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α-AMILASES DOS PEIXES *Colossoma macropomum*, (Cuvier, 1818) E *Oreochromis niloticus* (Linnaeus, 1758): PROPRIEDADES E APLICAÇÕES BIOTECNOLÓGICAS

Recife
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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de doutora em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

Orientador: Prof. Dr. Ranilson de Souza Bezerra
Coorientadora: Marina Marcuschi

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Às mulheres que, mesmo sob a sombra do patriarcado, resistem, criam e ocupam seus espaços.

Em especial, à Lia e Susi.

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"*Que o conquistador não seja escravo da sua conquista*"

(Autor desconhecido)

RESUMO

A recuperação e reutilização dos subprodutos do processamento pesqueiro têm sido discutidas como alternativas sustentáveis para manejo dos resíduos gerados pela aquicultura. Esses subprodutos são considerados potenciais fontes de biomoléculas com demanda biotecnológica e baixo custo. Diante dessa perspectiva, a presente tese descreve a obtenção e caracterização dos parâmetros físico-químicos, cinéticos e termodinâmicos das α-amilases dos peixes tilápia do Nilo e tambaqui, e propõe aplicações biotecnológicas para essas enzimas. No artigo 1 foram abordadas a purificação da α-amilase intestinal da tilápia (AMY-T), e sua caracterização cinética/bioquímica. A α-amilase mostrou atividade ótima no pH 5.0, e termoestabilidade até 50°C. Observou-se a importância do pH para o mecanismo catalítico, visto que os parâmetros cinéticos mudaram com a variação do pH. AMY-T foi fortemente ativada por Cl^- que, além de ativador alostérico, conferiu termoestabilidade à enzima. Brometo e iodeto também ativaram AMY-T, embora em menor proporção comparado ao cloreto, mostrando que o sítio de ligação do cloreto apresenta plasticidade para outros íons. A α-amilase foi resistente à EDTA e EGTA, sugerindo que sua atividade independe de cálcio. O artigo 2 avaliou a aplicação da α-amilase da tilápia no processamento enzimático de grãos nativos de amido de batata. A α-amilase adsorveu e hidrolisou amido, sob baixas temperatura e concentrações de enzima, e suspensões concentradas de amido (10% e 15%), alcançando grau de hidrólise de até 45%. Estudos microscópicos (MEV) confirmam a ação enzimática através das mudanças morfológicas causadas na estrutura dos grãos de amido. A α-amilase digeriu uma variedade de carboidratos, mostrando preferência por ligações glicosídicas α-1→4. Foi estável à ampla faixa de pH e vários solventes orgânicos durante 24h de incubação. Os principais produtos gerados foram glicose, maltose e maltodextrinas, indicando uma amilase com propriedades sacarificadoras, e sugerindo a aplicação dessa enzima no processamento industrial do amido. No artigo 3, α-amilases oriundas do intestino (AMY-1) e cecos pilóricos (AMY-2) do tambaqui foram parcialmente purificadas, caracterizadas e avaliadas como aditivo de limpeza em detergentes comerciais. Foi proposta uma metodologia para obtenção de enzimas digestivas, viabilizando o isolamento de α-amilases e tripsinas em um único procedimento. As α-amilases mostraram atividade ótima no pH 7.0 (AMY-1) e 8.5 (AMY-2), temperatura ótima de 60°C e termoestabilidade até 40°C durante 60 min. Foram inibidas por Cu^+ e Hg^+ e não foram ativadas na presença de Ca^{2+}, apresentando resistência a quelantes. Foi observada boa digestibilidade de diferentes substratos, exceto maltose,

confirmando a natureza amilácea das enzimas. A análise dos parâmetros cinéticos mostrou afinidade similar por amido, entretanto, AMY-2 apresentou melhor desempenho catalítico. Ambas foram resistentes aos agentes oxidantes e surfactantes, e compatíveis com todas as marcas de sabões comerciais testados, mesmo quando comparadas à α-amilase comercial de *Bacillus subtillis*, o que sugere a potencial aplicação dessas enzimas na indústria de detergentes. Os resultados obtidos através dessa pesquisa apontam para a utilização dos resíduos do processamento pesqueiro como fonte de α-amilases com perfil compatível com vários seguimentos industriais, e endossam a necessidade e importância de preencher uma lacuna existente no estudo das enzimas de peixes, sobretudo α-amilases.

Palavras-chaves: Enzimas. Resíduos pesqueiros. Tilápia do Nilo. Tambaqui.

ABSTRACT

The recovery and reutilization of fish processing waste have been considered as a sustainable alternative for the management of waste generated through aquaculture activities. These by-products are considered as potential sources of biomolecules with biotechnological demand and low cost. Thus, this work describes the obtaining and characterization of physico-chemical, kinetic and thermodynamic parameters of α-amylases from the tropical fishes Nile tilapia and tambaqui, and proposes biotechnological applications for these enzymes. On the chapter 1 the purification and kinetic/biochemical characterization of α-amylase from tilapia viscera (AMY-1) were evaluated. The α-amylase showed optimum activity at pH 5.0 and thermal stability until 50°C. It was observed the role of pH on catalytic mechanism, once the kinetic parameters were shifted according the pH. AMY-T was highly actived by chloride ion, which beside to acts as allosteric activator, enhanced the thermal stability. Bromide and iodide also acts as effectors of α-amylase, though in less extend in comparison to chloride, showing that the chloride site binding has plasticity toward other ions. In addition, it was observed resistance to chelating agents EDTA and EGTA, suggesting that AMY-T activity is calcium independent. The chapter 2 evaluated the application of tilapia α-amylase in enzymatic processing of raw potato starch. This enzyme was able to adsorb and hydrolyze raw starch, at low concentration of enzyme and temperature, and also high concentrated starch suspensions (10 and 15%), achieving a hydrolysis degree of 45%. The scanning electron microscopy confirms the enzymatic action by the morphological changes on the raw potato granules. The α-amylase was able to digest a variety of carbohydrates, showing preference to cleave glycosidic bonds α-1→4. It was highly stable at wide pH range and also to several organic solvents after 24h of incubation. The mainly products of hydrolysis were glucose, maltose and maltodextrins, suggesting a saccharifier enzyme and indicating the application of this enzyme on starch industry. On the chapter 3, α-amylases from intestine (AMY-1) and pyloric caeca (AMY-2) of tambaqui were partially purified and proposed as cleaning additive in commercial detergents. A new methodology to purify digestive enzymes was proposed, and it was possible to obtain α-amylases and trypsin in a single procedure. The α-amylases showed optimum activity at pH 7.0 (AMY-1) and 8.5 (AMY-2), optimum temperature of 60°C and thermal stability up 40°C for 60 min. Both were inhibited by Cu^{+} and Hg^{+}, they were not active by Ca^{2+} and showed resistance to chelating agents. The enzymes showed digestibility toward different substrates, except to maltose, confirming their amylaceus profile. They had

similar affinity to starch, but AMY-2 showed better catalytic performance. Both were highly resistant to oxidizing agents and surfactants, and showed compatibility to all brands of commercial laundry tested, even in comparison to commercial α-amylase of *Bacillus subtilis*, suggesting the potential application of these enzymes on laundry industry. The results obtained points to use of fish processing waste as source of α-amylases compatible with various industrial sectors, and emphasizes the need and importance of filling a gap in study of fish enzymes, mainly α-amylases.

Keywords: Enzymes. Fish processing waste. Nile tilapia. Tambaqui.

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1 INTRODUÇÃO

A maioria das reações biológicas que acontecem nos seres vivos é mediada por enzimas. Essas biomoléculas são, na sua maioria, de origem proteica e atuam catalisando reações permitindo assim o funcionamento metabólico em todos os organismos vivos. As enzimas são utilizadas em processos enzimáticos mesmo antes do conhecimento da sua natureza bioquímica, sendo seu uso relatado na produção de alimentos e bebidas desde o início da humanidade.

O avanço tecnológico permitiu que as enzimas fossem isoladas e estudadas, e com o advento da biotecnologia, aplicadas em vários segmentos. Atualmente, as enzimas estão presentes no nosso dia-a-dia em funções que variam desde a otimização de processos industriais à atuação como agentes terapêuticos (PANDEY, 2000). Dentre essas enzimas, a α-amilase se destaca por sua ampla presença e função em diversos organismos e setores industriais.

O primeiro relato sobre uma amilase data de 1831, quando Erhard Friedrich Leuchs descreveu a ação catalítica de uma enzima salivar. Dois anos depois, em 1833, Anselme Payen e Jean-François Persoz isolaram uma amilase a partir de um extrato de malte, e lhe atribuíram o nome de diastase – do grego, separar – devido à sua capacidade de clivar o amido em unidades de glicose (ROOHI, 2012). Em 1984, foi produzida a primeira enzima com fins industriais, a Taka amilase, utilizada como auxiliar digestivo (BOŽIĆ et al., 2017). Desde então, α-amilases têm sido estudadas sob as mais diversas perspectivas e utilizadas para diferentes fins biotecnológicos.

A α-amilase desempenha função primordial no metabolismo de carboidratos em diversos seres vivos, desde bactérias e fungos, a vegetais e animais. Devido à tamanha importância fisiológica, essa enzima tem sido utilizada para fins médicos, sendo sua atividade relacionada com diagnósticos e tratamentos de doenças humanas; e também empregada como indicativo metabólico/nutricional em sistemas de cultivos, formulação de rações e suplementação dietética de animais. No âmbito industrial, a α-amilase detém 1/3 do mercado global de enzimas, sendo a segunda enzima mais requerida em processos enzimáticos. Ela é amplamente utilizada nos setores de detergentes, alimentício, processamento de amido, têxtil, papel, biocombustíveis e bebidas (GUPTA et al., 2003).

Devido a facilidades no cultivo e manuseio, enzimas de bactérias e fungos ainda são as mais estudadas e, portanto, também as mais consumidas pelo mercado enzimático SIVARAMAKRISHNAN et al., 2006). No entanto, pesquisadores têm apontado para a necessidade de descobrir novas enzimas com potencial biotecnológico, sobretudo oriundas de fontes consideradas de baixo custo, destacando os subprodutos de processamentos agroindustriais, como por exemplo, os resíduos da atividade pesqueira, como uma alternativa para obtenção dessas biomoléculas (VAIDYA et al., 2015).

A aquicultura tem se destacado nos últimos anos como um dos setores agrícolas com maior expansão a nível mundial. Consequentemente, o aumento na produção e processamento de pescado tem levado à geração de uma alta quantidade de resíduos oriundos das atividades aquícolas (SOFIA, 2016). Esses resíduos são atualmente um obstáculo para aquicultura, pois seu descarte é altamente oneroso. Uma alternativa que tem se mostrado viável é a reutilização desses insumos para obtenção de moléculas bioativas (ALONSO, et al., 2010). Tal prática propicia a descoberta de biomoléculas com demanda biotecnológica e contribui para a redução da contaminação ambiental gerada pelo seu descarte. Essa proposta tem sido desenvolvida pelo Laboratório de Enzimologia da UFPE (LABENZ) e enzimas oriundas do processamento de diversas espécies de peixes têm sido isoladas, estudadas e relacionadas com aplicações biotecnológicas.

No entanto, estudos abordando α-amilases de peixes ainda são escassos quando comparados com outras enzimas digestivas, como a tripsina, por exemplo. Diante de tal perspectiva, este trabalho visa trazer algumas elucidações bioquímicas sobre as α-amilases dos peixes de maior expressão nacional, tilápia do Nilo (*Oreochromis niloticus*) e tambaqui (*Colossoma macropomum*), e junto à problemática do manejo de resíduos do processamento pesqueiro propor alternativas para, a partir desses insumos, avaliar a obtenção e utilização dessas enzimas em processos enzimáticos biotecnológicos e industriais.

2 OBJETIVOS

2.1 Objetivo Geral

Purificar e caracterizar α-amilases a partir das vísceras dos peixes *Oreochromis niloticus* e *Colossoma macropomum,* e avaliar seu potencial para aplicações industriais nos setores de processamento do amido e de detergentes comerciais.

2.2 Objetivos específicos

2.2.1 Artigo1

• Caracterizar a α-amilase da tilápia frente a seus parâmetros cinéticos;

• Investigar o papel do pH no mecanismo catalítico através da sua interferência nos parâmetros cinéticos;

• Examinar a presença e atuação dos sítios de cálcio de cloreto sobre a atividade da α-amilase;

2.2.2 Artigo 2

• Avaliar a aplicabilidade da α-amilase obtida a partir das vísceras da tilápia na hidrólise enzimática do amido;

• Avaliar os produtos oriundos da hidrólise enzimática dos grãos de amido relacionando com o potencial digestivo da α-amilase através de CLAE, CCD e MEV.

• Investigar a resistência da α-amilase frente à longa exposição a pH e solventes orgânicos;

2.2.3 Artigo 3

• Desenvolver uma metodologia para obtenção de α-amilases a partir das vísceras dos cecos pilóricos e intestino do tambaqui;

• Obter perfil bioquímico das α-amilases avaliando: pH e temperatura; especificidade ao substrato através da hidrólise de diferentes carboidratos; e ação de íons metálicos, quelantes e inibidor sobre sua atividade;

• Elucidar os parâmetros cinéticos e termodinâmicos das α-amilases;

• Avaliar a aplicabilidade das α-amilases como aditivo de limpeza em detergentes comerciais através da estabilidade frente a surfactantes, agentes oxidantes e detergentes comerciais.

3 FUNDAMENTAÇÃO TEÓRICA

3.1 α-Amilase

3.1.1 Família α-Amilase

As amilases são carboidrases amplamente distribuídas na natureza, podendo ser encontradas em animais, vegetais e micro-organismos. De acordo com a reação catalisada, as amilases se dividem em três tipos: α, β, γ-amilase. Essas enzimas são capazes de metabolizar uma alta variedade de carboidratos, atuando na hidrólise de ligações glicosídicas presentes no amido e seus derivados (JANECECK et al., 2013).

Tal característica reúne essas enzimas em um grande grupo, denominado glicosil-hidrolase – GH. Dentre as enzimas pertencentes a esse grupo, a α-amilase destaca-se por sua representatividade, sendo extensivamente demandada em estudos bioquímicos, bem como em aplicações industriais/biotecnológicas, detendo cerca de 30% do setor de enzimas (PANDEY, 2000; GUPTA et al., 2003).

Através do sistema de nomenclatura IUBMB (2017), as enzimas são classificadas conforme o tipo de reação que catalisam e sua especificidade pelo substrato. Para as glicosil-hidrolases (EC 3.2.1.x), os primeiros dígitos indicam a hidrólise de ligações glicosídicas, e o último corresponde ao substrato e/ou seu mecanismo molecular (VAN DER MAAREL et al., 2002). De acordo com o tipo de ataque enzimático, as amilases podem ser descritas como:

1- Endoamilases: hidrolisam ligações glicosídicas internas α-1,4, gerando como produtos carboidratos α-anoméricos;
2- Exoamilases: clivam ligações glicosídicas externas α-1,4 ou α-1,6, gerando como produtos sacarídeos α ou β-anoméricos;
3- Enzimas desramificadoras: hidrolisam exclusivamente ligações α-1,6 liberando polissacarídeos lineares longos; e
4- Transferases: clivam ligações glicosídicas α-1,4 e transferem parte da molécula doadora para um aceptor glicosídico, formando uma nova ligação glicosídica.

O avanço da Enzimologia e, consequentemente, uma maior disponibilidade de sequências de enzimas, despertaram a necessidade de melhor classificar essas carboidrases,

considerando aspectos estruturais e evolutivos que não eram contemplados pela IUBMB. Com auxílio do CAZy (Carbohydrate-Activy enZYmes - http://www.cazy.org/), um banco de dados online para carboidrases, as glicosil-hidrolases passaram a ser agrupadas segundo suas similaridades estruturais, estando dispostas individualmente em suas respectivas famílias, que totalizam 145 (HENRISSAT, 1991; JANECEK et al., 2013).

A α-amilase pertence à família GH-13 que, atualmente, é composta por enzimas de três grupos (hidrolases, transferases e isomerases) e compreende mais de 30 especificidades enzimáticas diferentes. Esta corresponde à maior família de GHs agregando mais de 52.438 membros que são subdivididos em 40 subfamílias, sendo estes predominantemente Bacteria ~48.854, seguido por Eukariota ~3.079, Archaea ~375 e não classificados ~130 (CAZy acessado em 29/01/2018).

Takata (1992), um dos pioneiros no estudo de α-amilases, elencou os seguintes critérios para a inclusão nessa família:

1- Atuar sob ligações α-glicosídicas através de hidrólise e produzir monossacarídeos ou oligossacarídeos α-anoméricos, ou formar ligações glicosídicas por transglicosilação;
2- Possuir quatro regiões altamente conservadas em sua estrutura primária (cadeias β3, β4, β5 e β7);
3- Possuir tríade catalítica formada por ácido aspártico, ácido glutâmico e ácido aspártico (correspondendo a Asp 212, Glu 248 e Asp 315 no peixe *Siganus canaliculatus* (XIE et al., 2016));
4- Apresentar domínio catalítico $(\beta/\alpha)_8$ β-barril ou "TIM-barril".

Devido à ampla diversidade de organismos presente nessa família e, consequentemente diferentes origens de α-amilases, a similaridade entre as sequências de aminoácidos dessas é bastante baixa, estando apenas a tríade catalítica (Asp, Glu e Asp) e um resíduo de arginina invariavelmente conservados (JANECEK, 2002). Essa semelhança, porém, é aumentada com a proximidade dos táxons.

α-Amilases de animais, inclusive humana, estão agrupadas na subfamília GH13-24, que, embora seja rica em multiplicidade de membros, é pouco estudada do ponto de vista estrutural. Dentre as estruturas terciárias de α-amilases de origem animal disponíveis na literatura, destacam-se a α-amilase pancreática de porco (BUISSON et al., 1987; QIAN et al., 1993; GILLES et al., 1996) e as α-amilases salivar e pancreática humanas (QIAN et al., 1994;

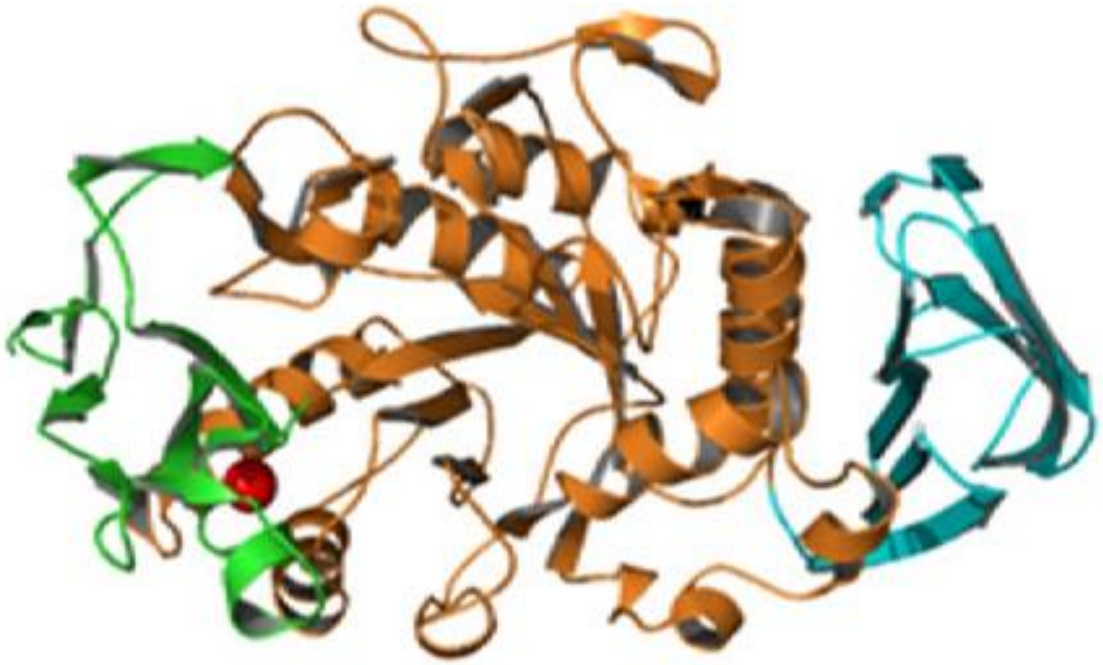
BRAYER et al., 1995; RAMASUBBU et al., 1996). Mizutani (2012) determinou a estrutura terciária da α-amilase do peixe *Oryzias latipes* e, ao comparar com demais membros da subfamília GH13-24, encontrou similaridades com os sítios superficiais de ligação de amido (SBS) e ativação alostérica pelo íon cloreto, descritos como características presente em α-amilases de origem animal.

Embora existam algumas diferenças entre as α-amilases de diferentes vertebrados, o alinhamento das sequências primárias dessas α-amilases mostrou haver uma alta similaridade entre elas. Entre as α-amilases de mamíferos e peixes foi observada homologia de 72% para α-amilase humana e 74% para α-amilase de porco, sendo essa similaridade ainda maior entre espécies de peixes (MIZUTANI et al., 2012; XIE et al., 2016).

3.1.2 Estrutura enzimática

A estrutura geral das α-amilases (Figura 1) consiste em uma única cadeia polipeptídica dobrada basicamente em três domínios: A, B e C.

Figura 1. Estrutura tridimensional da α-amilase pancreática de porco ilustrando os três domínios que compõem a estrutura da α-amilase. Domínio A – laranja; domínio B – verde; domínio C – azul.



Fonte: KUMARI et al., 2010.

O domínio A está localizado na porção N-terminal e é constituído por uma estrutura altamente simétrica, compondo um barril de oito β-folhas paralelas cercado por oito α-hélices $(\beta/\alpha)_8$ (Fig 2). Este corresponde ao domínio catalítico e é o mais conservado dentro da família α-amilase. Essa estrutura catalítica $(\beta/\alpha)_8$ é encontrada em outras enzimas, e foi primeiramente identificada por Banner (1975) na triose fosfato isomerase (TIM), oriunda de músculo de galinha e, portanto, denominada TIM-barril (PRAKASH & JAISWAL, 2010).

Fig 2. Estrutura catalítica da α-amilase de porco. $(\beta/\alpha)_8$-barril ou TIM-barril composta por oito α-hélices intercaladas por oito β-folhas.

Fonte: Mehta & Satyanarayana, 2016.

Já o domínio B é formado a partir de uma protrusão entre a terceira β-folha e a terceira α-hélice, compreendendo uma grande parte da fenda de ligação do substrato. Este domínio

apresenta uma estrutura irregular rica em configuração β, havendo variações de tamanho e estrutura entre as α-amilases. A variedade de conformação desse domínio confere à enzima especificidade pelo substrato.

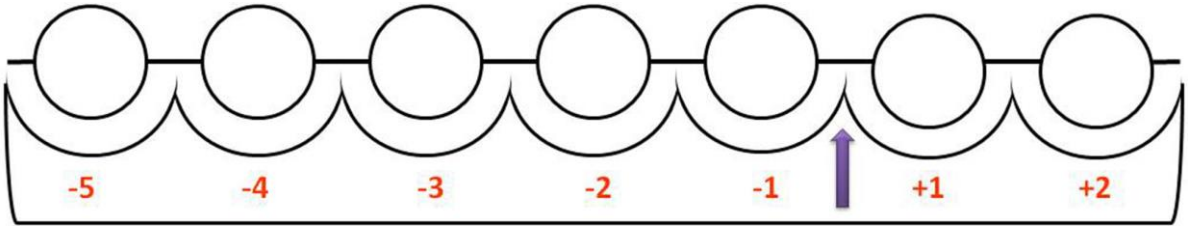
O domínio C corresponde a uma estrutura sanduíche-β e é formado por um motivo de chave-grega. Este se localiza na região C-terminal, situado opostamente ao domínio B. O domínio C está relacionado à ligação com o substrato e estabilização do domínio catalítico e se liga ao domínio A.

Outros domínios podem ser encontrados entre as enzimas da família GH-13. Algumas α-amilases possuem na sua porção C-terminal o domínio D, cuja função ainda é desconhecida (NIELSEN AND BORCHET, 2000). O domínio E está presente em cerca de 10% das enzimas da família α-amilases. Essa estrutura, também chamada de domínio de ligação do amido – SBD – (Starch Binding Domain), é composta por vários segmentos de β-folha formando um β-barril distorcido e ocorre também nas famílias β-amilase e glucoamilases (SVENSSON, 1994; JANECEK, 1999). Amilases que atuam na hidrólise de ligações glicosídicas α-1-6 de substratos ramificados podem ainda apresentar os domínios F, G e H localizados também na porção N-terminal (VAN DER MAAREL et al., 2002).

A estrutura primária da α-amilase apresenta até sete regiões conservadas com aminoácidos catalíticos e aminoácidos que são essenciais para a estabilidade do β-barril. No domínio catalítico (A) estão contidas as quatro regiões de aminoácidos conservadas invariavelmente em todas α-amilases. Esses resíduos são intimamente relacionados com o sítio ativo e correspondem às estruturas β-folha 3, 4 e 5 e o loop que conecta a β-folha 7 à α-hélice 7. Adicionalmente, mais três regiões conservadas foram identificadas e mostram conter resíduos de aminoácidos ligados à especificidade enzimática (JANECEK, 2002).

Análises estruturais, a partir de difração de raio-X, de α-amilases mostram que a fenda catalítica, localizada na interface entre os domínios A e B, na região C-terminal, pode acomodar de quatro até dez unidades de glicose, de acordo com o número de subsítios de ligação de glicose presentes na estrutura (Figura 3). Os subsítios situados na porção não redutora do substrato são enumerados negativamente e variam entre dois e sete, enquanto os que se ligam no terminal redutor são enumerados positivamente e totalizam de 2 a 3 subsítios (MACGREGOR, 1988; MEHTA & SATYANARAYANA, 2016).

Figura 3. Representação do sítio ativo de uma α-amilase ilustrando os seus subsítios. Cada subsítio interage com uma unidade de glicose do substrato. A fenda catalítica da enzima está indicada pela seta lilás. A quantidade de subsítios varia entre diferentes organismos.



Fonte: Mehta & Satyanarayana, 2016.

α-Amilases de vertebrados e alguns insetos apresentam ainda uma particularidade que as protege da ação de aminopeptidades. O resíduo de Gln localizado no N-terminal dessas enzimas passa pelo processo de ciclização, sendo modificado em pirroglutamato. Essa modificação bloqueia a degradação de seu N-terminal e impede que enzimas proteolíticas digiram a α-amilase (D'AMICO et al., 2000).

3.1.2.1 Sítios de cálcio

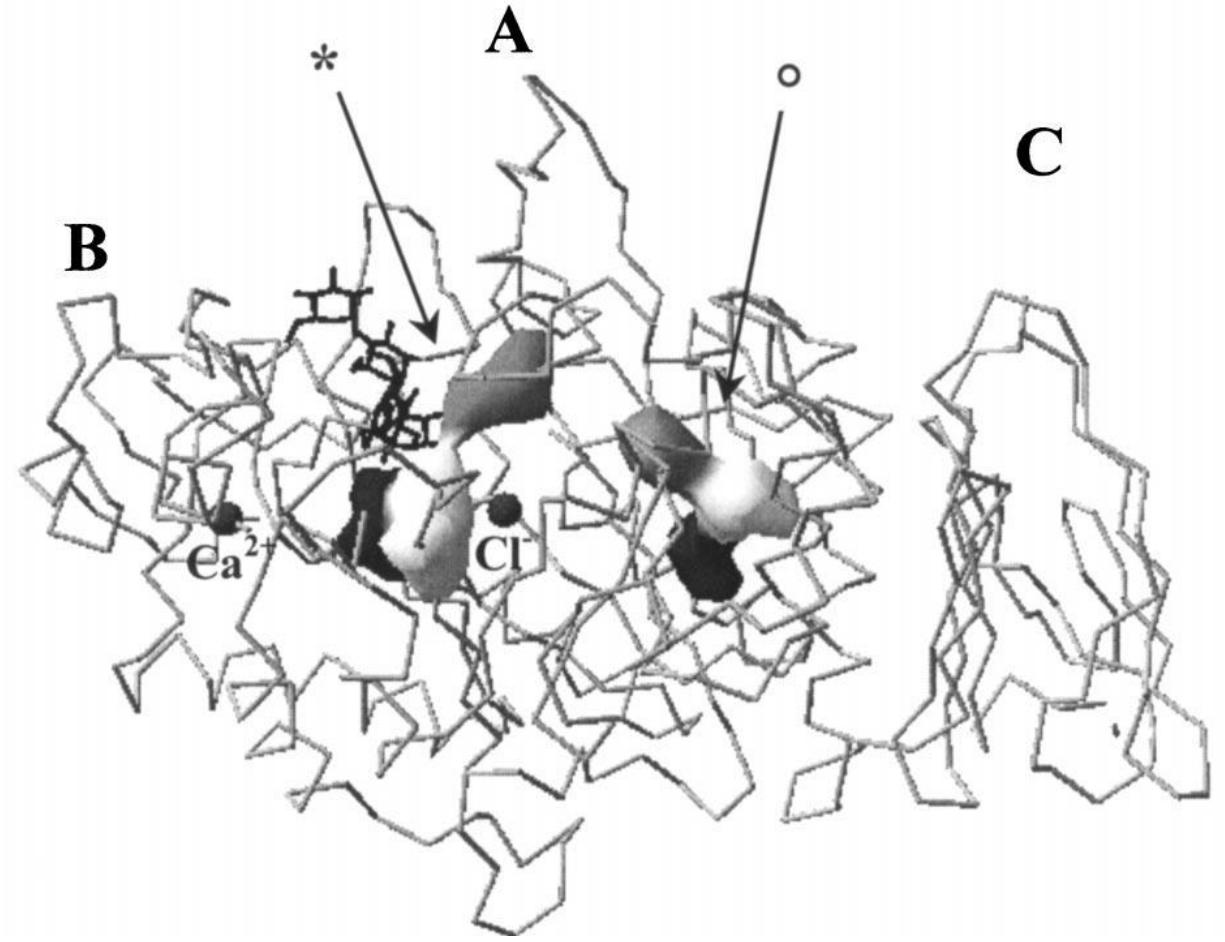
As α-amilases são descritas na literatura como metalo-enzimas que possuem de 1 a 10 íons Ca^{2+} conservados em sua estrutura. Esses íons se localizam na interface entre os domínios A e B e induzem a formação de uma ponte iônica entre esses domínios (Figura 4). Geralmente três resíduos conservados estão envolvidos na ligação ao cálcio, Asn, Asp e His, sendo o Asp oriundo do C-terminal do domínio B, o mais conservado (JANECEK, 1997).

Este íon é essencial para a atividade das amilases cálcio-dependentes. A remoção do Ca^{2+} da estrutura enzimática causa danos ao sítio de ligação do substrato, pois deixa livre o domínio B e os loops seguidos das β-folhas 3 e 4 presentes no $(\beta/\alpha)_8$-barril, levando ao enfraquecimento da interação entre o substrato e os resíduos catalíticos (BUISSON et al., 1987). A atividade amilolítica, no entanto, pode ser recuperada com a adição de Ca^{2+}.

Além disso, o cálcio também exerce papel na termoestabilidade enzimática. Ele confere rigidez à enzima através do efeito de salting out nos seus resíduos hidrofóbicos, o que causa uma compactação da sua estrutura (PRAKASH & JAISWAL, 2010; MEHTA & SATYANARAYANA, 2016).

O número de íons de cálcio conservados difere entre as enzimas, mas existe um sítio de ligação de cálcio para cada íon ligado na maioria das α-amilases. A maioria delas apresenta apenas um íon ligado, enquanto algumas amilases como a do *Bacillus* spp. contêm quatro íons de cálcio e uma tríade metálica cálcio-sódio-cálcio formando a ponte entre os domínios A e B (LEE et al., 2006). A α-amilase pancreática humana, por exemplo, interage com oito ligantes diferentes entre a cadeia principal, cadeia lateral e moléculas de água.

Figura 4. Estrutura a α-amilase pancreática de porco complexada com arcabose (preto) na fenda de ligação do substrato mostrando os domínios, resíduos catalíticos e sítios de cálcio e cloreto. Observam-se os domínios A, B e C, além dos resíduos catalíticos indicados por * (Asp197, cinza escuro, Glu233, cinza claro, Asp300, cinza médio), e a tríade protease-*like* (Glu27, cinza escuro, Ser340, cinza médio, His386, cinza claro) indicada por °.



Fonte: D'AMICO et al., 2000.

Além do sítio de ligação primário, o cálcio também se liga a sítios secundários localizados na fenda de ligação do substrato, o que justifica a comum inibição da α-amilase sob altas concentrações desse íon. Entretanto, esses sítios não estão presentes em α-amilases de origem animal (BRAYER et al., 1995, JANECEK, 1997).

Poucas amilases são descritas como cálcio-independentes. Essas enzimas apresentam resistência a agentes quelantes, como EDTA e EGTA, e devido a essa característica são cada vez mais demandadas na indústria. Elas não são ativadas nem tem sua termoestabilidade aumentada na presença de Ca^{2+}. Em algumas delas, como relatado na α-amilase cálcio-independente de *Bacillus* sp, há a substituição do Ca^{2+} pelo íon metálico Na^{+} (NONAKA et al., 2003).

Sajedi (2007), ao comparar as sequências primárias da α-amilase cálcio-independente de *Bacillus sp.* com as cálcio-dependentes BLA (*Bacillus licheniformis*) e BAA (*Bacillus amyloliquefaciens*), encontrou um alto grau de similaridade estrutural, ~90%, havendo substituição de apenas um resíduo envolvido no sítio de ligação de cálcio. Essa substituição parece influenciar as cargas negativas, induzindo a ligação de um íon monovalente como o Na^{+} ao invés do Ca^{2+}, divalente.

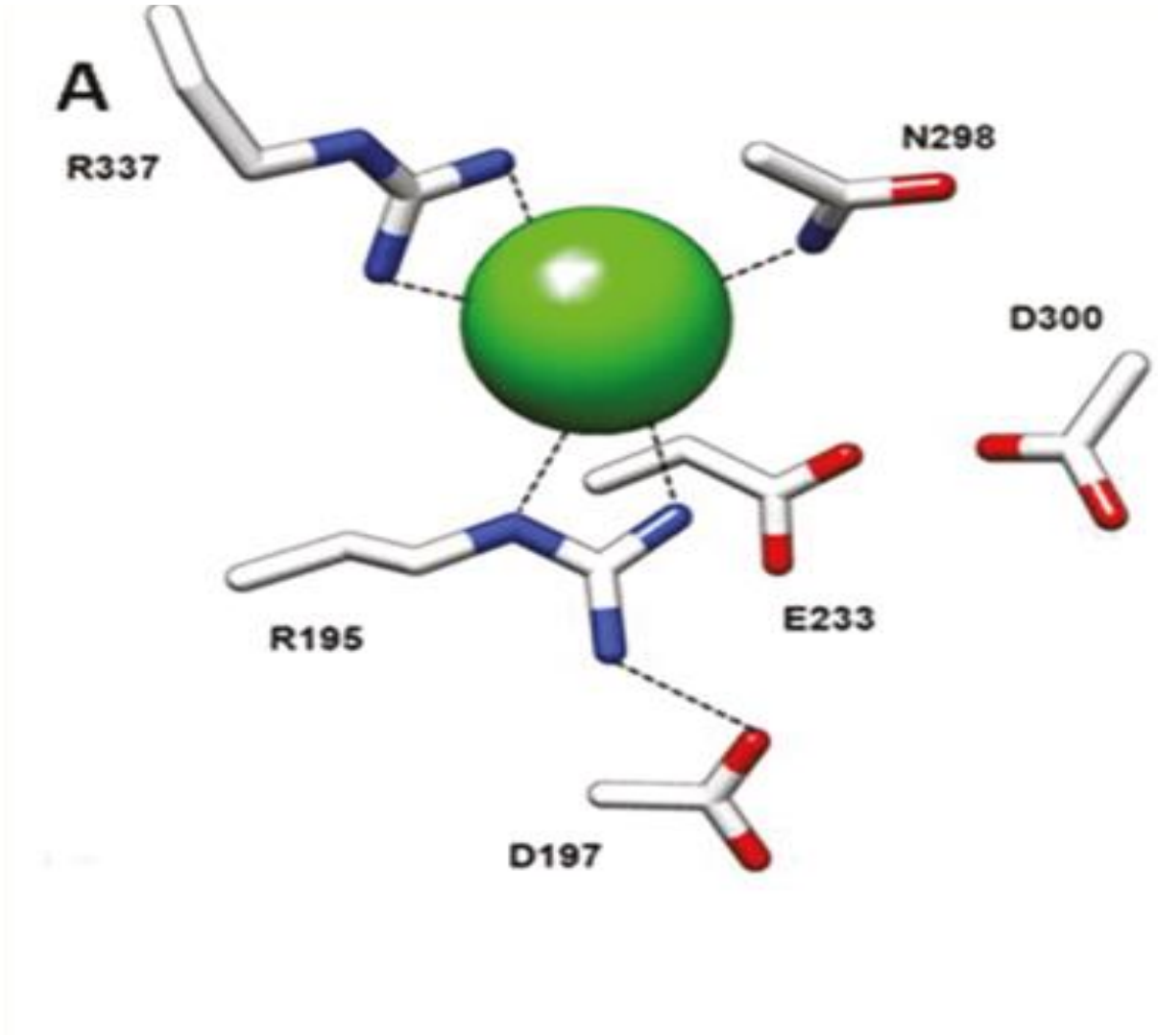
3.1.2.2 Sítio de cloreto

Algumas α-amilases apresentam também um sítio de ligação para o cloreto. A presença desse íon conservado na estrutura enzimática parece ser um evento evolutivamente recente, pois tal característica não é observada em α-amilases de organismos inferiores (BRAYER, 1995), estando presente principalmente em animais e algumas bactérias Gram-negativas (D'AMICO et al., 2000).

O sítio de ligação de cloreto foi identificado em análises de raio-X de várias α-amilases, como as de porco (QUIAN et al., 1993; LARSON et al., 1994), humanas (BRAYER et al., 1995; RAMASUBBU et al., 1996), *Alteromonas haloplancti*s (AGHAJARI et al., 1998) e *Tenebrio molitor* (STROBL et al., 1998) e sua função como ativador alostérico confirmada nesses organismos. Esse sítio está localizado próximo ao centro catalítico e ao sítio de ligação de cálcio, sendo constituído por Arg195, Asp298, Arg337, e uma molécula de água (numeração para mamíferos), sendo coordenado pelo resíduo de Asn através do modo

unidentado, e no modo bidentado pelos dois resíduos Arg (Figura 5) (BRAYER et al, 1995; D'AMICO et al., 2000; POKHREL et al., 2011).

Figura 5 – Sítio de ligação do cloreto ocupado pelo Cl^- na α-amilase pancreática humana. Esfera verde – íon de cloreto; Arg337, Asn298 e Arg195 resíduos formando o sítio de lição do cloreto; Arg197, Glu233 e Arg300 resíduos catalíticos da α-amilase.



Fonte: Pokhrel et al., 2011.

Ao se ligar ao seu sítio específico, o cloreto atua como ativador alostérico para as α-amilases, proporcionando sua atividade máxima. Ele fornece o ambiente eletrostático necessário para a catálise através da protonação do resíduo catalítico Glu, elevando o pKa do mesmo ao pH de sua atividade ótima (JANECEK, 1997). Larson (1994) propõe ainda que o

cloreto também aja neutralizando a carga do seu principal ligante, um resíduo de Arg positivamente carregado, permitindo assim a formação de uma ponte de sal com o resíduo catalítico Glu.

Além de atuar diretamente na atividade amilolítica, esse íon também promove mudanças conformacionais na região do sítio ativo. O alinhamento de sequências de α-amilases cloreto-dependentes revela a presença de uma tríade serinoprotease-*like* formada por Ser, His e Glu (Figura 4). A função dessa tríade ainda não é completamente elucidada, mas acredita-se que ela esteja relacionada com a estabilidade enzimática, uma vez que a substituição de um único aminoácido impede o dobramento da enzima (D'AMICO et al., 2000).

A ligação desse íon na estrutura enzimática confere à α-amilase uma atividade numa maior amplitude de pH, além de deslocar a atividade ótima de valores ácidos para neutros, estando mais próximo de valores fisiológicos. Tal comportamento pode ser considerado como evolutivamente vantajoso. Além disso, a ativação alostérica por cloreto não é uma característica muito comum, sendo observada, até então, em apenas duas enzimas além da α-amilase, na enzima conversora de angiotensina (ACE) e fotosistema II (PSII) (LEVITZKI et al., 1974; D'AMICO, 2000; POKHREL et al., 2011). Qian (2005) atribui esse deslocamento no pH ótimo à repulsão causada entre o íon e o resíduo catalítico Glu.

A remoção do cloreto diminui em 10 vezes o kcat da α-amilase, mostrando que amilases livres de cloreto apresentam atividade mais baixa e que essa atividade pode ser modulada através da variação na concentração do íon (QIAN et al., 2005).

3.1.1.3 Sítios de ligação do amido (SBS)

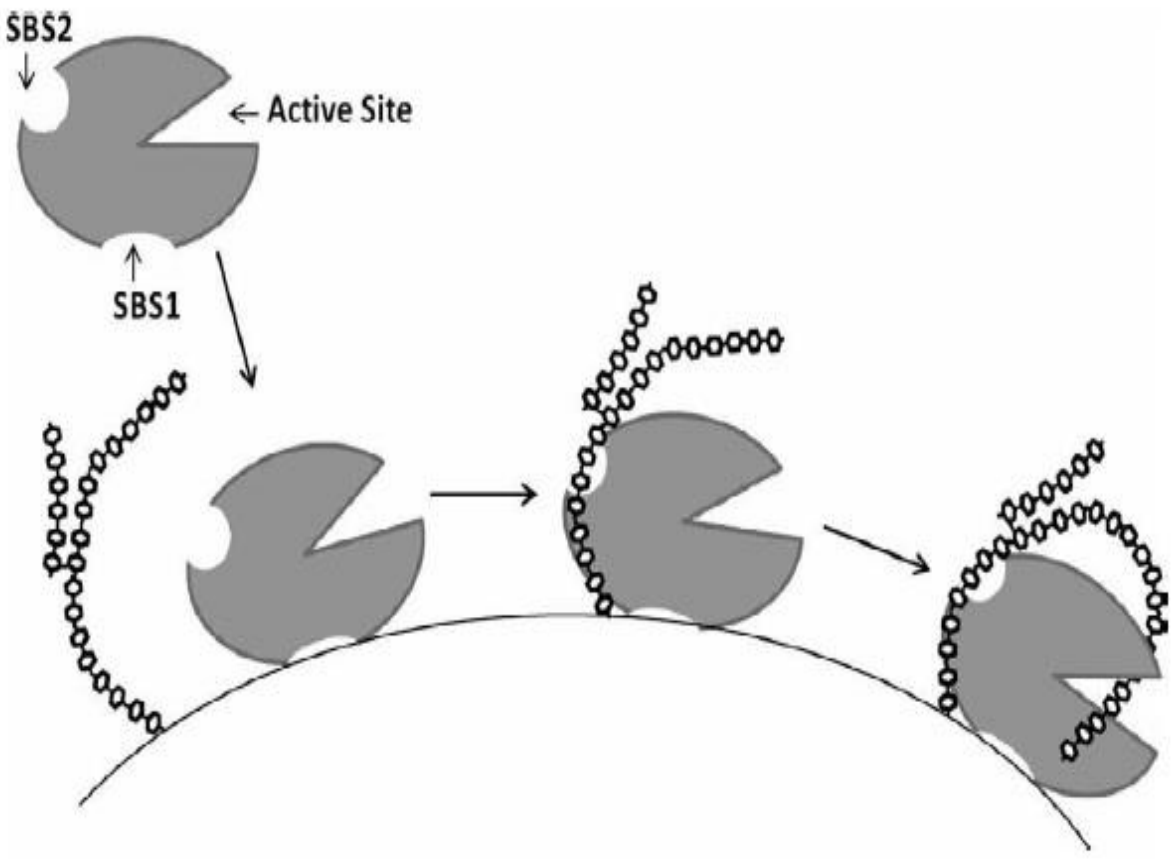
A ligação entre carboidrases e polissacarídeos, como o amido, geralmente é mediada por estruturas localizadas na superfície da enzima que podem ser domínios extras de ligação do amido (SDB) ou sítios específicos para ligação do amido (SBS). Essas estruturas têm sido identificadas em enzimas que metabolizam carboidratos, e suas respectivas funções elucidadas (JANECEK et al., 2013).

Os sítios de ligação de amido são estruturas encontradas principalmente na família GH13, sendo sua presença e número variantes entre α-amilases de diferentes espécies. A α-

amilase pancreática humana, por exemplo, possui até 10 sítios de ligação de ligação ao amido. Já a α-amilase de cevada apresenta dois sítios, os quais foram os primeiros a serem descritos na literatura (MØLLER et al., 2013; ZHANG et al., 2016).

Os SBSs são relacionados a um conjunto de funções especialmente necessárias para reações enzimáticas com macromoléculas biológicas e estruturas supramoleculares como encontradas em paredes celulares de plantas, quitina e grânulos de amido. Essas estruturas atuam como guia entre a enzima e o substrato e auxiliam no posicionamento das cadeias de amido para o sítio ativo, consequentemente, melhorando a hidrólise. Atuam ainda na regulação alostérica, liberação de oligossacarídeos e na interação com as paredes celulares (BÓŽIĆ et al., 2017). A figura 6 ilustra a atuação dos dois sítios de ligação de amido presentes na α-amilase da cevada na catálise enzimática.

Figura 6 – Modelo da ligação inicial entre a α-amilase da cevada e o grão de amido ilustrando a atuação dos sítios de ligação de amido (SBS) presentes na estrutura da enzima.



Fonte: Cockburn & Svensson, 2016.

O SBS1 serve como o ponto inicial de ligação ao grânulo de amido na face cristalino, enquanto o SBS2 tem um papel secundário de se ligar a uma cadeia de amilopectina, e em conjunção com o sítio ativo atua na degradação das cadeias de amilopectina. O modo de ação desses sítios varia de acordo com a quantidade de SBSs e também de cadeias de açúcar envolvidas na reação (COCKBURN & SVENSSON, 2016).

Essas estruturas estão ligadas principalmente a aminoácidos aromáticos, sendo seus principais ligantes triptofano e tirosina. A ligação entre SBS e um resíduo de triptofano foi bem identificada nas α-amilases pancreáticas humanas e de porco e na α-amilase da cevada (ZHANG et al., 2016). Substituições nos resíduos de triptofano em diferentes mutantes da α-amilase de cevada levaram à diminuição da afinidade entre a enzima e o amido e também de sua capacidade de hidrolisar o grão (MØLLER et al., 2013). No entanto, apesar auxiliar na ação enzimática, a presença de SBSs não é um pré-requisito para que a enzima seja capaz de hidrolisar macromoléculas (BÓŽIĆ et al., 2017).

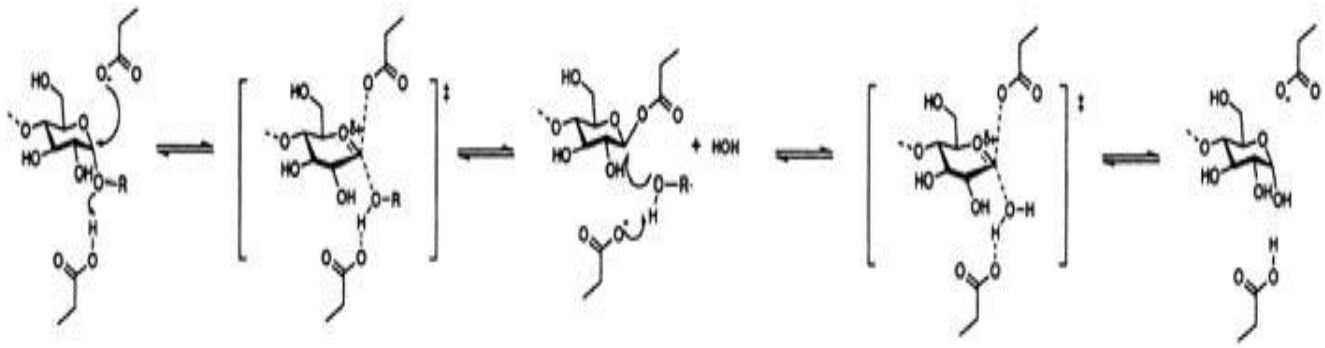
3.1.3 Mecanismo catalítico

O mecanismo catalítico da α-amilase (Figura 7), proposto por Koshland (1953), é dado através de duplo deslocamento α-retenção. Esse mecanismo acontece por cinco passos e envolve dois resíduos catalíticos presentes no sítio ativo: ácido glutâmico (Glu) que atua como catalisador ácido/base e um resíduo de aspartato (Asp) como nucleofílico.

1. Após a ligação do substrato no sítio ativo, Glu, na sua forma ácida, doa um próton ao oxigênio da ligação glicosídica, localizado entre duas moléculas de glicose no subsítio +1, enquanto o nucleofílico Asp ataca o C1 da glicose no subsítio -1.
2. É formado um estado de transição oxocarbono seguido pela formação de um intermediário covalente.
3. A molécula de glicose protonada no subsítio +1 deixa o sítio ativo enquanto uma molécula de água ou uma nova molécula de glicose entra no sítio ativo e ataca a ligação covalente entre a molécula de glicose e o Asp no subsítio -1.
4. Novamente há a formação de um estado de transição oxocarbono;
5. O resíduo Glu – na sua forma básica – recebe um hidrogênio da molécula de água ou de um outro resíduo de glicose recém entrados no subsítio +1. O oxigênio dessa

molécula de água ou glicose substitui a ligação oxocarbono entre a molécula de glicose no subsítio -1 e o Asp, formando: a) um novo grupamento hidroxil no C1 da glicose no subsítio -1, realizando uma hidrólise; ou b) uma nova ligação glicosídica entre a glicose do subsítio -1 e +1, caracterizando uma transglicosilação.

Figura 7 – Mecanismo catalítico da α-amilase.



Fonte: Prakash & Jaiswal, 2010.

O terceiro resíduo catalítico conservado, Asp, atua na ligação a grupamentos OH-2 e OH-3 presentes no substrato através de pontes de hidrogênio, e desempenha importante função na distorção do substrato. Os demais resíduos conservados não catalíticos atuam na orientação correta do substrato no sítio ativo, no posicionamento do resíduo nucleofílico, na estabilização do estado de transição e na polarização da estrutura do substrato (UITDEHAAG et al., 1999; VAN DER MAAREL et al., 2002).

O mecanismo de α-retenção está presente em toda família α-amilase, entretanto, essas enzimas apresentam uma grande variedade de especificidades por substratos e, consequentemente, uma ampla gama de produtos gerados. Essas diferenças podem ser atribuídas à presença de domínios anexos ao domínio catalítico (domínios D, E, F, G e H), bem como os subsítios extras de ligação de amido ou domínios de ligação de amido (SBS ou SBD) (SVENSSON, 1994; VAN DER MAAREL, 2002).

A ligação glicosídica é altamente estável, apresentando taxa de hidrólise espontânea de aproximadamente $2\times10^{-15}s^{-1}$ à temperatura ambiente. A α-amilase aumenta tanto essa taxa que é considerada uma das enzimas mais eficientes já conhecidas (VAN DER MAAREL et al., 2002).

3.2 ASPECTOS BIOTECNOLÓGICOS E APLICAÇÕES INDUSTRIAIS

O mercado global de enzimas movimentou em 2015 4,9 bilhões de dólares, sendo estimado para 2021 US$ 6,3 bi (BCC RESEARCH, 2017). Cerca de 30% desse setor é representado pela α-amilase, a segunda enzima mais utilizada em processos biotecnológicos (GUPTA, 2003). Devido a sua versatilidade, essa enzima atua nos mais diversos seguimentos, abrangendo desde aplicações clínicas a industriais.

No contexto clínico, essa enzima tem sido utilizada como molécula-alvo para diagnóstico e tratamento de doenças humanas como obesidade, diabetes tipo II, hiperlipidemias, pancreatites, fístula pancreática, cáries e estresse agudo (D'AMICO et al., 2000; KOH 2014; ELSAYED et al. 2014; PARTELLI et al. 2014; YAN & WU, 2016). Além disso, estão envolvidas com reações anti-inflamatórias desencadeadas pela liberação de histamina e substâncias semelhantes. Problemas de pele como psoríase, eczema, picadas de insetos, dermatites atópicas e herpes podem ser relacionados e diagnosticados através de deficiências na α-amilase. Mudanças na atividade amilolítica também são relacionadas com alguns tipos de câncer, como de colo do útero e de pulmão (SING et al., 2015).

Já no âmbito industrial, a α-amilase atua nos setores de detergentes comerciais através da remoção de manchas de origem amiláceas; nas indústrias de celulose e papel na remoção do amido aplicado durante o seu processamento; na indústria têxtil, onde atua como agente desengomador; refinamento de açúcares para obtenção de xaropes ricos em glicose e frutose; liquefação, sacarificação e processamento do amido, que é utilizado como processo base de várias aplicações industriais; na produção de biocombustíveis através da conversão de amido em oligossacarídeos fermentáveis em etanol; no tratamento de águas residuais minimizando o efeito poluente dos descartes industriais; e na indústria de alimentos para humanos e animais onde atuam no preparo de alimentos e também na suplementação dietética (VAN DER MAAREL et al., 2002; GUPTA, 2003; KUMAR et al., 2014).

Dentro do mercado enzimático, o setor alimentício tornou-se o mais rentável movimentando em 2016 mais de 1,6 bilhões de dólares (BCC RESEARCH, 2017). A α-amilase é a enzima mais utilizada nesse setor, sendo amplamente empregada na preparação de cervejas, sucos, pães e bolos (TOMASIK & HORTON, 2012).

O desempenho e viabilidade econômica de cada aplicação são influenciados pelas propriedades da enzima, como especificidade, estabilidade térmica e dependência de pH (VAIDYA et al., 2015). Nesse contexto, com o avanço da Biotecnologia e a crescente demanda por enzimas industriais, técnicas têm sido utilizadas para obtenção de enzimas com as características específicas requeridas nas suas aplicações. Essas modificações podem ser realizadas diretamente na proteína utilizando agentes químicos, através da engenharia enzimática, ou a nível genético com auxílio da mutagênese (ISMAYA et al., 2013).

Todavia, é importante considerar que ainda há uma limitação quanto às α-amilases utilizadas na indústria, visto que a fonte dessas se restringe a microrganismos. Além do mais, o número de processos envolvidos para produção dessas enzimas é intrínseco ao custo do produto final, o que torna necessária a busca por fontes alternativas de α-amilases com perfil industrial e que apresentem baixo custo (VAIDYA et al., 2015).

3.2.1 α-Amilases na indústria do amido

O amido é o segundo biopolímero mais abundante na natureza. Essa macromolécula é biodegradável, renovável, e amplamente disponível a baixo custo. A perspectiva da indústria química aponta o amido como um recurso promissor e versátil, que pode limitar ou mesmo eliminar a utilização de materiais de origem fóssil (OKKERSE & BEKKUM, 1996; TOMASIK & GLADKOWSKI, 2001). Devido a tais características, o amido tem sido objeto de estudos e aplicações biotecnológicas (TOMASIK & HORTON, 2012).

A despolimerização do amido pela ação de α-amilases é base de vários processos industriais e, em vista disso, este setor corresponde ao principal mercado para essas enzimas (RICHARDSON et al., 2012). A utilização de enzimas na degradação do amido foi a primeira aplicação em larga escala na indústria alimentícia (KHAN, 2012; PATEL et al., 2015). Enzimas hidrolisadoras de amido podem ser aplicadas na produção de etanol, xaropes ricos em frutose, maltose e glicose para aplicações farmacêuticas ou químicas, e nos setores de detergentes e alimentícios (MEHTA et al., 2016).

A hidrólise do amido pode ser dividida em dois estágios: liquefação e sacarificação. O processo de liquefação hidrolisa o amido em média 30-40%, e tem maltodextrinas como produtos, que são comercialmente valiosas. Devido às suas propriedades reológicas, esses

açúcares são usados na indústria alimentícia como estabilizantes e espessantes na composição de diversos alimentos. Já o processo de sacarificação procede a liquefação com um percentual de hidrólise de 95-97%, convertendo completamente maltodextrinas em glicose, maltose, isomaltose e outros açúcares menores (NOVOZYMES, 2013; TOMASIK & HORTON, 2012). Considerando os produtos gerados a partir da hidrólise, as α-amilases são classificadas como enzimas sacarificadoras ou liquefadoras.

Devido à sua insolubilidade e cristalinidade, o processamento industrial do amido requer o aquecimento dos grãos sob altas temperaturas para torná-lo solúvel. Essa etapa é altamente onerosa, considerando o alto consumo de energia. Contudo, algumas α-amilases são capazes de hidrolisar o amido solubilizado à baixa temperatura, ou até mesmo o grão nativo. Essas enzimas têm se mostrado uma alternativa economicamente superior ao processo convencional, pois elas minimizam o custo e reduzem problemas com viscosidade, otimizando assim o processamento do amido (GOYAL et al., 2005; LIAO et al., 2012).

3.2.2 α-Amilases como aditivo de limpeza em detergentes comerciais

Atualmente, o uso de enzimas na composição de detergentes é considerado como uma das aplicações biotecnológicas mais bem sucedidas, observando-se o consumo de cerca de 40% da produção enzimática mundial (IGARASH et al., 2003). No entanto, esta é uma prática antiga, havendo relatos da aplicação de um extrato bruto pancreático como aditivo de limpeza ainda em 1913 (RAO et al., 1998). Na década de 1960, houve a consolidação desse mercado com a utilização da Alcalase® (Novozymes), subtilisina oriunda do *Bacillus licheniformis* (MAURER, 2004). A partir daí outras enzimas passaram a ser introduzidas nesse setor, inclusive enzimas amilolíticas em 1975 (GUPTA et al., 2003).

Nessas formulações, as enzimas atuam como aditivo de limpeza através da redução da tensão superficial da água e pela degradação específica das manchas. Além disso, a utilização dessas biomoléculas dispensa o uso de produtos cáusticos, tornando a composição do detergente ambientalmente segura (HMIDET et al., 2009).

A α-amilase age sobre resíduos de amido comumente encontrados em molhos, frutas, chocolates etc. O amido atua como “cola” aderindo, além de carboidratos, manchas de outras naturezas bioquímicas, como proteicas e lipídicas. A hidrólise do amido pelas α-amilases

produz dextrinas de baixo peso molecular, oligossacarídeos e açúcares que são mais solúveis que o amido. Deste modo, a ação direta sobre manchas amiláceas otimiza o processo de lavagem, tornando mais fácil a remoção das manchas e impedindo a sua redeposição (ITO & HORIKOSHI, 2004; HMIDET et al., 2009; JURADO-ALAMEDA et al., 2015). α-Amilases, principalmente alcalinas, são o segundo tipo de enzimas utilizadas nesse setor, estando presente na formulação de 90% dos detergentes líquidos (MITIDIERI et al., 2006; VAIDYA et al., 2015).

Para ser aplicada nesse setor, as enzimas devem manter-se ativas frente aos componentes presentes na formulação de detergentes, como agentes oxidantes, surfactantes e quelantes, além de atuarem sob pH alcalino (ITO & HORIKOSHI, 2004; KIRK et al., 2002).

3.3 OBTENÇÃO DE ENZIMAS A PARTIR DO RESÍDUO PESQUEIRO

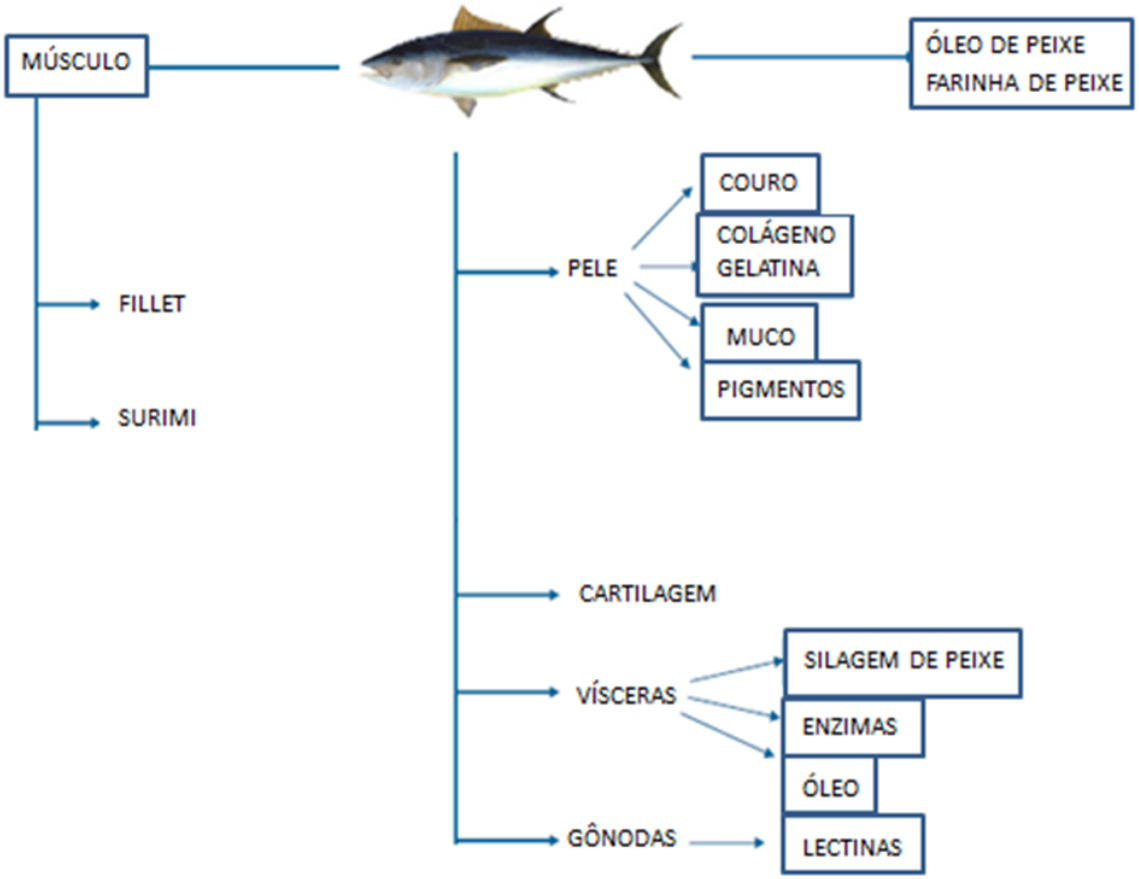
As α-amilases estão amplamente distribuídas na natureza atuando no metabolismo dos carboidratos em animais, vegetais e micro-organismos. Todavia, α-amilases de origem fúngica e bacteriana são as mais estudadas e utilizadas na indústria (GUPTA, 2003).

Apesar dos méritos da utilização de micro-organismos como fonte de enzimas, o monopólio de α-amilases bacterianas e fúngicas limita sua utilização a aplicações específicas, fazendo necessária a busca por novas fontes dessa enzima (SIVARAMAKRISHNAN et al., 2006). Pesquisadores sugerem que resíduos agroindustriais de baixo valor podem ser utilizados para obtenção de enzimas, auxiliando também na redução dos custos de produção e da poluição (SHAMALA et al., 2012; VAIDYA et al., 2015).

Devido à grande diversidade de organismos, o ambiente aquático possui um enorme potencial para descoberta de diferentes fontes de enzimas (SHAHIDI & KAMIL, 2001). No setor pesqueiro, o volume de resíduos pode chegar até 70% da produção total. Diante disso, no intuito de minimizar o impacto ambiental gerado pelo descarte desses resíduos no meio ambiente, pesquisadores têm buscado soluções para promover a reutilização desses valiosos insumos. Embora parte desse material já seja reaproveitado para produção de óleos e farinha de peixe, outras aplicações mais refinadas e rentáveis são possíveis e aumentariam o valor agregado ao produto (BLANCO et al., 2007).

Alonso (2010) afirma que a diversidade de produtos é tão grande quanto os setores industriais que se beneficiariam com as alternativas de valorização. Produtos como quitina, quitosana, colágeno, gelatina, ácidos graxos poli-insaturados, taurina, creatina, proteínas anticoagulantes, enzimas e condroitina sulfato vêm sendo obtidos a partir dos resíduos pesqueiros (LOPES et al., 2015). A utilização desses resíduos, mais propriamente as vísceras, corresponde a uma atrativa fonte de enzimas digestivas (BEZERRA et al., 2000; SOFIA, 2016).

Figura 8. Produtos e subprodutos obtidos através do processamento e utilização integral do pescado.



Fonte: Modificada de BLANCO et al., 2007.

O estudo acerca de enzimas de peixes vem crescendo nos últimos anos. Algumas dessas biomoléculas foram isoladas e caracterizadas e, de acordo com suas propriedades, relacionadas com aplicações nos mais variados setores. Autores sugerem a aplicação de proteases de ariocó, carpa, tambaqui e cioba nos setores alimentícios e de detergentes

(ESPÓSITO et al., 2009; ESPÓSITO et al., 2010; MARCHUSCHI et al., 2010; MEDEIROS et al., 2015), além de proteases de pescada-branca com propriedades colagenolíticas (OLIVEIRA et al., 2014).

Apesar do amplo leque de aplicações, não foram encontrados estudos relacionando a obtenção de α-amilases de peixes e aplicação em setores industriais. O estudo acerca dessa enzima ainda se restringe a investigações sobre fisiologia digestiva e formulação de cultivos e ração para peixes, portanto, uma maior atenção a essas enzimas é necessária.

3.3.1 α-Amilases de peixes

Assim como nos demais organismos em que está presente, nos peixes, a α-amilase desempenha papel crucial no metabolismo de carboidratos. Embora haja uma escassez de estudos abordando os processos bioquímicos envolvidos na digestão desses organismos, estudos qualitativos demonstram similaridades entre as enzimas digestivas oriundas de peixes e demais vertebrados (HIDALGO, 1999).

Nos peixes a expressão de α-amilases sofre influência de vários fatores, como idade, habitat e composição de dieta (JI et al., 2012). Peixes onívoros e herbívoros apresentam maiores níveis de atividade de α-amilase quando comparados com peixes carnívoros, apontando a íntima relação entre seus hábitos nutricionais e a expressão dessa carboidrase. Vários estudos levantam essa hipótese e endossam ainda que o aumento dos valores de carboidratos na dieta do peixe leva a uma maior atividade amilolítica (KUZ'MINA et al., 2003; AL-TAMEEMI et al., 2010; FALCÓN-HIDALGO et al., 2011; KIM et al., 2014; XIE et al., 2016). Outros autores, no entanto, contestam a relação entre atividade enzimática e hábito nutricional, e atribuem o perfil de atividade enzimática apenas ao habitat (CHAKRABARTI et al., 1995; LÓPEZ-VÁSQUEZ et al., 2009).

Hidalgo (1999) relata que existe uma correlação mais próxima entre as adaptações do sistema digestivo dos peixes e sua dieta do que com seu microambiente e categoria taxonômica, independentemente de seu hábito alimentar. Todavia, a α-amilase tem se mostrado mais dependente do hábito nutricional do que enzimas proteolíticas. Essa correlação também pode ser observada em outros vertebrados como pássaros, primatas, e cães (KOHL et al., 2011; PERRY et al., 2007; AXELSSON et al., 2013). A alta atividade da α-amilase em

herbívoros e onívoros pode ser considerada como uma especialização digestiva, visto que permite uma melhor utilização do amido, carboidrato mais abundante na dieta desses organismos.

Essas enzimas apresentam alta similaridade entre espécies de peixes, revelando-se uma estrutura bastante conservada nesses organismos. German (2016), em um estudo filogenético analisando α-amilases de 40 espécies diferentes de peixes observou, em média, identidade de 66,1%, sendo esta similaridade aumentada entre táxons mais próximos, chegando até 98%. Já o alinhamento das sequências de α-amilases de diferentes vertebrados mostrou similaridade de 72% entre o peixe *Siganus cauliculatus* e a α-amilase pancreática humana (XIE et al., 2016). A tabela 1 ilustra a caracterização bioquímica de α-amilases de peixes disponíveis na literatura:

Tabela 1 – Caracterização bioquímica de α-amilases de diferentes espécies de peixes disponíveis na literatura.

Espécie	**pH ótimo**	**Temperatura ótima (°C)**	**Estabilidade térmica (°C)**	**K_m (amido)**	**MM (kDa)**	**Referência**
Pagrus pagrus	7.0	45	50			Fernandez et al., 2001.
Boops boops	7.0	30	30			
Diplodus annularis	6.0 ; 9.0	40	60			
Pagellus erythrinus	7.0; 9.0	35	50			
Oreochromis niloticus	7.0	35			56.6	Moreal et al., 2001.
Sarotherodon melanotheron					55.5	
Taiwan tilapia	8.0	50	50		66.1	Wu et al., 2009.
Scophthalmus maximus	7.0 - 7.5	45				Munilla-Morán & Saborido-Rey, 1996.
Sebastes mentella	4.0-4.5	40				
Sparus aurata	7.0-7.5	40-45				
Barbus paludinosus	8.0 - 9.0					Cokson & Bourn, 1973.
Xiphister mucosus	8.0	50		0.36%		German et al., 2016.
Cebidichthys violaceus	7.5	40		0.33%		
Polyodon spathula					156.3	Ji et al., 2013
Labeo fimbriatus	4.5; 6.5-7.5	25	30		72, 68, 66 e 65	Kushwaha et al., 2012.
Catla catla	6.5				86	Roychan & Chaudari, 2001.
Sparus aurata	8.0	40			100	Alarcón et al., 2001.
Heterotis niloticus	8.45					Ugwumba, 1993.
Labeo fimbriatus	4.5; 6.5-7.0	25				Kushwaha et al., 2012.
Siganus canaliculatus	6.5	25		1.038 mg/ml		Sabaphaty & Theo, 1994

Fonte: Elaborada pela autora.

A presença de α-amilases foi detectada ao longo de todo trato digestivo de peixes, sendo identificada no fígado, esôfago, estômago, cecos pilóricos e secções do intestino de diferentes espécies (UGWUMBA, 1993; CHAKRABARTI et al., 1995; ALARCÓN et al., 2001; JI et al., 2012; IZVEKOVA et al., 2013; XIE et al., 2016).

Observa-se que as α-amilases de peixes mostram certa plasticidade quanto a seu perfil bioquímico, exibindo atividade em ampla faixa de pH, partindo desde faixas ácidas a alcalinas, além de serem termoestáveis sob temperaturas compatíveis com diversas aplicações em variados setores industriais.

Apesar das atrativas características, o estudo de α-amilases de peixes é escasso. Maiores investigações acerca dessa enzima proporcionariam recursos para uma melhor compreensão da bioquímica/fisiologia digestiva dos peixes, além de oferecer uma opção sustentável de fontes de biomoléculas.

3.4 AQUICULTURA

A crescente demanda mundial por pescado nas últimas décadas, gerada pelo aumento populacional atrelado com uma maior busca por hábitos nutricionais saudáveis, tem tornado a aquicultura uma das práticas mais viáveis e promissoras para o suprimento de proteína animal (DE et al., 2014). Estima-se que 16,7% de toda proteína animal consumida por humanos corresponda ao pescado, superando fontes como bovina, avícola, suína e etc. (FAO, 2014).

Em 2014 a produção aquícola mundial foi de 73.8 milhões de toneladas, com valor estimado de 160.2 bilhões de dólares. Quase toda produção aquícola é destinada à alimentação humana, havendo também utilização dos subprodutos para fins não alimentícios (SOFIA, 2016).

Dentre os países com maior potencial para a aquicultura, o Brasil se destaca por sua disponibilidade hídrica, clima favorável e ocorrência natural de espécies aquáticas que compatibilizam interesse zootécnico e mercadológico. A piscicultura continental, a carcinicultura marinha e a malacocultura são os ramos mais desenvolvidos da aquicultura brasileira, que em 2014 produziu 562.5 mil toneladas, conferindo ao país o posto de 14° produtor mundial (BRABO et al., 2016; SOFIA, 2016).

No cenário nacional, a região Nordeste aparece como maior produtora em 2013, com 140.748 toneladas, seguida pela região Sul com 107.448 toneladas, Centro-Oeste com 105.010 toneladas, Norte com 73.009 toneladas e Sudeste com 50.297 toneladas (MPA, 2013).

A piscicultura corresponde à prática com maior representatividade, tendo como espécies mais produzidas em âmbito nacional: a tilápia do Nilo (*Oreochromis niloticus*) (Linnaeus, 1758), o tambaqui (*Colossoma macropomum*) (Cuvier, 1818), o híbrido tambacu (♀ *Colossoma macropomum* x ♂ *Piaractus mesopotamicus*), a carpa-comum (*Cyprinus carpio*) e o pacu-caranha (*Piaractus mesopotamicus*) (MPA, 2013).

3.4.1 *Colossoma macropomum*

O tambaqui, espécie natural das bacias do rio Amazonas e Orinoco, encontra-se amplamente distribuído na parte tropical da América do Sul e Amazônia Central. Esta é a segunda espécie mais cultivada no Brasil, correspondendo a 135.96 mil toneladas em 2015, cerca de 28% da produção nacional (ARAÚJO-LIMA & GOULDING, 1997; IGBE, 2015).

Figura 9. Espécie juvenil de tambaqui *Colossoma macropomum*

Fonte: http://www.fishbase.org.

Pertence à classe Actinopterygii, ordem Characiformes e família Characidae. Pode chegar a medir 100cm e pesar 30kg, sendo o segundo maior peixe de escamas do mundo (GOULDING & CARVALHO, 1982).

Quanto ao seu hábito alimentar, o tambaqui é onívoro com tendência a filtrador. Os adultos se alimentam basicamente de frutos e sementes durante a cheia, e utilizam zooplâncton como complemento durante a seca. Possui fortes dentes molares e rastros branquiais longos, o que possibilita sua diversidade alimentar (GOULDING & CARVALHO, 1982; ARAÚJO-LIMA & GOULDING, 1997; SANTOS et al., 2006). Além disso, possui um elevado número de cecos pilóricos e apresenta um perfil de enzimas para cada secção do trato digestório, podendo ser observada a predominância de proteases no estômago, e presença de lipase e amilases ao longo de todo trato digestório. Adicionalmente, o perfil enzimático digestivo desse animal sofre influência da sua dieta (ALMEIDA et al., 2006).

O tambaqui apresenta alta capacidade adaptativa, tolerando águas com baixas concentrações de oxigênio (~1mg/L) e mudanças de pH, exibindo preferência por ambientes com pH ácido (pH 3.8 - 4.9), como as águas do rio Negro, além de ser bastante resistente a doenças (SILVA et al., 2007; ARIDE et al., 2007).

3.4.2 *Oreochromis niloticus*

A tilápia, *Oreochromis niloticus* (Linnaeus, 1758), espécie tropical e subtropical pertencente à ordem Perciformes e à família Cichlidae, é um peixe exótico de origem africana, introduzida no Brasil no ano de 1971. Ela corresponde à espécie com maior expressão mundial e nacional, sendo cultivada em 135 países. Em 2015 foram produzidas cerca de 219.3 mil toneladas de tilápia no Brasil, o que corresponde a 45,5% de toda produção pesqueira (FAO, 2014; IBGE, 2015).

Essa espécie habita águas quentes com temperatura entre 21 e 35°C, e pH entre 6 e 9, apresentando sinais de asfixia quando exposta a pH ácido. Além disso, apresenta uma considerável resistência a ambientes com baixas concentrações de oxigênio, alta salinidade e concentrações de amônia (KUBITZA, 2000; IGARASH, 2008; DELONG et al., 2009).

A tilápia apresenta hábito alimentar herbívoro e onívoro, se alimentando principalmente de fitoplâncton, pequenos invertebrados, algas, sementes e detritos. Adicionalmente, esse animal pode filtrar alimentos através da captura de partículas em suspensão, como bactérias e fitoplâncton. Pode alcançar até 5 kg (NAGASE, 1964; ROTTA, 2003).

Figura 10. tilápia do Nilo (*Oreochromis niloticus*)

Fonte: http://www.fishbase.org

Várias enzimas intestinais foram reportadas no trato digestório da tilápia, como amilases, pepsina, tripsina, esterases e fosfatase alcalina, mas assim como outros peixes herbívoros, a tilápia apresenta um perfil enzimático rico em carboidrases quando comparados com proteases, e baixa expressão de lipases (LI & FAN, 1997; TENGJAROENKUL et al., 2000).

KINETIC CHARACTERIZATION OF A α-AMYLASE FROM NILE TILAPIA (*Oreochromis niloticus*) PROCESSING WASTE

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Abstract

A α-amylase was purified from Nile tilapia processing waste, and characterized for kinetic parameters. It was most active at pH 5.0 to 6.5, and exhibited thermal stability until 50°C. The influence of pH for catalytic mechanism was evaluated, and was observed apparent k_m $8.47x10^{-5}$ and $1.455x10^{-4}$, k_{cat} $7.53x10^{-2}$ and $3.436x10^{-2}$ (s^{-1}), and k_{cat}/k_m $8.89x10^{6}$ and $2.361x10^{6}$ ($s^{-1}.M^{-1}$), respectively, for pH 6.0 and 7.4, showing the pH importance. The α-amylase was highly activated by chloride ion, which acts as allosteric activator, increasing the velocity, kinetic features and thermal stability of enzyme. In the presence of EDTA and EGTA (1mM) the activity was not lost, suggesting an independency of metal ions. The α-amylase of Nile tilapia showed interesting characteristics, which propound the study and recovering of enzyme from fish processing waste.

Keywords: chloride site; calcium site; allosteric activation; pH dependency

1 Introduction

The α-amylase (EC 3.2.1.1) belongs to glycosyl hydrolase family, and its function is to cleave internal 1,4-α-D-glycosidic bonds in starch and related carbohydrates, generating a wide variety of products, ranging from glucose and maltose to maltodextrins. This enzyme plays an imperative role in the carbohydrate metabolism, thus being ubiquitous in nature [1,2].

Its structure is composed of a single polypeptide chain folded in three large domains: A, B and C. The domain A contains residues folded into a $(\beta/\alpha)_8$-barrel structure, named TIM-barrel, the conserved catalytic core. Domain B is a protrusion between third strand and third helix of $(\beta/\alpha)_8$ barrel having irregular β-like structure. It is responsible for the substrate specificity and also for the stability of the enzyme. Finally, the domain C, located in the C-terminus, forms an antiparallel β-sheet that is thought to stabilize the catalytic site of the enzyme by shielding the hydrophobic patch [3–5]. Only 4 amino acid residues are invariantly conserved in the amylases, Arg 173 plus the three catalytic residues: Asp 212, Glu 248 and Asp 315 (numbering for fish *Siganus canaliculatus*). Despite the low homology between α-amylases from different organisms, in animals this enzyme have been highly conserved through evolution, showing similarity of 72% between human and fish, and 79-80% between some species of fishes [6] . Moreover, previous studies report the presence of specific biding sites for Ca^{2+} (located at the interface between domains A and B) and Cl^- (near the active site) in all α-amylases from animals [7,8].

In addition to its importance on carbohydrate metabolism, α-amylases corresponds to the second class of enzymes most required in the industry, equivalent to about 30% to the global market for enzymes [9]. They are widely used in many segments, like processing of starch, detergent, food, brewing, textiles, ethanol production, paper and in synthetic chemistry for the production of oligosaccharides. Furthermore, they can be exploited for the development and designing of therapeutic agents against type II diabetes, obesity, hyperlipidemia, caries and as antibiofilm agent [2,10–12].

Despite to be found in several organisms, α-amylases from bacteria and fungi are still the most studied, and consequently the most commercially applied. Due to this monopoly, there is few reports addressing the study and application of these enzymes from others organisms [13].

The study of digestive enzymes from fishes revealed molecules with interesting characteristics for industrial applications. These reports point to the necessity of elucidate the features of these enzymes, contributing for the knowledge of enzymatic mechanisms in these animals, and also for using these raw-materials in the recovery of biomolecules from the fish processing wastes, mainly viscera [14–17]. In addition, the use of these residues is environment friendly, contributes to the addition of value to the fish industry besides that promote the discovery of new enzymes with low cost.

Reports suggest the presence of several digestive enzymes in *Oreochromis niloticus* stomach and intestine, which may be exploited to recover and evaluate the potential of enzymes like trypsin, pepsin and amylase. [18]. Therefore, this work aimed to characterize the α-amylase obtained from *Oreochromis niloticus* processing waste according to their physico-chemical and kinetic parameters investigating the role of pH and chloride in their features.

2 Materials and methods

2.1 Purification

Viscera from *Oreochromis niloticus* was obtained at EAJ-UFRN (Escola Agrícola de Jundiaí, RN, Brazil). The tissue was homogenized in 10mM sodium-phosphate buffer, pH 7.5 (200 mg/mL) and centrifuged at 10.000xg for 25 min at 4°C to obtaining crude extract. The enzyme was purified through a three-step procedure: thermal treatment through incubation at 40°C for 30 minutes; saline fractionation by 30-60% ammonium sulfate; and gel filtration chromatography Superdex G-200 by Äkta FPLC Purifier (Akta Start, GE Life sciences, United Kingdom). The protein content was estimated according Bradford assay (Bradford, 1976). The purification was monitored with the aid of SDS-PAGE and zymogram.

2.2 SDS-PAGE and zymogram

Polyacrylamide gel electrophoresis (SDS–PAGE) was carried out following methodology described by Laemmli [19] using a 4% (w/v) stacking gel and a 12.5% (w/v) separation gel. After that, the gel was colored with silver stain. After electrophoretic run, zymogram was performed as described by Fernandéz [20] using starch 2% as substrate, and then the active band was revealed with iodide/iodine solution.

2.3 Enzyme assays and kinetics

The amylolytic activity was measured using the chromogenic substrate 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNP-G3) from Sigma®. Briefly, an aliquot of purified α-amylase was incubated with 10mM MOPS buffer and 200µM CNP-G3. The activity was monitored at 37°C and 405nm during 10 minutes with interval measurements from 10 seconds using the Synergy H1 hybrid reader spectrophotometer from Biotek®. The enzyme velocity was calculated fitting the slops through Grafit software (v.5.0, ERITHACUS Software).

2.3.1 Kinetics assays

The kinetic parameters were evaluated with the chromogenic substrate CNP-G3 at concentration ranging from 0 to 3mM at different pH (6.0 and 7.4) for 30 minutes. Besides that, the relationship with chloride and the kinetics features was evaluated. The Michaelis-Menten constant and the double reciprocal Lineweaver Burk was fitted by the Grafit software (v.5.0, ERITHACUS Software).

2.3.2 Effect of pH and temperature and salts

The effect of pH on purified enzyme was evaluated at 37°C in a range of 2-12 using a buffer systems of constant ionic strength (25mM glycine, 25 mM acetic acid, 25 mM MES and 75 mM Tris). The thermal study was carried at range 40°-70° at pH 6.0 and 7.4. The purified enzyme was incubated for 60 min in each temperature, subsequently cooled and measured the residual activities. The maximum activity was taken to 100%. In order to elucidate the relationship between the chloride and calcium sites and enzymatic activity and stability, the experiments above were also carried out in the presence of 1mM chloride (KCl) and calcium ($CaSO_4$).

2.2.3 Effect of metal ions and chelating agents

The relationship between amylolytic activity and metal ions was observed through the change in the velocity of the enzymatic reaction. In order to do so, the salts sodium acetate

($C_2H_3NaO_2$), NaBr, KCl and KI were used in various concentrations until the stagnation of velocity. To elucidate the relationship between the α-amylase activity and calcium binding site, enzymatic assays were carried with 1mM chelating agents (EDTA and EGTA).

2.3.4 Enzymatic inhibition

To investigate the enzymatic inhibition, the α-amylase assay was performed with addition of the specific inhibitor type 1 from *Triticum aestivum* (Sigma®) at concentrations ranging from 0-50 µg/mL. The amylolic activity was measured as described above, and then the IC-50 was calculated.

3 Results and Discussion

3.1 α-Amylase purification

The α-amylase from Nile tilapia (*Oreochromis niloticus*) intestine was purified through three steps: thermal treatment; saline fraction by ammonium sulfate (30-60%) and Superdex G-200 chromatography. The SDS-PAGE (Figure 1) revealed a majority band with about ~50 kDa, and the zymogram showed a single active band. The α-amylase was purified ~76 folds with yield of ~38.4% and specific activity of ~8265.9 (U/mg). Molecular weights of α-amylases from fishes available on literature vary from about 55.5 (*Sarotherodon melanotheron*) to 156.3 kDa (*Polyodon spathula*) [21,22].

Few reports of α-amylases from animals, particularly fishes, are available on literature when compared to microbial enzymes. Thus, the necessity to find new sources of enzymes is widely reported in the literature [23,24]. In this work from 1g of viscera (a fishery processing waste) it was possible to obtain 217.5 (U/mg) of α-amylase. Therefore, this enzyme may be considered a viable alternative to those bacterial and fungi enzymes, which are still the main sources for commercial amylases.

Many authors have pointed to the reuse of agroindustrial processing waste as an alternative source of enzymes [13,24]. The increase on industrial production and processing of fish has generated a great amount of residues, despite having high biotechnological value, are discarded leading to environmental and economic loss [25]. Thus, the utilization of fish

processing waste as source of enzymes is an attractive alternative to better manage the residues coming from fishing activity [13,26]. Many enzymes have been isolated from fish viscera, but so far there are few studies addressing the recovery of fish α-amylase [14–17], leading to the need of further studies.

3.2 Kinetic features

The kinetic parameters of α-amylase were elucidated and summarized on Table 1. In order to verify the action of pH at enzymatic mechanism, the assays were performed at pH 6.0 and 7.4. The α-amylase showed apparent k_m of 0.08mM at pH 6.0 and 0.14mM at pH 7.4; similarly to pancreatic and salivary human α-amylase, which showed k_m of 0.19 and 0.66mM at pH 6.0 [27,28]. These results have shown the interference of pH on the enzymatic affinity, once k_m was increased in almost two folds at a higher pH (7.4). In general the pH dependence of k_m reflects the involvement of groups that are essential to initial substrate binding events that precede catalysis [27]. Thus, at pH 6.0 the enzyme seems display a better affinity toward the substrate, probably due to the ideal ionization of these groups.

The pH variation also leads to change in turnover and specificity constant values (k_{cat} and k_{cat}/k_m). At pH 6.0 these parameters were enhanced, showing a better enzymatic performance in comparison to pH 7.4. Effects of pH on k_{cat} mainly reflect acid-base group involvement in the catalytic steps of substrate to product conversion; that is, these ionization steps occur in the enzyme-substrate complex [27]. Additionally k_{cat}/k_m as a function of pH reflect the essential ionizing groups of the free enzyme that play a role in both substrate binding and catalytic processing [29]. The catalytic activity of α-amylase has been known as limited at extreme pH, but so far the interference of intermediary values of pH on the catalytic mechanisms remain indeterminate[30]. These results suggest that all catalytic mechanism steps of α-amylase are influenced by pH.

It is known in the literature that chloride ion has some influence on amylase activity, acting as allosteric activator in chloride dependent α-amylases [8,31,32]. For Nile tilapia α-amylase, the presence of 1 mM chloride promoted an increasing on V_{max} of ~15 folds at pH 6.0 and ~19 folds at pH 7.4. Additionally, the k_m of α-amylase was increased, showing that chloride improves the V_{max} of enzyme, but did not enhance the affinity to the substrate. This finding is in disagreement with reported for human α-amylase, for which chloride ion does not change the affinity towards enzyme and substrate [32,33]. The chloride ions also enhanced the values of k_{cat} and k_{cat}/k_m ratifying its role as a natural allosteric effector for α-

amylases. Previous studies reported that the removal of chloride of α-amylase structure decrease the k_{cat} in 10 folds [32,34].

This activation is associated to increase in the pKa values of the catalytic residue Glu. The major residues binding for chloride are Arg or Lys, both having positive charge. So, in the presence of chloride, the pKa of catalytic residues Glu is fine-tuned for maximal activity because Cl^- is shielding these residues from the electrostatic influence of the protonated Arg/Lys residue. Among the catalytic residues, Glu is closest to chloride bound and is most likely affected by its removal. This ion allows Glu to be protonated at the pH of maximal enzymatic activity, leading to an alkaline pKa shift. Besides that, chloride site promote the correct positioning of the catalytic residues required for high activity [35,36].

The k_d of chloride, as seen on Table-1, ratify the strong affinity with this ion toward α-amylase from Nile tilapia. The results were similar to found for the fish *Oryzias latipes* and porcine α-amylases, 0.7 and 0.3-0.4 mM, respectively [37]. Additionally the k_d also undergo interference by change in pH values, since it was ~2 folds higher at pH 7.4 (0.517 mM) when compared to pH 6.0 (0.225 mM), suggesting that the chloride site is more accessible at slightly acid pH.

3.3 Physico-chemical characterization

3.3.1 pH and thermal studies

The α-amylase from Nile tilapia showed an activity peak at pH 5.0, as seen at Figure 2-A. The pH profile showed activity between 5.0 and 7.0, and revealed a preference for a slight acid pH, using CNP-G3 as substrate. At pH 8.0 the α-amylase was completely inactive. α-Amylases from other fish had optimum activity at pH varying from 4.0 to 9.0, showing no pattern among different species of fish [20,38].

The α-amylase active site consists of charged groups, a nucleophilic aspartate and the catalytic hydrogen donor glutamic acid. So, α-amylase catalysis is limited by the protonation of the nucleophile at low pH and by deprotonating of the hydrogen donor at high pH. This justifies the inactivation of α-amylase at extreme pH [30]. In addition, it is important to note thath the optimum pH can vary according to the substrate, due to interactions with the catalytic residues present in the active site [39].

Moreover, it is known that chloride usually broadens the pH-activity profile, shifting it

to alkaline media, an order to get physiological values [32]. However, the pH pattern of α-amylase from Nile tilapia did not show relevant changes when incubated with 1mM chloride. It was observed a slight shift in optimum pH (5.0 to 5.5 with 1mM chloride), but there was no increase in the pH-activity range, and maybe a higher concentration of ion is necessary to raise the pH range.

The α-amylase had maximum activity at 42°C and was thermostable until 50°C at pH 6.0 and 7.4 (Figure 2-B; C). At temperatures above that, activity was lost gradually. This result is in agreement with reported to α-amylases from other species of fish that shows thermostability between 30-60°C [20,40]. Some authors report that there is correlation between the environment temperature where the fish lives and the thermal profile of its digestive enzymes; usually it is higher than habitat temperatures [40]. However, it is important to consider that the enzyme thermostability is mainly associated with the amino acid pattern, e.g, a greater number of cysteine or methionine residues and then a greater number of disulfide bonds. Additionally, this thermal profile may categorize the α-amylase as an ITS (intermediate thermal stable) enzyme; a group of mesophilic enzymes, which has gained industry attention because does not require high temperatures to work [30].

The roles of chloride and calcium for α-amylase from Nile tilapia and the relationship of the ion with the maintenance of the enzyme structure were investigated. For this, the temperature tests were performed in 1mM KCl (Figure 2-B,C) and $CaSO_4$ (Figure 2-D,E). The results suggest that the chloride caused a slight increase in the thermostability of α-amylase. Overall, chloride increased the stability of α-amylase in 25% at temperatures above 50°C, suggesting a protective action against thermal denaturation. However, structural assays are required to ratify and to better understand this finding. It is already known that Ca^{2+} induce thermal protection to calcium-dependent α-amylases [41,42], however, the thermostability of α-amylase from Nile tilapia was not increased in the presence of 1mM Ca^{2+}.

Additionally, the α-amylase thermal profile seems do not have pH dependency, once it was not observed great difference between pH 6.0 and 7.4. The pH seems to interfere only with the chloride binding mechanism, binding more strongly to the enzyme at pH 6.0, and conferring higher stability to the enzyme at this pH.

3.3.2 Effect of ions and chelating

The α-amylase from Nile tilapia was highly activated in presence of chloride. An abrupt increase in enzyme velocity can be observed even at low concentrations of chloride (Figure 3-A). This ion has been reported as an important allosteric activator of some α-amylases [43]. Metal ions are commonly involved in a variety of important functions in proteins such as protein folding and catalysis; however, use of inorganic anions, particularly Cl^-, for protein function is rare [36]. Chloride enhances the catalytic efficiency of the enzyme by elevating the pKa of the hydrogen-donating residue in the active site, and acts in the correctly positioning of catalytic residues or other residues involved in the stabilizing the enzyme-substrate complex [36,41]. Previously, only mammalian α-amylases were known as chloride dependent, but some reports show that chloride dependence is archetypal of animals and in some Gram-negative bacteria [43]. Nevertheless, so far none study reports the relationship between α-amylases from fishes and chloride site.

Earlier structural studies of wild-type human pancreatic α-amylase suggested this site would likely be restricted to chloride binding, but other studies indicate that some alternative anions can induce some level of enzymatic activation despite very different atomic geometries, sizes, and polyatomic natures, attesting a plasticity of the chloride binding site [8]. The effect caused by chloride binding in Nile tilapia α-amylase was also observed in a lesser extent by other monovalent ions, such as Br^- e I^-. In the presence of these ions the velocity increased, but was needed higher ion concentrations. In Nile tilapia α-amylase, while the Cl^- (0.5 mM) increased the velocity more than 10 fold, Br^- (1mM) and I^- (50mM) increased in 6 and 9 fold the velocity of α-amylase, respectively. These results show that other ions could acts as effectors, but there is a preference for chloride on the α-amylase anion binding site. Besides that, it confirms that chloride dependent α-amylases need a negative charge to enhance its full activity [31]. Moreover, authors report that the removal of chloride decreases the k_{cat} of α-amylases, showing that chloride free α-amylases show less activity, and also that the variation on the ion concentration can modulate the enzyme activity [44].

As related to others reports, the anion acetate was not able to activate the enzyme, probably due to its size, which is stereochemically incompatible for fitting in the chloride binding site [8]. The decreased of velocity seen in the presence of acetate can be explain by distortion caused for its larger molecule. This distortion increase according the ion size, resulting in a less favorable orientation for catalysis. Thus, ions of the size of CH_3COO^-

cannot be fitted into the chloride site. In addition, it is known that the α-amylase possesses a cleft for the anion which can accommodate anions up to the size of 0.54 nm but is most suitable for the size of a chloride anion (0.35nm) [33].

Most of the α-amylases are described as metalloenzymes, which requires at least one ion of calcium to maintain enzymatic structure [45]. Consequently, chelating agents like EDTA and EGTA usually decrease the activity of these enzymes. Nevertheless, the α-amylase from Nile tilapia showed some characteristics suggesting being a putative calcium-independent enzyme. When incubated with EDTA and EGTA at 1mM, its activity does not decreased, showing a chelating resistance. This finding indicates that the calcium is not essential to activity and structure of α-amylase from Nile tilapia. This behavior has been observed in some bacterial α-amylases, and it has been considered an important characteristic for industrial proposes because these enzymes do not require addition of calcium to full work and would be resistant to chelating agents, which is an interesting feature aiming industrial application [30,46,47]. Calcium usually plays an important role on thermostability of several enzymes [41], although this ion was not able to increase the thermo resistance of α-amylase from Nile tilapia.

3.3.3 Enzymatic inhibition

The enzymatic inhibition of α-amylase was performed with of the specific α-amylase inhibitor type 1 from *Triticum aesvitum* (Sigma®). The inhibition curve was plotted and IC50 was calculated and found to be 3.35 μg/mL. At 20.0 μg/mL of inhibitor the enzyme activity was completely absent. This result corroborates that the enzyme is an α-amylase, and also provides information about the response of α-amylase toward several inhibition dose, which is usually applied in investigations for designers of therapeutic agents for diseases, such diabetes and obesity [48]. In addition the inhibition rate could to vary for α-amylases from different organisms. Different inhibition values for α-amylases from different fishes were found and correlated to specificity of each isoforms [20].

4 Conclusions

In this work an α-amylase from Nile tilapia intestine was purified in a three-steps method and characterized according its kinetics parameters. It was possible to observe an enzyme highly activated by chloride, which beside to be an allosteric activator, seems to improve the thermostability of α-amylase. This enzyme showed a mesophylic profile and pH dependence. It was chelating resistant, suggesting that calcium is not necessary for its activity. The technological methods used in extracted and application of fish enzymes is an interesting approach to turn a cheap-raw-material into valuable industrial grade new enzymes.

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Tables and Figures

Figure 1 – SDS-PAGE and zymogram of α-amylase from Nile tilapia. Line 1 – molecular weight marker; Line 2 – final purification step (superdex G-200 chromatography), showing a majority band of ~50 kDa; Line 3 – zymogram of α-amylase showing a single active band.

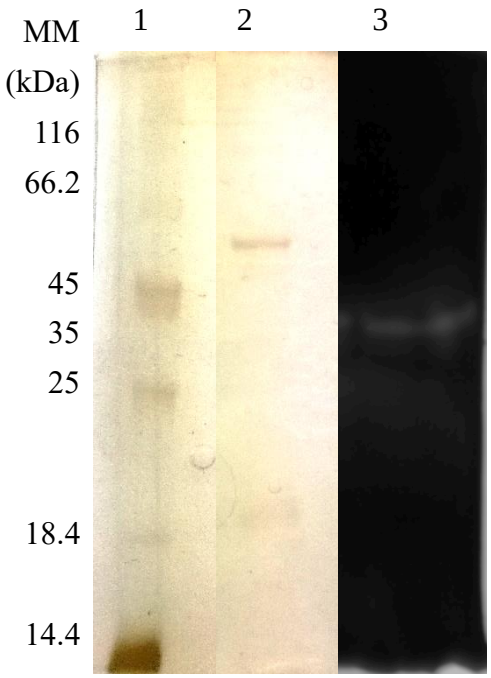


Table 1 – Kinetic parameters of α-amylase from Nile tilapia using CNP-G3 as substrate. The assays were carried at pH 6.0 and 7.4; and also with chloride 1mM. The Michaelis-Menten constant and the double reciprocal Lineweaver Burk was fitting by the Grafit software (v.5.0, ERITHACUS Software).

Parameter	**pH 6.0**	**pH 6.0 + Cl^-**	**pH 7.4**	**pH 7.4 + Cl^-**
K_m (M)	$8.470 \cdot 10^{-5}$	$2.251 \cdot 10^{-4}$	$1.455 \cdot 10^{-4}$	$5.176 \cdot 10^{-4}$
V_{max} (µMol/mL/s^{-1}/mg ptn)	0.0198	0.2975	0.009	0.1785
K_{cat} (s^{-1})	$7.533 \cdot 10^{2}$	$1.130 \cdot 10^{4}$	$3.436 . 10^{2}$	$6.78 . 10^{3}$
K_{cat}/k_m ($s^{-1}.M^{-1}$)	$8.890 \cdot 10^{6}$	$5.019 . 10^{7}$	$2.361 . 10^{6}$	$1.309 \cdot 10^{7}$
K_d (mM)	0.225		0.517	

Figure 2 – Physico-chemical features of α-amylase. Optimum pH of α-amylase (A); Thermostability of α-amylase pH 6.0 (B) pH 7.4 (C).; Native enzyme (▪); 1mM chloride ◦ ( ); 1mM calcium ( ).

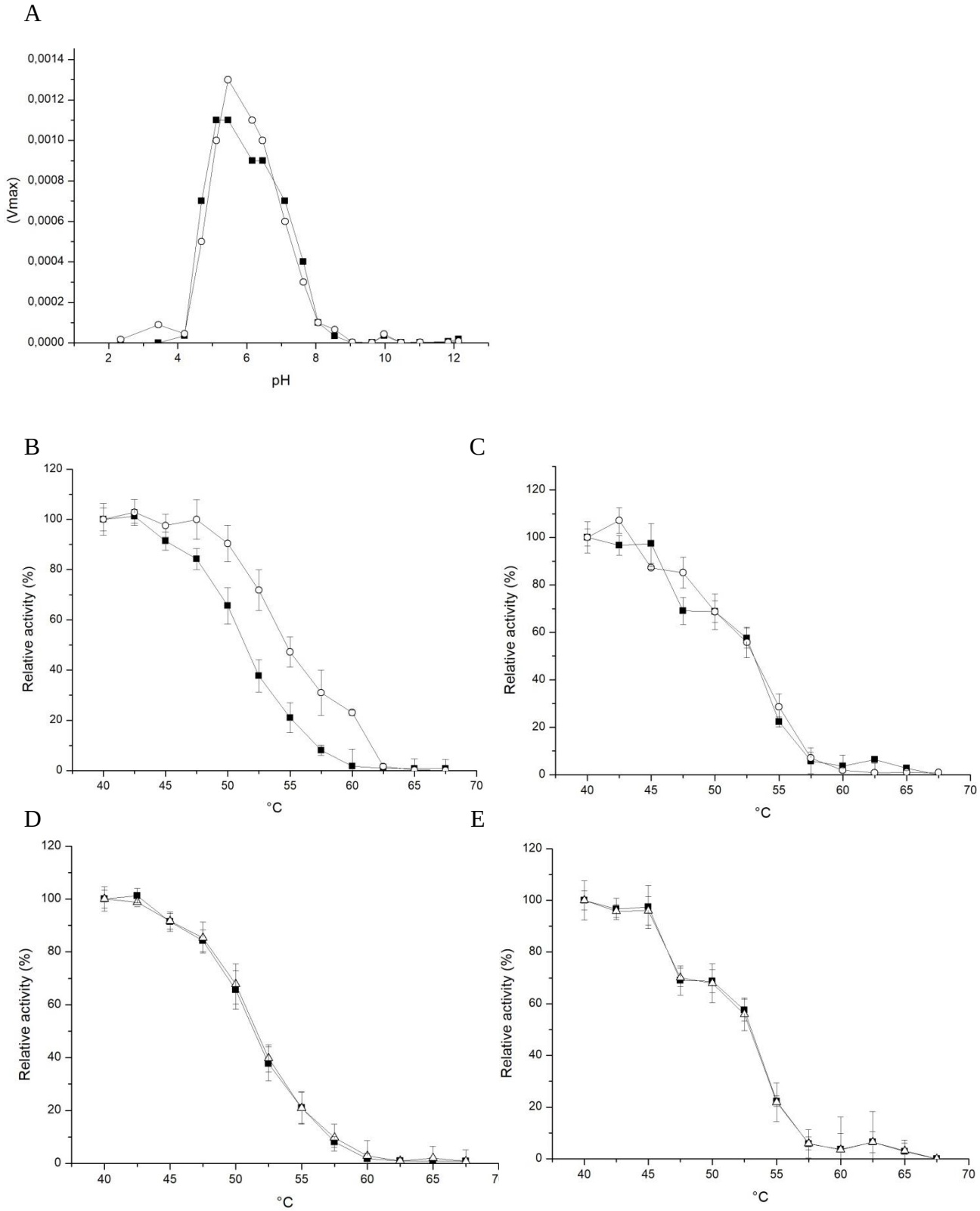


Figure 3 – Effect of different ions on velocity of α-amylase from Nile tilapia. A – potassium chloride; B – sodium bromide; C – potassium iodide; D – sodium acetate.

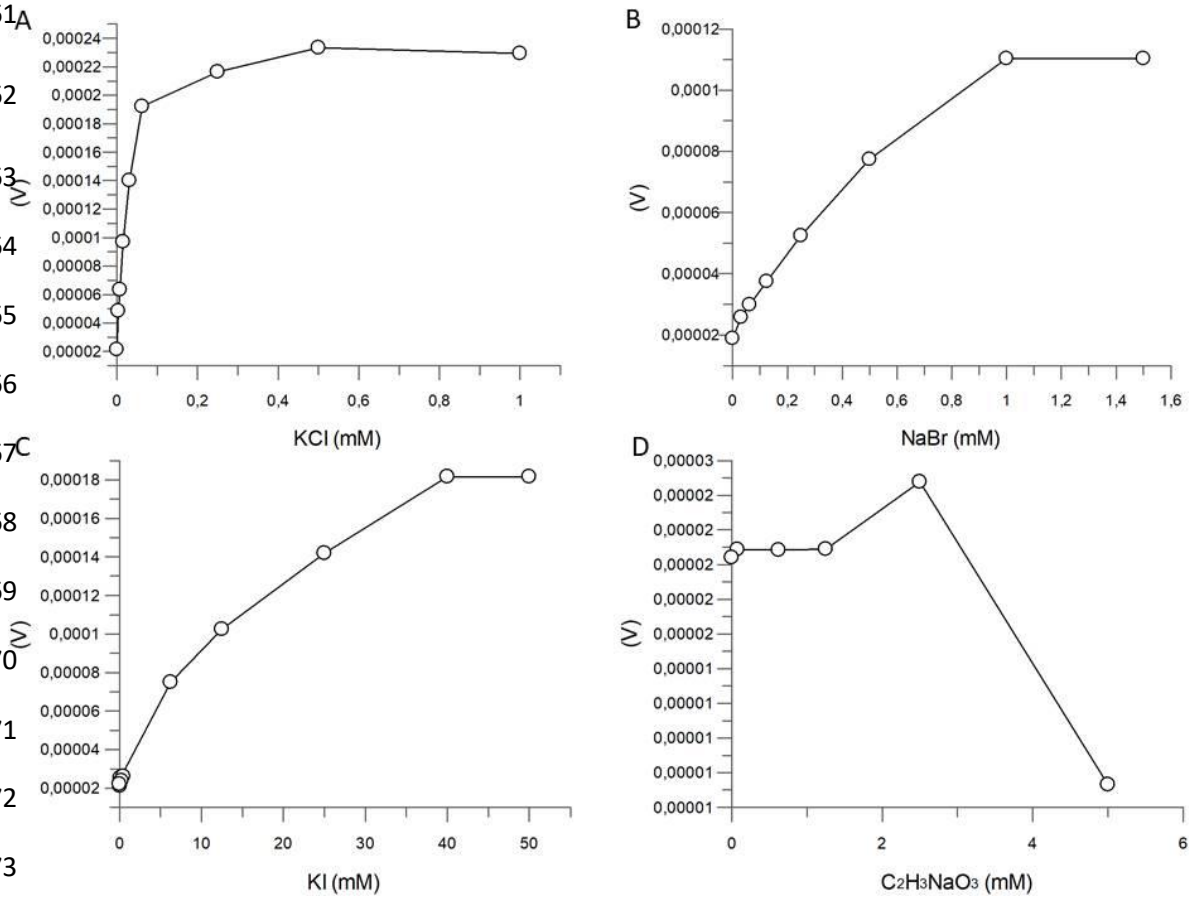


Figure 4 – Effect of chelating agents EGTA and EDTA (1mM) on activity of α-amylase.

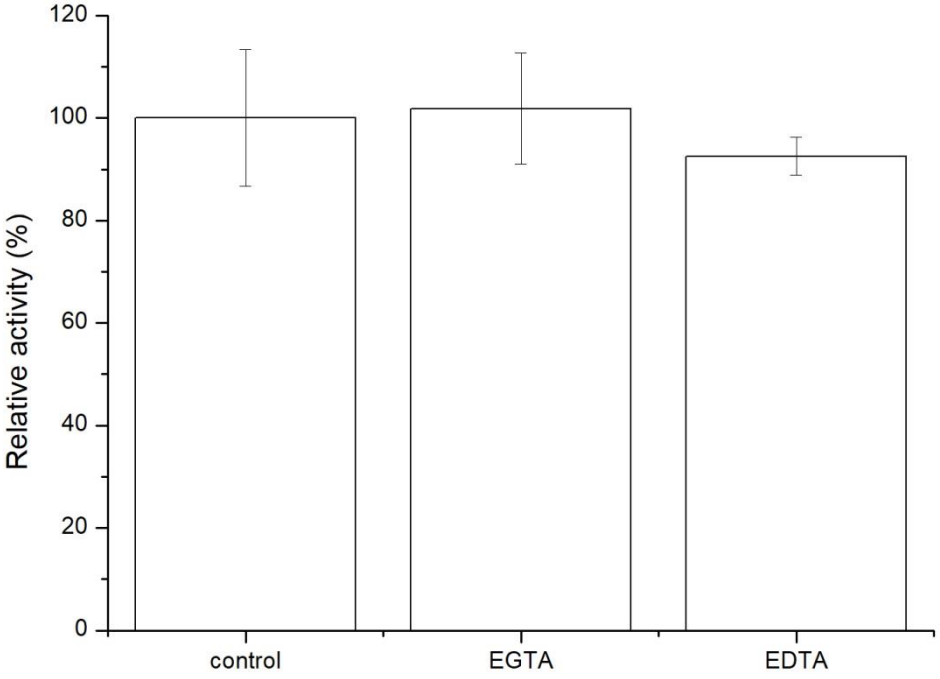
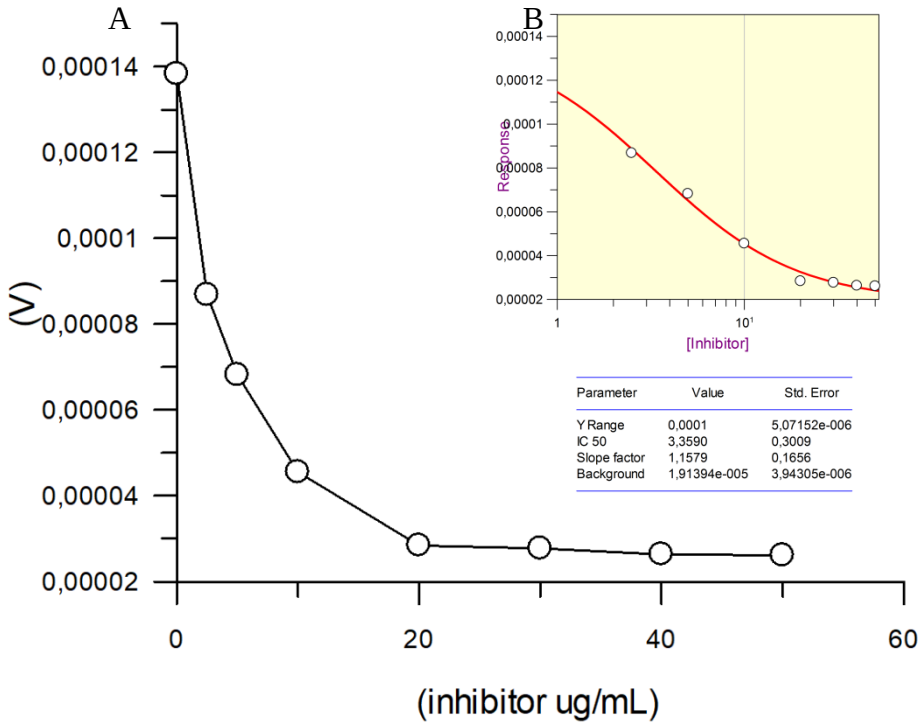


Figure 5 – Enzymatic inhibition of α-amylase (A) and IC-50 (B) of specific inhibitor of *Triticum aestivum*. The assay was carried out with inhibitor concentration ranging from 0 to 50 μg/mL.



A RAW STARCH DIGESTING α-AMYLASE FROM NILE TILAPIA (*Oreochromis niloticus*) VISCERA

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Abstract

In this work a potential raw starch digesting α-amylase from Nile tilapia processing waste was identified. The α-amylase (AMY-T) with estimated molecular weight of 50kDa was purified and characterized. AMY-T showed an apparent k_m 4.78 mg/mL and V_{max} 0.44 (mg/mL/min) to soluble starch. It was highly stable for 24 h over pH range from 3.0 to 10.0, and to the organic solvents: methanol, isopropanol, butanol, dimetylformamide, dimethyl sulfoxide and diethyl ether. AMY-T was able to digest different carbohydrates, showing preference for endo bonds. It was an effective enzyme in adsorbing and degrading raw potato starch with high hydrolysis degree (45%) and under temperatures above to related for others raw digesting α-amylases (40°C). The TLC and HPAEC-PAD analysis showed the end products of raw starch hydrolysis as glucose, maltose and maltodextrins, and identified the polymerization degree of oligomers ranging from 1 to 8. The SEM analysis of starch granule corroborates the enzymatic attack caused by AMY-T. These features suggest the application of AMY-T on the starch hydrolysis and modification for industrial uses.

Keywords: Digestive enzymes, fish processing waste, starch hydrolysis

1 Introduction

Starch is one of most important polysaccharides present in nature, being the major form of energy storage of higher plants. It is composed of two macromolecules: amylose and amylopectin. Amylose is a linear molecule composed of glucose units linked by α-1→4 glycosidic bonds and represents 20-30% of the polysaccharide, while amylopectin is highly branched consisting of numerous short chains of (1-4)-α-D-glucans that are interconnected by α-1-6 linkage, corresponding to 70-80% of starch structure. The starch granule is formed basically by two structures: an amorphous corresponding to amylose and a part crystalline made of amylopectin [1–3].

Enzymatic processing of starch was the first industrial enzyme-driven process carried in 1840, since then the starch market has grown, reaching several areas of application. Besides to its great importance and in human and animal food, starch is also widely used in many sectors, such as bioethanol, pharmaceutical, paper and textile industries [4,5].

Considering the crystallinity of raw starch granule, the most of these applications requires the disruption of its resistant structure. This process usually is carried out through high temperatures, acid, alkaline or enzyme treatments. The use of enzymes, mainly amylolytic enzymes, has gained attention for many reasons: their "green value" in manufacturing reduces energy costs, also provides better control of the products generated and allows understanding the structure of starch granules [6].

α-Amylases (E.C 3.2.1.1) are the main enzymes applied in starch processing. They act hydrolyzing α-1,4-glycosidic bonds in starch and related carbohydrates generating a wide variety of products with α-anomeric configuration, ranging from glucose and maltose to maltodextins. These enzymes are present in fungi, bacteria, plants and animals, playing an important role in carbohydrate metabolism [1,4]. α-Amylases for industrial purposes have been obtained mainly from bacterial and fungi sources, but even these microbial enzymes have limitations, so researchers together with industrial partners are interested in discovering alternative sources of enzymes, which shows compatibility with features required for industrial uses, like pH and temperature, and also offers low cost [7]. Since each different application requires amylases with unique properties, it is often necessary to search the biodiversity for new sources of these enzymes [8,9].

Some authors have reported the use of fishery processing wastes as source of biomolecules with biotechnological properties, and highlight that it corresponds to a valuable

input. Despite that, these byproducts are generally discarded generating a high environmental onus due to contamination of water streams and sea coast with these highly perishable processing wastes [9,10]. Studies addressing the recovery of these biomolecules point to obtaining of enzymes from the fish viscera, and suggest their application in various industrial sectors, like food and detergents industries, and environmental monitoring [11–14]. Thus, the recovery of these byproducts for biotechnological purposes is an important alternative for better management of fishing processing waste.

Nile tilapia (*Oreochromis niloticus*) is one of the fish with greatest expression on global aquaculture. The farming of tilapias is present in 135 countries on all continents, corresponding to the most widespread type of aquaculture in the world. The industrial processing of tilapia generated 2.765 million tonnes of waste and by-products in 2011, a high amount of residues which could find biotechnological utilization as a source of enzymes [15].

Therefore, considering the industrial demand for α-amylases and the necessity to better reuse the aquaculture processing waste, this work aims to recovery α-amylases from Nile tilapia viscera and to evaluate its application on raw starch hydrolysis and the potential of tilapia intestinal alpha-amylase as a starch-digesting enzyme.

2 Materials and Methods

2.1 α-Amylase purification

Viscera from *Oreochromis niloticus* was obtained at EAJ-UFRN (Escola Agrícola de Jundiaí, RN, Brazil). The tissue was homogenized in 10mM sodium-phosphate buffer, pH 7.5 (200 mg/mL) and centrifuged at 10.000xg for 25 min at 4°C to obtaining crude extract. The enzyme was purified through a three-step procedure: thermal treatment incubating the crude extract at 40°C for 30min; saline fractionation by 30-60% ammonium sulphate; and gel filtration chromatography Superdex G-200 by FPLC Äkta Purifier. The purification was monitored with the aid of SDS-PAGE and zymogram.

2.2 α-Amylase activity and kinetic parameters

The amylolytic activity was measured following the dinitro salicylic acid (DNS) method, using starch 2% (w/v) as substrate [16]. The assays were carried in 20mM sodium

phosphate buffer pH 6.0, 37°C for 10 min, following the DNS acid was added and then heating at 100°C for 10 min for color development and reducing sugars estimating. A calibration curve was constructed with maltose. One unit of enzyme was estimated as the amount necessary to produce 1µg from maltose per minute per mg of protein.

The kinetic parameters were evaluated varying the concentration of soluble potato starch from 0-12 mg/mL. The activity values were plotted at the software Grafit 5, and the Michaelis-Menten and the double reciprocal Lineweaver-burk were estimated.

2.3 Enzymatic activity using different substrates

The α-amylase digesting ability was evaluated through the measurement of enzymatic activity with different carbohydrates as substrates, namely potato starch, amylopectin, amylose, glycogen, wheat (flour), corn, and maltose at 2% (w/v) (all substrates were previously solubilized in water by heating). The assays were carried as described above. The potato starch value was taken as 100% and then the relative activity was calculated.

2.4 Resistance to pH and organic solvents

The resistance of α-amylase to pH was evaluated by the incubation of the enzyme at different pH values. For that, the universal system buffer strength (25mM glycine, 25 mM acetic acid, 25 mM MES and 75 mM Tris) was used in range to 3-10. An aliquot of α-amylase was incubated at each pH at 40°C, and after 24h the amylolytic activity was measured as described above.

To investigate the enzyme activity in the presence of organic solvents, methanol, isopropanol, butanol, dimetylformamide (DMF), dimethyl sulfoxide (DMSO) and diethyl ether, were used at concentration of 40% (v/v). Each solvent was prepared in the MOPS buffer (pH 6.0, 20 mM) and incubated with α-amylase at 40°C for 24h. After that, activity was measured under standard assay condition and the sample without organic solvent was considered as 100%.

2.5 Adsorbability

Determination of raw potato starch adsorbability of the α-amylase was studied by incubating 0.2 g of raw potato granules with 1mL of the enzyme at 37°C for 15 min. After centrifugation, the α-amylase activity of the supernatant was measured and the adsorption percentage was calculated as follows:

$$\text{Adsorption (\%)} = \frac{A-B}{A} \times 100$$

A is the original α-amylase activity and B is the α-amylase activity in the supernatant after adsorption on raw potato starch granules [17].

2.6 Raw-starch hydrolysis

The α-amylase ability of hydrolyzing raw potato starch was investigated. Aliquots of α-amylase containing 2.5 and 5 (U) were incubated with a dispersion of raw potato starch and MOPS buffer pH 6.0 20mM in a final concentration of starch 1, 5, 10 and 15%. Controls containing no enzyme are also performed. The samples were incubated in a shaker bath at 40°C for 24h. Aliquots were withdrawn at 1h, 3h, 6h, 12h and 24h and centrifuged at 10.000g for 5 min; the supernatant was used for estimating of reducing sugars, HPAEC-PAD and TLC analysis; and the starch precipitate used for SEM analysis. The degree of hydrolysis was calculated as described in the following reaction:

$$\text{Hydrolysis degree (\%)} = \frac{\text{reducing sugar value}}{\text{total carbohydrate}} \times 100$$

The reducing of turbidity of raw starch suspension was also measured. After the incubation time, an aliquot of the reaction mixture was diluted 20 folds in distilled water and read at 620 nm. The sample containing no enzyme was considered as 100% [4,18].

2.6.1 HPAEC-PAD and TLC analysis

After incubation at standard enzyme assay conditions, the samples were removed and cooled at room temperature for 10 min. The hydrolysis products present in the samples were subjected to thin layer chromatography (TLC) through a commercial pre coated silica plate ascending TLC in a chromatographic chamber (Silica gel 60, Merck, Germany) by using the

solvent system of butanol:ethanol:water (50:30:20). After TLC run, the bands were visualized by spraying the TLC plate with aniline reagent consisting of 1% aniline (v/v) and 1% diphenylamine (w/v) in a solution of acetone containing 10% of ortho-phosphoric acid and incubated at 100°C for 10 min.

Oligosaccharide composition was determined by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD, Dionex, Sunnyvale, CA, USA). Crude starch (1% and 5%, w/v) was hydrolysed as previously described using 2.5 U and 5 U amylase concentration. After hydrolysis, reaction tubes were centrifuged and supernatant and precipitate (insoluble) were collected separately and lyophilized. Samples were then diluted and filtered through 0.22 µm centrifuge tube filter. Chromatography was carried out using a Dionex CarboPac PA200 column (Dionex, Sunnyvale, CA, USA) at a flow rate of 0.35 ml/min, and injection volume 20 µL. A standard mixture run was performed before analysis of a batch of samples for verification of the response factors, using maltoligosaccharides from dp 1 to dp 8. Samples of 20µL oligosaccharides prepared as described above were injected and separated using a 1 mL min^{-1} flow rate, isocratic 150 mM NaOH, and the following NaOAc concentration gradient profiles: neutral oligosaccharides: 0–5 min linear gradient of 0–110 mM NaOAc; 5–130 min convex gradient (curve 4) of 110–350 mM NaOAc. Amylose in the neutral chain preparation had an extremely high affinity and was washed off the column after the gradient with 1 M NaOAc without NaOH. Preparative separation of linear neutral chains containing >80% of homodisperse chains ranging from dp 6 to dp 90 obtained from debranched potato amylopectin was performed using a semi-preparative CarboPac PA-1 column operated at 5 mL min^{-1} flow rate and the same program as used above for neutral oligosaccharides.

2.6.2 Scanning electron microscopy

In order to investigate the morphological changes in starch granule after hydrolysis, the pellets of residual starch were washed twice with pure ethanol followed by butanol, and centrifuged at 8000g for 5 min, and following all samples were then dry. The dried starch granules were attached to a SEM stub, gold sputtered and observed under scanning electron microscope (SEM) Jeol JSM-5600LV.

3 Results and Discussion

3.1 Purification of α-amylase from tilapia processing waste

The α-amylase from tilapia (AMY-T) was obtained through three-step purification; thermal treatment; saline fractionation by ammonium sulphate, and gel filtration chromatography with sephadex G-200. At last step, the yield and purification of purified α-amylase were 38% and 76 fold, respectively, with specific activity of 8265.9 (U/mg). The purification steps were monitored by SDS-PAGE and a major band with approximated molecular weight 50kDa was observed; and also zymograms which revealed a single active band (Figure 1).

The α-amylases available commercially are mainly produced by microorganism like bacteria and fungi, however there are some limiting aspects for utilization of enzymes from these organism, mainly in food industries [9]. Therefore, the search for others sources of enzymes are needed especially those that present low cost and advanced properties.

In this work was possible to obtain 217.5 (U/mg) of α-amylase from 1g of tilapia viscera. Considering that the amount of waste producing from fish industry corresponds to almost 50-70% of weigh of fish, a valuable input have been lost, plus that leading to high environmental onus [10,11].

The extraction of bioactive molecules from by-products allows fishery activity be more sustainable while adding value with new biotechnological products. Researches point the potential utilization of fish enzymes for many applications, like in food, detergent and environmental sectors [11–13,19,20]. However, studies and proposals of application for α-amylase from fishes at industrial sectors has not yet been prospected.

3.2 Kinetic features

The kinetic parameters of AMY-T were evaluated and summarized at Table-1. The enzyme showed a k_m from 4.78 (mg/mL) and V_{max} from 0.44 (mg/mL/min) to soluble potato starch, displaying a considerable affinity toward α-amylase and the substrate. AMY-T had a higher affinity to potato starch than α-amylase of *Bacillus sp.* I-3, *Nesterenkonia sp*. and *B. subtilis*, but showed less affinity in comparison with *B. licheniformis, Cryptococcus flavus* and *Bacillus sp.* GREI1.

3.3 Specificity of substrate

Aiming to to establish the specificity of AMY-T, the enzymatic activity was carried with different substrates consisting of different carbohydrates containing α-1,4 bonds or a mixture of α-1,4- and α-1,6-glucosidic bonds (Figure 2). Except for maltose, AMY-T was able to hydrolyse all carbohydrates in different extents. The digestibility profile of AMY-T against various substrates shows the versatility of this enzyme, which is able to cleave different sugars (starch, amylopectin, amylose and glycogen), and also sources of starch (potato, corn and wheat). It emphasizes the use of AMY-T on starch industry. Furthermore, it was seen a preference to cleave α-1→4 glycosidic bonds, when less activity was observed with glycogen and no activity for maltose, ratifying that it is an α-amylase [4].

3.4 pH and organic solvents resistance

Enzymes for industrial purposes should be active in a large range of pH for a long time, since that many processes, like starch hydrolysis, demand a considerable time of reaction [1]. In this work it was found that the enzyme was highly resistant to pH exposition, AMY-T showed stability toward acidic and alkaline media (3-10) for 24 h, which is an important feature to industrial applications (Figure 3A).

The pH profile of α-amylases varies greatly according the organism, but usually they loss their activity at very acid or alkaline ranges due the protonation/deprotonation of its catalytic residues (glutamic acid and aspartate). The maintenance of catalytic structure against huge range of pH and for long time is a desirable biotechnological feature [21].

From an economical point of view, this allows the minimized use of acids and bases for pH adjustment in processing of starch, for example the frequent use of acid to lower the pH from liquefying to saccharifying range [17,22]. Besides that, the action at lower pH values reduces the formation of some unwanted products which are usually generated at higher pH values, such as maltulose [23].

The influence of several organic solvents on the catalytic activity of AMY-T was assessed. When incubated with various organic solvents the α-amylase displayed a great compatibility with all them, as seen at figure 3B. AMY-T was highly resistant to all solvents used, in addition, showed an activating effect in presence of them, showing activity up for ~163% with DMF. Even when incubated with butanol, that is highly toxic for living

organisms and their macromolecules, the enzyme was not affected showing relative activity of ~130%, highlighting its high solvent compatibility [24].

Previous studies reporting solvent-tolerant α-amylases observed that the compatibility between enzyme and organic solvents depends of the polarity [25]. Water miscible organic solvents usually show more biological toxicity and lead to more inhibition of biocatalysts compared to immiscible organic solvents. This behavior was observed to some α-amylases from different bacteria [24,26–28], however AMY-T was highly tolerant to all organic solvents tested here (miscible and immiscible), showing a better performance than microbial α-amylases.

The demand for solvent-resistant enzymes has increased because of the use of organic solvents in enzymatic process, which have some advantages in comparison with aqueous systems, such as the enhancement of thermal stability, the suppression of undesirable water-dependent side reactions as well as decrease in microbial contaminations [25,29]. The resistance to organic solvents emphasizes the wide range of industrial applications of AMY-T, include such as treatment of carbohydrate-polluted industrial wastewater contaminated with organic solvents [28].

3.5 Adsorbability

The enzymatic catalysis of raw starch comprises a heterogeneous reaction which depends of many factors to succeed, including solid surface diffusion, adsorption of enzyme on granule and hydrolytic action of enzyme [4]. Although the adsorption is not the only determinant of catalytic rate and action, the adsorption of enzymes onto granule surfaces is a prerequisite for catalytic action, being a feature that indicates that enzyme can be applied in raw starch hydrolysis [30]. The adsorption ability of some α-amylases is correlated with the presence of SBD (starch binding domain) or SBS (starch binding site) on enzyme structure. In this work, AMY-T was capable to adsorb in raw potato starch to 78%, exhibiting an interesting affinity to potato starch.

Moreover, the adsorbability changes according the structure of starch once the distribution of the sites for enzyme binding depends of the origin of the grain [30]. Different levels of adsorbability were found for other raw starch digesting α-amylases on literature, such 71 and 57% respectively for α-amylase from porcine and *Cryptococcus sp.* [31]. Besides

that, an α-amylase from *Bacillus sp.* was reported to digest raw starch without adsorbing to the grain, but with very low hydrolysis degree ~10% [32]. Adsorbability is probably a desirable property for potential raw-starch hydrolyzing enzymes.

3.6 Raw starch hydrolysis

The potential of α-amylase in degrading raw potato starch was investigated at temperatures below the starch gelatinization (~50°C) and reported for others α-amylases (50-70°C). AMY-T showed to be able to hydrolyse raw starch at 40°C, a feature very attractive for starch industry, once the industrial starch processing usually display high temperatures for destroying the crystalline structure of raw starch (Table 2). Usually, the starch slurry is gelatinized at 100°C and then is liquefied at 50–60°C by amylases. This procedure is disadvantageous because lead to the increase of viscosity of starch slurry, rising of energy consumption, and consequently the production cost [23]. Because that, efforts have been aimed to found alternative enzymes that work at lower temperature.

The hydrolysis degree achieved by AMY-T suggests that it is a good choice to compete with some microbial enzymes, considering that they are not able to digest raw starch at low temperatures. The table 2 summarizes the results of raw potato starch hydrolysis related for bacterial and fungi α-amylases under similar conditions as used in this search. At 40°C AMY-T hydrolysed raw starch in 45% and 35%, respectively for the suspension containing 1 and 5% of starch (Figure 4-A,B). AMY-T was more effective in digest raw potato starch than related for other α-amylases.

It is important notice that some enzymes that are able to hydrolyse the raw starch at elevated degrees, but working at elevated temperatures, days of incubation, more concentrated enzyme doses and generally with aid of others enzymes, like glucoamylase [33]. Goyal and co-workers [17] achieved a hydrolysis degree (DH) of 80%, but at high temperature and enzyme doses. Under the temperature used (50°C) the raw starch potato was hydrolyzed in 20%.

In the sample 1% starch 5.0U, the maximum level of hydrolysis was reached at 12 h of incubation, and then it was observed stagnation on producing of reducing sugars (Figure 4-A,B). Probably the enzyme suffered product inhibition by maltose and/or glucose, or the residual raw starch available on sample is type resistant. Native starch granules exhibit two main allomorphic types (A or B type). The A type mainly occurs in cereal starches and the B

type in tubers and amylose-rich starches. It is well-known that the allomorphic B type structure, present in potato starch, is more resistant to enzymatic hydrolysis compared to the A-type structure [6]. Moreover, some factors could limit the raw starch hydrolysis, like the rheological properties of the substrate, ratio of its main building blocks, crystallinity level and the amylose-lipid complexes [34].

At higher raw starch concentrations the hydrolysis degree decreased, displaying 26 and 15% respectively for 10 and 15% raw starch suspensions (Figure 4-C,D). However, even with low hydrolysis degree, AMY-T was able to digest raw starch at elevated concentrations, generating a great value of reducing sugar. As reported for others studies, the enzymatic efficiency is highly dependent on the starch concentration [6]. Possibly, this hydrolysis degree could be increased with higher enzyme doses, furthermore is important to emphasize that was used less enzyme concentration in comparison to reported for others studies. Regarding change on turbidity of raw starch suspension, were observed decrease up of 67 and 75% respectively for samples containing 2.5U and 5U, ratifying the digesting property of AMY-T.

The ability to hydrolyse raw starch is usually associated with the presence of SBD (starch binding domain) or SBS (starch binding site). As have been known, α-amylases from animals does not have a domain to bind starch in their structure, nevertheless in human pancreatic α-amylase (HPA) it was found several sites for starch binding. Studies report that the binding of HPA to starch granules appears to be mediated primarily by SBS 7 with a hydrophobic patch composed of Tyr276 and Trp284, revealing the role of aromatic residues in starch binding [4]. Considering the high similarity between animal α-amylases, especially this feature between human and fishes of ~ 72% [35], this feature found to AMY-T could be associated to presence of this SBS's on its structure. However, structural studies are needed to ratify.

3.7 Analysis of α-amylase products from raw starch hydrolysis by TLC and HPAEC-PAD.

The HPAEC-PAD analysis of total end products from raw starch hydrolysis after 24h of incubation identified oligomers ranging from 1 to 8 DP. Maltose was the main product generated in all conditions tested, followed by maltotriose and glucose. The sample containing starch 1% and 2.5U was the most effective in hydrolysing the starch to G1 (Fig. 5-A).

These results were corroborated by TLC analysis (Fig. 5-B), when is possible to observe the pattern of products generated from raw starch hydrolysis by AMY-T at different times and concentrations. The hydrolysis products were detected and identified as glucose, maltose and maltodextrins, suggesting the endo mode of action of the amylase, which is the characteristic of α-amylase enzyme [23]. The pattern of carbohydrates is the same even at different conditions of time and concentrations of starch and enzyme dose, varying only in the intensity of spots; as time of incubation increases, the spots are more intense. At all times analysed glucose was found as product, suggesting a multi attack mode action. AMY-T show a great digesting performance in comparison to others raw starch amylases that just have glucose as product after 6 h of hydrolysis [2,31]. The variety of products generating by AMY-T ratify its potential use in starch industries, like food, chemistry and biofuel [1]. Besides that, the end product composition is in agreement with that of classical saccharifying-type α-amylase, which produces predominantly G1, G2, or G3 as end products [6].

3.8 Scanning electron microscopy

The raw potato starch was analysed by SEM in order to verify the morphological changes caused by α-amylase action. It was possible to observe the difference between untreated raw starch and the samples after enzymatic hydrolysis (Fig 6). The native starches show small and large granules and display a sphere or ellipsoid. Some starch granules are broken from the core of starch granule (2.5U 5% starch), and then they split in two. This images of 5.0U 5% starch show few granules are broken into some smaller parts than 2.5U 5% starch though they have similar break way. This image of 2.5U 1% starch shows fully broken starch granules, that we cannot see any spherical particles, indicates the starch granules are fully destroyed. There are similar changes between 5U 1% starch and 2.5U 1% starch, indicates 2.5U of the enzyme is enough to destroy the granular structure of starch.

The action of AMY-T caused large and deep holes with different sizes on the smooth surface of potato starch granules, showing that the modes of attack included both exo-corrosion and endo-corrosion [6]. Apparently, the raw potato starch has a surface ‘barrier’ which, once breached allows access of enzyme to the interior for all granule sizes. As known, raw potato starch is a resistant type, it is addressed to a combination of larger granule size and smoother surface, together with specific supramolecular properties providing the resistance of potato starch against enzymatic digestion [36].

However, the qualitative analysis of the granule after the enzymatic treatment show that AMY-T was efficient in damage the resistant structure of raw potato starch through a multiple-attack reaction which results in the formation of pits on the surface of starch granules. This AMY-T action mode could explain the ability of the enzyme in generating G1 as product, as previously described for other report [2].

4 Conclusions

The α-amylase from tilapia viscera showed a high potential for application on hydrolysis of raw-starch, achieving high degrees of hydrolysis at temperature and concentrations above to related for others enzymes. The characterization of hydrolysis products shows glucose, maltose and maltodextrins as main products, which meet industrial demand. This is the first report of the raw starch hydrolyzing ability of an α-amylase from fish. Therefore it is important to highlight the reutilization of fish processing waste as alternative source of enzymes, which beside to be environmental friendly and offers reducing costs, is a source of enzyme able to compete with microbial enzymes.

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Table 1 – Kinetic parameters of raw digesting α-amylase from Nile tilapia and microbial sources available on literature. The measurements were carried in triplicate using soluble potato starch 2% as substrate.

Origin	K_m	V_{max}	Reference
Oreochromis niloticus	4.78 (mg/mL)	0.44 (mg/mL/min)	This work
***B. licheniformis* AT-70**	1.203 (mg/mL)	0.01 (mg/mL/min)	Afrisham et al., 2016
***Bacillus sp* I-3**	3.44 (mg/mL)	0.45 (mg/mL/min)	Goyal et al., 2005
***Bacillus sp* GRE1**	4.98 (mg/mL^{-1})	42 (mg/min^{-1})	Haki et al., 2008
***Nesterenkonia sp.* strain F**	5.8 (mg/mL)	1.07 (mg/mL/min)	Shafiei et al., 2011
Cryptococcus flavus	0.37 (mg/mL^{-1})	-	Galdino et al., 2011
Bacillus subtilis	7.79 (mg/mL)	186.26 (mg/mL/min)	Konsula & Liakopoulou-Kyriakides, 2004

Table 2 – Raw potato starch hydrolysis by α-amylase from Nile tilapia and microbial sources available on literature.

Origin	Degree of hydrolysis / reducing sugar	Enzyme dose	Starch concentration	Incubation time	Incubation temperature (°C)	Incubation pH	Reference
Oreochromis niloticus	45%	2.5 (U)	1%	24h	40	6.0	This work
***Cryptococcus sp* S-2**	8%	3 (U/mL)	1%	24h	30	6.0	Iefugi et al., 1996.
***Bacillus sp* I-3**	20%	500 (U)	1%	5h	50	7.0	Goyal et al., 2005.
***Bacillus sp* GRE-1**	20%	10 (U)	1%	36h	40-70	6.0	Haki et al., 2008.
***Bacillus subtilis* S8–18**	12%	-	5%	24h	60	6.0	Kalpana and Pandian, 2013.
Bacillus subtilis	25%	15 (U)	3%	7h	60	7.0	Konsula and Liakopoulou-Kyriakides, 2004.
***Bacillus subtilis* IFO-3108– 1**	8%	16 (U)	0,50%	4 days	40	6.0	Mitsuiki et al., 2005
***Bacillus subtilis* IFO-3108 – 2**	10%	16 (U)	0,50%	4 days	40	6.0	Mitsuiki et al., 2005
Bacillus licheniformis	5%	5.2 (U)	1%	24h	37	7.0	Moore et al., 2015.
***Bacillus sp.* IMD434**	8%	1000 (U/g)	-	24h	40	6.0	Hamilton et al., 1998.

Figure 1 – SDS-PAGE and zymogram of α-amylase from Nile tilapia. Line 1 – molecular weight marker; Line 2 – final purification step (superdex G-200 chromatography), showing a majority band of ~50 kDa; Line 3 – zymogram of α-amylase showing a single active band.

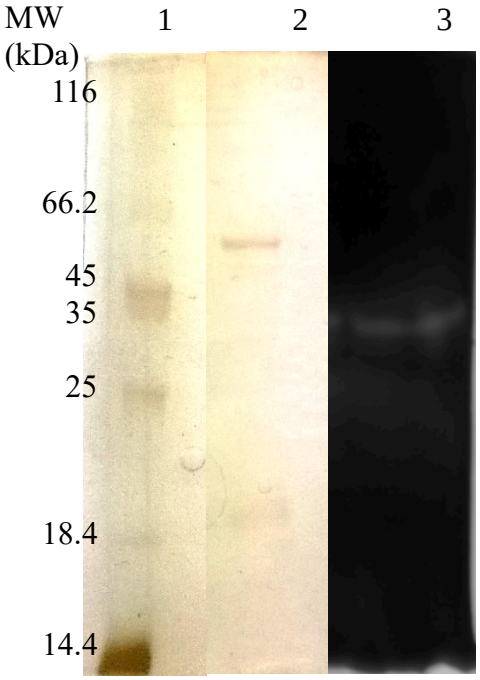


Figure 2 – Substrate specificity of α-amylase. Different carbohydrates were previously solubilized and used at 2% concentration. The α-amylase was carried at standard condition. All assays were performed at triplicate.

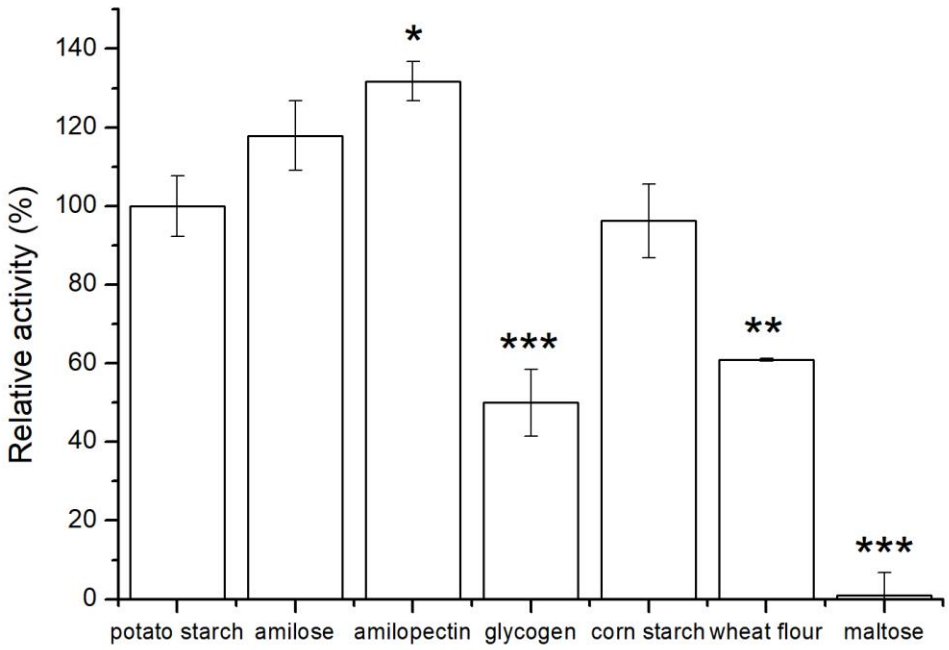
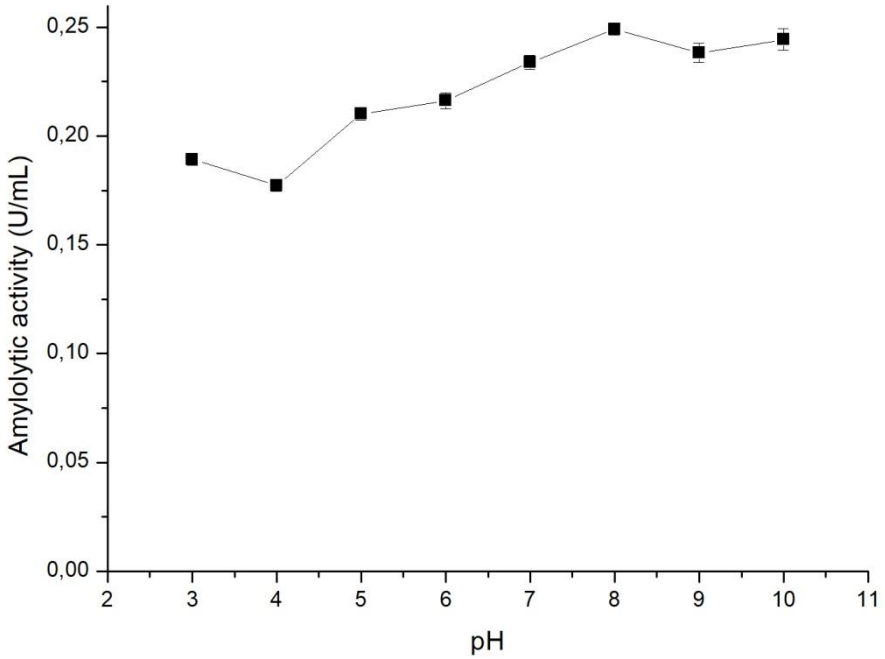


Figure 3 – pH (A) and organic solvent (B) resistance. The α-amylase was incubated at pH ranging from 3-10 and organic solvents for 24 hours at 40°C. After the incubation time, the enzymatic activity was assayed at standard conditions. The assays were performed in triplicate.

A



B

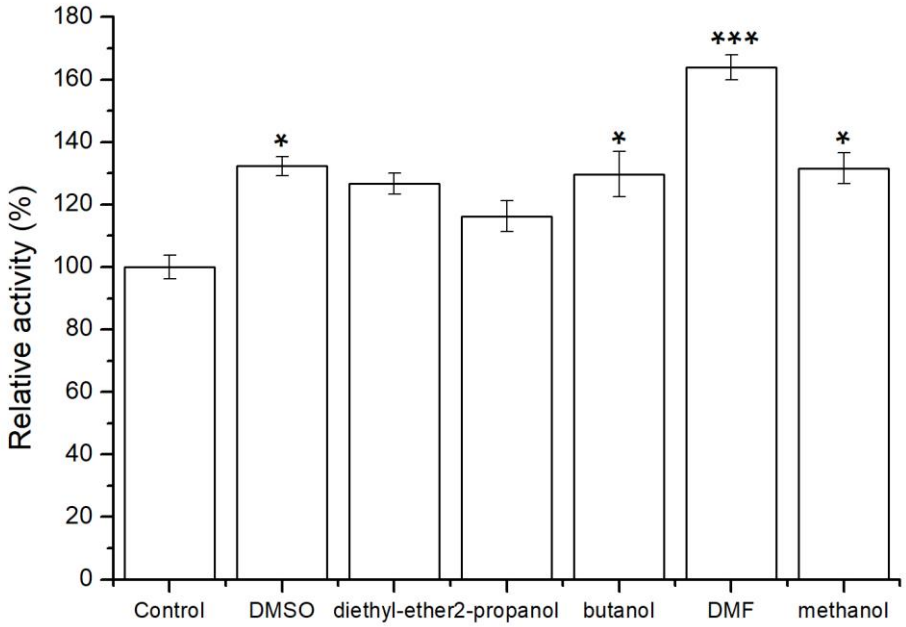
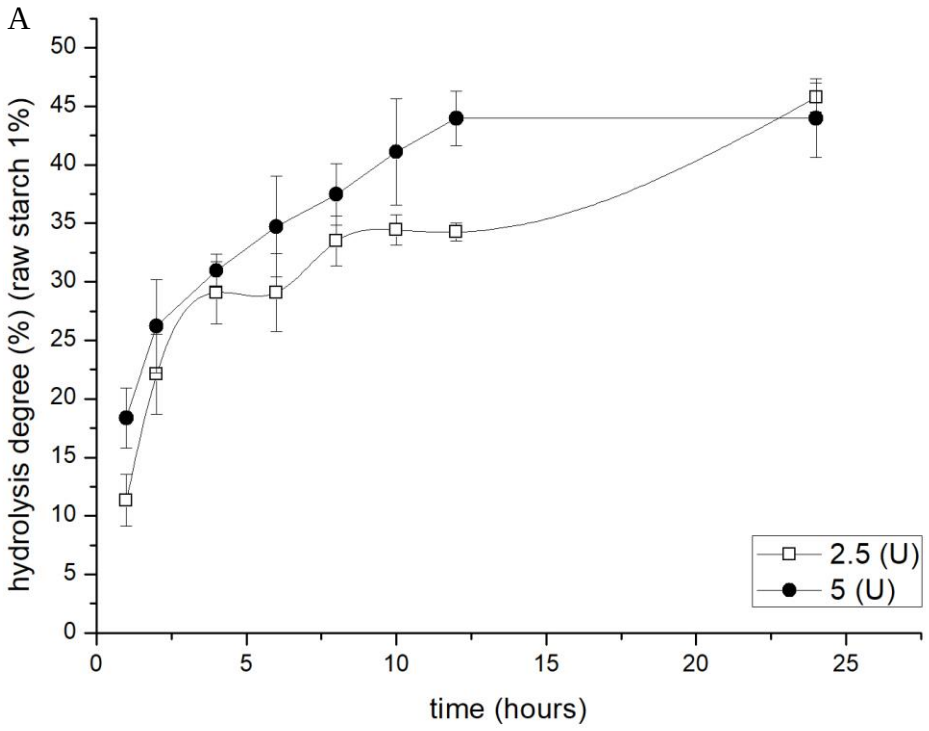
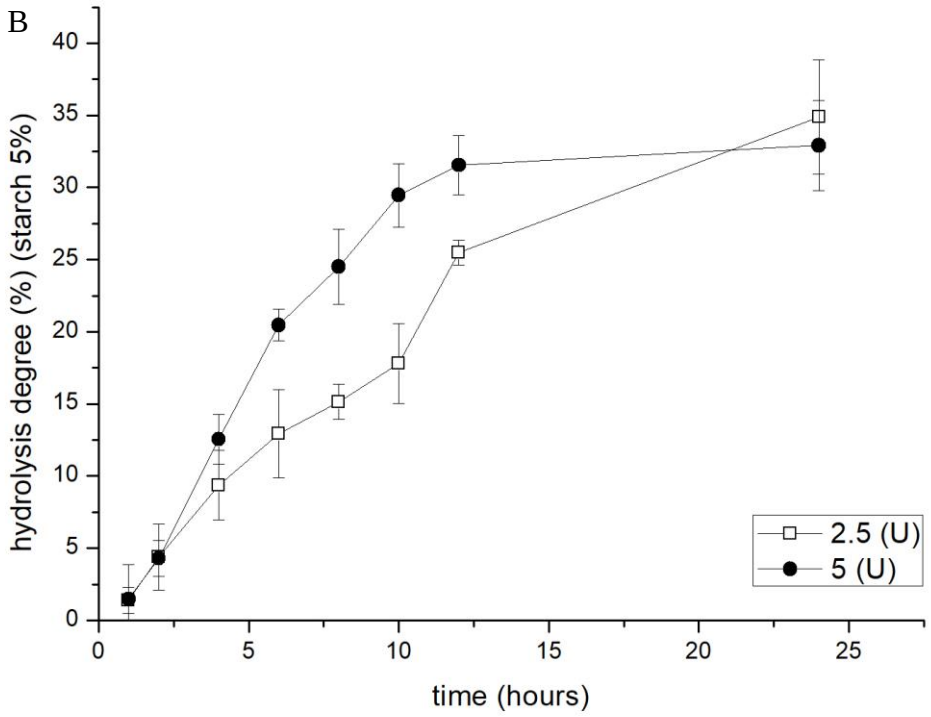
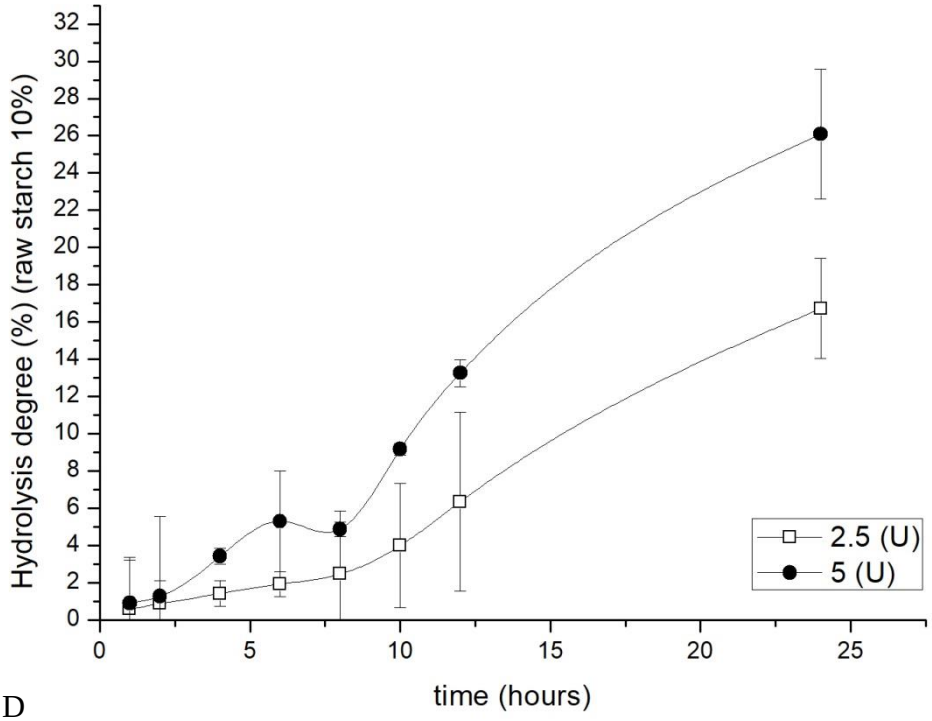


Figure 4 – Raw potato starch hydrolysis by α-amylase from tilapia viscera at different concentrations of suspensions starch. (A) 1% starch; (B) 5% starch; (C) 10%; and (D) 15% starch. Controls containing no enzyme also were performed. All assays were performed at triplicate.





C


Hydrolysis degree (%) (raw starch 10%)
time (hours)
2.5 (U)
5 (U)

D

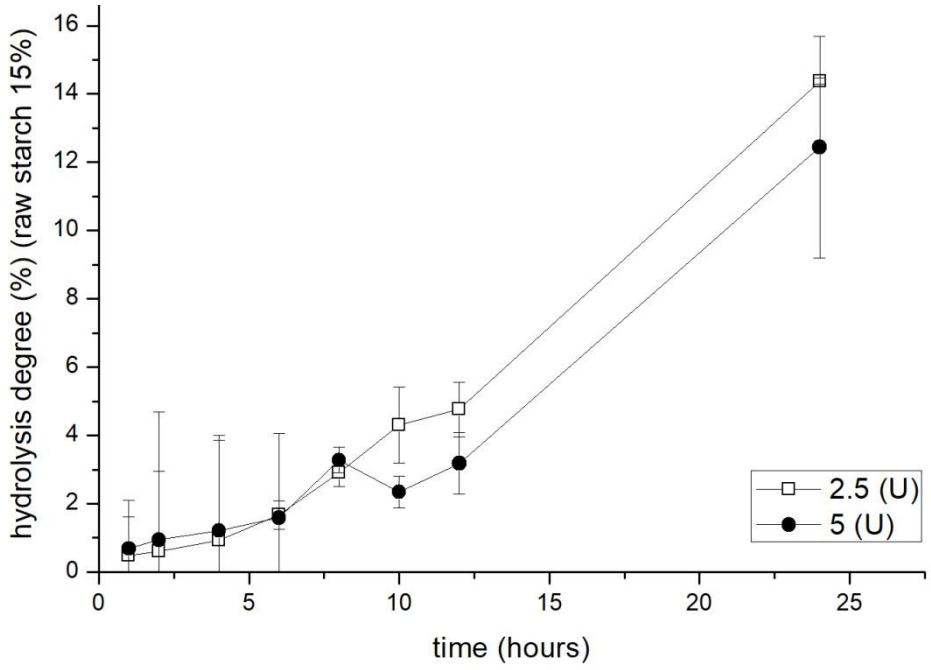
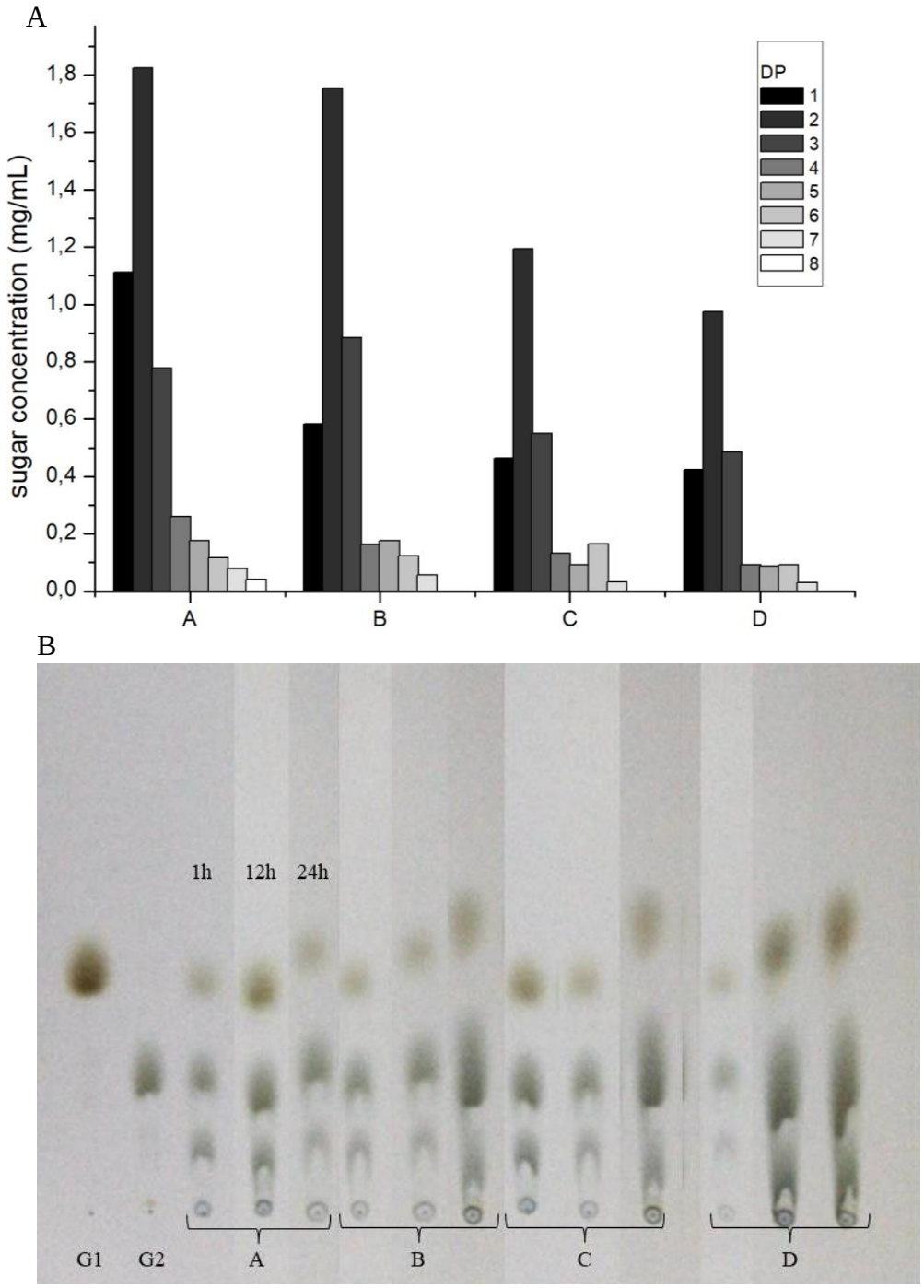

hydrolysis degree (%) (raw starch 15%)
time (hours)
2.5 (U)
5 (U)

Figure 5 – Analysis of end products of raw potato starch by AMY-T: (A) HPAEC-PAD analysis after 24h incubation – group A- starch 1% 2.5U; group B- starch 5% 5U; group C- starch 5% 2.5U; group D- starch 5% 5U; (B) Thin layer chromatography analysis after 1, 12, and 24h of incubation. G1- glucose standard; G2 – maltose standard; A- starch 1% 2.5U; B- starch 5% 5U; C- starch 5% 2.5U; D- starch 5% 5U.



714 Figure 6 – Scanning electron micrographs of native starch and enzyme-hydrolyzed raw potato
715 starch using AMY-T after 24h at 40°C.

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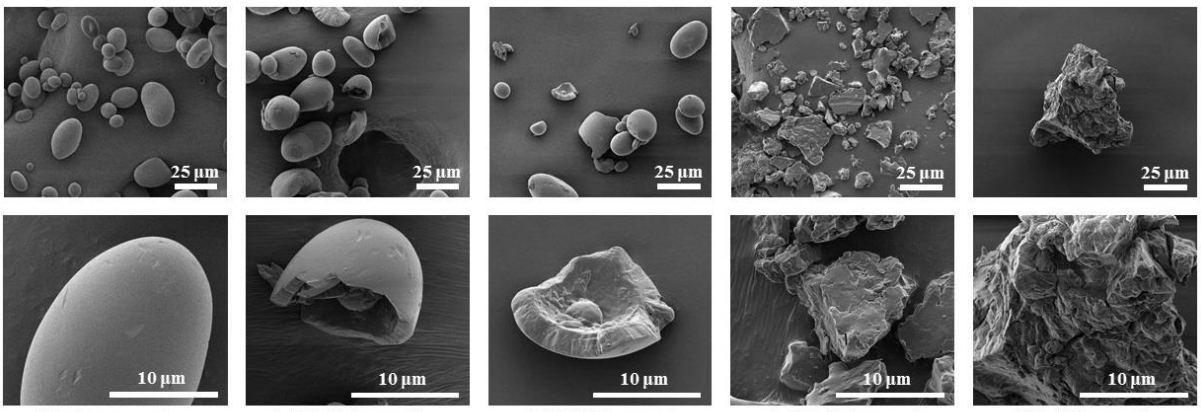

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FISH PROCESSING WASTE AS A SOURCE OF α-AMYLASE AND TRYPSIN FOR USE AS ADDITIVE FOR COMMERCIAL DETERGENTS

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Abstract

Tambaqui (*Colossoma macropomum*) processing waste was investigated as potential source of digestive enzymes. Using viscera, α-amylases were partially purified from intestine (AMY-1) and pyloric caeca (AMY-2). The method consisted of thermal treatment, salt fractionation and p-aminobenzamidine-agarose chromatography, and allowed for concomitant purification of trypsin. Biochemical characterization of the α-amylases revealed optimal activities at pH 7.0 and 60°C for AMY-1 and at pH 8.5 and 60°C for AMY-2, both were thermostable for 60 min at 40°C. The kinetic and thermodynamic studies showed apparent *Km* 2.8 and 2.2 mg/mL for starch, and *AE* 11.58 and 5.01 kcal/mol, respectively for AMY-1 and AMY-2. Their activity decreased in the presence of 5mM $CuCl_2$ and $HgCl_2$, while addition of 1mM EDTA had no effect, suggesting a calcium-independent amylase. These enzymes were stable in the presence of surfactants (1%), H_2O_2 (5%), and showed high compatibility with all the brands of commercial laundry detergents tested, suggesting their use as additives for cleaning. Furthermore, α-amylases from tambaqui viscera showed great performance in comparison with commercial amylases, emphasizes the fish processing waste as an important source of digestive enzymes.

Keywords: tambaqui, fish processing waste, enzyme characterization, laundry detergent.

1 Introduction

The α-amylase (EC 3.2.1.1) enzyme is one that hydrolyzes α-(1→4) glycosidic bonds in polysaccharides to produce mainly dextrins, maltose and other oligosaccharides. These enzymes can be obtained from several natural sources such as microorganisms, plants and animals and their role in carbohydrate metabolism is well known. Amylases correspond to about 30% of the world market of industrial enzymes and are widely used in the starch saccharification process, food, textile and brewing industries, bio-ethanol production, as well as helping increase the digestibility of animal feed and as a detergent additive to improve wash performance [1–3].

Detergent enzymes account for about 40% of global enzyme production, representing one of the largest and most successful industrial biotechnology applications [4]. α-Amylases have been used in laundry detergent powder composition since 1975. They are the second-most important enzymes used in detergent formulations and about 90% of all liquid cleaners contain amylolytic enzymes [5].

The use of enzymes in detergent formulations improves the removal of tough stains while also being environmentally safe. α-Amylase acts on carbohydrate stains by assisting in starch hydrolysis, which is insoluble at low temperatures and difficult to remove. Removal of starch from surfaces is also important in maintaining whiteness, since starch can be an attractant for many types of soil particles [6]. However, the use of enzymes is still problematic due to the difficulty of being stable in the presence of surfactant, oxidizing and chelating agents, which are common in cleaning product formulations [7].

Some authors have reported that enzymes can be obtained from fish processing waste, mainly viscera, which represents an important source of biomolecules with potential applications [8–10]. Therefore, alternative uses of these by-products are required in order to reduce contamination and generate value-added products.

The recovery and characterization of enzymes from fish have been achieved with some interesting applications [11]. However, amylases from fungal and bacterial sources still are the most used in industrial sectors, mainly due to their easy production and handling methods [1]. The purification processes are generally costly, so the choice of an enzyme for industrial purposes should consider some important aspects such as source, purification process and enzymatic characterization. When compared with bacterial and fungal proteins, the purification of animal enzymes still faces challenges in terms of research, developing and marketing due to the high cost of animal production [12]. Nevertheless, the use of fish waste as a source of enzymes minimizes production costs.

Furthermore, efforts have focused on developing low-cost methodologies for the use of fish waste as a source of enzymes. In the present work, we proposed a methodology to obtain two digestive enzymes from fish processing waste in a single procedure. The development of new procedures that improve the purification of these biomolecules is important as it allows the discovery of new enzymes that can meet market demands and are industrially valuable [13].

The tambaqui fish (*Colossoma macropomum*) is an important Amazonian fish, present in South America and in tropical and subtropical areas of other continents, currently being the second-most produced species in Brazilian aquaculture [14, 15]. Studies have reported the potential use of digestive enzymes of tambaqui, mainly proteases, in several industrial sectors, such as the food industry and in detergents [16]. Trypsins from the pyloric caeca of tambaqui have been purified and characterized, and their properties point to these applications [9, 10]. Despite to be an omnivorous fish, and expressing a great amount of carbohydrases, the potential of α-amylases from this species has not previously been studied.

Therefore, the present work aimed to obtain α-amylases from tambaqui viscera while developing a new protocol that reduces the costs of purification; elucidate the biochemical,

kinetic and thermodynamic parameters; and test the viability of their biotechnological use in detergent formulations.

2 Materials and methods

2.1 Enzyme purification

Viscera of tambaqui (*Colossoma macropomum*) were obtained from CODEVASF (Companhia de Desenvolvimento dos Vales do São Francisco e do Parnaíba). Intestine and pyloric caecum were collected and homogenized in 0.01M phosphate buffer pH 7.5 (200 mg/mL). The resulting homogenate was centrifuged at 10,000 x*g* for 25 min at 4°C to remove cell debris. The enzyme was purified from the supernatant by a three-step procedure: (1) incubation for 30 min at 45°C (heat treatment); (2) ammonium sulfate fractionation for 2 h at 4°C for a final salt saturation of 0–30% (fraction F1), 30–60% (fraction F2) obtaining a final supernatant (protein soluble in 60% salt concentration); (3) chromatography (2 cm^3 with 1 mL of p-aminobenzamidine–agarose, Sigma-Aldrich®). In this step, a joint purification of trypsins and amylases was performed using an affinity column for trypsin. Fractions with amylolytic activity were collected during elution of all samples. These fractions were pooled, dialyzed against 0.01 M phosphate buffer pH 7.5 for 24 h at 4°C and used in further steps.

2.2 Electrophoresis and zymograms

To evaluate the protein profile of the samples, SDS-PAGE electrophoresis was performed according to the methodology described by Laemmli (1975), using a 4% (w/v) stacking gel and 12.5% (w/v) separating gel under constant current of 12 mA per gel. After electrophoresis performed at 4°C, zymograms were carried out according to the modified methodology by Fernández et al. (2001), the gel was immersed in 2.5% Triton X-100 for 30 min at 4°C to remove the SDS. Next, the gel was washed three times with 10mM phosphate

buffer, and incubated with a starch solution (2% w/v) containing 10mM phosphate buffer, pH 7.5 for 60 min at 37°C to allow the digestion of starch by the active fractions. Finally, the gel was washed with distilled water and stained with an iodine/KI solution (100 $g.L^{-1}$) for 5 minutes. The gel was washed with distilled water to stop the reaction and then with an acetic acid solution (130 $g.L^{-1}$).

2.3 Determination of amylase activities

Amylase activity was determined according to Bernfeld (1955), using a 2% soluble starch solution as a substrate. Briefly, 20 µL of the enzyme preparation was mixed with 125 µL of 10 mM phosphate buffer, pH 7.5 at 37°C. The reaction was initiated by the addition of 125 µL of substrate and stopped 10 min later with the addition of 300 µL DNSA (3,5-Dinitrosalicylic acid). Activity was measured by estimating the reducing sugars released during this time using a standard curve of maltose. Blanks without enzyme extract were run simultaneously with the reaction mixture. One unit of activity was defined as the amount of enzyme able to produce 1µg of maltose per minute. All the assays were carried out in triplicate and all data was analysed using one-way analysis of variance (ANOVA) complemented with Tukey's test considering $p < 0.05$. The statistical program used was GraphPad Prism version 7.0.

2.4 Effect of pH and temperature

Optimum pH for amylase activity was determined using citrate-HCl, citrate-phosphate, Tris-HCl and glycine-NaOH (10mM), ranging from pH 2.5 to 11.5 at 37°C. Optimum temperature and thermal stability of amylase activity was determined by incubating the enzyme with a substrate pre-equilibrated at temperatures ranging from 25°C to 70°C, followed by measurement of residual activity. Assays were performed in triplicate.

151 2.5 Kinetic and thermodynamic studies

152 The kinetic parameters of α-amylases from *Colossoma macropomum* were determined
153 in 10 mM sodium phosphate buffer (pH 7.5) at 37°C. Assays was performed with potato
154 starch at final concentrations ranging from 0-11.5 mg/mL. V_{max} and k_m values were estimated
155 by fitting the initial rate data to the Michaelis–Menten equation using nonlinear regression
156 with Grafit 5 software (Erithacus Software, London, U.K.). All kinetic parameters presented
157 in this study are the means derived from triplicate measurements. The first-order rate constant
158 or turnover number (k_{cat}) and the second-order rate constant (catalytic efficiency - k_{cat}/k_m)
159 were determined according to Miller & Wolfenden (2002) and Assis et al. (2014). The ratio
160 between catalytic efficiency and the rate constant for the non-catalyzed reaction (k_{non}), named
161 catalytic proficiency [$(k_{cat}/k_m)/k_{non}$], was determined using data for spontaneous hydrolysis of
162 substrates according to Wolfenden & Yuan (2008).

163 Assays were carried out promoting the reaction under increasing temperatures (up to
164 the optimum temperature) from 25 to 60°C for the determination of activation energy (AE) by
165 plotting data as an Arrhenius graph (ln(k) vs 1/T) and using the Arrhenius equation
166 (logarithmic form):

167 $$\ln(k) = \ln(A) - \frac{AE}{R} \times \frac{1}{T}$$

168

169 where: k = rate constant; A = pre-exponential factor; AE = activation energy; R = gas
170 constant and T = absolute temperature.

171

172 The rate enhancement exerted by the enzyme in relation to the k_{non} of the reaction was
173 also determined according to Assis et al. (2014), using the following equation:

174

$$k = \left(\frac{\mathbf{k}T}{\mathrm{h}}\right) \mathrm{x}\, e^{\left(-\frac{AE}{R}\right)}$$ where: k = rate constant; **k** = Boltzmann constant; T = absolute temperature; h = Planck constant; AE = activation energy; R = gas constant.

2.6 Substrate specificity

To determine the substrate specificity of the amylase, starch of several sources (potato, wheat, corn) and also amylopectin, maltose and glycogen were used as substrates at 2% concentration during the enzyme assay. The highest activity was established as 100%.

2.7 Effect of metal ions and EDTA

The effect of metal ions on amylolytic activity was conducted in the presence of the ions $CaCl_2$, $CuCl_2$, $HgCl_2$, $AlCl_3$, $PbCl_2$, KCl, $ZnSO_4$, $BaCl_2$, $CdSO_4$ and NaCl at 5mM, and EDTA 1mM. An aliquot containing amylase and ion/chelator solution (1:1 v/v) was incubated for 30 min at 37°C and then the residual amylolytic activity was determined considering 100% as the activity in the absence of ions/chelator.

2.8 Effect of inhibitor

The effect of Type I specific α-amylase inhibitor (Sigma-Aldrich, USA) from *Triticum aestivum* on amylase activity was recorded according to Fernandéz et al (2001) . An aliquot (1:1) (v/v) was incubated with inhibitor (50µg/mL) for 30 min at 37°C. Afterwards, the amylolytic activity was measured. Controls were determined considering the activity in the absence of inhibitor as 100%.

2.9 Effect of surfactant and oxidizing agents

Stability towards ionic (sodium choleate and SDS) and non-ionic surfactants (Tween 20 and Tween 80) was investigated by incubation at solution concentrations of 1% (w/v) for

30 min at 40ºC, after which the enzyme activity was assayed. Hydrogen peroxide stability of the amylases was investigated by incubating samples with H_2O_2 (1:1) at 5% concentration at 40°C for 60 min.

2.10 Compatibility with commercial detergents

α-Amylase at a concentration of 0.20 mg/mL was incubated at 40ºC with several commercially available detergents: Ala® (Unilever); Ace® (Procter & Gamble); Brilhante® (Unilever), Roma® (Johnson&Johnson) and liquid detergents Tixan Ypê® and Ace Ação instantânea® (Procter & Gamble) to a final concentration of 7 mg/mL. Samples (20 μL) were removed after 60 min and the residual amylolytic activity in each sample was determined at 37ºC, assayed and compared with the control sample without detergent and blank containing no enzyme. Commercial α-amylase from *Bacillus subtillis* (Sigma® USA) was used as a standard parameter.

3 Results and discussion

3.1 Purification of α-amylases

The partial purification of α-amylases was performed via three steps: thermal treatment, salt fractionation and chromatography. Salt fractionation was performed using ammonium sulfate at concentrations of 0-30% and 30-60%. The 30-60% fraction showed the highest amylolytic activity in pyloric caeca and intestine and was used in the next step of purification. A chromatography column of p-aminobenzamidine-agarose (Sigma®) was used to increase the degree of purification of these enzymes. In this last step, the concomitant purification of trypsins and amylases was performed using an affinity column for trypsins. The yield of the purification, recovery and the total amylase specific activity was 37.94%,

47.03 and 2006.08 (U) for AMY-1 (isolated from intestine); and 22.11%, 23.5 and 1320.1 (U) for AMY-2 (isolated from pyloric caeca). To evaluate the protein profile of the samples, SDS-PAGE electrophoresis and zymograms were performed in which it was possible to observe the partial purification of these α-amylases, beside the presence of isoforms with estimated molecular masses for AMY-1(a) = 54.07 KDa and AMY-1(b) = 20.4 KDa, AMY-2(a) = 50.87 KDa and AMY-2(b) = 20.4 KDa. Other α-amylases from different fish species have molecular mass values varying from 55.5 (*Sarotherodon melanotheron*) to 156.3 KDa (*Polyodon spathula*) (Moreau et al., 2001; Ji et al., 2012).

Enzyme purification methods are generally onerous and particularly important to modern industrial processing because they directly relate to cost and efficiency [12]. In this work, through a single purification column, it was possible to obtain two different enzymes with important characteristics for industry using fish viscera as a source. Proteases and amylases correspond to the enzymes with the highest expression in the industrial market [25], and obtaining them from a cheap source, such as fish processing waste, is an interesting alternative source. In this study it was possible to obtain from one gram of viscera 771.57 (U) of AMY-1 and from pyloric caeca 471.47 (U) of AMY-2.

Furthermore, although bacterial and fungal enzymes are still the most commonly used in enzymatic processes, the use of these biomolecules also has some drawbacks. Enzymes from pathogenic bacteria would not be a good choice for some applications, like food. Besides that, single-cell organisms have fewer varieties of enzymes, limiting the applications of fungal and bacterial amylases [12, 13].

3.2 Biochemical characterization of the purified α-amylase from tambaqui

As mentioned, fish digestive enzymes have been studied and characterized. Among these enzymes, trypsin from tambaqui viscera was previously purified and characterized.

Bezerra et al. (2001) observed a thermostable trypsin from caeca pyloric. Other studies have investigated trypsin for industrial applications and properties compatible with those required by the food and detergent industries were found, like alkaline activity, thermal stability and compatibility with surfactant and oxidizing agents [9, 10]. However, despite the importance of α-amylase for metabolism, high expression and the wide range of industrial applications of this enzyme, its biochemical properties have not yet been elucidated.

3.2.1 Effect of pH and temperature

Some parameters are required in the choice of enzymes for commercial detergents, such as acting under alkaline pH and optimum temperatures compatible with washing temperatures (40~60°C) [7]. To better undertand the potential, the physical and chemical properties of the α-amylases partially purified here were investigated.

Both amylases had higher activity in neutral to alkaline media, showing discrete activity at extreme pH. AMY-1 presented an activity peak at pH 7.0, while for AMY-2 the optimum activity was at pH 8.5 (fig. 2-A). α-Amylases from digestive tract of several fish have shown optimum pH ranges from 6.5 to 9.0 [22, 23, 26–29]. Some authors report that α-amylases from fish are highly sensitive to acid pH, a fact that may be related to the digestion of carbohydrates in the gut which takes place mainly in an alkaline medium [22]. AMY-1 showed great activity in a pH range from 6.0 to 9.0, whereas AMY-2 still had 40% of its residual activity at pH 10.5, proving it to be more tolerant to alkaline conditions than AMY-1. For industrial purposes it is important that enzymes remain active under specific conditions; the pH effect on AMY1 and AMY2 suggest that they are suitable for their use with detergents.

Both α-amylases exhibited optimum temperature of 60°C, greater than that found for other fish species. At higher temperatures, AMY-1 and AMY-2 lost their activity (fig 2-B).

Structural conformation and amylolytic activity are highly related, so the loss of activity at elevated temperatures may be associated with structural changes caused by thermal denaturation. Many factors affect thermostability, these include the presence of calcium, substrate and other stabilizers, and it is known that amylases are more stable in the presence of substrate [3]. The thermostability study (Fig. 2-C) showed that heating at 40°C for 30 min exerted a stimulant effect on enzyme activity when residual activities of 104.02% and 132.12% for AMY-1 and AMY-2 respectively, suggesting that this enzyme may be suitable for the detergent industry. Heating beyond this temperature resulted in a gradual loss of activity. AMY-1 and AMY-2 retained 40 and 60% of their initial activity at 55°C after 30 min. α-Amylases from other fish species showed optimum temperature and thermostability between 25 and 50°C [27, 30, 31]. These results showed that both amylases isolated from tambaqui presented high activity in a wide range of pH and compatible temperatures for their use as additives in detergent.

3.2.2 Kinetic and thermodynamic studies

Michaelis-Menten kinetics revealed an apparent k_m of 2.82 mg/mL for AMY-1 and 2.24 mg/mL for AMY-2, similar to the fish *Siganus canaliculatus* (1.038 mg/mL) and bacterium *Bacillus subtilis* (1.9 mg/mL).When compared to other species, the k_m from AMY-1 and AMY-2 was greater than human pancreatic α-amylase (0.7mg/mL) and lower than α-amylase from *Aspergillus gracilis* (6.33 mg/mL), suggesting that although there is the same catalytic mechanism between the α-amylases, the substrate affinity varies among organisms [2, 26, 32, 33] The other parameters of catalytic performance are shown in Table1.

The thermodynamic studies revealed *AE* data analogous to those found for the fish trout (9.7 kcal/mol) and burbot (13.1 kcal/mol) [34] (Ponomarev, 1991). The activation energy of the enzyme from *C. macropomum* pyloric caeca (AMY-2) was higher than that from the

intestine (AMY-1) (11.58 and 5.01 kcal/mol, respectively). This apparently small difference in the activation energy values implies a difference of several orders of magnitude in rate enhancements. In fact, this was reflected in the smaller values of Rate Enhancement (6.2 x 10^4-fold) and V_{max} of AMY-2 in relation to AMY-1. Despite these findings and due to the lower k_m and lower total concentration of enzyme (Et) in the AMY-2 solution, the turnover number values (k_{cat}) showed that AMY-2 was able to hydrolyze more substrate per unit of time. And this tendency was corroborated by the other parameters such as Catalytic Efficiency (k_{cat}/k_m) and Catalytic Proficiency [$(k_{cat}/k_m)/k_{non}$]. The k_{cat} of AMY-2 reached 3.43 x 10^2 which is 55% higher than that obtained for AMY-1. The Catalytic Efficiency of AMY-2 was 95% higher than the one found for AMY-1. This is a parameter that considers the affinity between enzyme and substrate (unlike k_{cat}) and therefore can investigate the enzyme activity in physiological (sub-saturated) concentrations of substrate by describing the rate of product formation under such conditions.

Catalytic Proficiency is a parameter that not only describes the affinity between the enzyme and substrate but describes it during the transition state and the respective susceptibility to inhibitors that resembles the substrate in the transition state. According to the present findings, AMY-2 showed more affinity/susceptibility to substrates/inhibitors structurally similar to its active site during the transition state in relation to AMY-1 [19].

3.2.3 Substrate specificity

The specificity of α-amylase to different substrates is summarized in Figure 2-D. Both α-amylase displayed the highest specificity towards potato starch (100%) followed by corn (AMY-1 81.14% and AMY-2 73.78%), amylopectin (AMY-1 67.88% and AMY-2 45.14%), wheat (AMY-1 60.22% and AMY-2 51.1%) and glycogen (AMY-1 44.03% and AMY-2

325 32.39%). As expected, α-amylase could not degrade maltose, showing that it is not a
326 glucoamylase (α-glucosidase). Starches from different sources vary from each other by their
327 granule size and shape and the ratio and structure of the amylose and amylopectin molecules
328 [35]. These substrates consist of different glucose polymers containing α-1,4 linkages or a
329 mixture of α-1,4- and α-1,6-glycosidic linkages. Therefore the digestibility of various types of
330 starch varies with their source and their specificity towards the enzyme. In addition, for
331 industrial applications, it is important that the enzyme is able to degrade several substrates; in
332 this work AMY-1 and AMY-2 showed high digestibility efficiency against the substrates
333 used.
334
335 3.2.4 Effect of metal ions, EDTA and specific inhibitor
336 Similar interactions were observed between AMY-1 and AMY-2 and the metal ions to
337 which these enzymes have been exposed (Table 2). They were strongly inhibited by Cu^{2+}
338 (74.9% and 81.6%) and Hg^{2+} (87.9% and 84.4%). The inhibition by Hg^{2+} suggested the
339 presence of carboxyl and sulfhydryl groups in the enzyme molecule [35]. Similar results were
340 found for α-amylase from the fish *Labeo fimbriatus* [27] and from the yeast *Saccharomyces*
341 *cerevisiae* [36]. The ion Al^{3+} did not interfere in the amylolytic activity of the purified
342 enzymes. In the presence of 5mM Ca^{2+} AMY-1 and AMY-2 were inhibited by 17.6% and
343 12.4%, respectively. The affinity between metal ions and the α-amylase varies considerably
344 with the source of the enzyme. Many amylases are known to be calcium-dependent because
345 they contain a conserved Ca^{2+} binding site located at the interface between domains A and B.
346 At least one Ca^{2+} per molecule is required for the activation and stabilization of the enzyme
347 [37], however the findings here suggest that this amylase may not require calcium or maybe
348 the activity of Ca^{2+} in stabilization and activation is temperature-dependent and can only take
349 place at temperatures equal to or above the optimum temperature of the calcium-free enzyme

system [38]. Some α-amylases, like that from *Bacillus* sp., are calcium-free; these enzymes have calcium replaced by sodium in their molecular structure [39]. To elucidate the relationship between α-amylases from *Colossoma macropomum* and Ca^{2+}, EDTA 1mM was used as a chelating agent, and as shown in the Table 2, AMY-1 and AMY-2 remained active in the presence of EDTA. This result corroborates the hypothesis that they are not metalloenzymes. This is an important finding because chelators are a very common ingredient in most laundry detergents, so one may expect a denaturing effect on calcium-dependent enzymes [7]. The other ions reduced amylolytic activity, on average, by 10-30%. The influence by these metals may be due to their binding to catalytic residues in the active site of the enzyme, leading to an increase or decrease of activity.

The effect of a specific inhibitor of amylase from the plant *Triticum aestivum* on the amylolytic activity of *C. macropomum* was also evaluated. The reduction in activity caused by this inhibitor was similar in both α-amylases; around 46% inhibition of amylolytic activity could be observed (Table2). Studies addressing the characterization of α-amylases from several fish species showed different levels of inhibition (0-60%), attributing this difference to the presence of isoforms [22]. At concentrations of 200 and 50 μg/mL, this inhibitor supports inhibition rates from 40% to 60% against porcine and human salivary α-amylases, respectively [40]. The level of inhibition caused in AMY-1 and AMY-2 was consistent with the literature, certifying the amylaceous nature of the enzymes amylolytic.

3.3 Surfactant and oxidizing agents

Both enzymes showed significant stability in the presence of ionic and nonionic surfactant agents (Table 3). AMY-1 was more stable than AMY-2, showing increased activity in the presence of sodium choleate (110.69%), Tween 20 (107.22%) and Tween 80 (102.89%), but was inhibited in the presence of SDS, maintaining 67.37% of residual activity.

It has been reported that different ingredients of laundry detergents, such as anionic surfactants, bleaching agents and stabilizers influence the stability of enzymes [41]. Stable enzymes in the presence of anionic surfactants have been rarely observed. Most enzymes are unstable and lose biocatalytic activity in solutions of anionic surfactants, e.g. sodium dodecyl sulfate (SDS), caused by electrostatic and hydrophobic interactions leading to changes in the secondary and tertiary structures of the enzymes [42]. However, stability in the presence of detergents varies according to the source of the enzyme. The α-amylase from tambaqui was more stable than those from *Anoxybacillus beppuensis* (Tween 20 - 81.26%; Tween 80 - 85.62%; SDS - 72.18%) and *Aspergillus oryzae* (Tween 20 - 96.3%; Tween - 80 85.7; SDS - 32.2) [34, 43]. These results suggest that α-amylase from tambaqui is a promising alternative able to compete with bacterial and fungal enzymes.

In the presence of oxidizing agents, proteins may undergo inactivation due to oxidation of the side chains of their amino acid residues. In general, cysteine and methionine residues can be easily oxidized by H_2O_2. The oxidative stabilities of a few enzymes have been improved by replacing one methionine residue by a non-oxidizable amino acid residue via site-directed mutagenesis [44]. AMY-1 and AMY-2 are very stable when incubated with H_2O_2 (5%) for 60 min. They maintained activities (%) of 102.8±3.5 and 105.4±2.9, respectively, whereas α-amylase from *Bacillus licheniformis* had only 20% residual activity in the presence of H_2O_2 [45]. The activity loss depends on the location of these amino acids in the enzyme structure and is more susceptible when these are located on the surface of the enzyme. This is an important finding, because the inactivation by oxidation is a very common problem with enzymes used in detergents, and it is difficult to find enzymes compatible in extreme conditions.

3.4 Compatibility of α-amylase with commercial laundry detergents

α-Amylases are used in detergents to degrade the residues of foods (such as potatoes, gravies, custard, chocolate, etc.) to dextrin and other smaller oligosaccharides soluble in water. The suitability of any hydrolytic enzymes for inclusion in detergent formulations relies on their stability and compatibility with detergent components [5, 40]. An ideal detergent enzyme should be stable and active in the detergent solution for a long period. It should also be thermostable in order to maintain effectiveness through a wide range of washing temperatures [7]. Both α-amylases showed high compatibility with all the commercial laundry detergents tested in this study, retaining/increasing activity by up 116% and 152%, respectively for AMY-1 and AMY-2, during the washing time of 60 min at 40°C, as seen in Table 3. The increase in enzyme activity may be attributed to the stimulatory effect of some detergent components, for example ethoxylated surfactants and sucrose [40], but further studies are needed to elucidate the mechanism of this activation. AMY-2 was not inhibited by any detergent; however AMY-1 was slightly inhibited by ~13% in the presence of Tixan Ypê® and Ace®. This slight difference between the behaviors of the enzymes may be related to the better activity of AMY-2 in alkaline media.

Most enzymatic detergents use α-amylase of bacterial origin; however, the use of amylases in detergent formulations is problematic since the enzyme must offer stability and also an ideal level of activity in commercially used formulations [2]. AMY-1 and AMY-2 were greatly resistant to the detergent components, exhibiting, under some conditions, better results than commercial α-amylase of *Bacillus subtillis*. This finding highlights the potential use of α-amylases from tambaqui in this sector, and also reveals an important source of enzymes capable of competing with bacterial enzymes.

The amylases extracted from the digestive tract of tambaqui displayed high applicability as additives in the formulation of detergents, as they are resistant to surfactant

and oxidizing agents and their activity was not adversely affected by the presence of commercial detergents.

4 Conclusions

Fish viscera constitutes an important alternative source of enzymes with industrial applications. The recovery of purified α-amylases may represent a new way of utilization of fish enzymes with potential for biotechnological approaches. The use of fish viscera contributes to the sustainability of aquaculture making this activity more eco-friendly by reducing the amount of by-products, while adding value to the fish production chain by extracting molecules which can be applied in many different technological segments. In this work α-amylases could be obtained from the pyloric caeca and intestine of tambaqui through a heat treatment, saline fractionation and p-aminobenzamidine-agarose chromatography. The new method of purification developed in this study corresponds to an efficient and attractive technique that allows concomitant isolation of trypsins and α-amylases. When compared to other techniques, our methodology is an interesting alternative because it purifies in a few steps two different enzymes of biotechnological interest. Besides that, α-amylases from tambaqui viscera showed important biochemical characteristics, suggesting their potential use as additives to commercial detergents.

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583 Figure legends

584 Figure 1 - (A) SDS-PAGE and zymograms of purification of α-amylases (isolated from
585 tambaqui). 1 – Molecular-weight size marker; 2 – crude extract intestine (AMY-1); 3 – pooled
586 AMY-1; 4 – trypsin from intestine;5 – crude extract pyloric caeca (AMY-2); 6 – pooled
587 AMY-2; 7 – trypsin from intestine; 8 – zymogram AMY-1; 9 – zymogram AMY-2. (B)
588 Purification of amylase from intestine; (C) Purification of amylase from pyloric caeca.

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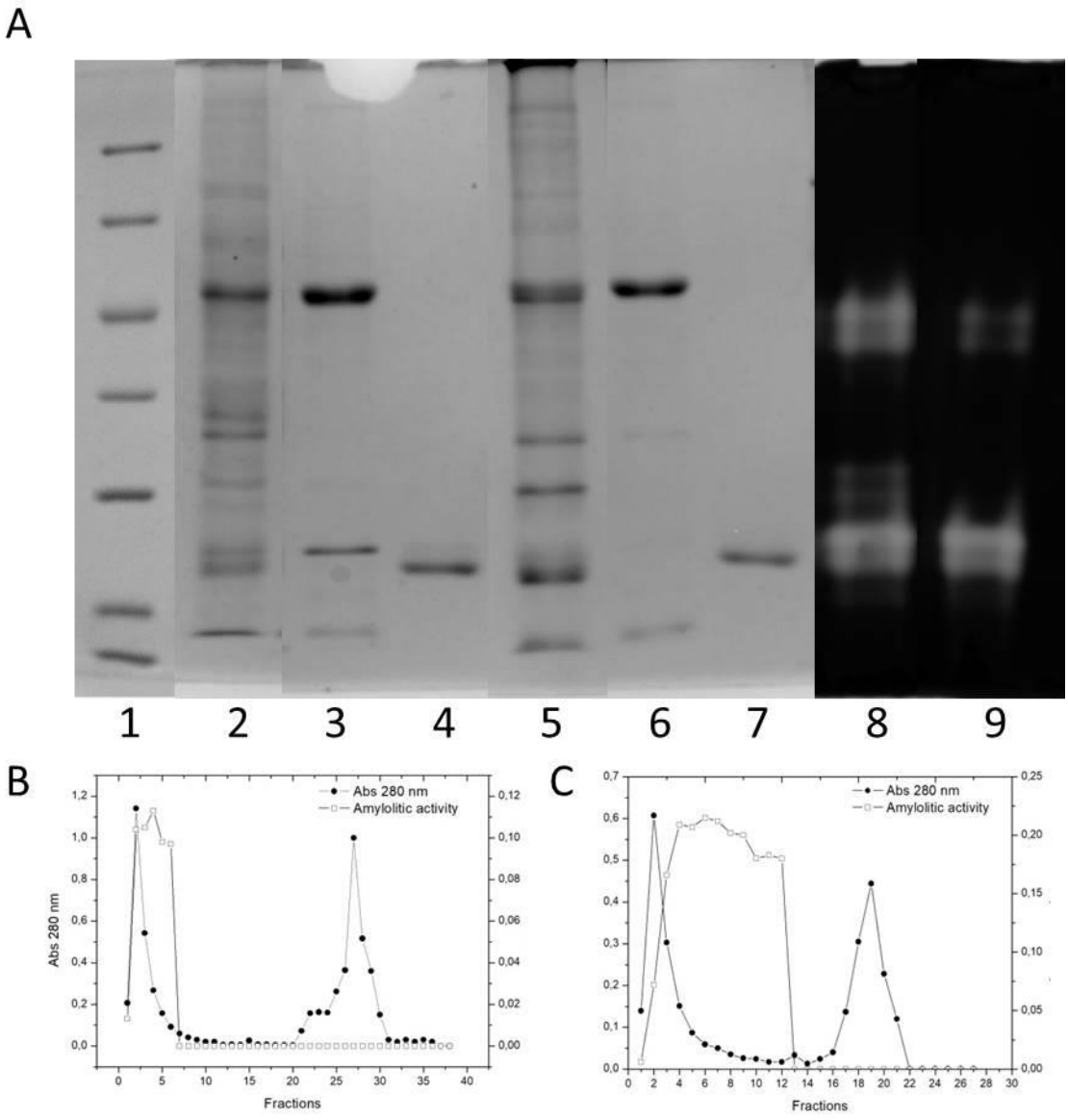
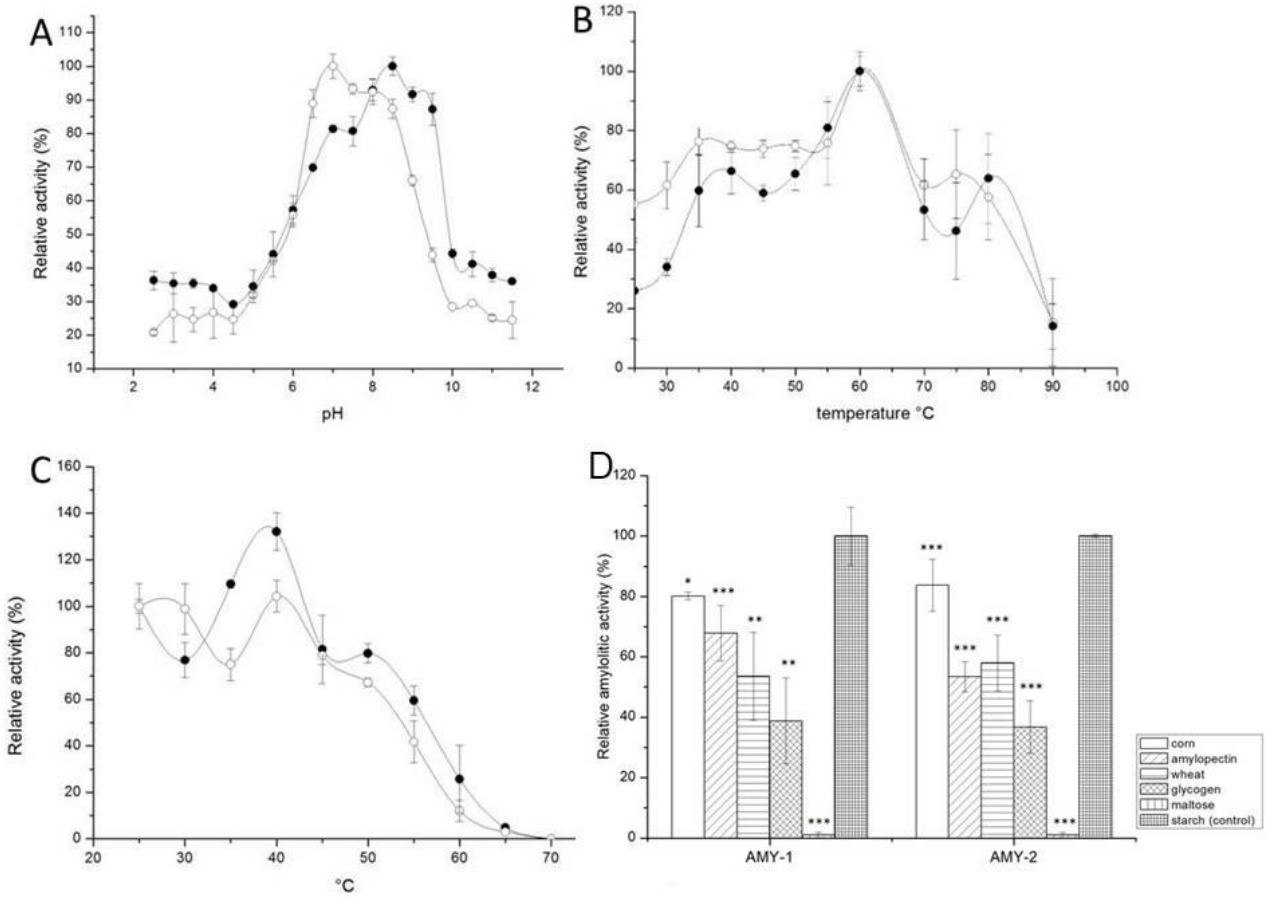


591

Figure 2 – Biochemical characterization of α-amylases from tambaqui (—○—: AMY-1; —●—: AMY-2). A – Optimum pH; B – Optimum temperature; C – Thermal stability; D – Substrate specificity.



Parameter	AMY-1 (from intestine)	AMY-2 (from pyloric caeca)
k_m (mg/mL)	2.82	2.24
V_{max} (mg maltose/mg ptn/min)	0.12	0.13
Rate Enh.	1.35E+23	2.18E+18
k_{cat} (s^{-1})	4.53E+03	4.66E-05
k_{cat}/k_m ($s^{-1}.M^{-1}$)	5.48E+05	7.11E+05
k_{non} (s^{-1})	1.00E-14; 1.90E-15	1.00E-14; 1.90E-15
$(k_{cat}/k_m)/k_{non}$ (M^{-1})	5.48E+19; 2.88E+20	7.11E+19; 3.74E+20
AE (Kcal/mol)	5.01	11.58

Table 1 – Kinetic and thermodynamic studies of AMY-1 and AMY-2.

Agent	AMY-1 (from intestine)	AMY-2 (from caeca pyloric)
Control	100	100
$CaCl_2$	82.3 ± 5.6 **	87.6 ± 3.8
$CuCl_2$	25.1 ± 7.0 ***	18.4 ± 4.6 ***
$HgCl_2$	12.1 ± 10.4 ***	15.6 ± 5.2 ***
$AlCl_3$	100.7 ± 0.9	101.1 ± 3.1
$PbCl_2$	78.3 ± 4.5 ***	81.2 ± 4.3 **
KCl	76.9 ± 0 ***	89.2 ± 3.6
$ZnSO_4$	73.9 ± 7.5 ***	78.2 ± 0.6 ***
$BaCl_2$	78.6 ± 6.1 **	81.2 ± 1.9 **
$CdSO_4$	63.2 ± 3.7 ***	68.4 ± 6.9 ***
NaCl	82.0 ± 2.6 ***	84.0 ± 3.9 *
EDTA	92.3 ± 4.01	101.8 ± 4.5
Amylase inhibitor	53.0 ± 7.9	54.3 ± 10.3

Table 2 – Effect of metal ions (5mM), EDTA (1mM) and amylase inhibitor (50µg/mL) on the activity of AMY-1 and AMY-2 isolated from tambaqui. Relative activity (%) values are mean ± SD of triplicate determinations.

Agent	AMY-1 (from intestine)	AMY-2 (from pyloric caeca)
Control	100 ± 1.2	100 ± 4.1
Choleate	110.68 ± 6.1	103.8 ± 0.4
Tween 20	107.21 ± 3.4	66.98 ± 3.9 **
Tween 80	102.88 ± 0.8	54.71 ± 5.8 ***
SDS	67.36 ± 6.9 **	57.25 ± 0.4 **
H_2O_2	102.85 ± 3.5	105.42 ± 2.9

Table 3 – Effect of surfactants (1%) and oxidizing agents (5%) on the activity of AMY-1 and AMY-2 isolated from tambaqui for 60 minutes at 40°C. Relative activity (%) values are mean ± SD of triplicate determinations.

Agent	AMY-1 (from intestine)	AMY-2 (from pyloric caeca)	commercial amylase
Control	100 ± 5.6	100 ± 3.1	100 ± 8.99
Ace®	88.74 ± 3.52	121.78 ± 3.1*	120.99 ± 2.44 *
Ala®	116.13 ± 5.73	122.5 ± 7.2*	126.33 ± 5.51 *
Brilhante®	100.75 ± 5.4	103.7 ± 7.9	119.66 ± 0.35*
Tixan ypê®	86.8 ± 2.55	117.2 ± 10.3	126.21 ± 4,73**
Ace®	112.3 ± 8.28	145.0 ± 8.9 ***	132.40 ± 3.57 ***
Roma®	108.2 ± 5.7	152.2 ± 4.5 **	86.04 ± 0.81

657

658 Table 4 – Effect of commercial detergents (7mg/mL) on the activity of AMY-1 and AMY-2
659 isolated from tambaqui and commercial amylase from *Bacillus subitillis* for 60 minutes at
660 40°C. Relative activity (%) values are mean ± SD of triplicate determinations.

5 CONSIDERAÇÕES FINAIS

- O estudo cinético da α-amilase da tilápia do Nilo revelou uma enzima cataliticamente eficiente e dependente de pH. Foi observada a interferência do pH em todos os parâmetros catalíticos, havendo melhores desempenhos no pH 6.0 em relação ao pH 7.4;

- A α-amilase da tilápia foi fortemente ativada por cloreto, tendo todos os parâmetros cinéticos aumentados na presença do íon. Pode-se observar que o íon aumenta a termoestabilidade da α-amilase em temperaturas acima de 50°C, mas apenas no pH 6.0. Foi observada ainda considerável plasticidade do sítio de cloreto, uma vez que outros íons como brometo e iodeto também levaram ao aumento da velocidade enzimática, mas em menor proporção comparado ao cloreto. O íon acetato, no entanto, causou declínio na velocidade enzimática, mostrando uma limitação quanto ao tamanho os íons comportados no sítio de cloreto; A enzima manteve-se ativa frente aos agentes quelantes EDTA e EGTA, o que sugere uma α-amilase independente de cálcio;

- A α-amilase da tilápia mostrou-se apta para aplicação no processamento enzimático do amido cru, hidrolisando amido de batata em diferentes concentrações sob baixa temperatura e dose de enzima. A α-amilase foi capaz de hidrolisar suspensões concentradas de amido (até 15%), mas obteve melhor grau de hidrólise nas reações com 1 e 5% de amido, alcançando até 45% de hidrólise; Os produtos finais de hidrólise mostram a produção de G1, G2 e maltodextrinas em todos os tempos de incubação, o que sugere uma enzima com ação sacarificadora, importante característica para aplicação na indústria do amido. A análise dos grãos de amido através da microscopia eletrônica de varredura mostra as mudanças morfológicas causadas na estrutura do amido após o tratamento enzimático, e confirma a capacidade de digestão da α-amilase;

- A α-amilase da tilápia foi altamente resistente à exposição a pH e solventes orgânicos, mantendo-se ativa após 24h na faixa de pH variando de 3 a 10, e em todos os solventes testados, o que ratifica sua aplicação em processos industriais.

- α-Amilases e tripsinas oriundas dos cecos pilóricos e intestino de tambaqui podem ser obtidas através de purificação conjunta, representando uma metodologia alternativa e econômica para obtenção dessas enzimas digestivas em um único procedimento; O perfil de eletroforese e zimograma mostram a purificação parcial das α-amilases e destacam ainda a presença de isoformas;

- As α-amilases obtidas do intestino e cecos pilóricos do tambaqui apresentaram características requeridas para aplicações industriais, como termoestabilidade até 60°C, compatibilidade com íons metálicos, sendo inibidas apenas por cobre e mercúrio; boa capacidade de digestão a diferentes substratos, mostrando preferência por clivar ligações α-1,4; e resistência a agentes quelantes, sugerindo ser uma amilase independente de cálcio;

- Quanto a seus parâmetros cinéticos, ambas apresentaram boa afinidade por amido, e foi possível observar ainda uma melhor eficiência catalítica de AMY-2 em relação à AMY-1;

- As α-amilases do tambaqui apresentaram alta compatibilidade com surfactantes, oxidantes e com sabões comerciais, o que sugere o potencial uso dessas enzimas como aditivo de limpeza no setor de detergentes.

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