudar o comportamento estrutural de uma proteína é através da fluorescência intrínseca. A fluorescência é uma característica típica de moléculas aromáticas, como por exemplo, os aminoácidos triptofano, tirosina e fenilalanina (LAKOWICZ, 2006). Por isso, as proteínas apresentam uma característica denominada fluorescência intrínseca, que pode ser mensurada e utilizada para se avaliar sítios de ligação a íons e moléculas orgânicas, a dinâmica de desnaturação da proteína, e as transições conformacionais em diferentes condições (VALEUR, 2001). O fenômeno da fluorescência envolve a absorção e liberação de energia por um elétron reativo. Sabe-se, por exemplo, que os resíduos de triptofano quando excitados em 295 nm, emitem fluorescência entre 310 e 400 nm. Se o pico de emissão for próximo de 310 nm, é indicativo que os resíduos de triptofano estão voltados mais para o interior da proteína, enquanto que um pico mais próximo de 400 nm indica que os resíduos estão mais expostos (Eftink, 1994). Há também o fenômeno de "quenching", no qual a presença de um ligante próximo a um triptofano pode modificar a fluorescência intrínseca, sendo assim possível estudar a interação da enzima com ligantes.

2.4.3 Técnicas aplicadas ao estudo bioquímico das enzimas

As enzimas são moléculas eficientes em catalisar reações específicas, facilitando a existência de vida na terra e atraindo interesse industrial, econômico e tecnológico. Para se entender como estas proteínas funcionam é preciso primeiro purificá-las, para então avaliar suas propriedades bioquímicas e biofísicas.

A eficiência da purificação de uma proteína depende de algumas de suas características químicas, físicas e biológicas como: massa molecular, hidrofobicidade, carga líquida da superfície, solubilidade, bioespecificidade e modificações pós-traducionais (SILVA Jr., 2004). Com base nisso, as metodologias mais comumente empregadas na purificação de proteínas são: **Precipitação fracionada** (as proteínas são separadas por fracionamento salino, com solventes orgânicos ou por mudança do pH do meio); **Cromatografia de exclusão molecular** (as proteínas são separadas de acordo com suas massas moleculares); **Cromatografia de troca iônica** (as proteínas são separadas de acordo a carga líquida de suas superfícies); **Cromatografia de afinidade** (as proteínas são separadas por interação com um ligante específico, *e.g.* antígeno, inibidor ou substrato).

Durante a purificação de uma proteínas é importante que se acompanhe o processo através de eletroforeses em gel de poliacrilamida (PAGE) e dosagens da atividade biológica.

A eletroforese é comumente utilizada para uma primeira avaliação, mais grosseira, da pureza e massa molecular das proteínas (SILVA Jr, 2001). Para uma análise mais fina é preciso utilizar técnicas como espectrometria de massa ou HPLC. Já no que se refere ao acompanhamento da atividade biológica das proteínas, os parâmetros mais importantes a serem observados são os cinéticos (constante de Michaelis (K_m), velocidade máxima (V_{max}), constante catalítica (k_{cat}) e eficiência catalítica (k_{cat}/K_m)) (BERG et al, 2008); e os parâmetros físico-químicos (efeito de pH, temperatura, inibidores e cofatores). Para tanto, existem várias metodologia que podem ser utilizadas. No presente trabalho, foram usados os substratos proteolíticos sintéticos azocaseína e o BAPNA (Hidroclorodrato de Na-Benzoil-D,L-arginina p-nitroanilida). O produto de hidrólise destes substratos confere cor ao meio e o aumento da intensidade desta cor pode ser acompanhado por espectrofotômetros.

Adicionalmente, podem ser utilizados substratos fluorescentes, quais usualmente utilizam o fenômeno de Transferência Ressonante de Energia de Fluorescência (FRET). O FRET é um fenômeno eletrodinâmico no qual há um grupo doador de energia (molécula no estado excitado) e um grupo aceptor de energia (molécula no estado fundamental). O grupo doador emite naturalmente ondas curtas de fluorescência, que se sobrepõem ao espectro de absorção do aceptor (LAKOWICZ, 2006). Assim, se estes grupos estiverem unidos através de um oligopeptídio, quando o doador for afastado do aceptor (hidrólise), a fluorescência emitida pelo primeiro passa a ser detectável.

3 Objetivos

3.1 Geral

Realizar um estudo aprofundado da estrutura e função da tripsina-símile do tambaqui (*Colossoma macropomum*) e investigar a aplicação da mesma em aditivos pré-lavagem de roupas.

3.2 Específicos

- Purificar a tripsina-símile do tambaqui;
- Amplificar, clonar e sequenciar o RNA mensageiro do gene que codifica a tripsina no ceco pilórico do tambaqui;
- Modelar a estrutura terciária da tripsina a partir de sua sequência primária de aminoácidos;
- Realizar dinâmicas moleculares em diferentes temperaturas com o modelo desenhado;
- Validar as dinâmicas moleculares através de análises de dicroísmo circular;
- Caracterizar a tripsina-símile quanto à sua interação com o íon cálcio;
- Avaliar a compatibilidade da enzima purificada frente a tenso-ativos e detergentes comerciais;

Capítulo 1: Serine proteases as pre-wash additives: a comparative study between fish and commercial enzymes

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Abstract

The efficiency of five enzymes as pre-wash additives, two fish trypsins (tambaqui and Nile tilapia) and three commercial proteases (subtilisin, porcine trypsin and bovine chymotrypsin) was investigated in this work. All enzymes were stable at the alkaline pH range (7.2 to 11.0) and temperatures up to 30°C. However, only tambaqui and Nile tilapia trypsin were able to remain stable at temperatures as high as 50°C. The anionic surfactant SDS inhibited more than 50% of all enzymes activities at 25°C, except for subtilisin, that remained 91.2% active. However, at 40 °C while all enzymes were inhibited, tambaqui trypsin was able to retain 70.5% of its activity. Circular dichroism spectra indicated that the α-helix content from the porcine trypsin structure increases significantly in the presence of SDS; however, this effect was less intense on tambaqui trypsin. Moreover, Tambaqui, Nile tilapia and subtilisin stability to powder detergent was higher than porcine trypsin and bovine chymotrypsin, but as a pre-wash additive, all enzymes seemed to produce a similar visual effect on the blood stains removal. Thus it can be stated that the trypsin purified from tambaqui and Nile tilapia present characteristics compatible with laundry detergent and pre-wash formulations.

Keywords**:** alkaline proteases, laundry detergent, stain removal, tropical fish; aquaculture processing waste.

1. Introduction

The first application of enzymes in the detergent industry was made in 1913 by Röhm and Haas who used swine pancreas extracts (Wolfgang, 2007). This was an important breakthrough for detergent formulation, since enzymes are biodegradable and able to remove stains at lower temperatures, what makes them more advantageous than surfactants (Jegannathan and Nielsen, 2013). However, it was only in the 1960s, when subtilisin Carlsberg from Bacillus species was introduced, that the use of proteases in cleaning products became technically and economically feasible (Maurer, 2004). This development allowed the market of detergent enzymes to grow and become the largest industrial applications of proteases, both in terms of volume and value (Kirk et al, 2002).

Today, the main protease used in the detergent industry continues to be subtilisin from microorganisms (Li et al, 2013), but this fact does not preclude scientists to search for new sources of detergent enzymes. The main features sought in these enzymes are high catalytic efficiency, stability at alkaline pH, stability in the presence of chelants and oxidant agents, and ability to hydrolyze proteins bounded to insoluble surfaces, such as cloth, dentures and contact lenses (Rawlings et al, 2007; Wolfgang, 2007). In this context, an yet underexploited source of enzymes suitable for application as detergent additive is fish viscera (Ali et al, 2009; Espósito et al, 2009a; 2009b; 2010; Jellouli et al 2009; Ktari et al, 2012; Nasri et al, 2012).

The processing of fish generates two main products: the edible (fillet and flitch) and the inedible (viscera, skin, scales, bones and carcass) parts. The edible portion of commercial fish is the most valuable and it may vary from 30 to 60% of the animal weight, depending on the species, growth condition, gender and processing method (Borderías and Sánchez-Alonso, 2011). The inedible portion, on the other hand, is

sometimes used in the manufacturing of low value products, such as fish meal, oil, fertilizer, silage and hydrolysates (Blanco et al 2007; Bougatef, 2013); or it is simply treated as urban solid waste. This neglected material, however, is rich in biomolecules like serine proteases, which could be better used to increase the market value of fish waste and turn its recovery into profite.

Therefore, the aim of this study was to purify trypsins from the two main freshwater fish cultured in Brazilian, tambaqui (*Colossoma macropomum*) and Nile tilapia (*Oreochromis niloticus*) (MPA, 2013), and compare them to three commercial serine proteases (subtilisin, porcine trypsin and bovine chymotrypsin).

2. Materials and Methods

2.1. Tissue extraction and crude extract preparation

Viscera from juvenile tambaqui (*Colossoma macropomum*) and Nile tilapia (*Oreochromis niloticus*) specimens were obtained from local fish farming companies. Tambaqui and Nile tilapia presented average weight of 1.8 kg ±0.2 and 1.1 kg ±0.1 and average length of 41.4 cm ±2.7 and 35.6 cm ±1.5, respectively. Tambaqui pyloric caeca (4.9 g ±0.7) and Nile tilapia intestines (16.1 g ±3.9) were homogenized in 10 mM Tris-HCl pH 8.0 with 15 mM NaCl (200 mg of tissue per buffer mL), using a tissue homogenizer (IKA RW 20D S32, China). Resulting homogenates were centrifuged (Sorvall RC-6 Superspeed Centrifuge, North Carolina, USA) at 10,000 x g for 15 min at 4 ^{o}C to remove cell debris and nuclei. Supernatants (crude extract) were used in the further purification steps.

2.2. Enzyme purification

Throughout the purification, tryptic activity was assayed with N-a-benzoyl-L-arginine p-nitroanilide (BApNA, Sigma-Aldrich®) prepared in dimethylsulfoxide (DMSO, Vetec). The assay followed the methodology described by Erlanger et al (1961), with volumes adapted for microplate reader (Bio-Rad X-Mark spectrophotometer, California, USA). Trypsin purification from both fish followed the methods described by Marcuschi et al (2010) modified for the present work, in which only three purification steps were used: (1) crude extract heating for 30 min at 45 °C; (2) ammonium sulfate fractioning (with higher trypsin activity found on fraction 30-60%), (3) and affinity chromatography, p-Aminobenzamidine–Agarose (Sigma-Aldrich®). In addition to the purified trypsins, three commercial enzymes were used

(subtilisin from *Bacillus licheniformis*, trypsin from *Sus scrofa* and chymotrypsin from *Bos taurus*, all purchased from Sigma-Aldrich®).

2.3. Total proteolytic activity and protein determination

Nonspecific proteolytic activity was assayed using 1% ($w \cdot v^{-1}$) azocasein according to Bezerra et al (2005). Released azo-dye (product) was measured at 450 nm using a microplate reader (Bio-Rad X-Mark™ spectrophotometer, California, USA). One unit (U) of proteolytic activity was defined as the amount of enzyme required to hydrolyze azocasein and cause an increase in the absorbance of 0.001 per min. Total protein content was estimated with Pierce™ BCA Protein Assay Kit (Thermo scientific), following manufacturers recommendations.

2.5. Temperature and pH effect on enzyme activity

Optimum temperature was assayed by incubating enzymes with 1% ($w \cdot v^{-1}$) azocasein pH 8.0 in a water bath (Tecnal TE-056 Mag, Brazil) for 60 min at temperatures ranging from 0 to 80 ^{o}C. For thermal stability the enzymes were incubated at temperatures ranging from 0 to 70 ^{o}C for 60 min without substrates, and then had their activity measured at 25 ^{o}C. Optimum pH was assayed at 25 ºC using 1% ($w \cdot v^{-1}$) azocasein prepared in different 0.2 M buffer solutions (Tris-HCl, pH 7.2-9.0; and NaOH-glycine, pH 9.5-11.0). pH stability was measured by incubating the enzymes at a 1:1 proportion with different buffers for 60 min and then measuring their residual activity at 25 ºC, pH 8.0.

131 *2.6. Enzyme stability to surfactants, oxidants and commercial detergents*

132 Proteases stability was assayed according to Moreira et al (2002) with: surfactants
133 agents, anionic (SDS, sodium choleate), zwiterionic (Chaps) and non-ionic (Triton X-
134 100, Tween 20) at final concentration of 0.5% and laundry detergents, Ala® (Procter &
135 Gamble), Bem-te-vi® (Alimonda), Ace®, Surf® (UniLever) and Assim® (Assolan) at
136 final concentration of 7 $mg \cdot mL^{-1}$. The proteases were incubated with each reagent at 25
137 ^{o}C and 40 ^{o}C. For each test, 30 µL aliquots were withdrawn after 60 min incubation (in
138 triplicate) and were assayed for proteolytic activity at 25 ^{o}C with 1% ($w \cdot v^{-1}$) azocasein,
139 as previously described. Two controls were prepared and submitted to the same
140 procedures: protease sample incubated with distilled water instead of reagents (100%
141 activity), and reagents incubated with distilled water instead of protease sample (blank).
142

143 *2.7 Circular dichroism spectroscopy*

144 Circular dichroism (CD) spectra were obtained using Jasco J-815 Circular CD
145 Spectropolarimeter (Japan). The instrument was calibrated with d-10-camphorsulfonic
146 acid (Sigma-Aldrich®) at 1 $mg \cdot mL^{-1}$ in water. The purified trypsin samples were
147 solubilized at a concentration of 0.1 $mg \cdot mL^{-1}$ in 10 mM borate buffer, pH 7.8.
148 Measurements were made at the Far-UV (190-250) spectrum, with and without SDS
149 (final concentration of 0.5%) in a 2 mm path length cell, at 25 °C. Reading speed was
150 50 nm·min and maximum accepted voltage threshold was 700 V. Each spectrum is
151 composed of three consecutive scans, which were averaged to increase the signal-to-
152 noise ratio. CD spectra values (millidegrees) were converted to mean residue ellipticity
153 [Θ] ($deg \cdot cm^{2} \cdot dmol^{-1}$), with the following formula:

154 $[\Theta] = ((\Theta \cdot 0.1) * (MW/Res-1))/(l \cdot [prot])$, where: **Θ** is the machine units in
155 millidegrees; **MW** is the protein molecular weight; **Res** is number of residues in the

156 protein; ***l*** is the cell pathlength in centimeters; and **[prot]** is protein concentration in
157 $mg \cdot mL^{-1}$.
158
159 *2.8 Removal of blood stains from cotton cloths*
160 Cotton cloth pieces (6 x 6 cm) were stained with 250 µL of chicken blood and
161 were allowed to dry at room temperature for 24 h. Stain removal assay was tested as a
162 pre-wash additive, by incubating the stained cloths with 25 mL solution with 0.1 $U \cdot mL^{-1}$
163 at pH 8.0 of each enzyme (in triplicate) for four hours at room temperature (25 °C). A
164 control wash was performed with only distilled water. Afterward the cloths were
165 washed in a 1kg washing machine (Brastemp Eggo, Brazil) for 33 min at room
166 temperature using 7 g of Bem-te-vi® powder detergent, and were left to dry for 24 h at
167 room temperature.
168 In order to reduce the subjectivity from the visual analysis of the stain removal
169 efficiency between the washed cloths, the CIELAB method for color difference
170 measurement was used. A Konica Minolta Chroma Meters CR-400 (made in Japan) was
171 used to obtain the L*, a* and b* parameters from each cloth. These parameters were
172 then inserted in the the Colour-Difference Formula, CIEDE2000, to calculate the ΔE_{00}
173 (Luo et al, 2001).
174
175 *2.9. Statistical analysis*
176 Statistical analysis was performed using OringPro 8SRO software program, from
177 OriginLab Corporation using one-way analysis of variance (ANOVA), followed by
178 Tukey's test ($p<0.05$).
179

3. Results and discussion

In the present work it was observed that viscera corresponds to 12.3% ± 2.1 and 11.2% ± 3.9 of tambaqui and Nile tilapia total mass, respectively. The joint production for these fish in 2011 in Brazil was about 365,000 ton (MPA, 2013), what can be estimated to have generated circa of 45,000 ton of viscera. From each 53 kg of tambaqui pyloric caeca and 40 kg of tilapia intestines, 1g of high purity grade trypsin can be obtained. Considering that these organs correspond to circa of 2.5% of total viscera waste, it can be estimated that about 28 to 21kg of pure trypsin could have been obtained from these neglected material.

One of the main reasons for introducing enzymes in laundry detergents is to lower the washing temperature, thus saving energy and allowing for environmental sustainability (Kirk et al, 2002; Jegannathan and Nielsen, 2013). Enzymes are normally found in powder detergents as dust-free granulates covered by sugars salts, waxy materials and hydrophilic builders that protect them against detergent components (Otzen, 2011). But, once the enzymes are dissolved in water, they must be able to remain active in harsh conditions. Laundry machines can work under different temperatures, and in most cold countries a “hot wash” is still the most common option. Therefore, enzymes must remain active in a broad temperature range in order to be used as a detergent additive. Tambaqui trypsin and subtilisin were more active at temperatures around 60 °C, whereas Nile tilapia proteases, porcine trypsin and bovine chymotrypsin presented optimum temperature around 40 °C (Table 1).

Tambaqui and Nile tilapia proteases were the most thermalstable enzymes, maintaining up to 80% of their activity after 1 h incubation at 60 and 50 °C, respectively. Subtilisin and porcine trypsin, on the other hand, were stable only up to 30 and 40 °C, respectively (Table 1). These results are consistent with the fact that

proteases from tropical fish are usually more stable than those from homoeothermic and psychrophilic animals (Kishimura et al, 2008). Moreover, mammal trypsins are more prone to autolysis, and their thermalstability is calcium-dependent, while the same is not observed for tropical fish trypsin (Bezerra et al, 2005; Papaleo et al, 2005; Souza et al, 2007). Considering that most detergents also have metal chelators, the use of metalloproteases, as well as any metal-dependent enzyme, becomes hampered (Otzen, 2011). This may have been the main problems with the early use of mammal trypsins in laundry detergent. However, low thermal stability and metal dependence are not observed in fish trypsin, making them more viable for such applications.

Laundry detergent proteases must be active in alkaline pH, since powder detergents' pH range is usually between 10 and 12 when dissolved in water at 25 °C. The maximum activity for the trypsins purified from both fish was found between pH 8.0 and 9.0 (Table 1), but the enzymes still retained more than 70% of their activity up to pH 12 (data not shown). Porcine trypsin was more active at neutral pH, and bovine chymotrypsin and subtilisin between pH 8.0 and 10.5. Furthermore, all enzymes studied were stable to the alkaline pH range (7.2 to 11.0).

Surfactant is a common component of detergents, and they can be anionic, zwiterionic and non-ionic. Usually, non-ionic and zwitterionic surfactants do not denature proteins, whereas ionic surfactants do even at very low concentrations (Otzen, 2011). From all surfactants tested, only SDS significantly inhibited the proteases (Table 2). Tambaqui, Nile tilapia and porcine trypsin residual activity after 1 h incubation with 0.5% SDS at 25 °C was of 27%, 43.5% and 8.1%, respectively. Similar response to 1 h incubation with 0.5% SDS at 25 °C has been reported for trypsin from *Balistes capriscus* (Jellouli et al, 2009), *Lithognathus mormyrus* (Ali et al, 2009) and *Salaria basilisca* (Ktari et al, 2012), that retained 23.8%, 23.5% and 31% of their initial activity,

respectively. Subtilisin, on the other hand, was the most stable at 25 °C, maintaining 91.2% of its initial activity. Subtilisin Carlsberg from *Bacillus licheniformis* was one of the first enzymes to be known as SDS-resistant, and it has since been used as a model for SDS resistance in enzymes (Maurer, 2004; Otzen, 2011). Interestingly, tambaqui trypsin was more active in the presence of SDS when the temperature was raised to 40 °C (Table 2). The same was not observed for any of the other enzymes, and subtilisin activity decreased significantly at 40 °C in the presence SDS.

To better understand the effect of SDS on the structure of tambaqui trypsin, a circular dichroism analysis was performed (Figure 1). The way tambaqui trypsin interacts with SDS was noticeably different from that shown by porcine trypsin. The later showed an evident increase in α-helix content and a shift of the negative peak to 210 nm, similarly to results reported by Ghosh (2008) for bovine trypsin. According to Ghosh (2008), excess SDS can penetrate the globular structure of trypsin and interact with its hydrophobic backbone causing repulsion and ultimately the partial unfolding of trypsin. Moreover, Parker and Song (1992) demonstrated that some proteins, even though not rich in helical structures, may have high potential to form amphiphilic α-helixes in the presence of anionic detergents, such as SDS, and that this increase in helicity may be linked to protein denaturing.

These amphiphilic patterns that are commonly found in mammal are possibly not present in tambaqui trypsin, since it maintained roughly the same secondary structure with and without SDS. This result is similar to those found by Shaw and Pal (2007) for subtilisin Carlsberg. These authors observed that the structural perturbation of SDS on the subtilisin was insignificant, indicating that the enzyme did not form any complex with the micelle. Nevertheless, tambaqui trypsin activity was significantly inhibited by

SDS at 25 °C, suggesting that this inhibition may not caused by structural loss, but by some impediment for the substrate to reach the enzyme active site.

To further investigate the enzymes in the present work, their compatibility to powder detergents was assayed. Tambaqui, Nile tilapia and subtilisin maintained their activity above 80% after 1h incubation with powder detergents at 25 °C (Table 3), whereas porcine trypsin had activity below 25% and bovine chymotrypsin was completely inhibited. At 40 °C a decrease was seen in all enzymes activity in the presence of laundry detergents. Similar results have been found for trypsin from the fish *B. capriscus* (Jellouli et al, 2009) and *L. mormyrus* (Ali et al, 2009). On the other hand, subtilisin demonstrated considerable stability to laundry detergent even at 40 °C.

Another important feature sought in laundry detergent enzymes is the ability to remove stains bound to solid surfaces. Figure 2 portrays the effect that the enzymes studied in the present work had as pre-wash additives for the removal of blood stain. A colorimetric method (CIELAB) was used to analyze the stain removal from the cloths. This method was developed by the International Commission on Illumination (CIE), and it consists of three main parameters L*, a* and b*, in which L* represents the difference between light (L*=100) and dark (L*=0); a* represents the difference between green (-a*) and red (+a*); and b* represents the difference between yellow (+b*) and blue (-b*). CIELAB is most commonly used to compare colors of wines, fruits, and printouts and to the best of our knowledge it has not yet been used to compare stain removal. However, it was useful to the present work, since it allowed for a more objective analysis.

All cloths that were pre-washed with enzymes showed L*, a* and b* values significantly different form the buffer only control. The cloths pre-washed with enzyme presented L* values statistically similar to the unstained cloth, but a* and b* values

were statistically different. Additionally, a variable that measures the difference between two colors (ΔE_{00}) was calculated for each pair of pre-washed cloths from the present work (Supplementary table 1). The higher the ΔE_{00} the more different the color of the two samples being compared is. All cloths pre-washed with enzymes, when compared amongst themselves, showed ΔE_{00} values lower than 1. However, this value was higher than 1 when compared to the buffer control and the unstained cloth. The lower values of ΔE_{00} for Nile tilapia and tambaqui trypsin were found when compared to subtilisin, a reference detergent enzyme. These numeric results show that all enzymes tested were more efficient at removing blood stains than plain buffer, but unable to completely remove the stains. Even though it has been shown that different proteases that can easily remove blood stain from cloth pieces (Abidi et al, 2008; Haddar et al, 2010; Jellouli et al, 2011), it must be kept in mind that these works used temperatures as high as 50 °C, and freshly stained cloths. In the present work, on the other hand, the stains were dry, and the removal was performed at room temperature. These results show that tambaqui and Nile tilapia trypsins were noticeably efficient in removing blood stains adhered to cotton cloths and would be viable detergent additives.

4. Conclusion

The present work reported on two trypsins extracted from commercially important fish and compared their activity to three commercial enzymes. The fish trypsins were thermalstable and remained active in the presence of several denaturing agents that are commonly found in detergent formulations. Tambaqui trypsin secondary structure behaved differently with SDS in comparison to porcine trypsin, indicating that this fish trypsins is less disturbed by anionic surfactants than mammal ones. Moreover, the enzymes, when tested as pre-wash additive, were able to remove stains from cotton cloths, with efficiency similar to that found for subtilisin. These results suggest the fish trypsins here studied have great potential to be employed as additive in the laundry industry.

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399 Figure 1. Circular Dichroism spectra from tambaqui (A) and porcine trypsin (B) incubated without and with 0.5% SDS at 25 °C.

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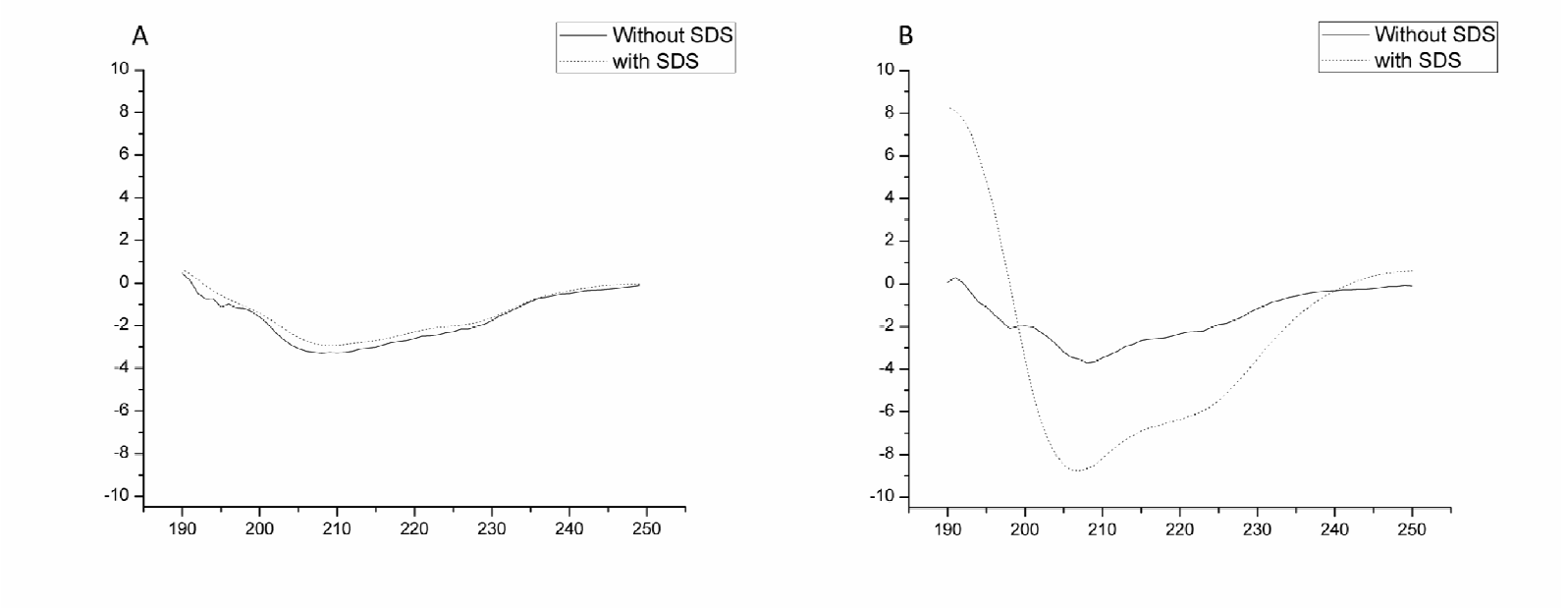

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405 Figure 2. Blood stain removal from white cotton cloth pieces, by each enzyme studied and buffer control.

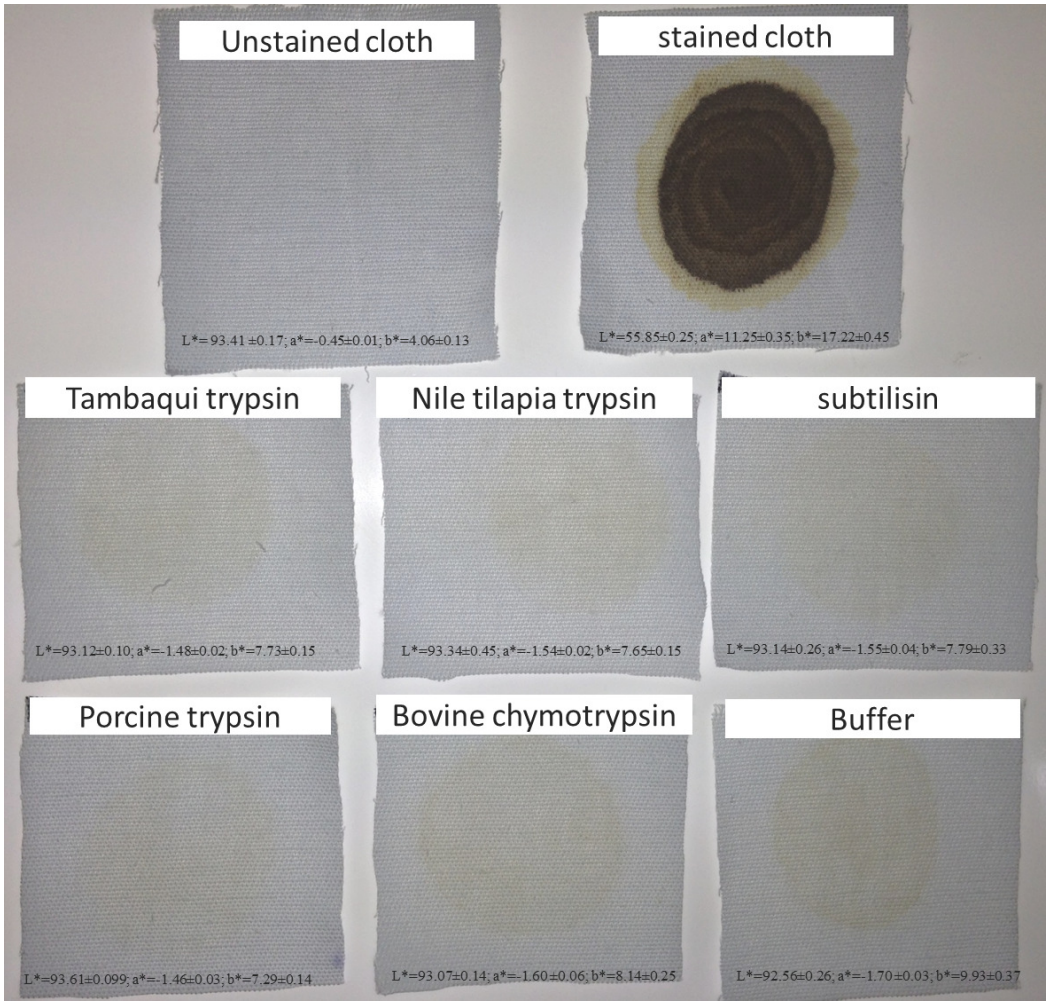

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Table 1. Effect of temperature and pH on the activity of the enzymes.

	Residual Activity (%)				
	tambaqui trypsin	**Nile tilapia trypsin**	**subtilisin**	**porcine trypsin**	**bovine chymotrypsin**
Optimum temperature (°C)	50-60	40-50	60	40	40
Thermal stability (°C)*	60	50	30	40	40
Optimum pH	8.5	8.0-9.0	10.5	7.2	8.0-10.0
pH stability**	7.2-11.0	7.2-11.0	7.2-11.0	7.2-11.0	7.2-11.0

* temperature in which the enzyme maintained at least 80% of its initial activity;

** pH in which the enzyme maintained at least 80% of its initial activity;

Table 2. Average residual activity and respective standard deviation (SD) found for the enzymes after 60 min incubation with surfactants 0.5% at 25 and 40 °C.

	Residual Enzymatic Activity(%)				
Surfactants	**Tambaqui trypsin**	**Nile tilapia trypsin**	**subtilisin**	**Porcine trypsin**	**Bovine chymotrypsin**
25 °C					
Control	100.0^{c} (4.5)	100.0^{c} (3.0)	100.0^{c} (2.7)	100.0^{a} (4.1)	100.0^{d} (3.1)
SDS	27.5^{d} (6.9)	43.5^{d} (5.0)	91.2^{c} (4.8)	8.1^{b} (4.1)	18.2^{e} (8.3)
Triton X-100	146.6^{a} (9.9)	110.8^{b} (2.4)	144.9^{a} (6.4)	98.4^{a} (2.4)	111.5^{c} (2.8)
Sodium Choleate	115.8^{b} (7.1)	122.9^{a} (2.9)	143.1^{a} (4.7)	94.4^{a} (2.1)	100.7^{d} (3.7)
Chaps	128.6^{b} (6.0)	124.2^{a} (1.4)	149.5^{a} (4.9)	99.8^{a} (4.8)	133.6^{a} (2.8)
Tween 20	119.4^{b} (5.1)	98.2^{c} (3.6)	124.1^{b} (4.1)	99.1^{a} (3.8)	116.4^{b,c} (4.4)
40 °C					
Control	100.0^{b} ±4.7	100.0^{a} (5.3)	100.0^{d} (3.6)	100.0^{a} (2.0)	100.0^{a,b} (4.5)
SDS	70.5^{c} ±4.0	6.0^{d} (7.6)	24.2^{e} (0.0)	9.6^{c} (4.4)	5.9^{e} (9.6)
Triton X-100	115.7^{a} ±3.0	94.8^{a,b} (5.7)	313.2^{c} (5.3)	102.3^{a} (4.6)	92.7^{c} (2.6)
Sodium Choleate	99.4^{b} ±2.5	76.0^{c} (4.5)	393.2^{a} (6.6)	67.8^{b} (4.7)	61.0^{d} (4.2)
Chaps	111.0^{a} ±2.5	72.8^{c} (3.0)	389.1^{a,b} (5.5)	100.4^{a} (4.9)	92.6^{c} (2.8)
Tween 20	111.7^{a} ±4.5	89.3^{b} (4.3)	356.4^{b} (6.7)	103.2^{a} (5.0)	95.5^{b,c} (1.9)

^{a,b,c,d,e} Different letters indicates statistical difference between values within each column ($p<0.05$). Same letter indicates no statistical difference between values within each column ($p>0.05$).

Table 3. Average residual activity and respective standard deviation (SD) found for the enzymes after 60 min incubation with powder detergents (7 mg·mL^{-1}) at 25 and 40 °C.

	Residual Enzymatic Activity(%)				
Commercial Detergent	**Tambaqui trypsin**	**Nile tilapia trypsin**	**subtilisin**	**Porcine trypsin**	**Bovine chymotrypsin**
25 °C					
Control	100.0^{a,b,c} (2.7)	100.0^{c} (12.7)	100.0^{a} (2.4)	100.0^{a} (4.4)	100.0^{a} (6.7)
Ala®	109.0^{a} (4.1)	81.5^{d} (8.0)	104.6^{a} (2.8)	3.4^{d} (3.3)	0.0^{b} (0.0)
Bem-te-vi®	79.5^{d} (6.1)	133.3^{b} (4.8)	85.1^{b} (2.2)	23.1^{b} (1.1)	0.0^{b} (0.0)
Ace®	95.3^{b,c} (4.2)	194.1^{a} (8.7)	106.4^{a} (4.6)	19.7^{b,c} (1.2)	0.0^{b} (0.0)
Surf®	101.1^{a,b} (6.3)	84.4^{c,d} (8.0)	102.6^{a} (4.8)	16.5^{c} (1.2)	0.0^{b} (0.0)
Assim®	90.9^{c} (7.3)	142.8^{b} (4.2)	102.9^{a} (6.5)	5.0^{d} (8.9)	0.0^{b} (0.0)
40 °C					
Control	100.0^{a} (3.4)	100.0^{a} (2.4)	100.0^{c} (2.7)	100.0^{a} (4.2)	100.0^{a} (3.9)
Ala®	69.6^{b} (2.1)	31.2^{c} (4.5)	126.7^{a} (3.6)	1.2^{e} (0.0)	40.9^{b} (2.7)
Bem-te-vi®	54.9^{d} (2.8)	68.0^{b} (4.6)	101.3^{c} (1.5)	6.4^{e} (5.9)	0.0^{c} (0.0)
Ace®	65.4^{c} (3.3)	25.4^{d} (3.4)	118.6^{b} (2.2)	25.2^{c} (5.3)	0.0^{c} (0.0)
Surf®	64.1^{c} (2.6)	12.7^{e} (4.7)	120.3^{b} (2.7)	14.2^{d} (2.9)	0.0^{c} (0.0)
Assim®	70.2^{b} (4.8)	0.0^{f} (0.0)	126.1^{a} (3.7)	43.1^{b} (1.8)	0.0^{c} (0.0)

^{a,b,c,d,e} Different letters indicates statistical difference between values within each column ($p<0.05$). Same letter indicates no statistical difference between values within each column ($p>0.05$).

Supplementary table 1. Color comparison (ΔE_{00}) of the stained cloths that were prewashed with different enzymes. ΔE_{00} was calculated using the Colour-Difference Formula: CIEDE2000, by an on-line CIE2000 Calculator (colormine.org/delta-e-calculator/cie2000)

ΔE_{00}	Tambaqui trypsin	Nile tilapia trypsin	Subtilisin	Porcine trypsin	Bovine Quimotrypsin	Buffer	Unstained cloth	Stained cloth
Tambaqui trypsin	-	-	-	-	-	-	-	-
Nile tilapia trypsin	0.169	-	-	-	-	-	-	-
Subtilisin	0.100	0.160	-	-	-	-	-	-
Porcine trypsin	0.444	0.325	0.477	-	-	-	-	-
Bovine Quimotrypsin	0.509	0.584	0.458	0.897	-	-	-	-
Buffer	1.616	1.707	1.575	2.015	1.123	-	-	-
Unstained cloth	3.151	3.122	3.222	2.915	3.651	4.694	-	-
Stained cloth	31.168	31.331	31.221	31.450	31.156	30.826	31.085	-

Capítulo 2: Calcium dependency in trypsin from tropical fish tambaqui (*Colossoma macropomum*)

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Abstract

The effect of calcium on tambaqui and porcine trypsin was compared by biochemical, eletrophoretical and mass spectrometry analysis. Tambaqui trypsin was more active in the presence of EDTA at temperatures up to 60°C, but the enzyme was only able to remain active at temperatures higher than that when in the presence of $CaCl_2$. Porcine trypsin was much more sensible to autolysis, when in the presence of EDTA even at lower temperatures. On the other hand, porcine trypsin was capable of remaining active in the presence of calcium up to 65 °C. Intrinsic fluorescence results indicated that calcium can make the enzyme structure become more compact and rigid, thus contributing to the decrease in tambaqui trypsin activity and increase in its stability. Tambaqui trypsin also presents a conserved calcium loop, fairly similar to porcine trypsin, but not as negatively charged.

Keywords: Autolysis, fish protease; intrinsic fluorescence; Structure flexibility; Thermalstability.

Introduction

The name trypsin was first used by Kühne, in 1876, to describe the "amorphous ferments" with proteolytic activity found in pancreatic extracts. Afterwards, several aspects of the trypsin structure, activity and mode of action were described, and currently this enzyme is classified as a serineprotease with specificity to hydrolyze peptide bonds on the carboxyl side of arginine and lysine residues (Hedstrom, 2002).

The influence of various ligands on trypsin structure and activity has been extensively studied, among which calcium ion stands out as one of the most important. Trypsin, while not a metalloprotease, has a specific loop for binding calcium ions, which is located between amino acids 68 and 80 (numbering system for bovine chymotrypsin, Hartley and Kauffman, 1966) and connects two antiparallel β-strands in the N-terminal domain (Bode and Schwager, 1975). Calcium can affect trypsin activity, by accelerating the conversion of trypsinogen to trypsin (McDonald and Kunitz, 1941), and by protecting it against autolysis and degradation at high temperatures (Sipos and Merkel, 1970).

While the protective effect of calcium is well established for mammalian enzymes, it has not been found to show the same effect among fish. It has been observed that some fish trypsin are indifferent or even slightly inhibited by calcium ions (Bezerra et al, 2005; Cao et al, 2000; Freitas-Júnior et al, 2012; Jellouli et al, 2009), whereas other fish trypsin thermal stabilities are highly dependent on calcium ions (Fuchise et al, 2009; Khaled et al, 2011; Kishimura et al., 2006; 2007, 2008; Klomklao et al., 2006; 2007; 2009; 2011). In this context, the present work intends to shed light onto the difference of calcium interaction between Amazonian fish tambaqui and porcine trypsin.

Material and Method

Crude extract preparation and Enzyme purification

Juvenile specimens of tambaqui (*Colossoma macropomum*) were kindly provided by the rearing units of Embrapa Semiárido (Petrolina, Brazil). Pyloric caeca and intestines of tambaqui were desiccated and homogenized in 10 mM Tris-HCl pH 8.0 with 15 mM NaCl (200 mg of tissue per buffer mL), using a tissue homogenizer (IKA RW 20D S32, China). The resulting homogenates were centrifuged (Sorvall RC-6 Superspeed Centrifuge, North Carolina, USA) at 10,000 g for 15 minutes at 4 ^{o}C to remove cell debris and nuclei. Tambaqui trypsin was purified by a three step method, modified from Marcuschi et al (2010): (1) crude extract heating for 30min at 45 °C; (2) ammonium sulfate fractioning (with higher trypsin activity found in fraction 30-60%), (3) and affinity chromatography, p-Aminobenzamidine–Agarose (Sigma-Aldrich®). Commercial porcine trypsin (Sigma-Aldrich®) was also subjected to purification through affinity chromatography, to ensure that all contaminant proteins were removed.

Tryptic activity

Tryptic activity was assayed with 4mM N-a-benzoyl-L-arginine p-nitroanilide (BApNA, Sigma-Aldrich®) prepared in dimethylsulfoxide (DMSO, Vetec). The assay followed the method described by Erlanger et al (1961), with volumes adapted for a microplate reader (Bio-Rad X-Mark spectrophotometer, California, USA). One unit (U) of enzyme specific activity was defined as the amount of enzyme capable of hydrolyzing 1 mmol of BAPNA per minute, using 9.6 as the millimolar extinction coefficient of p-Nitroaniline at 405nm.

Effect of Calcium on the enzimatic activity and thermal stability

The effect of the calcium ion on trypsin activity was assayed by incubating each trypsin with either 10 mM $CaCl_2$ or 10 mM EDTA (Ethylenediamine tetraacetic acid) (final concentration) for one hour on ice. Afterward, the activity of the samples, as well as their thermal stability was measured with 4 mM BAPNA in a range of 10 to 80°C, with a 5°C increase. For the thermal stability the enzymes were incubated in each temperature for 1 hour, and then had their residual activity measured at 25 °C.

Intrinsic fluorescence

Intrinsic fluorescence spectra from tambaqui and porcine trypsin were obtained with a Microplate Fluorescence Spectrophotometer Hitachi F-7000 (Japan). Excitation wavelength was fixed at 295 nm, and emission was measured from 300 to 400 nm. Excitation and emission slits were both set at 5.0 nm and the scan speed was 60 $nm \cdot min^{-1}$. Samples of porcine trypsin were incubated with either 10 mM $CaCl_2$ or 10 mM EDTA (final concentration) for one hour on ice. Then, they were incubated for one hour at temperatures ranging from 20 to 80°C, with a 10°C increase. Afterward, their intrinsic fluorescence was measured at 25°C

Determination of the Arrhenius activation energy (Ea)

In order to calculate the Ea of tambaqui and porcine trypsin, the enzymes were incubated with 10 mM $CaCl_2$ or 10 mM EDTA for one hour on ice and then used in substrate kinetics (with Bapna 150 to 9600 µM, final concentration) at temperatures ranging from 20 to 50 °C. The activation energy was calculated by ploting the Ln k_{cat} vs 1/T and using the Arrhenius formula (slope=−Ea/R), in which the gas constant R is 8.314 joules K^{-1} mol^{-1}.

Evaluation of protein autolysis

Samples of tambaqui and porcine trypsin were incubated with 10 mM $CaCl_2$, 10 mM EDTA, or 1mM TLCK (final concentration) for one hour on ice. Afterward, these samples were incubated at -20, 30 or 60°C for 24h. Every four hours the samples activity was checked with 4mM BapNa. After 24h incubation, the samples were loaded into a reducing polyacrylamide gel electrophoresis (SDS–PAGE) carried out according to Laemmli (1970), with a 4% ($w \cdot v^{-1}$) stacking gel and a 15% ($w \cdot v^{-1}$) separation gel. The gel was silver stained using the protocol described by Blum et al (1937). The gel image was acquired with GelDoc™ EZ Imager (Bio-Rad, USA), and analyzed using ImageLab™ Software, version 4.0 (Bio-Rad, USA). Additionally, a MALDI-TOF(/TOF) mass spectrometer (Autoflex smartbeam, Bruker Daltonics, USA) was used in order to obtain a mass profile from the autolysis.

Statistical analysis

Statistical analysis was performed using OringPro 8.0 software program, from OriginLab Corporation using one-way analysis of variance (ANOVA), followed by Tukey's test ($p<0.05$).

Results and Discussion

In order to test the effect of Calcium on the autolysis of tambaqui and porcine trypsin, both enzymes were incubated at three different temperatures (-20, 30, 60 °C) for 24h. Tambaqui trypsin lost all of it activity after only 1h incubation with EDTA at 60°C; but when incubated with $CaCl_2$ it kept 60.5%±5 of its initial activity after 6h, and 32.3%±5.9 after 24h. When incubated at 30°C for 24h with both $CaCl_2$ and EDTA, tambaqui trypsin remained 100% active. Porcine trypsin, lost more than 80% of its

initial activity after only 3h incubation at 30°C with EDTA and at 60°C with $CaCl_2$ and EDTA, remaining 100% active after 24h when incubated with $CaCl_2$ at 30°C.

The results from these incubations were evaluated by SDS-PAGE (Figure 1). Almost all lanes in the gel for tambaqui trypsin show a main band of 26.6 kDa, as well as two low-weight bands (14kDa). The only lane that shows signs of complete autolysis was EDTA for incubation at 60°C. Strong hydrolysis was also observed for incubation with $CaCl_2$ at 60°C, with the formation of an 18kDa band (indicated with the black arrow in Figure 1). The same band can be seen, more faintly, in the samples incubated at 30°C with both $CaCl_2$ and EDTA.

Porcine trypsin gel (Figure 1) has a main band of 25.1 kDa in all lanes. Evident signs of autolysis can be seen at the lanes for EDTA at 30°C, as well as at the lanes for $CaCl_2$ and EDTA at 60°C. It is possible to see signs of autolysis in the samples of porcine trypsin that were incubated with TLCK, while the same was not observed for tambaqui trypsin. One possible explanation is that porcine trypsin remained 20% active in the presence of TLCK, while tambaqui trypsin remained only 5% active. It can be observed that porcine trypsin yield different sizes peptides during its autodigestion, as shown by the white arrows in Figure 1B, with band of size 23.9; 22.5 and 17.1 kDa. This difference in band sizes can indicate that tambaqui and porcine trypsin may present different sites for autolysis, thus producing different sizes of peptides. Comparison of the activity in the samples with their profile in the gel indicates that the lost of activity was mainly due to autolysis and not unfolding. This corroborates with previous works that state that calcium ions prevent trypsin to undergo autolysis, but do not avoid structural unfolding (Sipos and Merkel, 1970; Bode and Schwager, 1975; Várallyay et al, 1998).

Site of autolysis in trypsin may be defined as any peptide bond that has an arginine or lysine residue in the carboxyl side which is accessible to other trypsins. However not all potential autolysis site is available in the native trypsin, in which the main ones are K145, K61, K154, K188 and R117 (Papaleo et al, 2005; Várallyay et al, 1998). The amino acid K145 is the split site from the so-called autolysis loop. This site is found only in a few mammalian trypsins, having the main function of converting β-trypsin into α-trypsin, what does not necessarily lead to autolysis (Várallyay et al, 1998). The amino acids K61 and K188 are present in both mammal and fish trypsin, whereas K154 is only found in fish homologues (Papaleo et al, 2005).

The amino acid R117 is considered to be one of the main autolysis sites for mammalian trypsin, so much so that its absence is associated with human hereditary pancreatitis (Whitcomb et al, 1996). Várallyay et al (1998) showed that a mutation on R117 gives trypsin the same resistance to autolysis as it presents when associated with calcium ions. One interesting factor is that fish trypsins in general have an R117Y mutation, therefore showing higher resistance to autolysis (Papaleo et al, 2005). Pancreas in teleost fishes is usually not a define organ, but a diffused mass along the mesentery and fat that surrounds the digestive organs. Even so, the pancreatic cells that produce the digestive enzymes in fish (acinar cells) are similar to those found in other vertebrates groups (Genten et al, 2009). Moreover, there is no work that shows that fish in general suffer from pancreatitis, and one must be careful when assigning the cause of pancreatitis to a single mutation, since there is a conjunction of factors that enable the development of this disease (Graf and Szilágyi 2003).

With respect to the trypsin activity at different temperatures in the presence of 10 mM $CaCl_2$ and EDTA, it was observed that at lower temperatures, tambaqui trypsin activity was higher when incubated with EDTA than it was with calcium (Figure 2A);

189 however at 60°C the activity begins to fall significantly in the presence of EDTA, while
190 calcium enables the enzyme to remain active up to 70°C. Porcine trypsin, on the other
191 hand, indicating a shift in its optimal activity from 20 to 35°C with EDTA to 55 and 65
192 °C, with $CaCl_2$.

193 Several researchers have investigated how the presence of calcium in the trypsin
194 structure affects the enzyme activity. What has been observed was that calcium does not
195 directly affect the trypsin active site. In fact, the almost regular octahedron complex that
196 calcium makes inside its loop turns it into a more rigid structure, preventing the
197 cleavage of neighboring regions, thus increasing activity because of the reductions on
198 the rate of autolysis (Bode and Schwager, 1975; Várallyay et al, 1998; Papaleo et al,
199 2005). This goes in accordance to Fontana et al (1986) that showed that a peptide bond
200 will be more prone to a proteolytic attack if it is located in a more flexibility location.

201 Tambaqui trypsin was more thermalstable when incubated with calcium.
202 Nevertheless, it is interesting to note that heating tambaqui trypsin for 60 min. at mild
203 temperatures (30 to 45°C) in the presence of EDTA has increased its residual activity at
204 room temperature (Figure 3). Since this increase in activity was not observed in the
205 presence of calcium, it is possible that the "loosening" of the structure be connected to
206 the higher activity. Cold adapted enzymes, for example, usually present a more flexible
207 structure, therefore being more active at low temperatures and more unstable at higher
208 temperature (Somero, 2004). The presence of calcium ions and the consequent rigidity
209 of the structure may account for the retention of activity at higher temperatures from
210 both tambaqui and porcine trypsin.

211 To better understand the effect of calcium on the structure of these trypsins when
212 they undergo heating, the intrinsic fluorescence of tryptophan was investigated. It is
213 known that a tryptophan residue when excited at 295 nm, will emit fluorescence at a

maximum emission peak closer between 310 nm (“red” wavelength) and 400 nm (“blue” wavelength). An emission peak closer to 310 nm indicates that the tryptophan residues are more buried in the structure and that probably the structure is more compact. On the other hand, a peak shift towards 400 nm shows that the tryptophan residues are more exposed (Eftink, 1994). However, one must be aware of molecular quenching, since shifts in the fluorescence emission peak commonly occur when there is a ligand, like calcium, nearby a tryptophan. Therefore, the interpretation of intrinsic fluorescence must be made carefully when examining ligands and temperature change in conjunction, especially in proteins with more than one tryptophan, such as trypsin.

Nevertheless, it is noticeable that calcium ions provoked some changes in the structure of tambaqui trypsin, so that it becomes more compact in the presence of $CaCl_2$, as temperatures rises (table 1). The same was not observed in the presence of EDTA. Regarding the porcine trypsin, it was observed that the enzyme kept a more compact structure up to 50°C in the presence of calcium, while with EDTA, from 40°C forward it started to show some loosening in the structure.

Figure 4 shows the structure modeled from tambaqui trypsin sequence (JQ437817.1) in gray aligned with the porcine structure (1S81) in blue. The calcium loop from tambaqui and porcine trypsin were highlighted in green and yellow, respectively. The calcium loop goes from the amino acid residue 69 to 80 (Bode and Schwager, 1975). The amino acid composition of the calcium loop from tambaqui and porcine trypsin are respectively (GEHNI**Q**V**T**E**NT**E) and (GEHNI**D**V**L**E**GN**E). It can be observed that the residues that bind to the calcium ions (underlined residues) are conserved in both enzymes. However, there was an exchange of a negatively charged amino acid (D) for an uncharged one (Q) in tambaqui trypsin.

238 Kanno et al (2011) observed that the calcium loop from frigid zone fishes have a
239 higher incidence of positive charged amino acids than temperate and tropical zones
240 fishes. A more positive charge can lead to a reduction in calcium binding efficiency.
241 This result was consistent with the concept that the presence of calcium makes the
242 trypsin structure more rigid, thus being unfavorable for enzymes that need to work with
243 high catalytic efficiency at low temperatures, therefore needing a more flexible
244 structure.

245 The tryptophan residues were highlighted for tambaqui and porcine trypsin in
246 red and purple, respectively (Figure 4). Tambaqui trypsin has 6 tryptophan residues,
247 while porcine trypsin presents only 4. One of the extra residues from tambaqui was
248 found in the n-terminal loop, seeming to be more exposed to the surface than the others
249 residues. Moreover, both enzymes have a tryptophan residue near the calcium loop.
250 These structural features may account for some of the differences seen in the intrinsic
251 fluorescence profiles from the enzymes (table 1).

252

Conclusion

Tambaqui trypsin was more thermal stable than porcine in the absence of calcium ions. This most likely occurs because the important autolysis site, R117, commonly found in mammalian trypsins, was absent in fish, thus making fish enzymes less prone to autolysis. Even so, calcium still exerts a protective effect on tambaqui trypsin during heating at temperatures higher than 60°C, probably due to increase in structural rigidity. A collateral effect of this rigidity was a reduction in the enzymatic activity at temperatures lower than 60°C. Moreover, the compactness of porcine trypsin structure may also account for its lower enzymatic activity in comparison to tambaqui.

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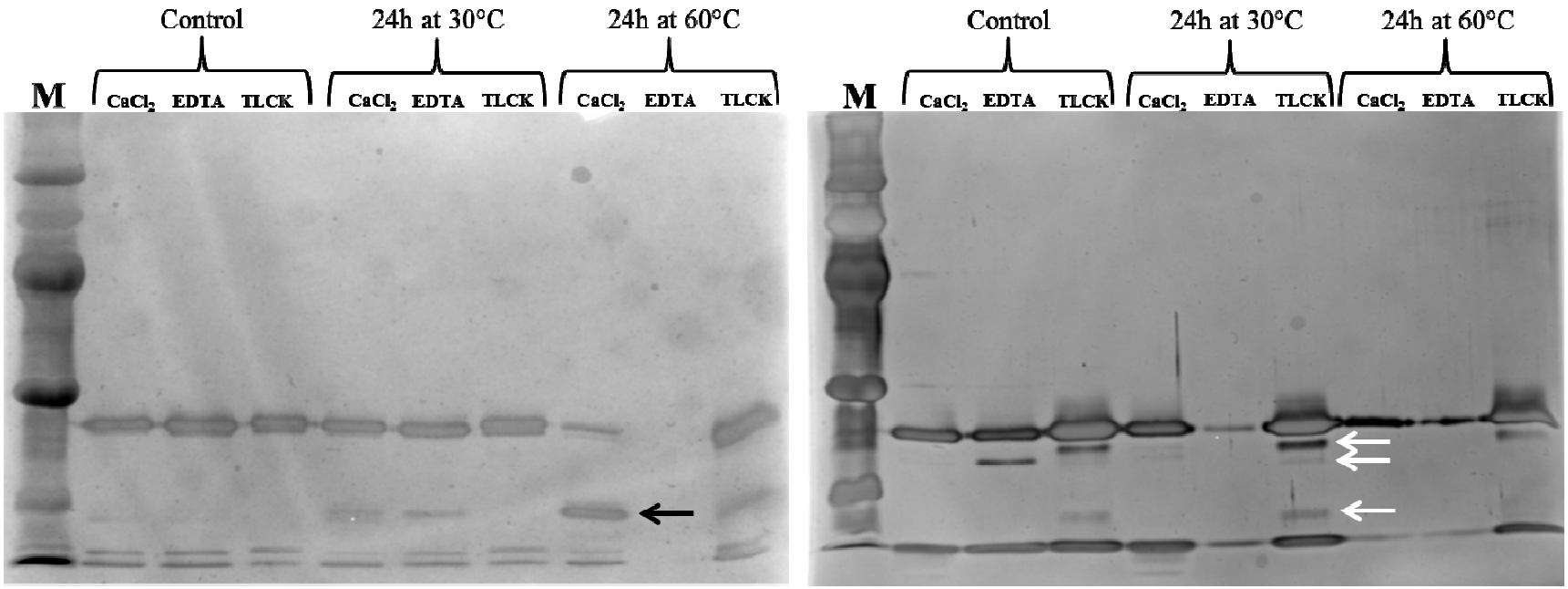
376

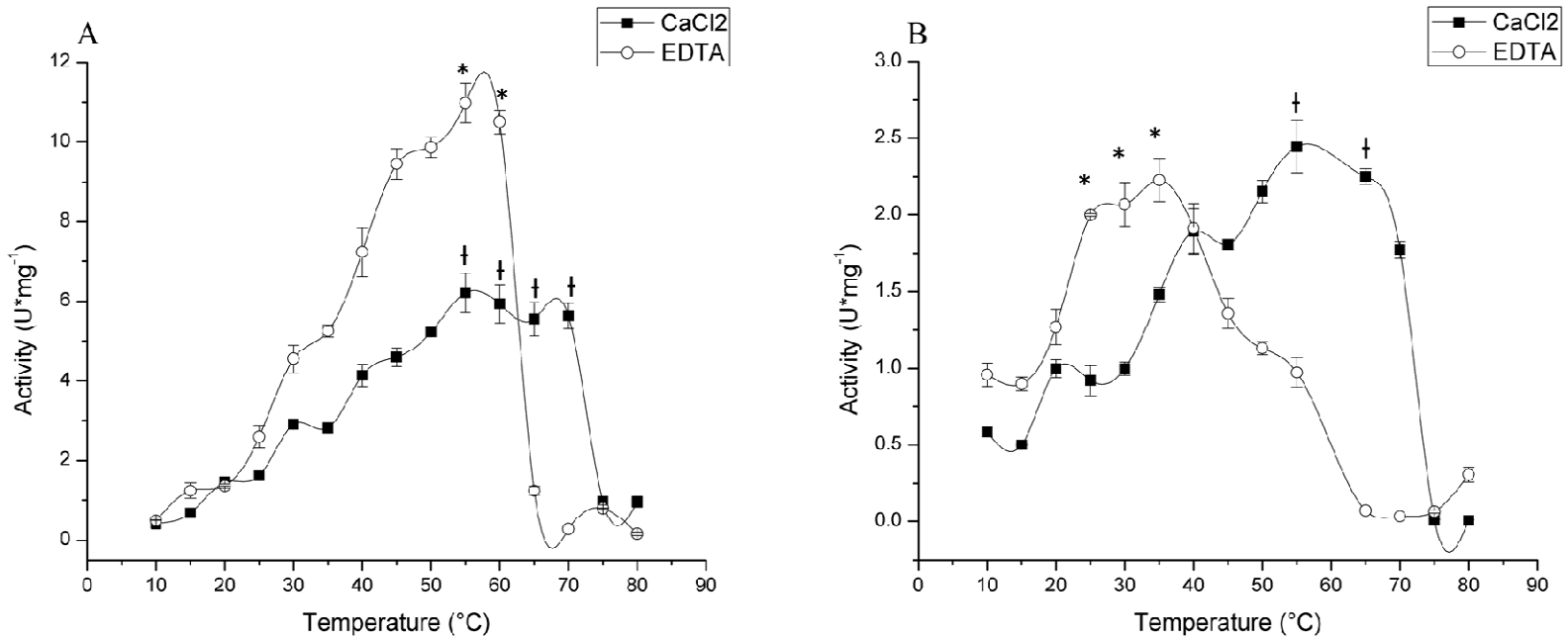
Figure 1. Polyacrilamide Gel electrophoresis (SDS-PAGE) from tambaqui and porcine trypsin. (M) Marker; ($CaCl_2$) trypsin incubated with 10 mM $CaCl_2$; (EDTA) trypsin incubated with 10 mM EDTA; (TLCK) trypsin incubated with 1 mM TLCK. Control stands for the enzymes kept at -20°.



382 Figure 2. Enzymatic activity from (A) tambaqui and (B) porcine trypsin at temperatures ranging from 10 to 80 °C with 10mM EDTA and 10mM

383 $CaCl_2$.

384



385

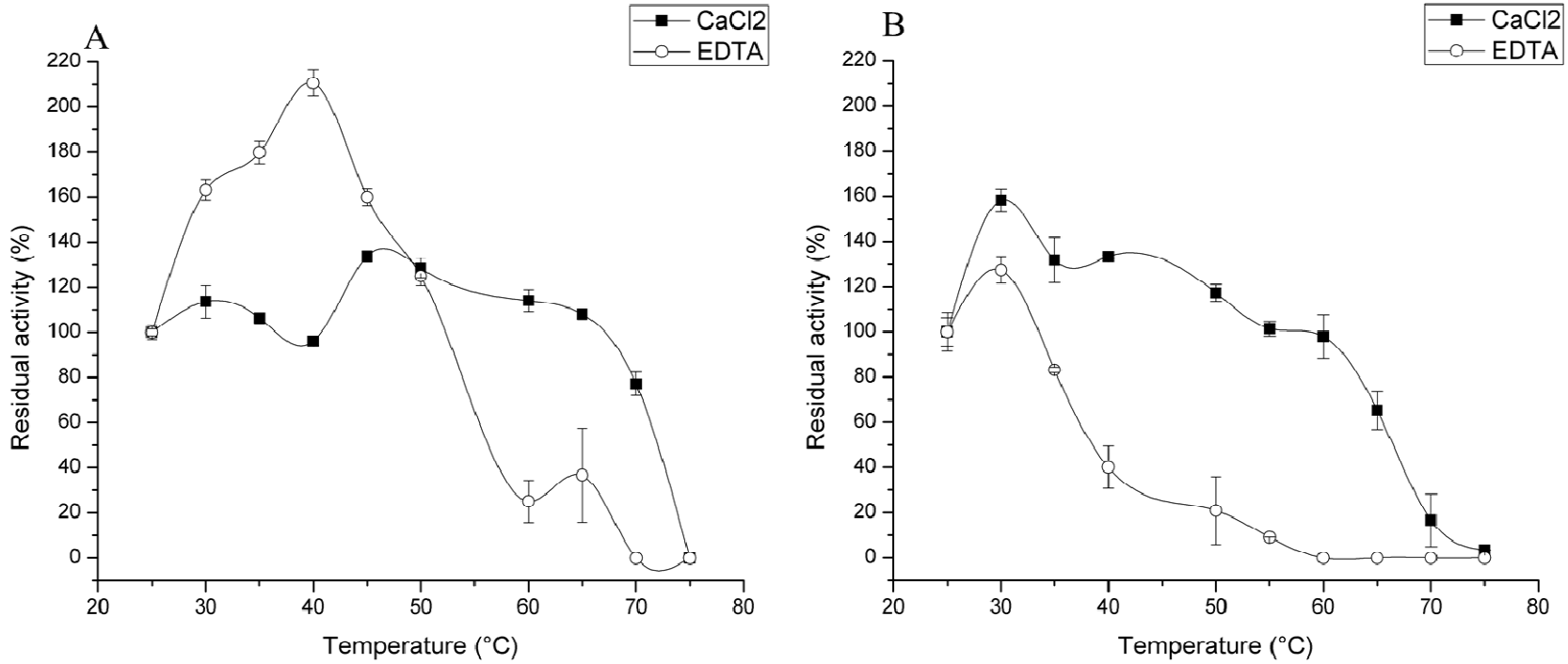
386 ǂ Trypsin activities that are statistically equal in the presence of 10 mM $CaCl_2$ (One way Anova tukey mean comparison test, $p > 0.05$).

387 * Trypsin activities that are statistically equal in the presence of 10 mM EDTA (One way Anova tukey mean comparison test, $p > 0.05$).

388

389 Figure 3. Thermal stability from (A) tambaqui and (B) porcine trypsin incubated for 1h at temperatures ranging from 25 to 75 °C 10mM EDTA

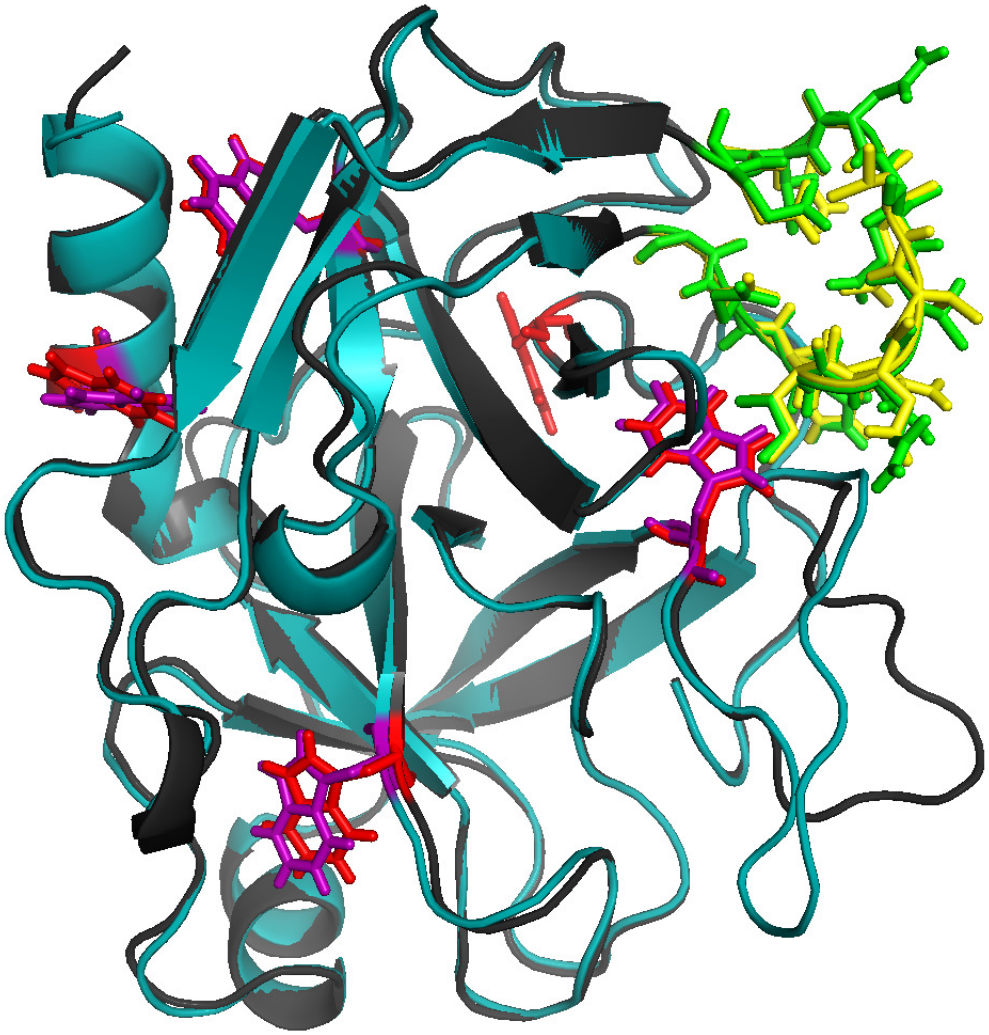
390 and 10mM $CaCl_2$.



391

392

Figure 4. Alignment of tambaqui (Gray) and porcine (blue) trypsin structures. Calcium loops are highlighted in green (tambaqui) and yellow (porcine). Tryptophan residues are highlighted in red (tambaqui) and purple (porcine).



Tables

Table 1. Maximum emission wavelength (λ_{max}) from intrinsic fluorescence scan of tambaqui and porcine trypsin after incubation at different temperatures for 60 min Scan readings were obtained using a fixed excitation wavelength of 295 nm and emission wavelength between 310 to 400 nm, at 25°C.

	Tambaqui trypsin (λ_{max})		Porcine trypsin (λ_{max})	
Temperature (°C)	10 mM CaCl2	10 mM EDTA	10 mM CaCl2	10 mM EDTA
No heating	340.4	340.4	331.0	334.0
30	340.4	340.2	333.0	335.6
40	335.0	340.4	332.4	334.4
50	335.2	340.8	332.2	344.0
60	336.2	340.4	340.8	348.6
70	337.0	344.2	346.2	348.8
80	344.6	348.8	347.4	349.4

Capítulo 3: Analysis of tambaqui trypsin model, a mesophilic enzyme with some cold-adapted features

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Abstract

Fishes have long been used as models for studding the adaptation of vertebrates' metabolism to temperature. Since trypsin is a well conserved protease, much of the research in this field was done by investigating variations in its activity and structure among various organisms. The present work used molecular dynamics and circular dichroism to analyses the structure of tambaqui, porcine and salmon trypsin subjected to temperature variations. Moreover, a database of anionic and cationic trypsin sequences from fish and homeothermic animals was put together in order to better access the differences among these groups. Even though the structures of cold adapted salmon trypsins were 85% similar, they behave differently under temperature variation. The active site of salmon trypsin is more flexible and easily disrupted than tambaqui and porcine. Moreover, tambaqui is also able to endure heating at temperatures higher than porcine trypsin, probably due to the tendency of mammal trypsins to suffer autolysis at temperatures close to 40°C. Thus, even though tambaqui trypsin structure is more flexible than porcine, it can endure a greater range of temperatures, due to its low propensity for auto digestion.

Keywords: Anionic trypsin; Cationic trypsin; Molecular dynamics

Introduction

Fishes are poikilothermic animals, and yet they are able to live in tropical (25 to 40 °C), cold (6 to 17°C) and even freezing water (around -1.9 °C) (Ficke et al, 2007, Somero, 2004). Even with a body temperature equal to the environmental, fishes are still able to have the same metabolic pathways that homeothermic animals do (Halver and Hardy, 2002). According to Somero (2004), in order to compensate for lower temperatures, fishes can: increase enzyme concentration; genetically alter the kinetic properties of enzymes; and modulate the activities of preexisting enzymes. Among these strategies, the altering of kinetic properties is one of the most interesting to be analyzed comparatively between species.

Much of the research made on temperature on enzymatic activity has showed that if an enzyme is highly active at low temperatures, it is more likely to be unstable at higher temperatures. This happens mostly because in order for an enzyme to be efficient at low temperatures it must have a flexible structure, what leads to low stability (Feller et al, 1996; Somero, 2004; Leiros et al 1999; 2000; Papaleo et al, 2008). Among the enzymes whose cold adaptation has been extensively studied is trypsin. This serine protease is interesting for comparative biology studies, because it has a well conserved structure (Hedstrom, 2002) and it can be found in almost every organism, from virus to mammals (Pólgar, 2005).

Leiros et al (1999; 2000) and Papaleo et al (2008) compared the structures from psychrophilic and non- psychrophilic trypsin from salmon trypsins and observed that the main features of the cold adapted trypsins are: reduced hydrophobicity, with overall lower ratio of (I+L)/(I+L+V); lack of seven isoleucines; higher number of methionine residues,; low stability of the C-terminal portion; mutation of P152 to G152 and deletion of Y151, and mutation of the amino acid residue P28 and Y29.

While the study on the structure of cold-adapted trypsins is prominent, fish mesophilic trypsins receives less attention. A good example of a well-studied mesophilic trypsin is the one from tambaqui (*Colossoma macropomum,* Cuvier, 1818), which is an enzyme stable at temperatures up to 55°C (Bezerra et al, 2001; Marcuschi et al, 2010). Tambaqui is a teleost fish from the characiformes order considered to be a "living fossil", since evidences found in the Miocene deposits from Magdalena River indicate that it has remained almost morphologically unaltered for at least 15 million years (Lundenberg et al, 1986; Goulding and Carvalho, 1982).

Considering that several structural characteristics considered to be typical of cold adapted trypsins have also been found in tambaqui trypsin, the present work aimed to modell the structure of tambaqui trypsin and compares it to a cold-adapted salmon and an pork trypsin. Moreover, to better access which features are more likely connected to temperature adaptations and which ones are typical of anionic fish trypsins instead, a large database of trypsins sequences was also analyzed.

Methodology

Crude extract preparation and Enzyme purification

Juvenile specimens of tambaqui (*Colossoma macropomum*) were kindly provided by the rearing units of Embrapa Semiárido (Petrolina, Brazil). Pyloric caeca and intestines of tambaqui were desiccated and homogenized in 10 mM Tris-HCl pH 8.0 with 15 mM NaCl (200 mg of tissue per buffer mL), using a tissue homogenizer (IKA RW 20D S32, China). The resulting homogenates were centrifuged (Sorvall RC-6 Superspeed Centrifuge, North Carolina, USA) at 10,000 g for 15 minutes at 4 ^{o}C to remove cell debris and nuclei. Tambaqui trypsin was purified by a three step method, modified from Marcuschi et al (2010): (1) crude extract heating for 30min at 45°C; (2) ammonium sulfate fractioning (with higher trypsin activity found in fraction 30-60%), (3) and affinity chromatography, p-Aminobenzamidine–Agarose (Sigma-Aldrich®). Commercial porcine trypsin (Sigma-Aldrich®) was also subjected to purification through affinity chromatography, to ensure that it was free of any other contaminant proteins. Fractions with tryptic activity were assayed with 4mM N-a-benzoyl-L-arginine p-nitroanilide (BApNA, Sigma-Aldrich®) prepared in dimethylsulfoxide (DMSO, Vetec). The assay followed the method described by Erlanger et al (1961), with volumes adapted for a microplate reader (Bio-Rad X-Mark spectrophotometer, California, USA).

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were obtained using Jasco J-815 Circular CD Spectropolarimeter (Japan). The instrument was calibrated with d-10-camphorsulfonic acid (Sigma-Aldrich®) at 1 mg·mL^{-1} in water. The purified trypsin samples were

517 solubilized at a concentration of 0.1 $mg \cdot mL^{-1}$ in 10 mM boric buffer, pH 7.8.
518 Measurements were made at the Far-UV (200-250) spectrum, in a 0.2 cm path length
519 cell, at temperatures ranging from 10°C to 80°C, with a complete scan every 5°C.
520 Reading speed was 50 $nm \cdot min^{-1}$ and maximum accepted voltage threshold was 700 V.
521 Each spectrum was composed of three consecutive scans, which were averaged to
522 increase the signal-to-noise ratio. CD spectra values (millidegrees) were converted to
523 mean residue ellipticity [Θ] ($deg \cdot cm^2 \cdot dmol^{-1}$), with the following formula:

524 $[\Theta] = ((\Theta \cdot 0.1)*(MW/Res-1))/(l \cdot [prot])$, where: **Θ** is the machine units in
525 millidegrees; **MW** is the protein molecular weight; **Res** is number of residues in the
526 protein; ***l*** is the cell path length in centimeters; and **[prot]** is protein concentration in
527 $mg \cdot mL^{-1}$. Data deconvolution was carried out on Dichroweb on-line software
528 (Whitmore and Wallace, 2008), using the analysis program CONTINLL (Provencher
529 and Glockner, 1981) and reference set number 4 (optimized for 190-240nm).
530
531 *RNA isolation cDNA synthesis*
532 Fresh pyloric caeca from juvenile tambaqui were obtained from the fishing base
533 of *Universidade Federal Rural de Pernambuco* and conserved in liquid nitrogen. The
534 total RNA was extracted with trizol® (Invitrogen), following manufacture's protocol.
535 The cDNA was produced with SuperScript® III Reverse Transcriptase (Life
536 technologies).
537
538 *Trypsin gene cloning and sequencing*
539 The primers used in the amplification of the trypsin gene were designed from
540 conserved regions of fish trypsin sequences found in the NCBI data base. The Forward
541 and reverse primers were designed with ECO RI and NOT I cleavage site, respectively.

Forward primer: 5' ACATAAGAATTCAATHGTYGGAGGCTACGAGTGC 3' and reverse primer: 5' ACATAAGCGGCCGCAAATCAACCTCCGGTACCA 3'. The PCR products were cloned into PTZ 57 R/T vectors (Thermo Scientific®) that were transformed into *E. coli* (DH5α) and sequenced in an ABI 3130 sequencer (Applied Biosystems). The Genebank ID for the obtained tambaqui trypsin sequence is JQ437817.1.

Construction of protein models and structures analysis

Suitable templates of trypsin structure were searched on the PDB database (Berman et al, 2000) through BLASTp from NCBI. The primary sequence of tambaqui trypsin was used as query to perform the search and five structures that showed sequences homology of 85% were selected. Then, a total of 25 three-dimensional structures of tambaqui trypsin were predicted through homology-modelling using the software Modeller version 9.11 (Eswar et al 2006). The quality of the models was analyzed with Swiss Model Workspace (Arnol et al, 2006), using QMEAN (Benkert et al, 2011) and Procheck (Laskowski et al, 1993). The best model chosen was one predicted using a salmon trypsin structure (1HJ8, Leiros et al, 2001) as template. Additionally, were also analyzed the trypsin structures from salmon and pork, PDB entries 1BIT (Berglund et al, 1995) and 1S81 (Transue et al 2004) respectively.

Molecular dynamics simulations

The Molecular Dynamics simulations were performed using Gromacs software version 4.5.4 and Gromos-96 53a6 force field (Spoel et al 2005), in a cubic box with SPC water. In order to neutralize the overall charge of the system, a number of water molecules equal to the protein net charge was replaced by Cl− (in porcine trypsin) or

567 Na+ ions (in tambaqui and salmon trypsins). Energy minimization was done using
568 steepest descent method (100,000 steps), followed by an optimization step of 60 ps from
569 50 to 373 K. To improve conformational sampling, three independent 100ns simulations
570 were carried out for each protein and temperature (300 and 328 K). The root mean
571 square deviation (RMSD) was computed for protein atoms using as reference the
572 starting structure of each MD simulations. The root mean square fluctuation (RMSF)
573 per residue was calculated on protein atoms as well. The secondary structures variations
574 throughout the simulation were calculated using DSSP program (Joosten et al, 2011).
575 The visual analysis of protein structures was carried out using Pymol™ Molecular
576 Grafic System, version 1.5.
577
578 *Selection of trypsin amino acid sequences*
579 NCBI and MEROPS (Rawlings et al, 2012) databanks were used for selecting
580 trypsin sequences from vertebrates. Trypsin from animals of the class agnathas,
581 chondrichthyes, and osteichthyes were grouped as “fish”, whereas mammals and birds
582 were grouped as “homeotherms”. Other poikilothermic animals (reptiles and
583 amphibians) were not included due to the low number of sequences available.
584 Sequences with similarity higher than 99%, as well as trypsin Y were excluded. The
585 sequences’ theoretical pI were calculated with JVirGel website (Hiller et al, 2006), and
586 based on that, they were subdivided in anionic (pI<6.9) and cationic (pI>7.0), thus
587 forming four analysis groups: “Anionic fish trypsin” (AFT), “Cationic fish trypsin”
588 (CFT), “Anionic homeotherm trypsin” (AHT), and “Cationic homeotherm trypsin”
589 (CAT), being composed by 61, 18, 80, 68 sequences, respectively. The sequences were
590 aligned using ClustalW and analyzed with Mega 5 software (Tamura et al, 2011).
591 Amino acid composition was calculated by Bioedit software (Hall, 1999), the grand

592 average of hydropathy (GRAVY) was calculated by the method developed by Kyte and
593 Doolittle (1982), using SMS on-line software (Stothard, 2000), and sequence Logos
594 were made on WebLogo website (Crooks et al, 2004). A supplementary material
595 containing the alignment of all sequences used is available at the end of this work.
596
597 *Statistical analysis*
598 Statistical analysis was performed using OringPro 8.0 software program, from
599 OriginLab Corporation using one-way analysis of variance (ANOVA), followed by
600 Tukey's test ($p<0.05$).
601
602

Results and Discussion

Amino acids composition

According to Feller et al (1996; 1997) it is possible to draw some conclusions on whether a protein is more or less stable by analyzing their amino acid content. Cold-adapted enzymes are usually less stable than meshophilic and thermophilic ones, due to greater structural flexibility. That flexibility is acquired by displaying some characteristics, namely: reduction of the number of weak interactions involved in the folded state; lower hydrophobicity of the hydrophobic clusters forming the core of the protein; deletion or substitution of proline residues in loops or turns; improvement of solvent interactions with a hydrophilic surface; occurrence of glycine clusters close to functional domains; and looser coordination of Ca^{2+} ions (Feller et al, 1997). Not all of these features will be found in every cold adapted enzyme, nor be exclusive to them, but that is the most common case. When comparing the amino acids composition from the four main groups in the present work a few patterns were observed (Table 1).

Hydrophobic residues

Some hydrophobic residue, such as Ala, Leu and Ile were more frequent in the homeothermic groups, whereas there were more Met in fish. According to Leiros et al (2000), even though methionine is a hydrophobic residue, the placing of them in fish trypsins structure seems to introduce additional flexibility to them. Two good methods for measuring hydrophobicity in proteins are the Grand average of hydropathicity (GRAVY) and the aliphatic index, and both values were significantly lower in fish than in homeothermic animals. Leiros et al (1999) analyzed the amino acids sequences of cold-adapted trypsins and observed that the hydrophobic residues content is much lower

628 than in mesophilic trypsin. Nevertheless, it is possible to say that there is a tendency for
629 fish trypsin, as group, to be less hydrophobic than homeothermic ones, regardless of
630 cold adaptation, as can be observed on the example of the tambaqui sequence (Table 1).
631
632 *Polar residues*
633 The presence of glycine residues in loops close to the active site helps facilitate
634 the flexibility of the protein backbone, because the absence of a side chain results in a
635 lower torsion barrier for the rotation around the ψ and ϕ bonds (Gombos et al, 2008).
636 Even though AFT has lower Gly content, the glycine in hinges positions (G19, G142,
637 G184, G193) are all conserved, therefore not posing a problem for enzymatic activity.
638 Cysteine is another structurally important residue, and it is fairly conserved in all
639 trypsin. The 12 Cys residues are most likely involved in forming six conserved disulfide
640 bonds, which help to keep the structure firm (Leiros et al, 1999). Asn and Ser residues
641 are higher in cationic trypsins than anionic, especially in CFT.
642
643 *Charged Residues*
644 Fish trypsin also showed a higher content of charged residue, with the main
645 difference between cationic and anionic trypsins resting in the concentration of
646 negatively charged amino acids (Table 1). A higher concentration of Arg residues in
647 fish enzymes is also contradictory to a looser structure, since proteins with more
648 flexible structures usually have less Arg residues, since they are usually involved in
649 forming multiple salt bridges and hydrogen bonds (Georlette et al, 2004).
650 By analyzing the amino acid content of tambaqui, it does not present the main
651 features of cold adapted enzymes. For instance, its Proline content is higher than most
652 trypsins and it has low glycine and serine residues content. That is consistent with the

653 fact that this enzyme endures heating up to 70 °C, and does not show high activity at
654 low temperatures (Marcuschi et al, 2010). However as it is going to discussed, tambaqui
655 trypsin shares some structural features that are considered to be exclusive of cold
656 adapted enzymes.
657
658 **Sequences analysis**
659 Vertebrates trypsins have a fairly conserved structure, being characterized for
660 two β-barrels, connected by loops, with a serine catalytic triad (His57/Asp102/Ser195)
661 in the middle, a substrate recognition site (Asp189/Gly216/Gly226), an oxyanion hole
662 (formed by a salt bridge between I16 and D194), as well as a calcium loop (residues 68
663 to 80) and an autolysis loop (residues 141 to 155) (Hedstrom, 2002; Polgar, 2005; Bode
664 and Schwager, 1975).
665 As the enzymes were further analyzed it was possible to notice that the anionic
666 trypsin fish seem to be the most conserved group of all, with a total of 222 amino acids
667 (Figure 1). According to Leiros et al (1999) most cold adapted fish trypsins are anionic,
668 but since there are also anionic mesophilic trypsisn, it can be difficult to differentiate the
669 features that are linked to protein charge from cold adaptation. Even so, there are
670 several point mutations along the sequence (Figure 1), that can indicate the differences
671 that allow each enzyme to work better at a particular temperature. It is also noticeable
672 that AFT show many differences to CFT (Figure 2), while AHT (Figure 3) and CHT
673 (Figure 4) are much more similar between them.
674 The deletion of residue T151 and the substitution of residue P152 are mutation
675 that have long been associated with cold adaptation in fish trypsin (Feller et al, 1996;
676 Kanno et al, 2011; Leiros et al, 1999; 2000; Gombos et al, 2008). These mutations are
677 considered to promote a better access of the substrate to the active site, as well as

678 increase this site flexibility (Feller et al 1996). However, as it can be observed in figure
679 1, this mutation is common to all anionic fish trypsin, including the mesophilic
680 tambaqui trypsin.

681 As a consequence, this mutation also changes the conformation of the autolysis
682 loop, which is located between residues 140 and 155 (Leiros et al, 1999; 2000). A
683 mutation K145M (hydrolysis site of the autolysis loop) is seen in all AFT, whereas a
684 K145S is more common in CFT. However, it must be noted that a K145L mutation is
685 also common in homeothermic trypsins, so that only a few species have this autolysis
686 site, which is associated with converting β-trypsin into α-trypsin. According to Feller
687 1996 one more mesophilic determinant in the autolysis loop is the presence Gly148,
688 which is substituted by b-branched residue in cold-adapted enzyme. And according to
689 papaleo 2008, the aminoacids P130 and P140 are conserved only in cold adapted serine
690 proteases. However, once again this is a feature common to all anionic trypsins,
691 including tambaqui.

692 Another interesting point mutation is R117, a crucial site for autolysis found in
693 mammalian trypsins, but non-existent in fish where it is mutated for a tyrosine. This
694 autolysis site has long been associated to pancreatitis (Whitcomb et al, 1996; Varallyay
695 et al, 1998) but, as far as it is known, fish as a group do not suffer from chronic
696 pancreatitis. This mutation is possibly associated with allowing fish trypsin to remain
697 active longer in the digestive tract, since these animals are highly dependent on protein
698 for their growth and energy.

699 It is also an interesting fact that calcium is known to be more effective in
700 protecting trypsin from autolysis when Arg 117 is present (Varallyay et al 1998).
701 However the presence of calcium in the enzyme structure can also make it more rigid,
702 thus less active (Bode and Schwager, 1975). That may explain the trend seen in frigid-

zone fishes to have a higher incidence of positive charged amino acids in the calcium loop, thus reducing the strength of the ligation to Calcium ion (Kanno et al, 2011). The calcium binding loop is one of the regions with greater variation among AFT (Figure 1), what may indicate that this is one of the best sites to look for cold-adaptation traces.

Structure analysis and molecular dynamics

The RMSD values calculated for the protein atoms of tambaqui, salmon and porcine trypsin showed that the dynamics were stable after 20 ns (Suplementary figure 1), and to ensure that calculated parameters reflect the intrinsic properties of each system, the analyses of MD trajectories were carried out from 80 to 100 ns.

The Flexibility of the structures after the dynamics is detailed by the b-factor coloring from figure 6. These results reflect the RMSF (available in Supplementary figure 2) values from, and indicate the regions in which the residues have fluctuated the most. It is noticeable that the loops close to the active site, as well as the c-terminal helix become more flexible as temperature rises. Comparing the structures to the amino acid sequences, displayed in figure 5 with the structures from figures 6 and 7 it is possible to notice that the core of the enzymes are well conserved among them, while the surface tends to be more hydrophilic on the fish enzymes.

The region close to the residues from the active center from tambaqui and porcine trypsin are not very flexible, even at high temperatures. However, the active site becomes disorganized in salmon trypsin, even at temperature as low as 27°C (Figure 7). This can be illustrated by the distances displayed for the residues Ser195 and His57, which is 7.0 Å for salmon and 2.5 Å for tambaqui. The increase in distance between the active site residues is probably caused due to greater flexibility of the active site in cold

adapted enzymes (Somero, 2004; Feller et al, 1996). However this distancing can diminish the strength of the hydrogen bonds, thus affecting the enzyme activity.

Papaleo et al (2008) suggested that some regions of the psychrophilic serine proteases are more flexible, thus being more likely that they are involved in enhancing the total structure flexibility. Some of the residues in these regions are conserved between salmon and tambaqui trypsin, but different in porcine trypsin. Manny of the variations in flexibility observed in the present work were also observed by Papaleo et al 2008, and Arvizu-Flores et al 2012, and the main ones are discriminated bellow:

(1) The N-terminal α-Helix, behaved in the same way in all enzyme, but the residue K23 was more flexible in tambaqui and salmon trypsin; (2) Calcium binding loop was more flexible in porcine trypsin; (3) autolysis loop (residues 140-151), was more flexible in fish trypsin, mainly due to the backbone configuration, including the deletion of residue 151; (4) The region 116-118, main site of autolysis in mammals was more flexible in porcine trypsin; (5) The internal helix (residues 91-98) was more flexible in the fish enzymes, with salmon trypsin showing even higher flexibility. Interestingly, flexibility in this site does not increase in tambaqui trypsin, with the increase of temperature, as it is observed in salmon and porcine trypsin. This is an important helix, since it is close to the active site and the disruption of its configuration can be one of the reasons for the loss of activity associated with increase of temperature. (6) Loop of Residues 161-170, was more flexible in fish trypsin.

It must also be noted that even though tambaqui trypsin bears similarities to salmon trypsin, with a more flexible structure, it is less prone to denaturation by heating than porcine trypsin. This is noticeable by analyzing the circular dichroism spectra (Figure 8), that shows the denaturation of fish porcine trypsin at lower temperatures than tambaqui trypsin. However it must also be observed that secondary structure

content does not seem to change significantly throughout the enzymes heating. Moreover the comparison of both structures secondary composition obtained by circular dichroism with the obtained by modeling showed difference in the amount of turns and beta-structures, but were equal in unordered and helix structures. This may be an artifact of dichroism analysis, since trypsin does not yield very sharp spectras, as it can be observed by other works on trypsin dichroism (Ghosh 2008; Wang et al, 2013).

Conclusion

Tambaqui trypsin has many mutations that are considered typical of cold adapted trypsin. However, these mutations are better related to anionic trypsins than cold-adaptation. Since that tambaqui is a “living fossil”, it is also possible that it had to endure colder temperatures in the past, and as well as other mesophilic fishes, it still carries some mutations destined for better activity at low temperatures. Thus, even though fish trypsins are structurally less stable than homeothermic, the first are much less prone to autolysis than the latter, what ends up allowing fish trypsin to endure a greater range of temperatures.

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896

897

898

899 **Figure Captions**

900 Figure 1. Sequence Logo made from the alignment of fish anionic trypsin genes. (•)
901 Residues involved in the salt bridge that forms the oxyanion hole; (Ŧ) Residues involved
902 in the catalytic triad; (*) Residues involved in the ligation with the calcium ion; (ɣ)
903 Classical trypsin autolysis sites; (ŧ) Residues involved in substrate recognition.

904 Figure 2. Sequence Logo made from the alignment of fish cationic trypsin genes;

905 Figure 3. Sequence Logo made from the alignment of homeotherm anionic trypsins
906 genes.

907 Figure 4. Sequence Logo made from the alignment of homeotherm cationic trypsins
908 genes.

909 Figure 5. Alignment of the amino acid sequences from tambaqui trypsin with the
910 psychrophilic salmon trypsin and porcine trypsin. Arrows indicate β-structures, straight
911 lines indicate turns and bends, and up and down lines indicate helical structures.

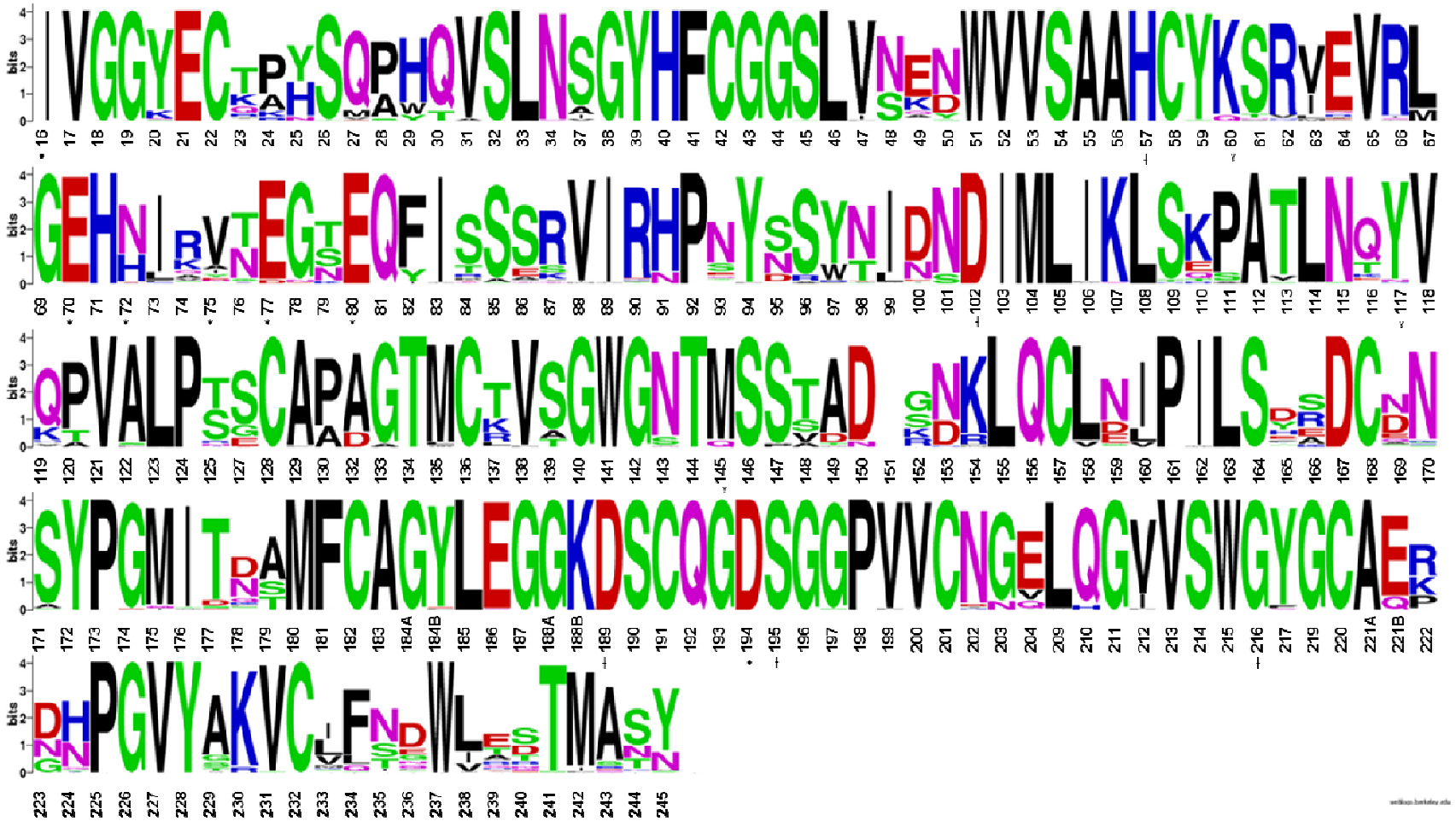
912 Figure 6. Three dimensional structure of tambaqui, salmon and porcine colored
913 according to the β-factors from low flexibility (blue) to high flexibility (red) referring to
914 the 100ns molecular dynamics at 27, 55 and 100 °C.

915 Figure 7. Three dimensional structure of tambaqui, salmon and porcine after 100ns
916 molecular dynamics at 27, 55 and 100 °C. Calcium loop is marked in yellow, active site
917 residues in red and autolysis sites in purple.

918 Figure 8. Circular Dichroism spectra from (A) tambaqui and (B) porcine trypsin, at 25,
919 30, 55 and 80 °C.

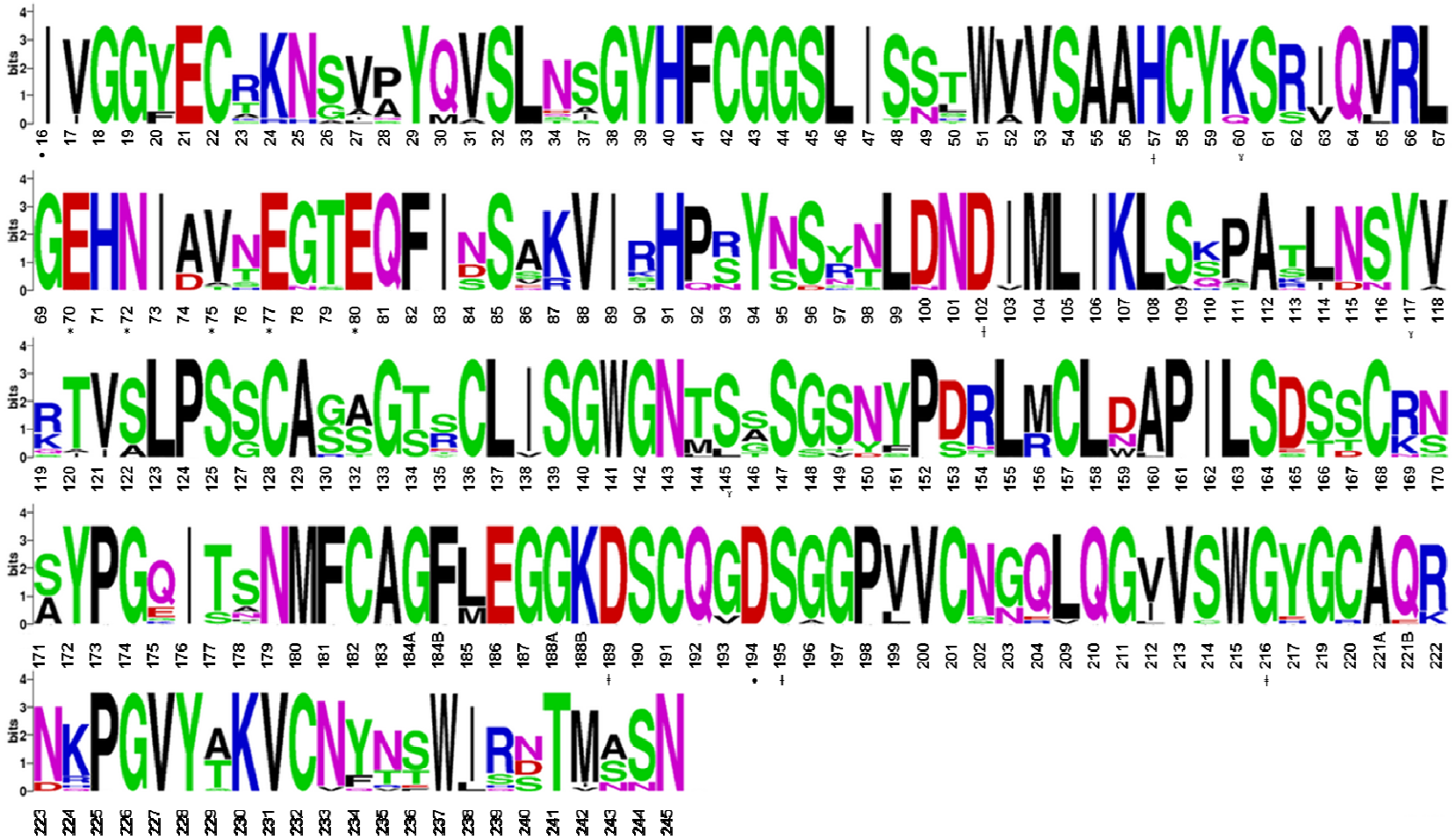
920

921 Figure 1



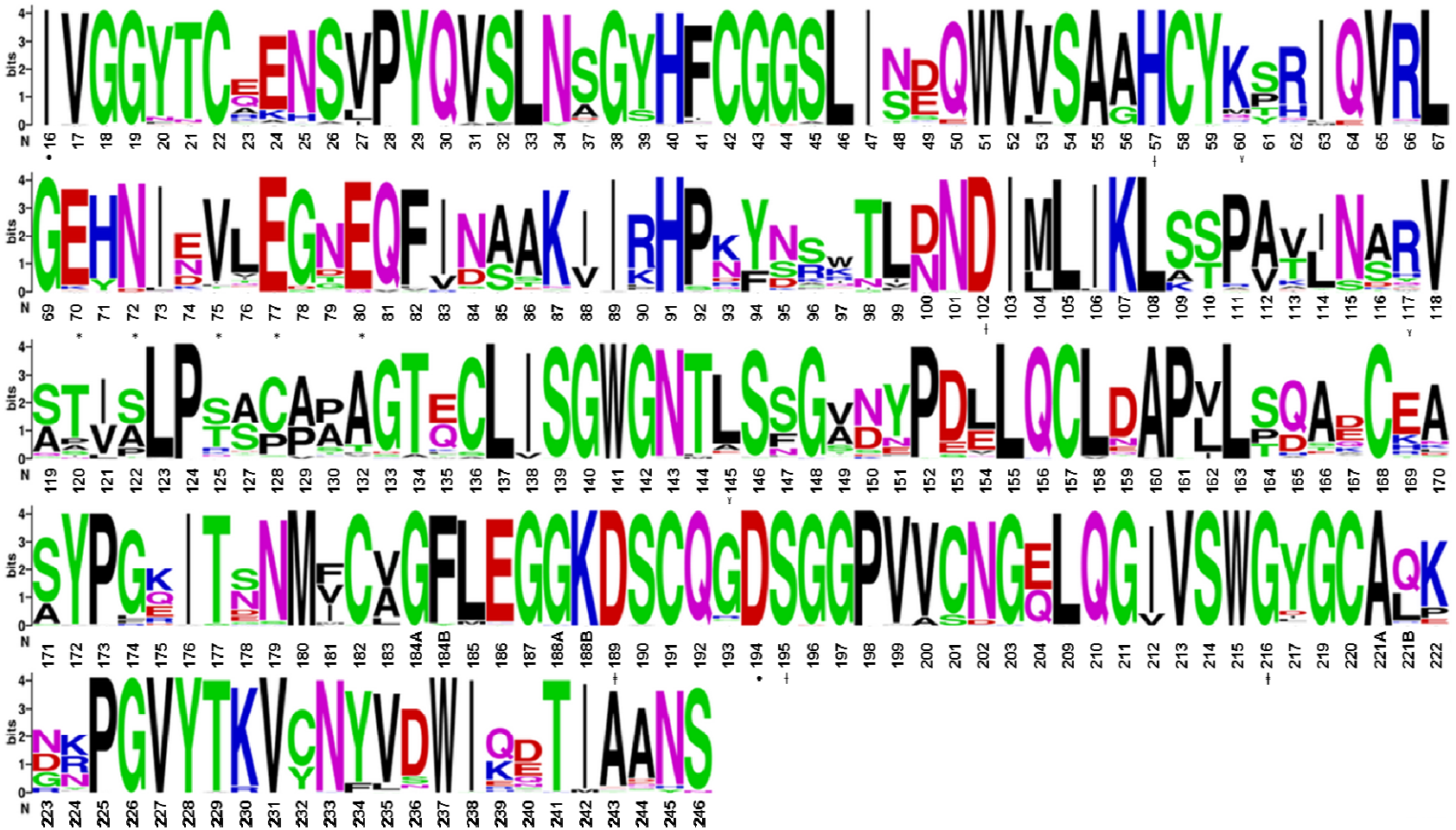
922

923 Figure 2



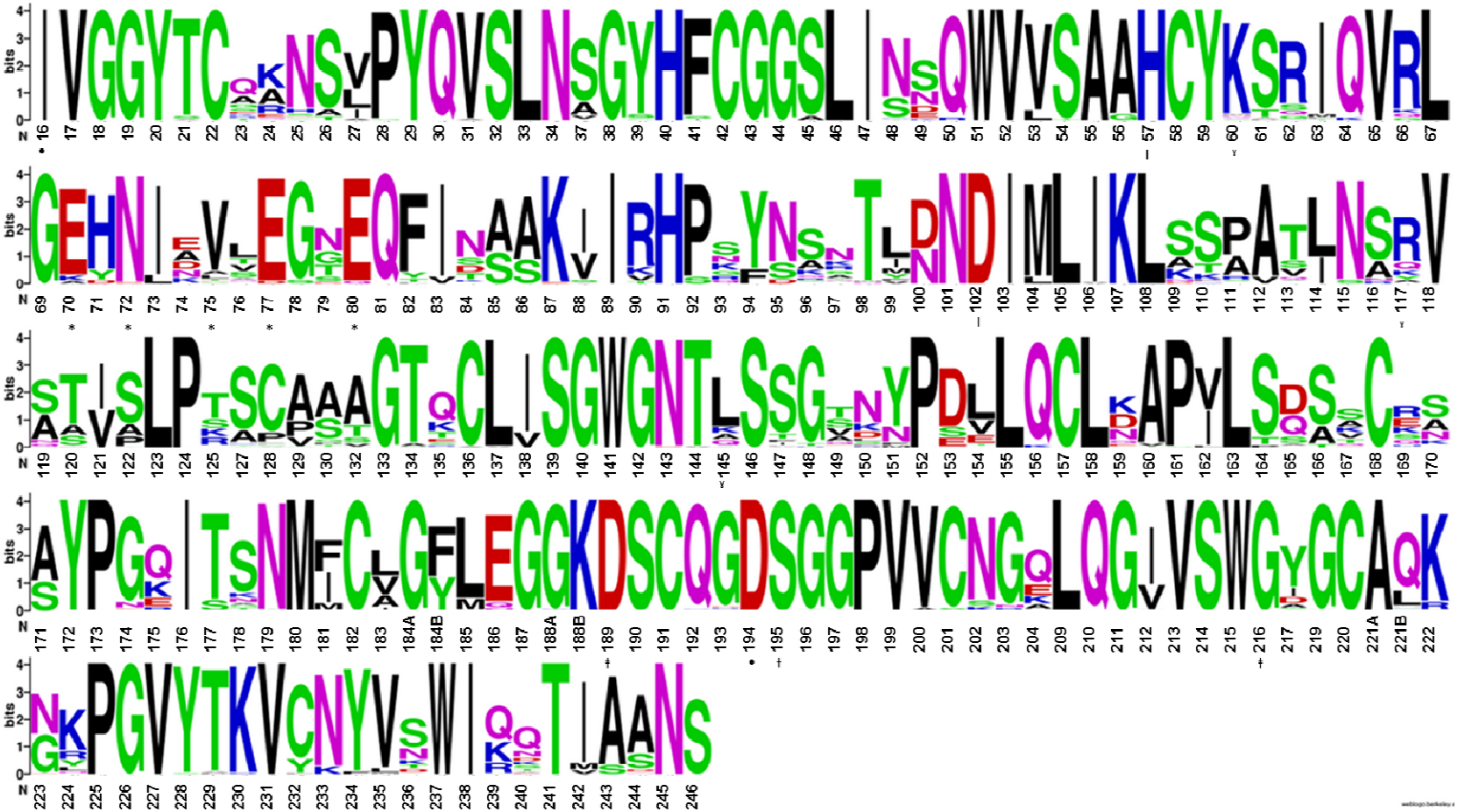
924

925 Figure 3



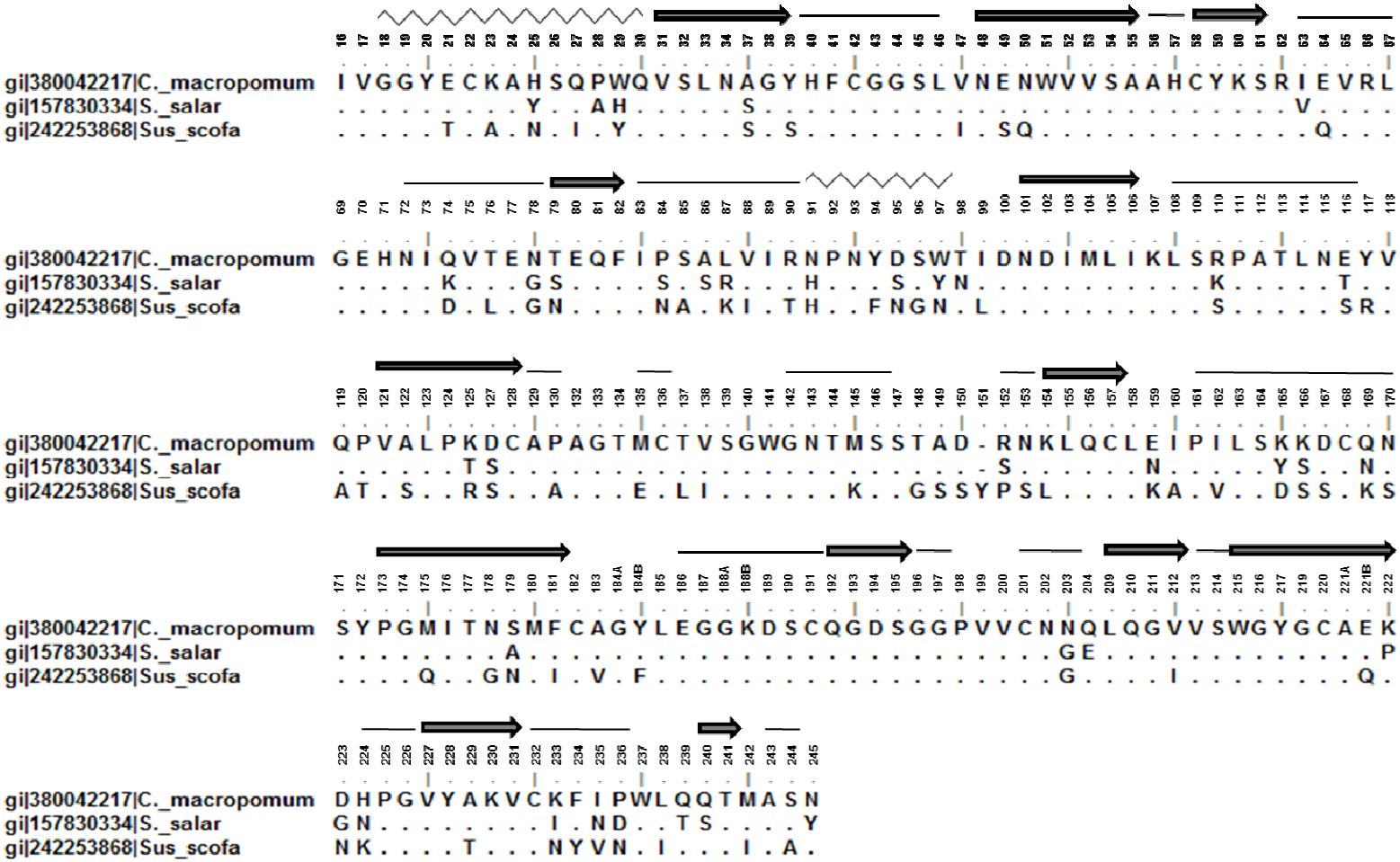
926

927 Figure 4



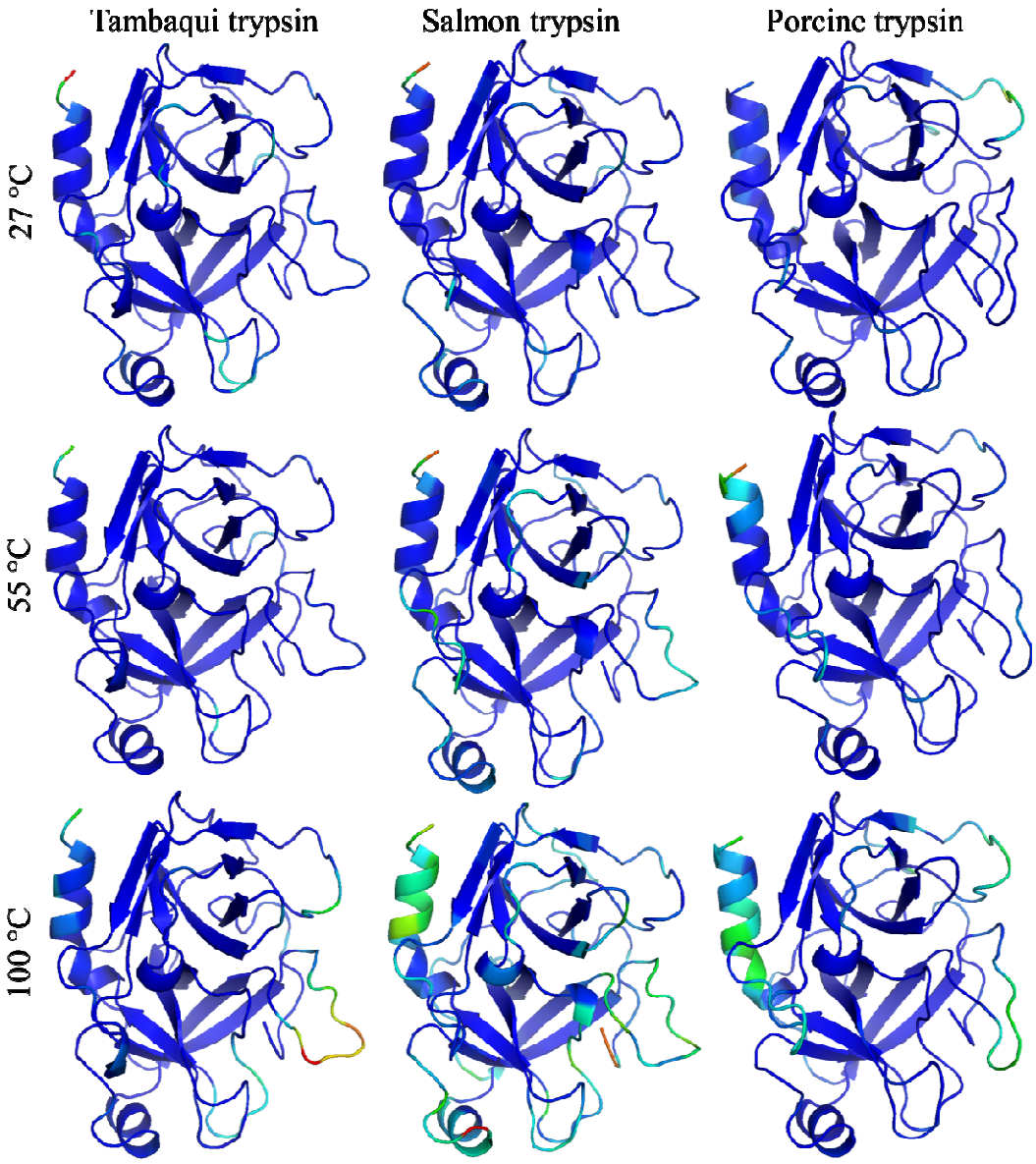
928

929 Figure 5



930

931 Fig 6



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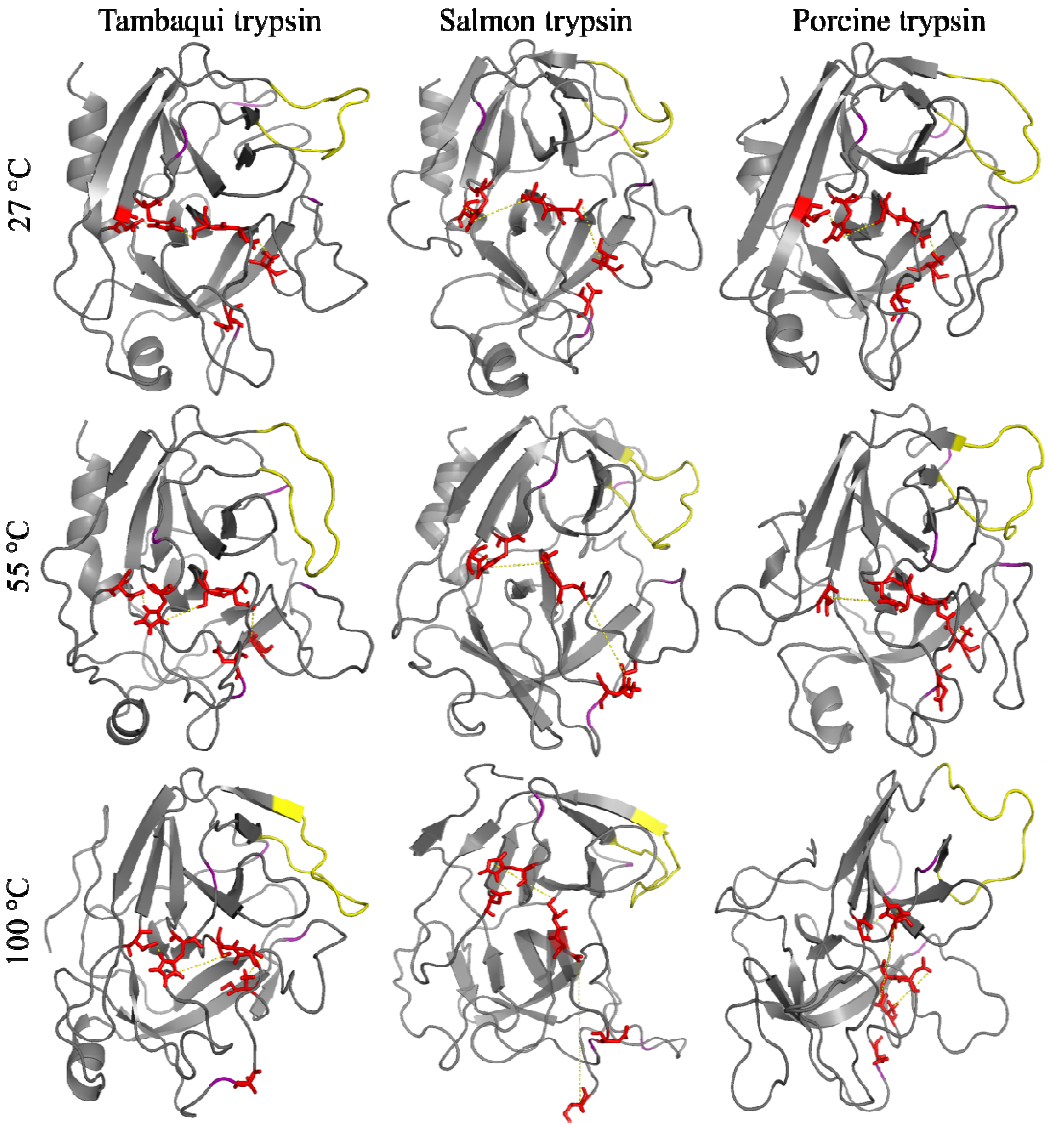
934 Fig 7



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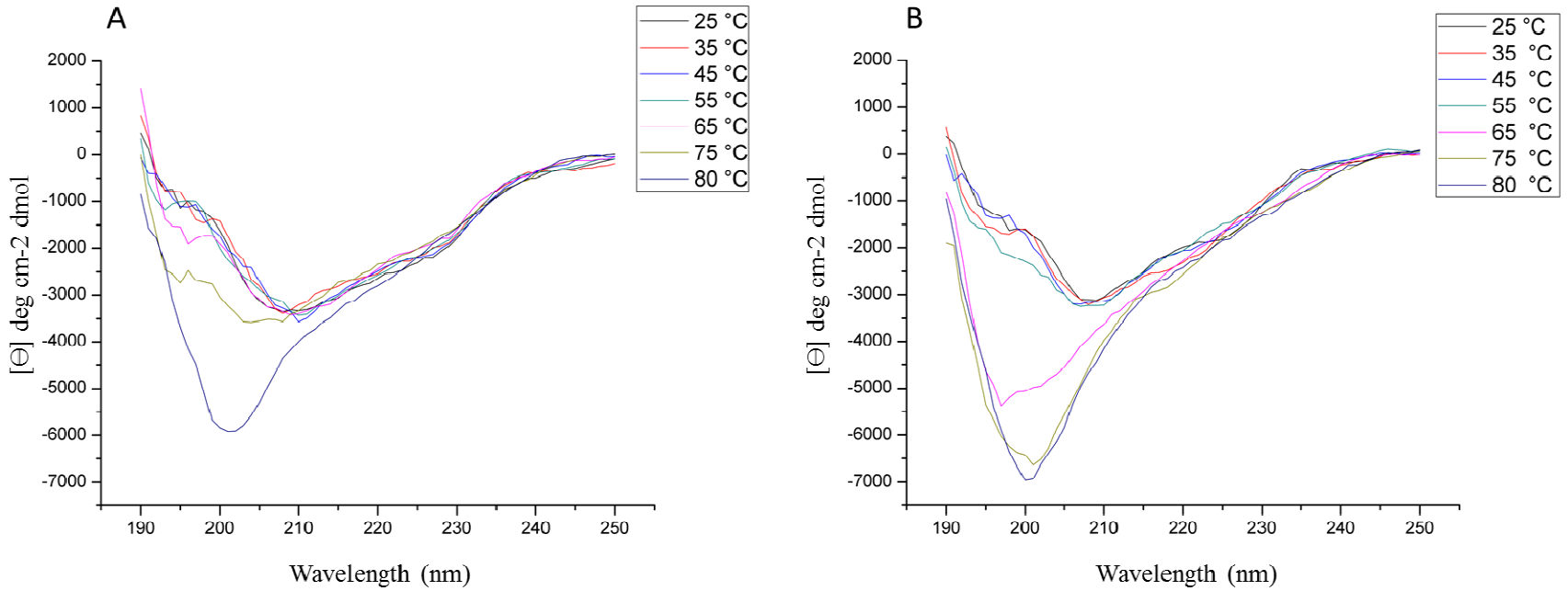
937 Fig 8



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946 **Tables**

947 Table 1. Average (standard deviation) of the amino acids frequency and Gravy score from tambaqui trypsin and the other trypsin groups.

Charge	Amino acids	Tambaqui trypsin	Anionic fish trypsin	Cationic fish trypsin	Anionic homeotherm trypsin	Cationic homeotherm trypsin
Nonpolar, Hidrophobic	A	14	12.8 (1.3)^{b}	11.8 (1.2)^{b}	15.2 (2.0)^{a}	14.8 (2.2)^{a}
	V	17	18.1 (1.6)^{a}	15.5 (1.7)^{c}	17.3 (1.8)^{b}	16.9 (1.6)^{b}
	L	14	13.7 (1.0)^{c}	14.4 (1.0)^{c}	17.4 (1.8)^{a}	16.2 (1.6)^{b}
	I	12	10.7 (1.6)^{c}	12.5 (1.3)^{b}	15.1 (1.8)^{a}	15.1 (1.7)^{a}
	F	4	3.8 (0.7)^{b}	4.5 (0.7)^{a}	4.0 (0.9)^{ab}	3.2 (1.1)^{c}
	W	6	4.3 (0.8)^{a}	4.2 (0.4)^{ab}	4.4 (0.6)^{a}	4.0 (0.0)^{b}
	M	6	6.2 (0.8)^{a}	4.3 (1.0)^{b}	2.1 (0.9)^{c}	2.5 (0.9)^{c}
	P	12	10.0 (1.4)^{b}	8.3 (0.7)^{c}	10.8 (1.3)^{a}	9.1 (1.2)^{c}
	G	20	22.9 (1.0)^{c}	23.8 (0.8)^{ab}	23.3 (0.8)^{b}	23.9 (1.1)^{a}
Polar, Uncharged	C	12	12.0 (0.1)^{a}	12 (0.0)^{a}	11.2 (1.4)^{b}	11.6 (0.9)^{ab}
	S	18	23.7 (2.2)^{c}	32.1 (3.1)^{a}	19.6 (2.9)^{d}	25.5 (5.2)^{b}
	T	10	10.0 (1.7)^{ab}	8.9 (1.7)^{b}	9.5 (1.8)^{b}	10.8 (2.2)^{a}
	N	16	14.4 (2.4)^{c}	17.3 (2.5)^{a}	15.4 (2.9)^{bc}	15.9 (2.0)^{b}
	Q	12	8.6 (1.6)^{c}	8.4 (1.1)^{c}	10.8 (1.7)^{b}	12.1 (1.8)^{a}
	Y	9	11.4 (1.5)^{a}	10.9 (1.0)^{ab}	9.8 (1.5)^{c}	10.2 (1.2)^{bc}
Polar Acidd	D	9	9.4 (2.0)^{a}	7.4 (1.1)^{b}	9.4 (1.6)^{a}	6.3 (1.4)^{b}
	E	10	9.8 (1.7)^{a}	5.6 (0.8)^{b}	9.7 (2.4)^{a}	5.3 (2.0)^{b}
Polar Basic	H	5	6.4 (1.7)^{a}	4.3 (0.6)^{bc}	4.6 (1.1)^{b}	3.9 (0.7)^{c}
	K	11	7.6 (1.6)^{c}	7.9 (1.4)^{bc}	8.7 (1.8)^{b}	10.7 (2.3)^{a}
	R	5	6.1 (1.7)^{b}	8.7 (2.0)^{a}	4.7 (1.4)^{c}	4.9 (2.1)c
Gravy score		-0.25	-0.19 (0.06)^{a}	-0.19 (0.04)^{a}	-0.07 (0.07)^{b}	-0.06 (0.075)^{b}
Aliphatic index		74.19	72.42 (2.4)^{c}	72.70 (1.6)^{c}	86.03 (2.9)^{a}	83.3 (3.32)^{b}

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949 Table 2. Secondary structures composition obtained from molecular dynamics.

Percentage of secondary structure from circular dichroism									
Tambaqui trypsin					Porcine trypsin				
°C	α-helix	β-strand	turns	unordered	°C	α-helix	β-strand	turns	unordered
25	10	34	22	34	25	11	34	22	34
35	10	34	22	34	35	9	35	22	34
45	11	33	22	34	45	10	34	22	34
55	10	34	22	34	55	9	34	23	34
65	9	35	22	34	65	8	34	24	35
75	9	35	22	34	75	8	33	24	34
80	9	32	24	34	80	8	33	24	35

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Considerações finais

A presente tese reportou a purificação, clonagem e sequenciamento de uma tripsina do tambaqui, bem como a caracterização de sua estrutura e frente ao íon cálcio, tenso-ativos e sabão em pó. A enzima sequenciada apresentou similaridade próxima a 85% das tripsinas aniônicas de salmão, tendo sido possível produzir um modelo por homologia da estrutura desta enzima. As dinâmicas moleculares apontaram uma estrutura mais flexível que a da tripsina do porco, porém menos flexível que a do salmão. Isso indica que apesar da alta similaridade entre as enzimas de peixe, mutações pontuais permitiram que o tambaqui tivesse uma estrutura que suporta melhor o aquecimento. Adicionalmente, apesar de ter uma estrutura mais flexível que a da tripsina de porco, a tripsina do tambaqui se mostrou não ser muito propensa à autólise. Consequentemente, o íon cálcio se mostrou quase que desnecessário para a sua atividade, permitindo que a enzima do tamaqui possa permanecer em solução à temperatura ambiente por longos períodos, sem ter uma queda acentuada em sua atividade. Assim, é possível afirmar que a tripsina do tambaqui é uma enzima com potencial para a aplicação em processos industriais, como o de aditivo para produtos de pré-lavagem de roupas.

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Apêndice A – Purification, characterization and substrate specificity of a trypsin from the Amazonian fish tambaqui (*Colossoma macropomum*)

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Purification, characterization and substrate specificity of a trypsin from the Amazonian fish tambaqui (*Colossoma macropomum*)

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ABSTRACT

An enzyme was purified from the pyloric caecum of tambaqui (*Colossoma macropomum*) through heat treatment, ammonium sulfate fractionation, Sephadex® G-75 and *p*-aminobenzamidine–agarose affinity chromatography. The enzyme had a molecular mass of 23.9 kDa, NH_2-terminal amino acid sequence of IVGGYECKAHSQPHVSLNI and substrate specificity for arginine at P1, efficiently hydrolizing substrates with leucine and lysine at P2 and serine and arginine at P1′. Using the substrate z-FR-MCA, the enzyme exhibited greatest activity at pH 9.0 and 50 °C, whereas, with BAPNA activity was higher in a pH range of 7.5–11.5 and at 70 °C. Moreover, the enzyme maintained ca. 60% of its activity after incubated for 3 h at 60 °C. The enzymatic activity significantly decreased in the presence of TLCK, benzamidine (trypsin inhibitors) and PMSF (serine protease inhibitor). This source of trypsin may be an attractive alternative for the detergent and food industry.

1. Introduction

The term trypsin was coined by W.F. Küne in 1876 to describe the proteolytic activity found in the pancreas of animals. Currently, trypsin (EC 3.4.21.4) is known as the serine endoprotease that hydrolyzes peptide bonds at the carboxylic end of the amino acid residues arginine (R) and lysine (K) [1]. These enzymes are found in several organisms (animals, bacteria and viruses) and play a pivotal role in digestive physiology [2]. In most teleost fish trypsin is synthesized in the cells of the pyloric caecum as an inactive precursor (trypsinogen), which is then secreted into the intestinal lumen and activated by enteroproteases [3].

Fish viscera are a common by-product of the fishery industry and a possible enzyme source for biotechnological application. Tropical fish proteases are known to be thermostable, have a long shelf life and be highly active over a wide range of pH [4–7]. In Northern Brazil, one of the most important tropical freshwater fish is the tambaqui (*Colossoma macropomum*). It is a characid found mainly in rivers, lakes and floodplains of the Amazon [8]. Tambaqui acid and alkaline proteases were first characterized by Bezerra et al. [9]. Proteases extracted from the viscera of *C. macropomum* have been proposed as a source of alkaline proteases for laundry detergent [6]. Moreover, a thermostable trypsin-like enzyme of 38.5 kDa from the pyloric caecum of this same fish was already partially purified and characterized [4].

In the present work this enzyme was further purified and its NH_2-terminal amino acid sequence was determined. Furthermore, this enzyme was characterized with fluorogenic and chromogenic substrates and its substrate specificity was investigated by using Fluorescence Resonance Energy Transfer (FRET) peptides.

2. Materials and methods

2.1. Enzyme purification

Juvenile specimens of tambaqui (*C. macropomum*), with an average weight of 316.7 ± 73.2 g and length of 24.9 ± 2.2 cm, were kindly provided by the rearing units of the *Universidade Federal Rural de Pernambuco* (Brazil). The specimens were sacrificed in an ice bath and had their pyloric caecum (0.7 ± 0.14 g per fish) collected and homogenized in 10 mM Tris–HCl, 15 mM NaCl pH 8.0 (200 mg/mL). The resulting homogenate was centrifuged (Herolab UniCen MR Centrifuge, Germany) at 10,000g for 15 min at 4 °C to remove cell debris. The enzyme was purified from the homogenate supernatant (crude extract) through a four-step procedure: (1) incubation for 30 min at 45 °C (heat treatment), as described by Bezerra et al. [4]; (2) ammonium sulphate fractionation for 2 h at 4 °C for the final salt saturation of 0–30% (fraction F_1), 30–60% (fraction F_2) and final supernatant (protein soluble in 60% salt

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concentration); (3) size exclusion filtration chromatography (120 cm^3 with 9 g Sephadex® G-75, Sigma®) at a flow rate of 0.4 mL/min of 100 mM Tris–HCl pH 8.0; (4) affinity chromatography (2 cm^3 with 1 mL of *p*-aminobenzamidine–agarose, Sigma®) at a flow of 0.5 mL/min of 100 mM Tris–HCl pH 8.0 as the binding buffer and then of 500 mM KCl–HCl pH 2.0 as the elution buffer. For each 1 mL fraction collected, 60 μL of 1.5 M Tris–HCl pH 8.0 buffer was added to neutralize the sample pH. These fractions were pooled, dialyzed against 100 mM Tris–HCl pH 8.0 for 24 h at 4 °C and used in the following assays. The total protein content of the samples was estimated following the procedure described by Bradford [10], using bovine serum albumin (BSA) as the standard protein.

2.2. Specific trypsin activity with chromogenic substrate

The tryptic activity was assayed with the chromogenic substrate *N*-α-benzoyl-L-arginine *p*-nitroanilide (BAPNA) prepared in dimethylsulfoxide (DMSO), according to Bezerra et al. [4]. The release of *p*-nitroaniline (product) was monitored at λ 405 nm in a microplate reader (Bio-Rad X-Mark spectrophotometer, California, USA). One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing 1 μmol of BAPNA per min under the established conditions.

2.3. Specific trypsin activity with fluorimetric substrate

Tryptic activity was also assayed using the fluorescence substrate carbobenzoxy-Phe-Arg-7-amido-4-methylcoumarin (z-FR-MCA) prepared in DMSO. The reaction mixture used to assay the effect of pH and temperature consisted of 100 μM z-FR-MCA (0.2 μL), 100 mM Tris–HCl pH 8.0 (1 mL) and sample (10 μL). The mixture was kept under agitation in a Hitachi F-2500 (Tokyo, Japan) fluorimeter for 25 min and the release of free MCA (fluorophore) was continuously measured at λ_{EX} 380 nm and λ_{EM} 460 nm. The absorbance values were used to calculate the apparent second-order rate constant (k_{cat}/K_m) assayed under pseudo-first-order conditions, in which $[S] \ll K_m$, using the software Grafit 5.0.0 [11]. These values were later converted to a relative percentage of k_{cat}/K_m.

The thermal stability and the effect of inhibitors were obtained considering residual enzymatic activity values, by measuring the initial enzymatic velocity. In this assays the reaction mixture was composed of 1 mM z-FR-MCA (1 μL), 100 mM Tris–HCl pH 8.0 (1 mL) and sample (1–2 μL). The release of free MCA was monitored for 90 s at 25 °C. One unit (U) of enzyme activity was defined as the amount of enzyme capable of hydrolyzing one μmol of z-FR-MCA per second under the established conditions. The results were reported as the activity relative to the non-treated samples.

2.4. SDS–PAGE and mass spectrometer

Polyacrylamide gel electrophoresis (SDS–PAGE) was carried out following the procedure described by Laemmli [12] using a 4% (w/v) stacking gel and a 12.5% (w/v) separation gel. The molecular mass and purity of the purified enzyme was also checked by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF, TofSpec-E, Micromass).

2.5. Effect of synthetic inhibitors

The effect of protease inhibitors on the pure trypsin was evaluated using z-FR-MCA according to Beynon and Bond [13] and BAPNA, according to Bezerra et al. [4]. The inhibitors used in this assay were: tosyl lysine chloromethyl ketone (TLCK), phenylmethylsulfonylfluoride (PMSF), tosyl phenylalanine chloromethyl ketone (TPCK), benzamidine, *O*-phenanthroline, E-64, ethylenediamine tetraacetic acid (EDTA) and β-mercaptoethanol.

2.6. Determination of NH_2-terminal amino acid sequence

The NH_2-terminal sequence was determined by the Edman degradation method with a protein sequencer PPSQ-23 (Shimadzu Tokyo, Japan) and an isocratic HPLC system.

2.7. Effect of pH and temperature

The effect of pH on the purified enzyme was evaluated with BAPNA and z-FR-MCA (apparent second-order rate constant) at 25 °C in a range of 4–11 (100 mM citrate–phosphate, Tris–HCl and Glycine–NaOH). The effect of temperature was evaluated in a range of 4–80 °C using 100 mM Tris–HCl pH 8.0 as the buffer. Thermal stability of the purified enzyme was evaluated at temperatures ranging from 4 to 70 °C for 3 h. Samples were incubated at each temperature. An aliquot was collected every hour and mixed with 100 mM Tris–HCl pH 8.0 and BAPNA or z-FR-MCA to assay the residual activities.

2.8. Determination of cleavage specificity

Two series of fluorescence resonance energy transfer (FRET) peptides containing ortho-aminobenzoyl (Abz) and 2,4-dinitrophenyl (Dnp) were used to determine the substrate specificity of the purified enzyme. The general sequences were Abz-XRFK(Dnp)-OH and Abz-RXFK(Dnp)-OH, in which X denotes the position of the altered amino acid. The assay mixture was comprised of the sample (1 μL), 100 mM Tris–HCl pH 8.0 (1 mL) and substrate in an increasing final concentration ranging from 0.25 to 20.00 μM. The activity for each substrate concentration was assayed for 120 s at 25 °C (λ_{EX} 320 nm and λ_{EM} 420 nm). The kinetic parameters K_m and V_{max} were calculated by non-linear regression data analysis using the program Grafit 5.0.0 [11]. The specificity of the peptide bond cleavage was monitored through a HPLC System (Shimadzu LC-10AD, Tokyo, Japan) using a C-18 column (5 μm, 4.6 × 150 mm).

3. Results and discussion

A single band protein of 27.5 kDa on the SDS–PAGE (Fig. 1A) and 23.9 kDa as the main peak in the mass spectrum (Fig. 1B) was purified from the pyloric caecum of tambaqui by using a four-step procedure: (1) heat treatment; (2) ammonium sulphate fractionation (30–60% fraction); (3) size exclusion chromatography and (4) affinity chromatography. The purification yield and specific activity (using BAPNA as substrate) were 30.0% and 2263 U mg^{-1} (approximately 370-fold higher than that of the crude extract), respectively. It was possible to obtain one gram of purified enzyme from five kg of pyloric caecum. Other trypsins from different fish species have similar molecular mass values, such as 24 kDa in *Gadus macrocephalus*, 24 kDa in *Eleginus gracilis* [14] and 23.2 kDa in *Balistes capriscus* [15].

The effect of different protease inhibitors on the activity of this enzyme is presented in Table 1. The enzyme was strongly inhibited by the trypsin inhibitors TLCK and benzamidine as well as the serine protease inhibitor PMSF. Similar results have been reported for the trypsin from other fish [16–20]. The chelating agent EDTA and the reducing agent β-mercaptoethanol slightly inhibited the tambaqui trypsin activity as also reported for other fish [17,21–23]. The inhibitors TPCK (chymotrypsin inhibitor), *O*-phenantroline (metallo-proteases inhibitor) and E-64 (cystein protease inhibitor) had no significant effect on the tambaqui trypsin, as reported in the liter-

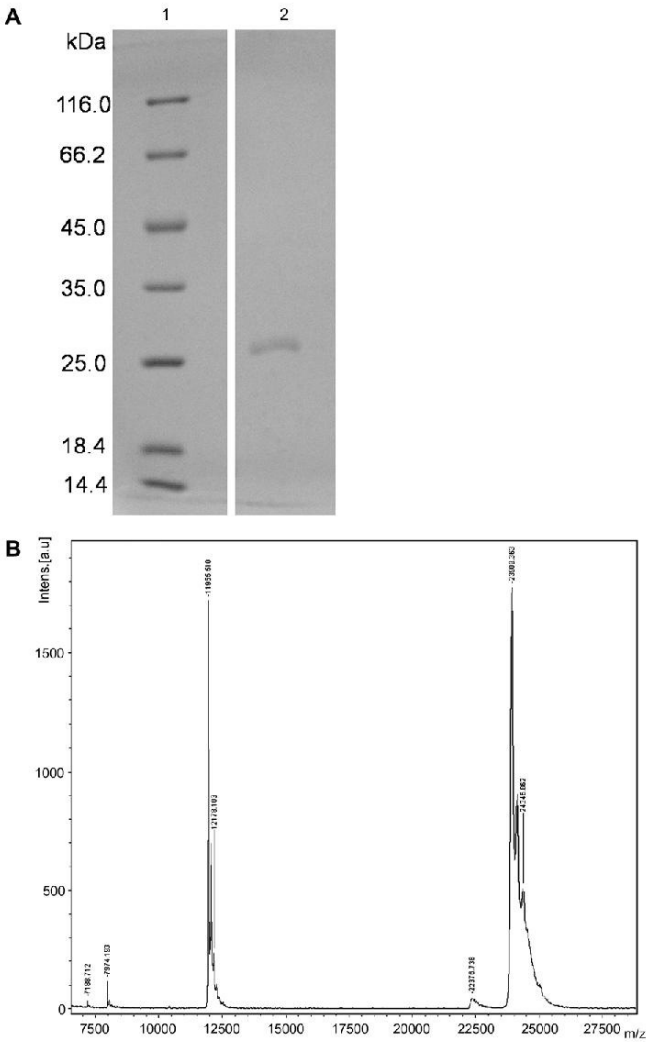

Fig. 1. Molecular mass of the purified trypsin from the tambaqui; SDS–PAGE of the purified trypsin from the tambaqui; Line 1 – pattern of standard proteins bands; Line 2 – final purification step (affinity chromatography), showing a single band of 27.5 kDa (A); mass spectrum from the purified enzyme was comprised of two main peaks – one with 24 kDa and the other with half this value (12 kDa) (B).

ature for other fish trypsin [16,19–23]. Due to the above features this tambaqui enzyme can be named as trypsin.

Twenty amino acid residues (IVGGYECKAHSQPHVSLNI) were identified in the NH_2-terminal sequence of the tambaqui trypsin (Fig. 2). For the numbering applied here, the NH_2-terminal isoleucine was considered amino acid number one. In comparison to other vertebrates trypsin, the tambaqui enzyme exhibited greater NH_2-terminal homology to the fish *Thunnus albacores* [22], *Katsuwonus pelamis* [20,24], *Sebastes schlegelii* [16] and *Sardinops melanostictus* [25]. The alignment in Fig. 2 indicates that the first seven NH_2-terminal amino acid residues (IVGGYEC) and the residues between positions 15 and 19 (QVSLN) are conserved in the trypsin of all vertebrates. However, in mammals, glutamic acid (E) in position 6 is replaced by a threonine (T) [26,27]. The conservation of the NH_2-terminal amino acid residues (isoleucine) is very important to trypsin activity, since it forms a salt bridge with the amino acid Asp-179 that promotes a molecular rearrangement, enabling the active conformation of the oxyanion hole in the trypsin [2]. An-

Table 1
Effect of synthetic inhibitors on the trypsin from the tambaqui using 10 nM z-FR-MCA and 0.6 mM BAPNA as substrate at 25 °C in 100 mM Tris–HCl pH 8.0. The results are represented by mean ± standard deviation.

Inhibitors				
Inhibitors	Concentration	Residual activity (%) BAPNA	Concentration	Residual activity (%) z-FR-MCA
TLCK	1 mM	0.00 ± 0.10	100 μM	10.04 ± 0.09
Benzamidine	1 mM	1.05 ± 1.24	NT*	NT*
PMSF	1 mM	14.55 ± 1.47	1 mM	27.20 ± 3.41
EDTA	NT*	NT*	10 mM	82.97 ± 4.31
β-Mercaptoethanol	1 mM	86.09 ± 1.41	NT*	NT*
TPCK	1 mM	96.29 ± 1.64	100 μM	113.58 ± 5.31
E-64	NT*	NT*	10 μM	102.99 ± 2.85
O-Fenantroline	NT*	NT*	10 μM	110.69 ± 9.18

*NT – Not tested.

Species	10 20	Reference
Colossoma macropomum	IVGGYECKAHSQPHQVSLNI	Present work
Thunnus albacores A	.....Q.....A	[22]
Katsuwonus pelamis A	.....Q.....S	[24]
Thunnus albacores B	.....Q.....P.....A	[22]
Katsuwonus pelamis B	.....Q.....P.....A	[20]
Katsuwonus pelamis C	.....Q.....P.....S	[20]
Sebastes schlegelii	.....PY.....S	[16]
Sardinops melanostictus	.....Y...W.....S	[25]
Gadus macrocephalus	.....TR...A.....S	[14]
Eleginus gracilis	.....PR...A.....S	[14]
Alcichthys alcicornis	.....TP...A.....S	[16]
Theragra chalcogramma	.....TK...A.....S	[17]
Pomatomus saltatrix	.....PK.A.V.....L	[23]
Pleuroprammus azonus	.....TP.T.A.....S	[25]
Balistes capriscus	.....TPN.T	[15]
Sardina pilchardus	.....QKY..	[18]
Porcine	.....T.A.N.V.Y.....S	[26]
Bos taurus	.....T.AEN.V.Y.....A	[27]
Homo sapiens	.....T.EEN.V.Y.....S	[26]

Fig. 2. Alignment of the NH_2-terminal amino acid sequence of the trypsin from the tambaqui (*Colossoma macropomum*) compared to trypsin sequences from other vertebrates. The dots represent the amino acid residues that are identical to the query sequence (tambaqui trypsin) and letters indicate the different residues.

Table 2
Kinetic parameters from the hydrolysis of two series of synthetic fluorogenic peptide substrates by trypsin from the tambaqui – Abz-RXFK-EDDnp (X represents P1′) and Abz-XRFK-EDDnp (X represents P2); Activities were assayed for 120 s at 25 °C in 100 mM Tris–HCl pH 8.0 with a final substrate concentration ranging from 0.25 to 20.00 μM.

Abz-R↓XFK-Eddnp				Abz-XR↓FK-Eddnp			
Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
RGFK	9.15	15.94	0.57	GRFK	31.94	16.30	1.960
RVFK	4.59	18.31	0.25	VRFK	46.57	2.87	16.22
RPFK	0.10	3.10	0.03	FRFK	8.87	0.53	16.68
RSFK	16.63	1.03	16.30	LRFK	53.23	1.01	52.60
RYFK	10.65	1.66	6.40	YRFK	27.72	5.57	4.98
RNFK	16.63	4.93	3.38	NRFK	30.61	7.44	4.12
RQFK	13.31	7.38	1.80	QRFK	12.64	8.93	1.42
RDFK	2.33	8.08	0.29	WRFK	6.32	3.26	1.94
REFK	7.49	12.89	0.58	ERFK	17.30	6.86	2.52
RRFK	26.61	1.69	16.46	KRFK	53.23	0.58	91.58
RHFK	8.32	1.85	4.49	HRFK	21.07	6.76	3.11
RTFK	18.30	3.25	5.63				

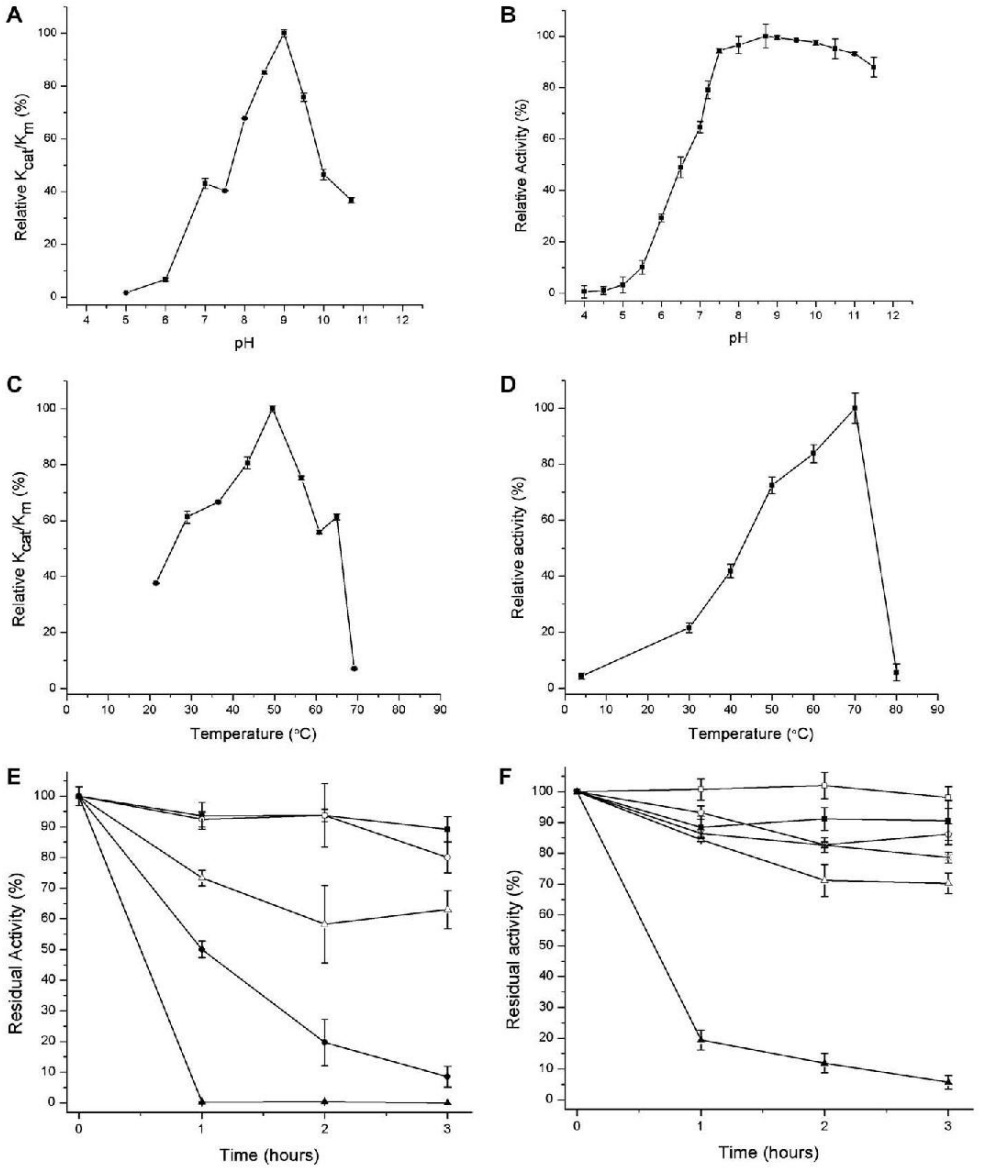

Fig. 3. Physicochemical characterization of the trypsin from the tambaqui using z-FR-MCA and BAPNA as substrates: Effect of pH on second-order kinetic parameters (k_{cat}/K_m) using z-FR-MCA as substrate (A); effect of pH on activity using BAPNA as substrate (B); effect of temperature on second-order kinetic parameters (k_{cat}/K_m) using z-FR-MCA as substrate (C); effect of temperature on activity using BAPNA as substrate (D); thermal stability at temperature of 40 °C (-■-), 55 °C (-◇-), 60 °C (-△-), 65 °C (-●-), 70 °C (-▲-) for 3 h (E); activity measured at 25 °C using z-FR-MCA as substrate; thermal stability at temperatures of 4 °C (-□-), 30 °C (-○-), 40 °C (-■-), 50 °C (-✳-), 60 °C (-△-), 70 °C (-▲-) for 3 h (F); activity measured at 25 °C using BAPNA as substrate.

other important feature for proteins structures is the disulfide bonds. There may be up to six bonds in the trypsin of vertebrates, one of which occurs between Cys-7 and Cys-142 [26]. The conservation of a cysteine residue in position 7 is an indicator of the possible existence of a similar bond in the enzyme purified from tambaqui.

A convenient tool for the study of peptidases specificity is FRET peptides substrate, as it allows monitoring of the reaction on a continuous basis, providing a rapid method for the determination of enzymatic activity [28]. Table 2 displays the kinetic parameters: Michaelis constant (K_m), catalytic constant (k_{cat}) and catalytic efficiency (k_{cat}/K_m) for the hydrolysis of two series of synthetic fluorogenic tetrapeptides (Abz-R↓XFK-Eddnp and Abz-XR↓FK-Eddnp). These substrates were used to determine the cleavage preferences of the tambaqui enzyme for positions P1′ and P2 (nomenclature according to Schechter and Berger [29]). Tambaqui trypsin preferably hydrolyzed substrates presenting a lysine (K) or an arginine (R) at P1. In the present study, the HPLC analysis (data not shown) confirmed that the tambaqui enzyme cleaved only peptide bonds on the carboxyl site of arginine (R) and occasionally of lysine (K), when it was available. The tambaqui enzyme exhibited greater efficiency at hydrolyzing substrates with serine (S) and arginine (R) at P1′. Lesser affinities were found for glycine (G), valine (V), glutamine (Q), aspartic acid (D), glutamic acid (E) and proline (P) at P1′. One of the most interesting results was that the enzyme was able to hydrolyze the substrate with proline (P) at P1′. The turnover value for proline (P) at this position was low, but so was the K_m, which indicates that this enzyme is able to bind to this substrate, but does not cleave it in a rapid or efficient fashion. Regarding the P2 position, the tambaqui enzyme exhibited a preference for leucine (L) and lysine (K), whereas trypsin from rats [30] and cockroaches [31] exhibit very low affinities for these residues at this same position. The tambaqui trypsin also exhibited low affinity for the substrates with glycine (G), glutamine (Q), tryptophan (W) and glutamic acid (E) at position P2.

In the assay using the fluorogenic substrate (z-FR-MCA), the purified enzyme was highly active in the pH range from 8.0 to 9.5, with the greatest activity at pH 9.0 (Fig. 3A). Using the chromogenic substrate (BAPNA), the enzyme was more active in the pH range from 7.5 to 11.5 (Fig. 3B). Using both substrates, enzyme activity at pH 6.0 was approximately 70% lower. A possible explanation for this is that the activity of trypsin-like enzymes is dependent on an unprotonated histidine in their active site, which presents pK_R value around 6.0 [32]. On the other hand, a high pH value also reduces the catalytic activity by promoting the deprotonation of the NH_2-terminal isoleucine, thereby disrupting the active center conformation [32]. However, when BAPNA was used the catalytic activity of the enzyme was not significantly reduced with the increase in pH value. Similar results have been seen for other fish when this same substrate was used [15,23,32].

When z-FR-MCA was employed the tambaqui enzyme was highly active at temperatures from 30 to 65 °C, with the greatest activity at 50 °C (Fig. 3C). This is the same trypsin optimal temperature found in a number of temperate fish, such as *Sardinops sagax caerulea* [21], *Alcichthys alcicornis* [16], *G. macrocephalus* and *Eleginus gracilis* [14]. However, when BAPNA was used (Fig. 3D), the greatest activity occurred at 70 °C. It is possible that the substrate itself provided thermostability to the enzyme when this same substrate was used as trypsin from other fish have also exhibited optimal activity at high temperatures, such as 60 °C for *Katsuwonus pelamis* [24] and 60 °C for *Oncorhynchus tshawytscha* [33].

The thermal stability of the tambaqui enzyme was similar when assayed with z-FR-MCA (Fig. 3E) and BAPNA (Fig. 3F). This result corroborates the hypothesis that the substrate BAPNA may help to stabilize the trypsin activity in higher temperatures. The enzyme maintained more than 60% of its initial activity after 3 h at 60 °C. However, the activity was reduced to 50% after 1 h at 65 °C and was completely lost after 3 h at 70 °C, possibly due to enzyme denaturation. Likewise, trypsin from other tropical fish such as *Thunnus albacores* [22] and *Katsuwonus pelamis* [24,20] are very stable at temperatures up to 60 °C, but rather unstable at temperatures higher than 70 °C. On the other hand, trypsin from the subtropical fish *S. Pilchardus* [18], *S. caerulea* [21] and *B. capriscus* [15] as well as the temperate fish *S. schlegelii*, *A. alcicornis* [16] and *G. macrocephalus* [14] is stable at temperatures below 40 °C, but loses more than 80% of its activity at temperatures higher than 60 °C.

Kishimura et al. [17] found a direct correlation between the temperature of the fish habitat and the thermal stability of trypsin. According to Gudmundsdóttir and Pálsdóttir [34], trypsins from fish adapted to cold environments have greater catalytic efficiency and lesser thermal stability due to their molecular flexibility. On the other hand, trypsins from tropical fish are more stable due to the lesser surface hydrophilicity and stronger hydrophobic interactions in the protein center [20,24]. The stability at higher temperatures of the tambaqui enzyme may be related to thermal selection performed by the heat treatment step carried out during the purification process. This is an interesting characteristic for the detergent industry, since this type of application requires enzymes with a wide range of thermal stability for long periods [6,7,35].

It is worthwhile to register that proteases extracted from the pyloric caeca and intestines of *C. macropomum* have been already proposed as a source of alkaline proteases for laundry detergent [6]. These proteolytic enzymes remained stable in the presence of non-ionic (Tween 20 and Tween 80) and ionic surfactants (saponin and sodium choleate). They also revealed high resistance (60% residual activity) when incubated with 10% H_2O_2 for 75 min. Furthermore, the preparation retained approximately 80% of its proteolytic activity after incubation for 1 h at 40 °C with commercial detergent. Further biotechnological applications can be proposed for this trypsin such as in food industry.

4. Conclusions

The enzyme purified from tambaqui in the present study had molecular weight of 23.9 kDa, was inhibited by TLCK, benzamidine and PMSF, exhibited a conserved trypsin NH_2-terminal amino acid sequence and showed specificity to substrates with arginine at P1. Moreover, this enzyme presented stability at temperatures up to 60 °C and in a broad pH range. Considering these results, the enzyme from *C. macropomum* may be classified as trypsin. This source of trypsin may also be an attractive alternative for the detergent and food industry.

Acknowledgments

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Anexo A – Material suplementar to capítulo 3

Supplementary Figure 1. Plot of the RMSD calculated for the molecular dynamics repetitions. (A) tambaqui trypsin model, based on PDB structure entry 1HJ8; (B) salmon trypsin with PDB structure entry 1BIT (C) porcine trypsin with PDB structure entry 1S81.

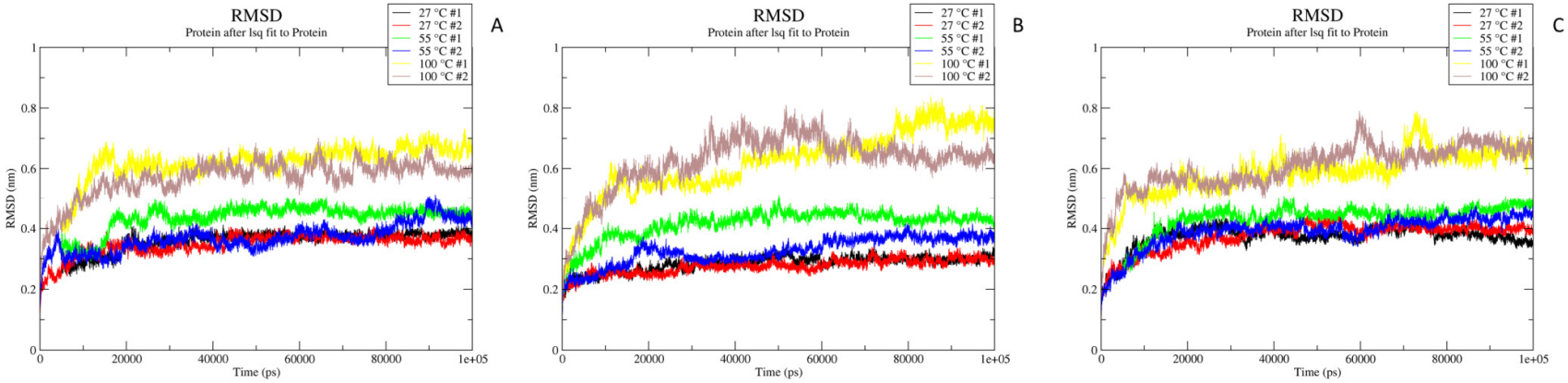
Supplementary Figure 2. RMS fluctuation calculated for each molecular dynamic from 80 to 100 ns. (A) tambaqui (B) salmon (C) porcine.

Supplementary Figure 3 Plot of the secondary structure variation from all trypsins throughout the 100ns MD. (A) Tambaqui trypsin at 27 °C; (B) Tambaqui trypsin at 55 °C; (C) Tambaqui trypsin at 100 °C; (D) Salmon trypsin at 27 °C; (E) Salmon trypsin at 55 °C; (F) Salmon trypsin at 100 °C; (G) Porcine trypsin at 27°C; (H) Porcine trypsin at 55°C; (I) Porcine trypsin at 100°C.

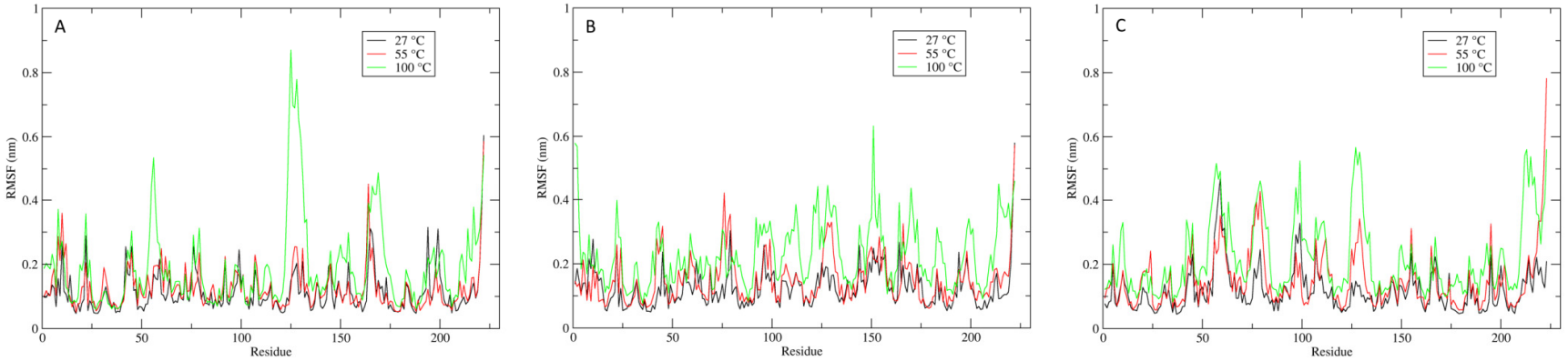
Supplementary Figure 4. Procheck analysis of the structures used in the present work. Plots were made at swiss model Workspace. (A) tambaqui trypsin model, based on PDB structure entry 1HJ8; (B) Salmon trypsin with PDB structure entry 1BIT (C) porcine trypsin with PDB structure entry 1S81.

Supplementary Figure 5. Alignment of all sequences used in the analysis. (A) fish anionic trypsin genes; (B) fish cationic trypsin genes; (C) homeotherm anionic trypsins genes; (D) homeotherm cationic trypsins genes.

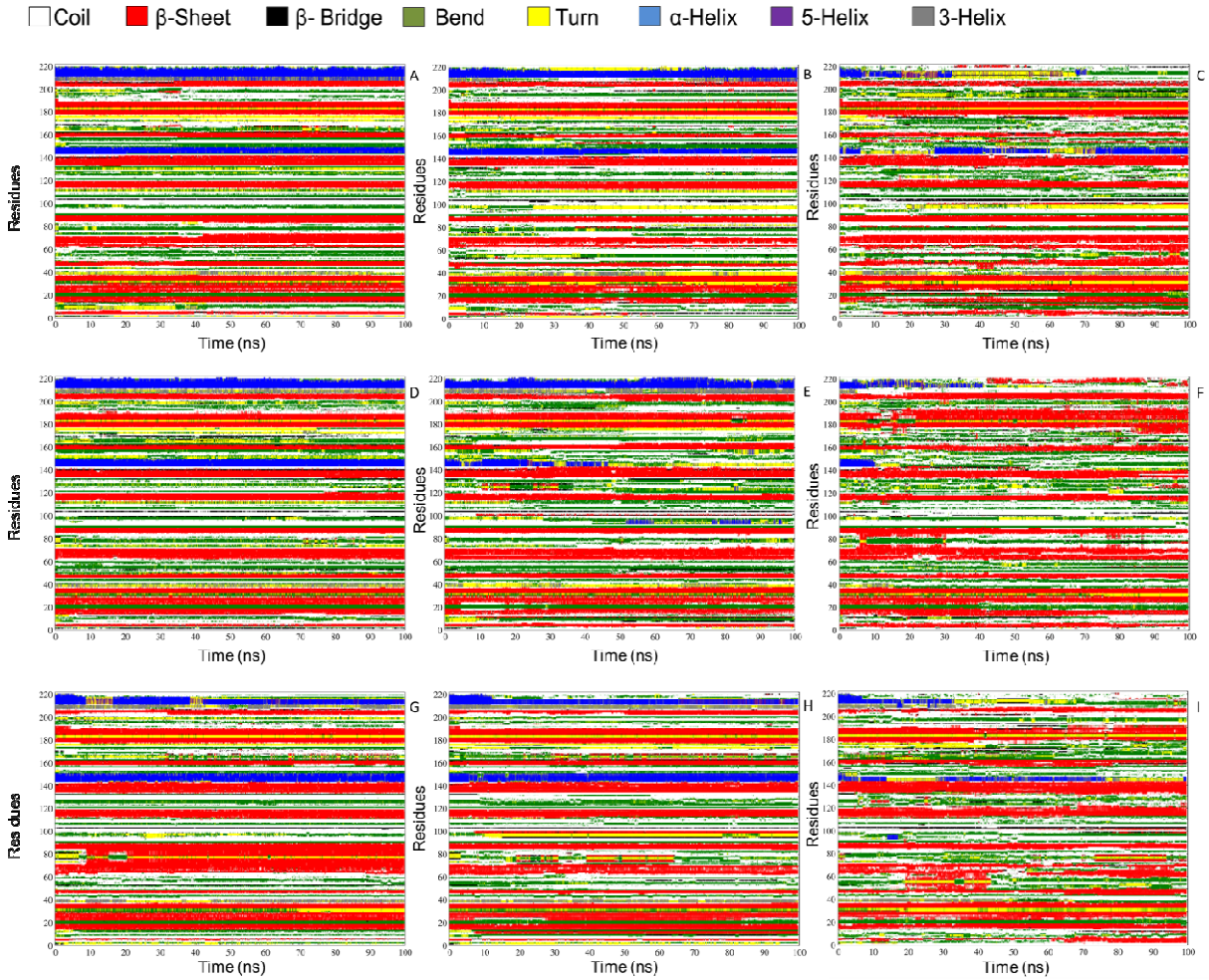
Supplementary Figure 1



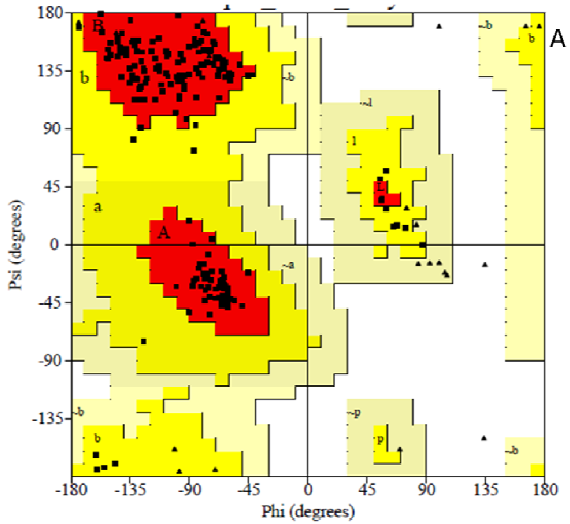
Supplementary Figure 2



Supplementary Figure 3



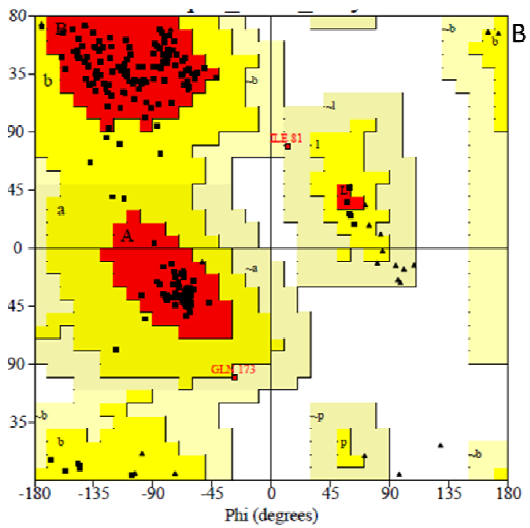
Supplementary Figure 4



Plot statistics

Residues in most favoured regions [A,B,L]	170	90.9%
Residues in additional allowed regions [a,b,l,p]	17	9.1%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	187	100.0%
Number of end-residues (excl. Gly and Pro)	3	
Number of glycine residues (shown as triangles)	20	
Number of proline residues	12	
Total number of residues	222	

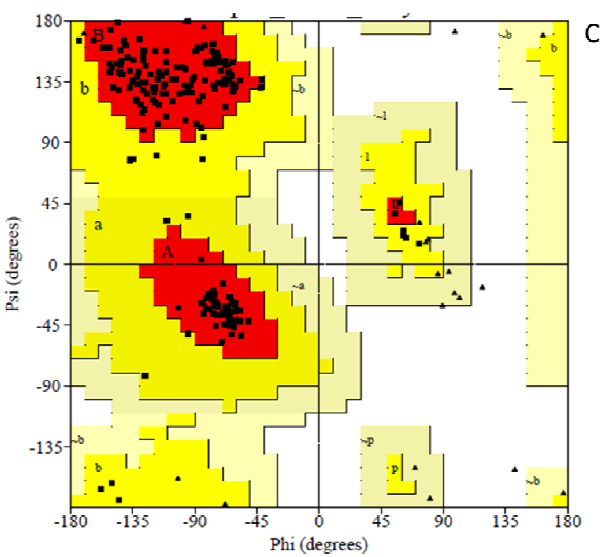
Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.



Plot statistics

Residues in most favoured regions [A,B,L]	164	88.6%
Residues in additional allowed regions [a,b,l,p]	19	10.3%
Residues in generously allowed regions [~a,~b,~l,~p]	2	1.1%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	185	100.0%
Number of end-residues (excl. Gly and Pro)	3	
Number of glycine residues (shown as triangles)	23	
Number of proline residues	10	
Total number of residues	221	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

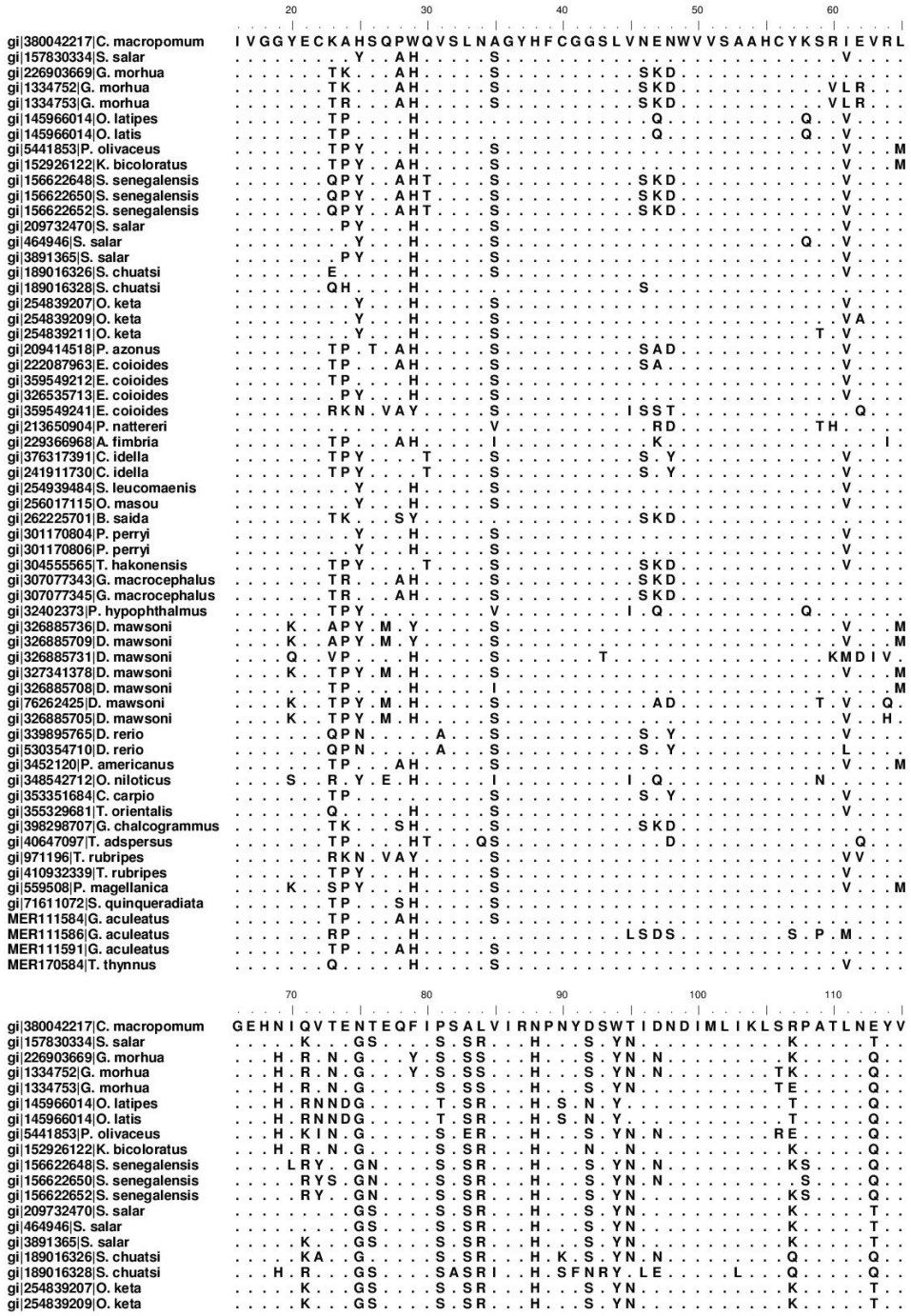


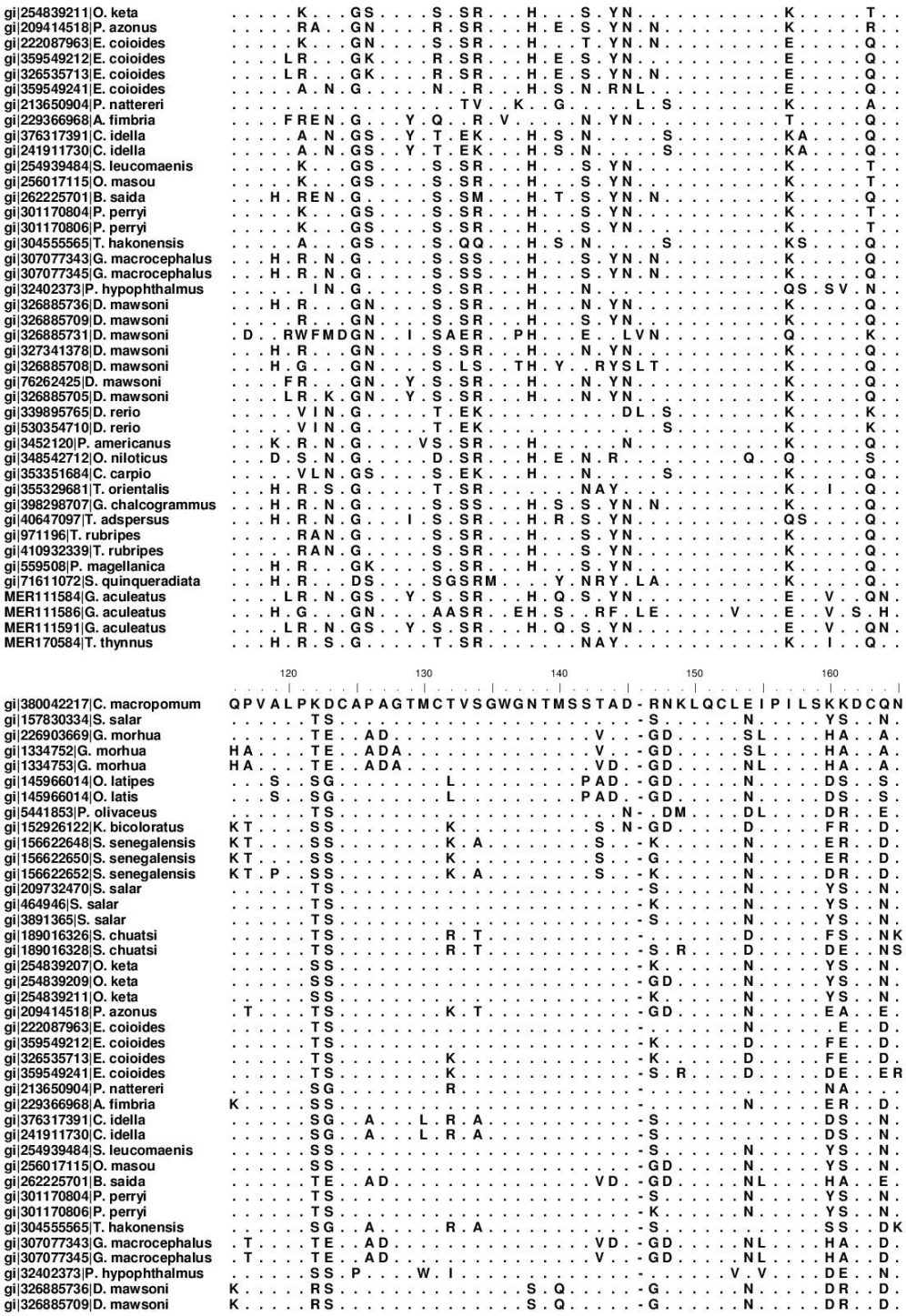
Plot statistics

Residues in most favoured regions [A,B,L]	170	90.9%
Residues in additional allowed regions [a,b,l,p]	17	9.1%
Residues in generously allowed regions [~a,~b,~l,~p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	187	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	25	
Number of proline residues	9	
Total number of residues	223	

Based on an analysis of 118 structures of resolution of at least 2.0 Angstroms and R-factor no greater than 20%, a good quality model would be expected to have over 90% in the most favoured regions.

Supplementary Figure 5A

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gi|359549241|E. coioides
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gi|229366968|A. fimbria
gi|376317391|C. idella
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gi|254939484|S. leucomaenis
gi|256017115|O. masou
gi|262225701|B. saida
gi|301170804|P. perryi
gi|301170806|P. perryi
gi|304555565|T. hakonensis
gi|307077343|G. macrocephalus
gi|307077345|G. macrocephalus
gi|32402373|P. hypophthalmus
gi|326885736|D. mawsoni
gi|326885709|D. mawsoni
gi|326885731|D. mawsoni
gi|327341378|D. mawsoni
gi|326885708|D. mawsoni
gi|76262425|D. mawsoni
gi|326885705|D. mawsoni
gi|339895765|D. rerio
gi|530354710|D. rerio
gi|3452120|P. americanus
gi|348542712|O. niloticus
gi|353351684|C. carpio
gi|355329681|T. orientalis
gi|398298707|G. chalcogrammus
gi|40647097|T. adspersus
gi|971196|T. rubripes
gi|410932339|T. rubripes
gi|559508|P. magellanica
gi|71611072|S. quinqueradiata
MER111584|G. aculeatus
MER111586|G. aculeatus
MER111591|G. aculeatus
MER170584|T. thynnus
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150
160
gi|380042217|C. macropomum
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gi|226903669|G. morhua
gi|1334752|G. morhua
gi|1334753|G. morhua
gi|145966014|O. latipes
gi|145966014|O. latis
gi|5441853|P. olivaceus
gi|152926122|K. bicoloratus
gi|156622648|S. senegalensis
gi|156622650|S. senegalensis
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gi|464946|S. salar
gi|3891365|S. salar
gi|189016326|S. chuatsi
gi|189016328|S. chuatsi
gi|254839207|O. keta
gi|254839209|O. keta
gi|254839211|O. keta
gi|209414518|P. azonus
gi|222087963|E. coioides
gi|359549212|E. coioides
gi|326535713|E. coioides
gi|359549241|E. coioides
gi|213650904|P. nattereri
gi|229366968|A. fimbria
gi|376317391|C. idella
gi|241911730|C. idella
gi|254939484|S. leucomaenis
gi|256017115|O. masou
gi|262225701|B. saida
gi|301170804|P. perryi
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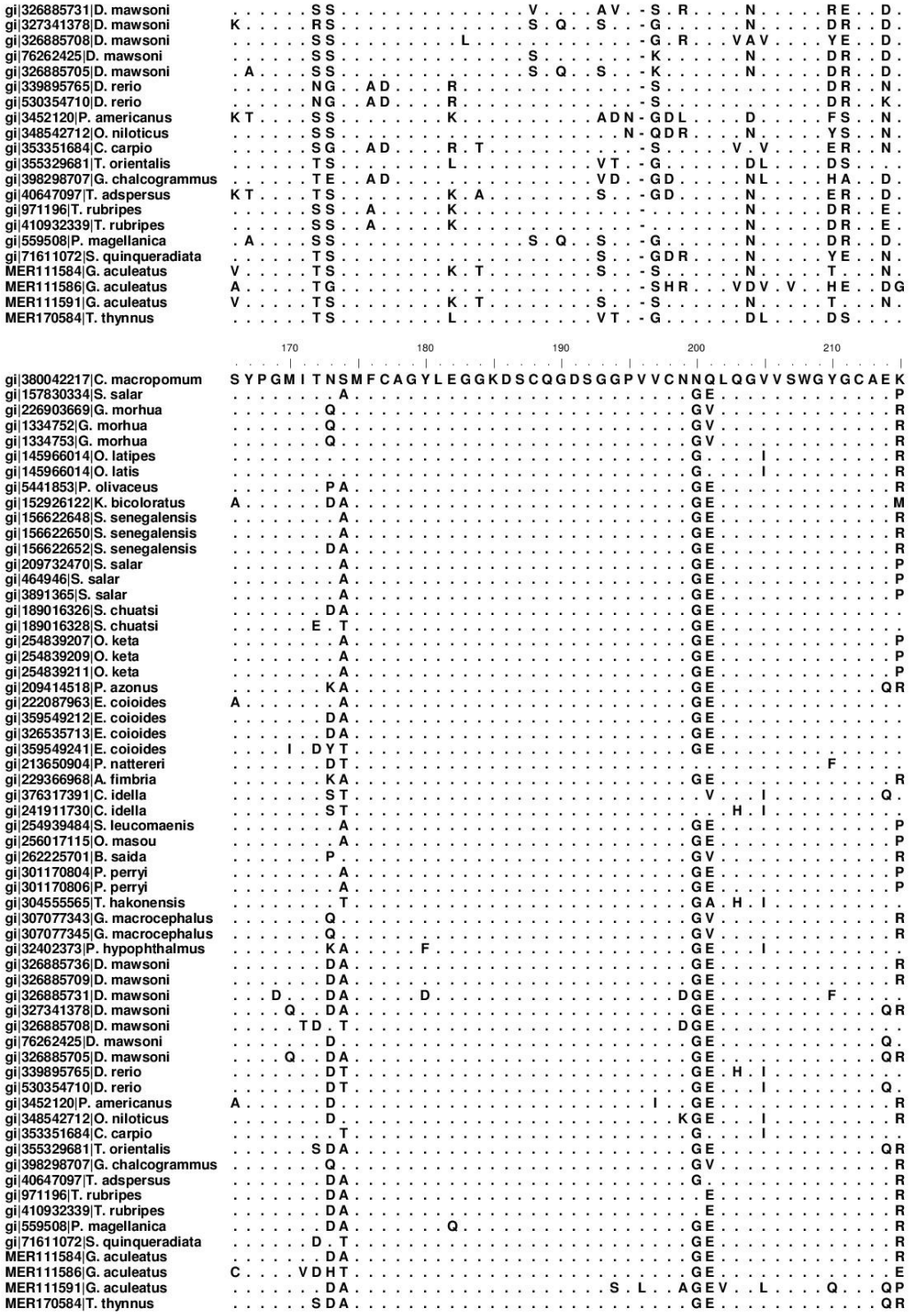
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gi|559508|P. magellanica
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MER111591|G. aculeatus
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gi|189016328|S. chuatsi
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gi|254839211|O. keta
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gi|326535713|E. coioides
gi|359549241|E. coioides
gi|213650904|P. nattereri
gi|229366968|A. fimbria
gi|376317391|C. idella
gi|241911730|C. idella
gi|254939484|S. leucomaenis
gi|256017115|O. masou
gi|262225701|B. saida
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gi|301170806|P. perryi
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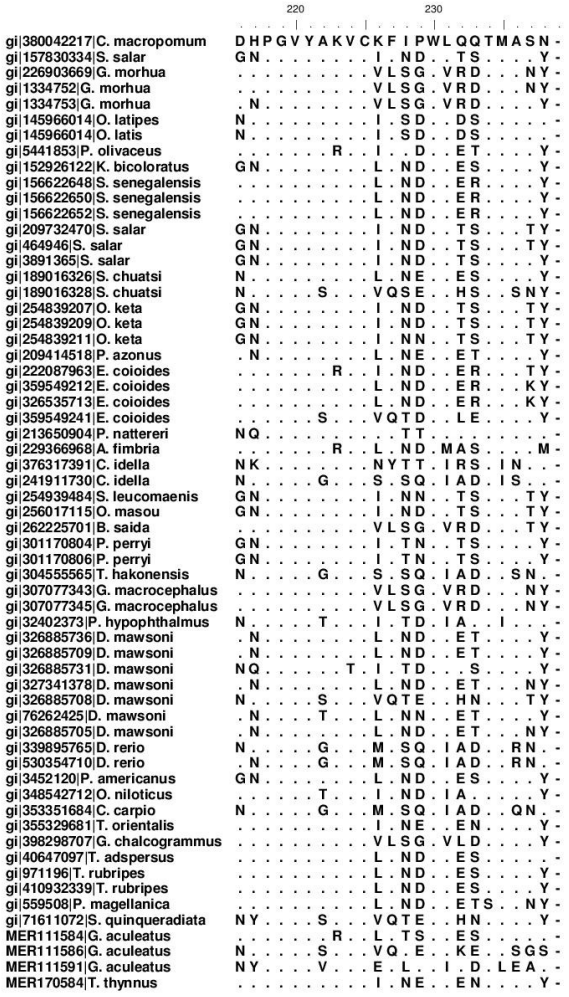
MER170584|T. thynnus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gi|359549212|E. coioides . . . . . L . N D . . E R . . . K Y -
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gi|213650904|P. nattereri N Q . . . . . T T . . . . . -
gi|229366968|A. fimbria . . . . . R . . L . N D . M A S . . . . M -
gi|376317391|C. idella N K . . . . . N Y T T . I R S . I N . . -
gi|241911730|C. idella N . . . . . G . . . S . S Q . I A D . I S . . -
gi|254939484|S. leucomaenis G N . . . . . I . N N . . T S . . . T Y -
gi|256017115|O. masou G N . . . . . I . N D . . T S . . . T Y -
gi|262225701|B. saida . . . . . V L S G . V R D . . . T Y -
gi|301170804|P. perryi G N . . . . . I . T N . . T S . . . . Y -
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gi|353351684|C. carpio N . . . . . G . . . M . S Q . I A D . . Q N . -
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gi|398298707|G. chalcogrammus . . . . . V L S G . V L D . . . . Y -
gi|40647097|T. adspersus . . . . . L . N D . . E S . . . . . -
gi|971196|T. rubripes . . . . . L . N D . . E S . . . . Y -
gi|410932339|T. rubripes . . . . . L . N D . . E S . . . . Y -
gi|559508|P. magellanica . . . . . L . N D . . E T S . . N Y -
gi|71611072|S. quinqueradiata N Y . . . . S . . . V Q T E . . H N . . . . Y -
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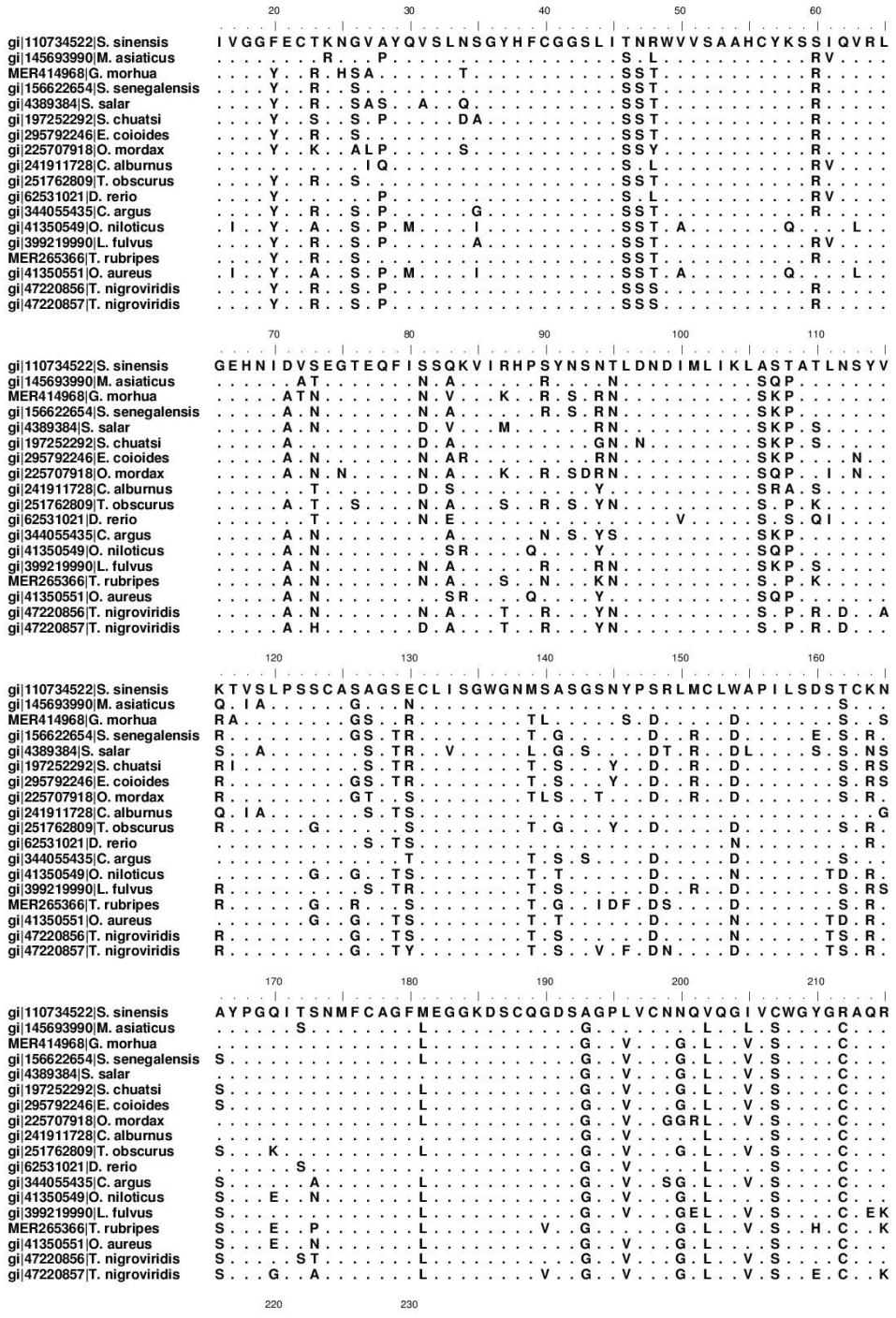
MER111586|G. aculeatus N . . . . . S . . . V Q . E . . K E . . S G S -
MER111591|G. aculeatus N Y . . . . V . . . E . L . . I . D . L E A . -
MER170584|T. thynnus . . . . . I . N E . . E N . . . . Y -

Supplementary Figure 5B

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gi|197252292|S. chuatsi . . . . . T . . . . . S . . . N . M S . . -
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gi|225707918|O. mordax . . . . . V S . . Q S . M S . . -
gi|241911728|C. alburnus . Q . . . . . F T T . . . N . M S . . -
gi|251762809|T. obscurus . . . . . T . . . . F . S . . . . . M A . . -
gi|62531021|D. rerio . . . . . F T T . . . N . M . . . -
gi|344055435|C. argus . . . . . T . . . . . T . . . N . M S . . -
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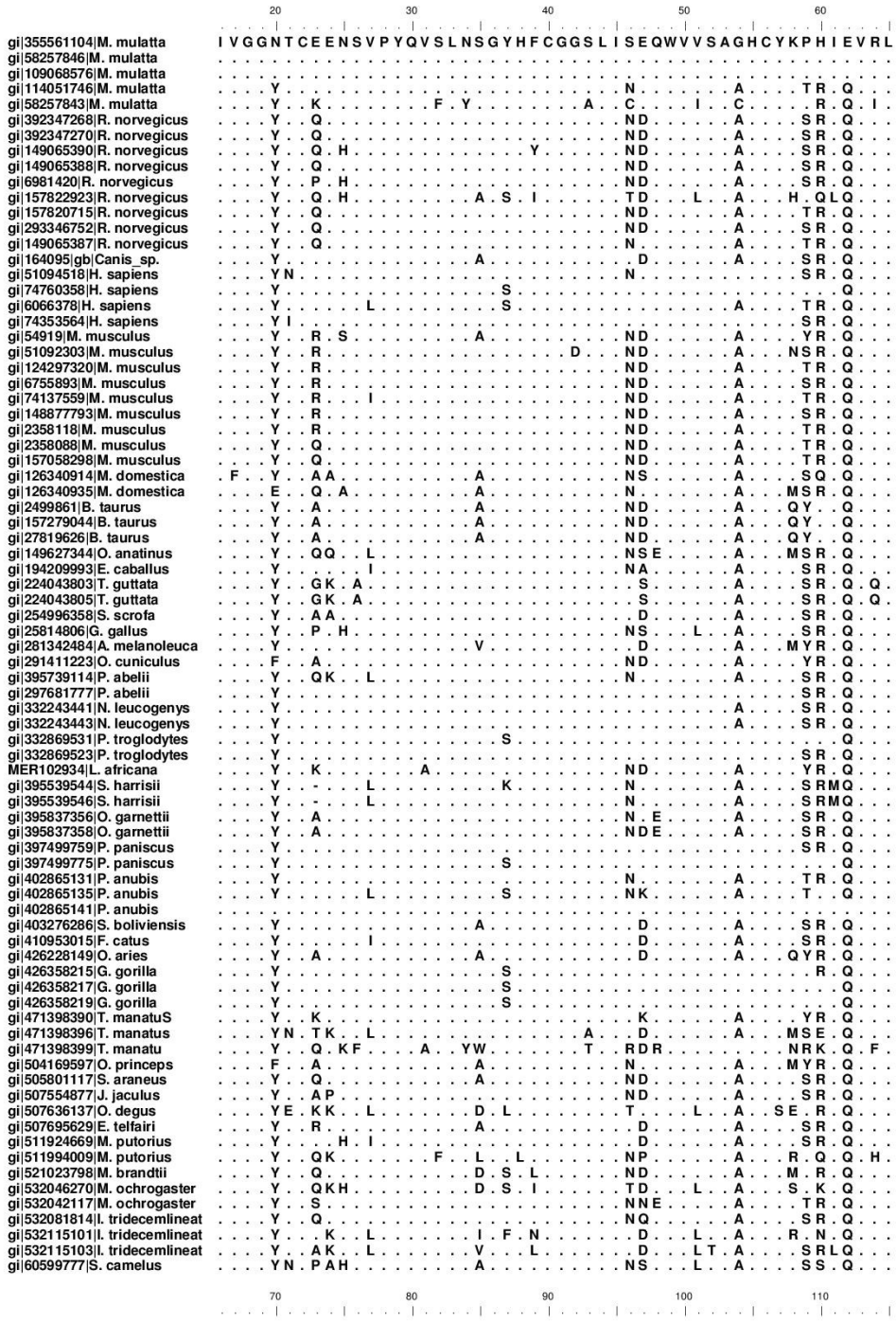
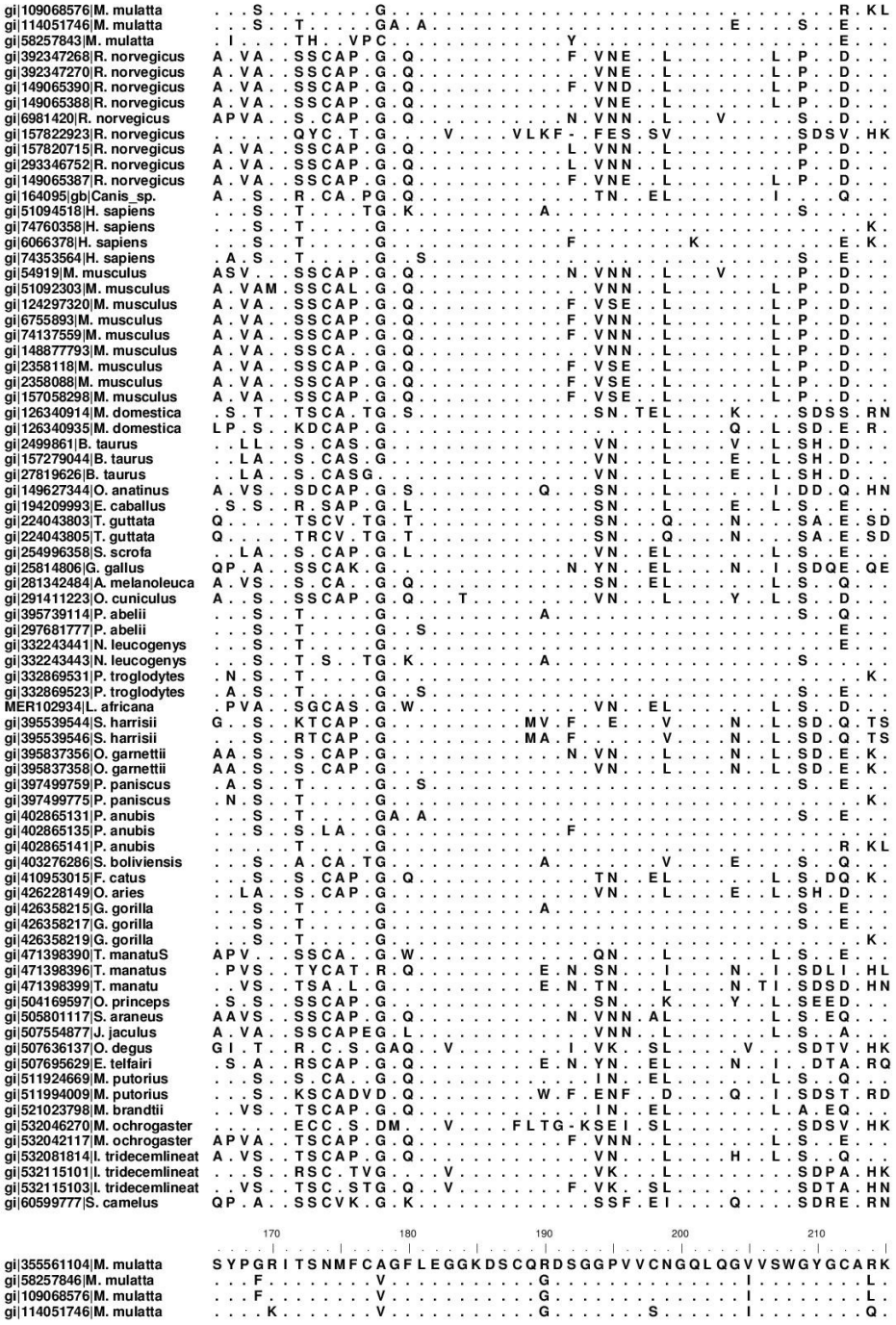
Supplementary Figure 5C

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gi|6981420|R. norvegicus
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gi|293346752|R. norvegicus
gi|149065387|R. norvegicus
gi|164095|gb|Canis_sp.
gi|51094518|H. sapiens
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gi|157058298|M. musculus
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gi|426358215|G. gorilla
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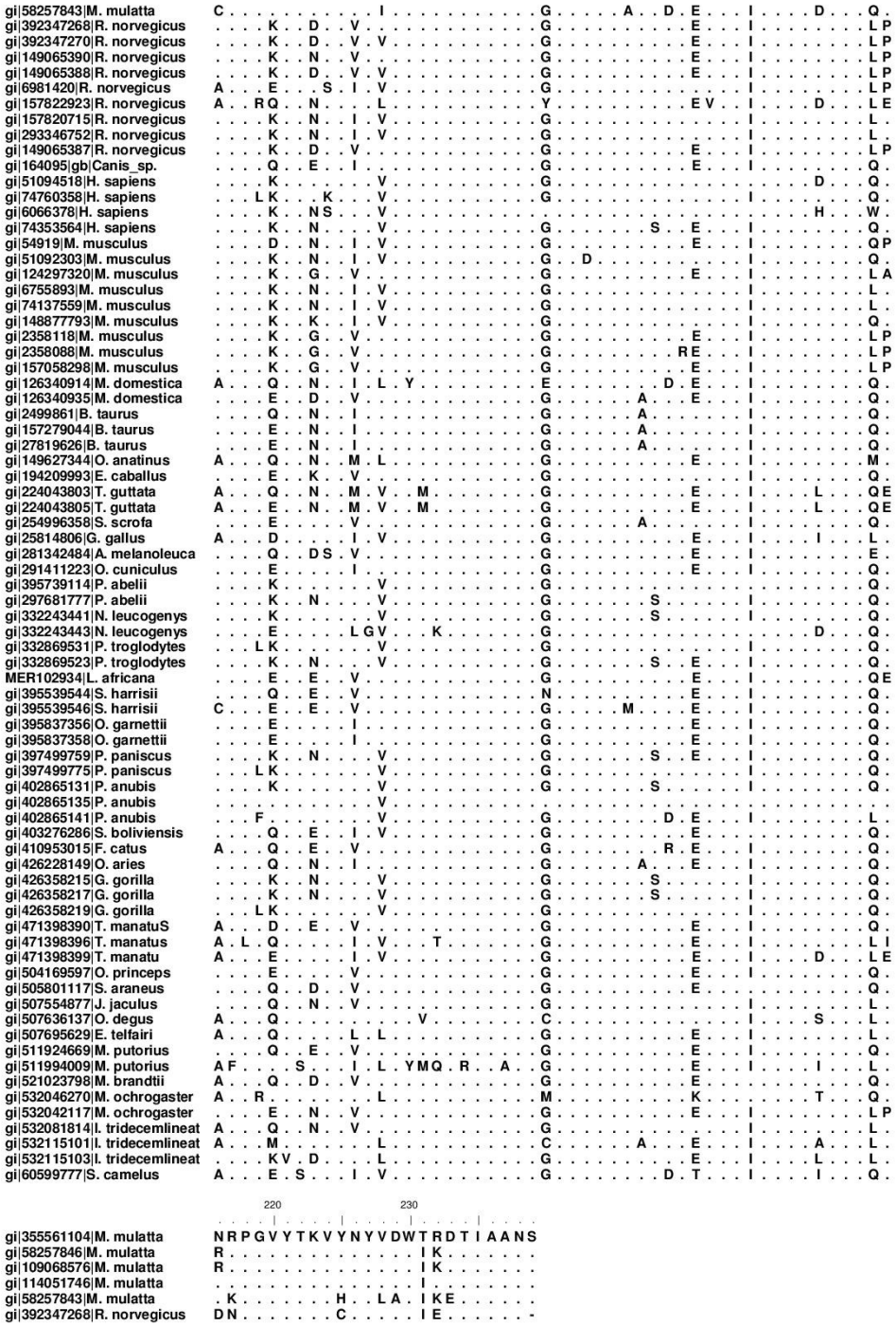
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gi|471398399|T. manatu
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gi|505801117|S. araneus
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gi|60599777|S. camelus
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230
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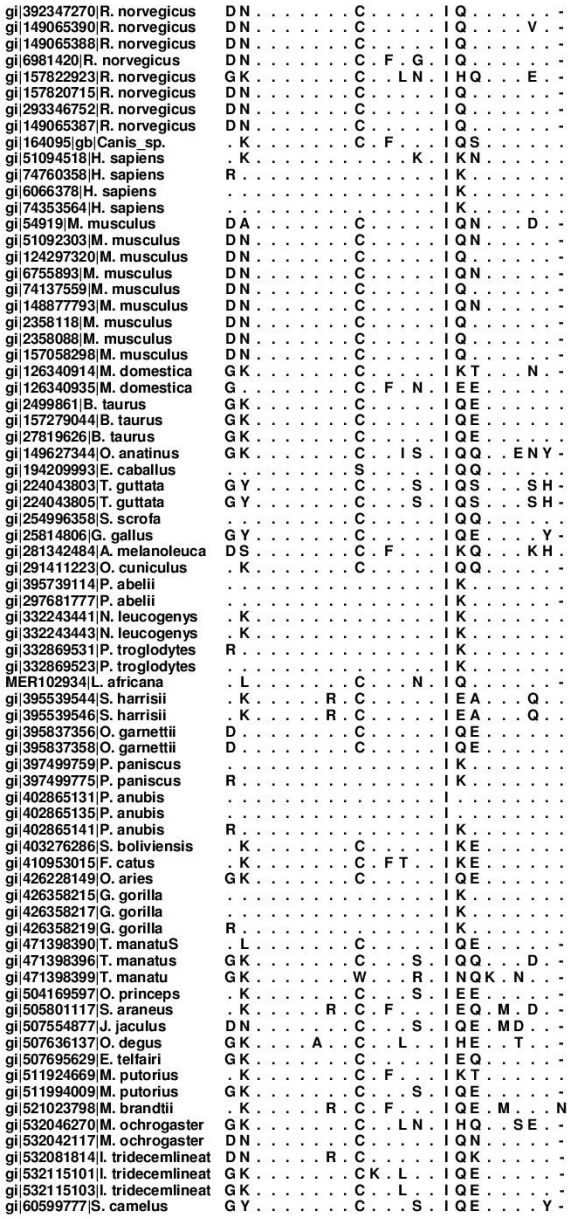
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gi|157820715|R. norvegicus DN.....C.....IQ.....-
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gi|164095|gb|Canis_sp. .K.....C.F...IQS.....
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gi|74760358|H. sapiens R.....IK.....
gi|6066378|H. sapiens .....IK.....
gi|74353564|H. sapiens .....IK.....
gi|54919|M. musculus DA.....C.....IQN...D.-
gi|51092303|M. musculus DN.....C.....IQN.....-
gi|124297320|M. musculus DN.....C.....IQ.....-
gi|6755893|M. musculus DN.....C.....IQN.....-
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gi|148877793|M. musculus DN.....C.....IQN.....-
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gi|126340935|M. domestica G.....C.F.N.IEE.....
gi|2499861|B. taurus GK.....C.....IQE.....
gi|157279044|B. taurus GK.....C.....IQE.....
gi|27819626|B. taurus GK.....C.....IQE.....
gi|149627344|O. anatinus GK.....C..IS.IQQ..ENY-
gi|194209993|E. caballus .....S.....IQQ.....
gi|224043803|T. guttata GY.....C...S.IQS...SH-
gi|224043805|T. guttata GY.....C...S.IQS...SH-
gi|254996358|S. scrofa .....C.....IQQ.....
gi|25814806|G. gallus GY.....C.....IQE....Y-
gi|281342484|A. melanoleuca DS.....C.F...IKQ...KH.
gi|291411223|O. cuniculus .K.....C.....IQQ.....-
gi|395739114|P. abelii .....IK.....
gi|297681777|P. abelii .....IK.....-
gi|332243441|N. leucogenys .K.....IK.....
gi|332243443|N. leucogenys .K.....IK.....
gi|332869531|P. troglodytes R.....IK.....
gi|332869523|P. troglodytes .....IK.....
MER102934|L. africana .L.....C...N.IQ.....-
gi|395539544|S. harrisii .K.....R.C.....IEA...Q..
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gi|395837356|O. garnettii D.....C.....IQE.....
gi|395837358|O. garnettii D.....C.....IQE.....
gi|397499759|P. paniscus .....IK.....
gi|397499775|P. paniscus R.....IK.....
gi|402865131|P. anubis .....I.....
gi|402865135|P. anubis .....I.....
gi|402865141|P. anubis R.....IK.....
gi|403276286|S. boliviensis .K.....C.....IKE.....
gi|410953015|F. catus .K.....C.FT..IKE.....
gi|426228149|O. aries GK.....C.....IQE.....
gi|426358215|G. gorilla .....IK.....
gi|426358217|G. gorilla .....IK.....
gi|426358219|G. gorilla R.....IK.....
gi|471398390|T. manatuS .L.....C.....IQE.....-
gi|471398396|T. manatus GK.....C...S.IQQ...D.-
gi|471398399|T. manatu GK.....W...R.INQK.N...-
gi|504169597|O. princeps .K.....C...S.IEE.....-
gi|505801117|S. araneus .K.....R.C.F...IEQ.M.D.-
gi|507554877|J. jaculus DN.....C...S.IQE.MD...-
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gi|532046270|M. ochrogaster GK.....C..LN.IHQ..SE..-
gi|532042117|M. ochrogaster DN.....C.....IQN.....-
gi|532081814|I. tridecemlineat DN.....R.C.....IQK.....-
gi|532115101|I. tridecemlineat GK.....CK.L..IQE.....-
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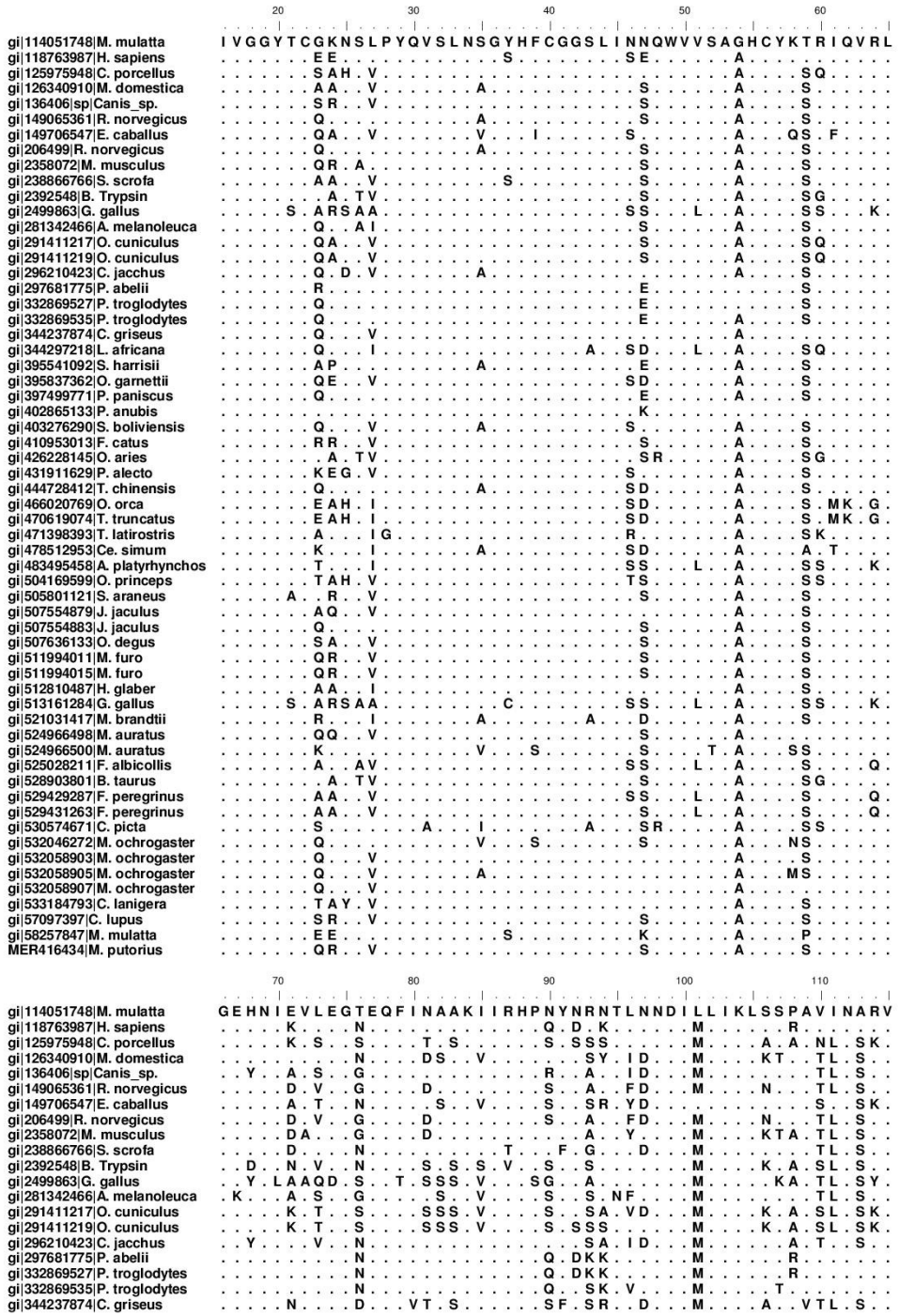
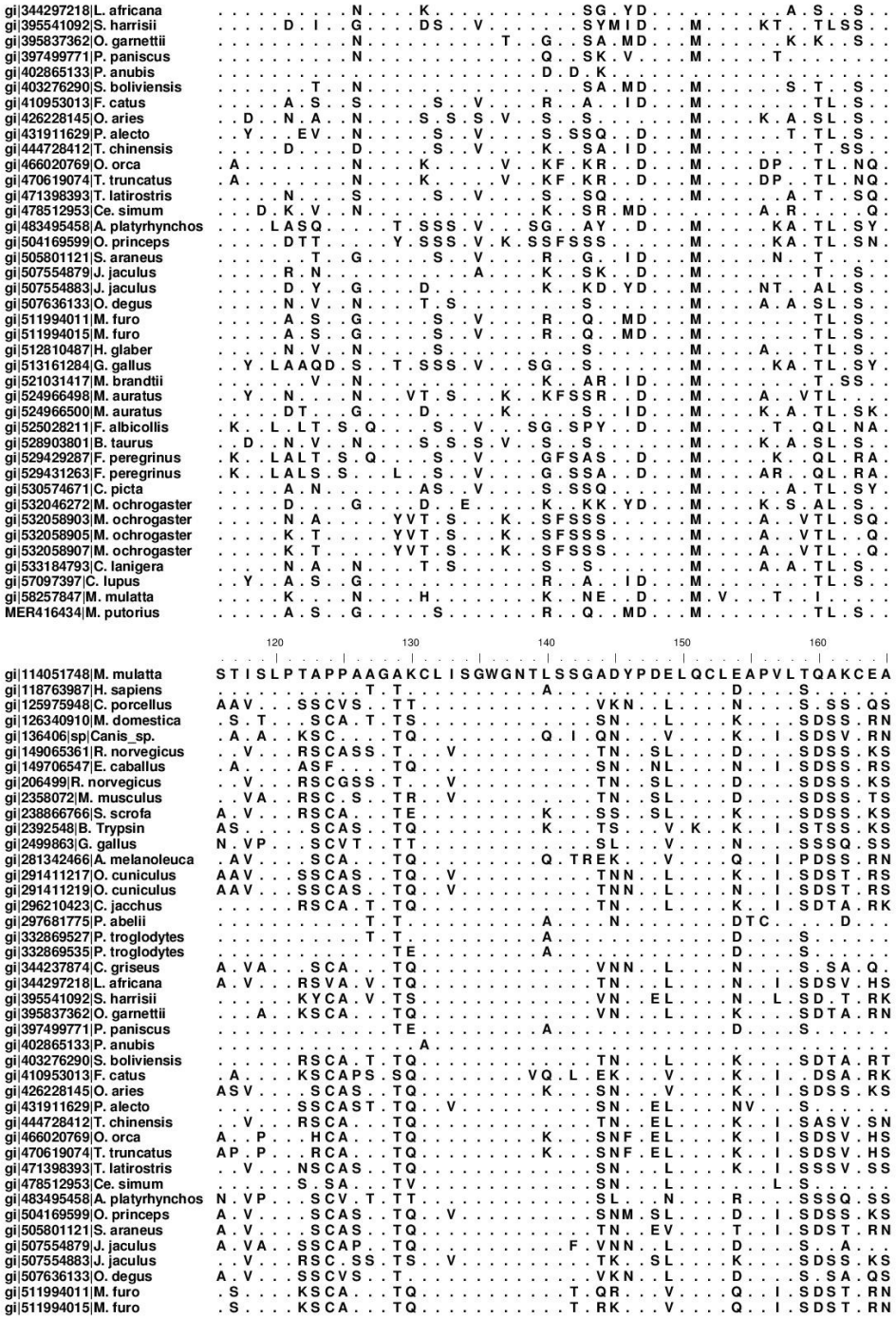
gi|60599777|S. camelus GY.....C...S.IQE....Y-

Supplementary Figure 5D

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gi|410953013|F. catus
gi|426228145|O. aries
gi|431911629|P. alecto
gi|444728412|T. chinensis
gi|466020769|O. orca
gi|470619074|T. truncatus
gi|471398393|T. latirostris
gi|478512953|Ce. simum
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gi|511994015|M. furo
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gi|57097397|C. lupus
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MER416434|M. putorius
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130
140
150
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gi|2392548|B. Trypsin
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gi|281342466|A. melanoleuca
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gi|291411219|O. cuniculus
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gi|332869535|P. troglodytes
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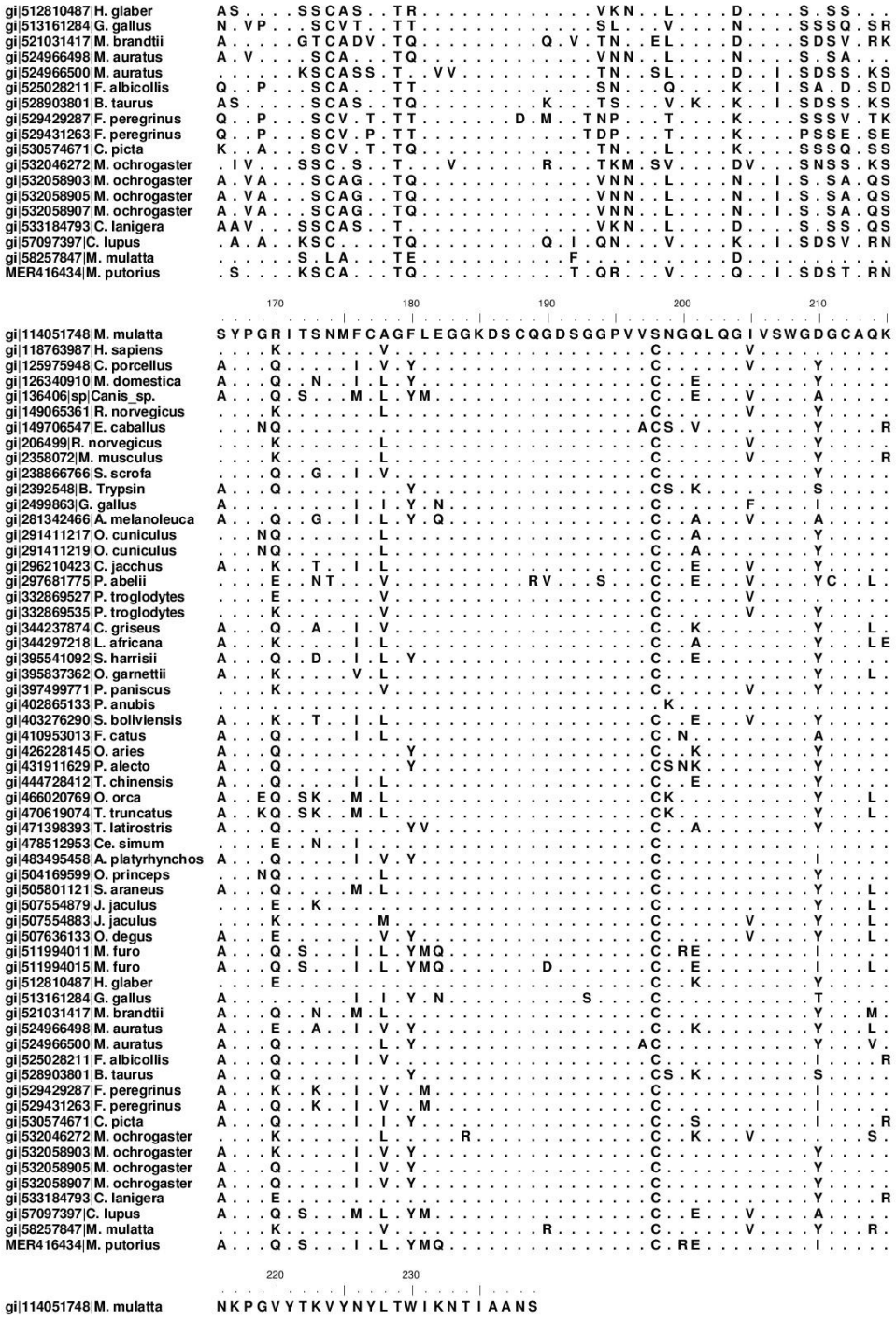
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gi|529429287|F. peregrinus
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gi|530574671|C. picta
gi|532046272|M. ochrogaster
gi|532058903|M. ochrogaster
gi|532058905|M. ochrogaster
gi|532058907|M. ochrogaster
gi|533184793|C. lanigera
gi|57097397|C. lupus
gi|58257847|M. mulatta
MER416434|M. putorius
170
180
190
200
210
gi|114051748|M. mulatta
gi|118763987|H. sapiens
gi|125975948|C. porcellus
gi|126340910|M. domestica
gi|136406|sp|Canis_sp.
gi|149065361|R. norvegicus
gi|149706547|E. caballus
gi|206499|R. norvegicus
gi|2358072|M. musculus
gi|238866766|S. scrofa
gi|2392548|B. Trypsin
gi|2499863|G. gallus
gi|281342466|A. melanoleuca
gi|291411217|O. cuniculus
gi|291411219|O. cuniculus
gi|296210423|C. jacchus
gi|297681775|P. abelii
gi|332869527|P. troglodytes
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gi|344237874|C. griseus
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gi|444728412|T. chinensis
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gi|470619074|T. truncatus
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gi|478512953|Ce. simum
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gi|57097397|C. lupus
gi|58257847|M. mulatta
MER416434|M. putorius
220
230
gi|114051748|M. mulatta

```
gi|118763987|H. sapiens          . . . . . V K . . . . . .
gi|125975948|C. porcellus        . . . . . C . . V S . . R Q . . . S . -
gi|126340910|M. domestica        G . . . . . C . . V D . . . T . . . N . -
gi|136406|sp|Canis_sp.           G . . . . S P . . C K . V S . . Q Q . . . . . -
gi|149065361|R. norvegicus       G . . . . . C . . V N . . Q Q . V . . . -
gi|149706547|E. caballus         . . . . . V N . . R Q . . . . . -
gi|206499|R. norvegicus          G . . . . . C . . V N . . Q Q . V . . . -
gi|2358072|M. musculus           G . . . . . C K . V N . . Q Q . . . . . -
gi|238866766|S. scrofa           . . . . . C . . V N . . Q Q . . . . . -
gi|2392548|B. Trypsin            . . . . . C . . V S . . . Q . . . S . -
gi|2499863|G. gallus             G Y . . . . . C . . V S . . . T . M S S . -
gi|281342466|A. melanoleuca      G . . . . . C . . V S . . R Q . . . . . -
gi|291411217|O. cuniculus        . . . . . C . . V S . . R Q . . . . . -
gi|291411219|O. cuniculus        . . . . . C . . V S . . R Q . . . . . -
gi|296210423|C. jacchus          . R . . . . . C . . V K . . Q E . . . . . -
gi|297681775|P. abelii           . R . . . . A . . . . . M N . . . D . . . . . .
gi|332869527|P. troglodytes      . . . . . V N . . . . . .
gi|332869535|P. troglodytes      . R . . . . . V K . . . D . . . . . .
gi|344237874|C. griseus          . N . . . . . C . . V S . . Q S . . . . . -
gi|344297218|L. africana         G . . . . . S . . Q Q . . . . . -
gi|395541092|S. harrisii         G . . . . . C . . V N . . . K . . . E . -
gi|395837362|O. garnettii        G R . . . . . C . . V Q . . Q Q . . . . . -
gi|397499771|P. paniscus         . R . . . . . V K . . . D . . . . . .
gi|402865133|P. anubis           . . . . . .
gi|403276290|S. boliviensis      . R . . . . . C . . V K . . Q Q . . . . . -
gi|410953013|F. catus            G . . . . . C . . V S . . Q Q . . . . . -
gi|426228145|O. aries            . . . . . C . . V S . . Q Q . . . S . -
gi|431911629|P. alecto           . . . . . C K F V D . . . K . V . S . -
gi|444728412|T. chinensis        G . . . . . C . . V D . . . Q . . . . . -
gi|466020769|O. orca             G . . . . . C . . V N . . Q K . . . D . -
gi|470619074|T. truncatus        G . . . . . C . . V N . . Q K . . . D . -
gi|471398393|T. latirostris      D . . . . . C . . V S . . Q Q . . . . . -
gi|478512953|Ce. simum           . R . . . . . F . . V N . . Q Q . . . . . .
gi|483495458|A. platyrhynchos    G Y . . . . . C . . V S . . Q S . . . . . -
gi|504169599|O. princeps         . . . . . C . . V S . . Q Q . . . . . -
gi|505801121|S. araneus          G . . . . . C . . V S . . Q Q . . . S . -
gi|507554879|J. jaculus          . S . . . . . C . F V S . . Q A . . . . . -
gi|507554883|J. jaculus          G . . . . . C . . V N . . Q Q . . . S . -
gi|507636133|O. degus            . . . . . C . . V S . . Q Q . . . . . -
gi|511994011|M. furo             G . . . . . C . . V S . . R Q . . S . . -
gi|511994015|M. furo             G . . . . . C . . V S . . Q E . . . . . -
gi|512810487|H. glaber           . L . . . . . C . . V S . . Q Q . . . S . -
gi|513161284|G. gallus           G Y . . . . . C . . V S . . . T . M S S . -
gi|521031417|M. brandtii         G . . . . . C . . V R . . Q D . M . . . -
gi|524966498|M. auratus          . N . . . . . C . . V S . . Q S . . . . . -
gi|524966500|M. auratus          G . . . . . C . . V S . . Q K . . . . . -
gi|525028211|F. albicollis       G Y . . . . . C . . V S . . R S . M . . . -
gi|528903801|B. taurus           . . . . . C . . V S . . . Q . . . S . -
gi|529429287|F. peregrinus       G Y . . . . . C . . V S . . R S . M S S . -
gi|529431263|F. peregrinus       G Y . . . . . C . . V S . . T A . . S . . -
gi|530574671|C. picta            G Y . . . . . C . . V S . . Q . . . . . -
gi|532046272|M. ochrogaster      G . . . . . C K . V S . . Q Q . . . . . -
gi|532058903|M. ochrogaster      . L . . . . . C . . V . . . . . -
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gi|532058907|M. ochrogaster      . L . . . . . C . . V . . . . . -
gi|533184793|C. lanigera         . . . . . C . . V S . . Q Q . . . S . -
gi|57097397|C. lupus             G . . . . . R . C K . V S . . Q Q . . . . . -
gi|58257847|M. mulatta           . R . . . . . V D . . R D . . . . . .
MER416434|M. putorius            G . . . . . C . . V S . . R Q . . S . . -
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Supplementary table 1 QMEAN analysis of the structures used in the present work. Analysis were made at Swiss Model Workspace.

Scoring function term	Tambaqui model		Salmon (1BIT)		Porcine (1S81)	
	Raw score	Z-score	Raw score	Z-score	Raw score	Z-score
C_beta interaction energy	-94.96	-0.74	-73.39	-1.29	-89.09	-0.82
All-atom pairwise energy	-4417.46	-1.6	-3602.11	-2.02	-4077.24	-1.74
Solvation energy	-16.12	-0.84	-11.17	-1.43	-13.85	-1.12
Torsion angle energy	-83.28	1.3	-81.52	1.19	-69.42	0.41
Secondary structure agreement	93.20%	1.77	91.00%	1.39	92.80%	1.7
Solvent accessibility agreement	86.00%	1.14	90.10%	1.88	89.70%	1.81
QMEAN6 score	0.936	1.79	0.987	2.35	0.962	2.08