YAREMIS BEATRIZ MERIÑO CABRERA

MODELAGEM MOLECULAR E CINÉTICA ENZIMÁTICA: ANÁLISES DA INIBIÇÃO DE TRIPSINAS DE PRAGAS LEPIDOPTERA POR PROTEÍNAS ENVOLVIDAS NA DEFESA DE PLANTAS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada, para obtenção do título de *Doctor Scientiae*.

Orientadora: Maria Goreti de Almeida Oliveira

Coorientadores: Tiago de Oliveira Mendes
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Porque é se orgulhar da escolha que te oferecu mil tesouros e se odiar pela mesma escolha que te subtraiu outras mil pedras preciosas.

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RESUMO

MERIÑO-CABRERA, Yaremis Beatriz, D.Sc., Universidade Federal de Viçosa, novembro de 2019. **Modelagem molecular e cinética enzimática: análises da inibição de tripsinas de pragas Lepidoptera por proteínas envolvidas na defesa de plantas**. Orientadora: Maria Goreti de Almeida Oliveira. Coorientadores: Tiago Antonio de Oliveira Mendes, Maria Ligia Rodrigues Macedo, Glaucia Cordeiro e Joel Antônio de Oliveira.

Inibidores de proteases (IPs) são considerados uma alternativa mais sustentável aos pesticidas inorgânicos comumente utilizados no controle de pragas. A atuação de IPs nas tripsinas digestivas de herbívoros gera impactos negativos em parâmetros do ciclo de vida destes, como sobrevivência, peso larval e oviposição. Embora existam IPs naturais com atividade inseticida satisfatória, muitos destes são superados pelos insetos através da expressão de novas proteases. Além disso, inibidores de tripsinas naturais são moléculas grandes, aumentando as chances de serem clivados e inativados por outras enzimas proteolíticas. Sendo assim, o desenvolvimento de inibidores de proteases orgânicos de pequenas cadeias de resíduos de aminoácidos e alta especificidade a tripsinas do intestino médio de herbívoros, pode auxiliar no controle de pragas agrícolas. Assim, neste estudo foi testado a capacidade de inibição de duas proteínas inhibitorias, ApTI (do inglês Adananthera pavonina trypsin inhibitor) e ILTI (do inglês Inga laurina trypsin inhibitor), sobre tripsinas das espécies *Spodoptera cosmioides* e *Anticarsia gemmatalis*, ambas as espécies pertencem à ordem Lepidoptera. O sítio de ligação e o potencial de inibir tripsina-*like* destas especies pelos inibidores foram avaliados por *docking* e dinâmica molecular, ensaios para caracterização de parâmetros cinéticos e ensaios biológicos, misturando os inibidores em dieta artificial. O objetivo foi analisar o potencial inibiotório destas proteínas e a partir dos resultados estruturais e bioquímicos obtidos, desenvolver peptídeos com alta capacidade inseticida, que possam ser utilizados como modelo para a produção de peptídeos miméticos, serem pulverizados nas plantas, ou gerar dados para o desenvolvimento de plantas transgênicas. Os inibidores ILTI e ApTI apresentam alto potencial biotecnológico como agentes contra insetos fitófagos Lepidoptera, inibindo tripsinas por inibição tight-binding, com características competitivas e não competitivas, respectivamente. A ação de ApTI e ILTI no desenvolvimento de larvas de *S. cosmioides* e *A. gemmatalis* mostra que estes inibidores influenciam a sobrevivência larval, indicando seu potencial tóxico. Os peptídeos derivados do sitio reativo do inibidor ILTI apresentam afinidade pelas enzimas tripsinas de *S. cosmioides* e reduzem sua atividade biológica, confirmando o potencial inibitório destes compostos.

Palavras-chave: Proteínas-inibidores. Simulação de acoplamento molecular. Insetos. Peptídeos. Enzimas proteolíticas.

ABSTRACT

MERIÑO-CABRERA, Yaremis Beatriz, D.Sc., Universidade Federal de Viçosa, November, 2019. **Molecular modeling and enzymatic kinetics: Analyzes of Lepidoptera pests trypsin inhibition by proteins involved in plant defense**. Adviser: Maria Goreti de Almeida Oliveira. Co-advisers: Tiago Antonio de Oliveira Mendes, Maria Ligia Rodrigues Macedo, Glaucia Cordeiro and Joel Antônio de Oliveira.

Protein protease inhibitors (PIs) are considered a more sustainable alternative to inorganic pesticides commonly used for pest control. The action of PIs on herbivorous digestive trypsins negatively impacts parameters of their life cycle, such as survival, larval weight and oviposition. Although there are natural IPs with satisfactory insecticidal activity, many of these are overcome by insects through the expression of new proteases. In addition, natural trypsin inhibitors are large molecules, increasing the chances of being cleaved and inactivated by other proteolytic enzymes. Thus, the development of organic protease inhibitors of small amino acid residues chains and high specificity to herbivorous midgut trypsins may help control agricultural pests. Thus, in this study we tested the inhibitory capacity of two inhibitory proteins, ApTI (Adananthera pavonina trypsin inhibitor) and ILTI (Inga laurina trypsin inhibitor), on trypsins of *Spodoptera cosmioides* and *Anticarsia gemmatalis* species, both species belong to fear Lepidoptera. The binding site and the trypsin-like inhibiting potential of these species by inhibitors were evaluated by docking and molecular dynamics, assays for characterization of kinetic parameters and biological assays, mixing the inhibitors in artificial diet. The aim was to analyze the inhibitory potential of these proteins and from the structural and biochemical results obtained, develop peptides with high insecticide capacity, which can be used as a model for the production of mimetic peptides, be sprayed on plants, or generate data for development of transgenic plants. ILTI and ApTI inhibitors have high biotechnological potential as agents against Lepidoptera phytophagous insects, inhibiting trypsins by tight-binding inhibition, with competitive and non-competitive characteristics, respectively. The action of ApTI and ILTI on the development of *S. cosmioides* and *A. gemmatalis* larvae shows that these inhibitors influence larval survival, indicating their toxic potential. The peptides derived from the ILTI inhibitor reactive site have affinity for *S. cosmioides* trypsin enzymes and reduce their biological activity, confirming the inhibitory potential of these compounds.

Keywords: Docking. Protein-inhibitors. Docking. Insects. Peptides. Proteolitic enzymes.

SUMÁRIO

1. INTRODUÇÃO GERAL 11
2. OBJETIVOS 14
Objetivo Geral 14
3. DELINEAMENTO EXPERIMENTAL 15
4. REVISÃO DE LITERATURA 15
4.1 INIBIDORES DE PROTEASES 15
4.1.1 Inibidor de tripsina *Inga laurina* (ILTI) 17
4.1.2 Inibidor de tripsina de *Adenanthera pavonina* (ApTI) 19
4.2 MODELAGEM MOLECULAR 19
4.2.1 Modelagem comparativa de proteínas 19
4.2.2 Phyre2 e Modelagem de proteínas 21
4.2.3 Qualidade e Validação dos Modelos tridimensionais de proteínas 21
4.3 DOCKING MOLECULAR 22
4.3.1 *Docking* Proteína-proteína 24
4.4 PEPTÍDEOS 25
5. REFERÊNCIAS 26
Capitulo I: 33
Inibição de tripsinas digestivas mediante proteínas de planta tipo Kunitz reduz a viabilidade das larvas de *Spodoptera cosmioides* (Lepidoptera:Noctuidae) 33
Abstract 34
Introduction 35
Materials and methods. 36
In silico Assay 36
Obtaining the sequences 36
Molecular modeling and docking 37
In vitro assay 38
Trypsins purification of *Spodoptera cosmioides* 38
Inhibition kinetic 39
In vivo assay 39
Biological material 39
ApTI and ILTI inhibitors effect on development and survival of *Anticarsia gemmatalis* 40

Results 40
Molecular Docking 40
Trypsins purification of *Spodoptera cosmioides* 41
Inhibition kinetic 42
Survival Assay 42
Discussion 43
Acknowledgements 47
References 47
Capitulo II: 63
Desenho racional de peptídeos miméticos baseados na interação entre *Inga laurina trypsin inhibitor* e tripsinas para o controle da praga*Spodoptera cosmioides* 63
Abstract 64
1. Introduction 65
2. Materials and Methods 66
3. Results 70
4. Discussion 73
5. Conclussion 77
References 78
Capitulo III: 87
Inibidor de protease extraído de sementes da planta *Adenanthera pavonina*: efeitos sobre tripsinas digestivas de *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) 87
Abstract 88
1. INTRODUCTION 89
2. MATERIALS AND METHOD 90
2.1 *In silico* docking of ApTI inhibitor and trypsin 90
2.1.1 Sequence and structure retrieval 90
2.1.2 Protein-protein Docking 91
2.2 *In vitro* inhibitory activity assay 91
2.2.1 Trypsin enzyme extraction 91
2.2.1.1 Biological material 91
2.2.1.2 Preparation of gut extracts 91
2.2.1.3 Chromatography and SDS-PAGE 92
2.2.2 Adenanthera pavonina Trypsin Inhibitor obtainment 92
2.2.3 Enzyme inhibitory assays 92

2.3 ApTI inhibitor effect on survival of *Anticarsia gemmatalis*.....93
3. RESULTS.....93
DISCUSSION.....98
ACKNOWLEDGEMENTS.....101
REFERENCES.....101
CONCLUSÕES GERAIS.....109
ANEXO 1.....110

1. INTRODUÇÃO GERAL

O desenvolvimento biológico de insetos é afetado pela quantidade e qualidade de alimento consumido. Os insetos têm como exigências nutricionais básicas aminoácidos, vitaminas, sais minerais, carboidratos, lipídeos e esteróides e a alimentação deles influencia diretamente a taxa de crescimento, tempo de desenvolvimento, sobrevivência, fecundidade, longevidade, movimentação e capacidade de competição de adultos (Wang et al. 2018; Pannizi and Parra, 2009).

Como a maioria dos organismos vivos, os insetos produzem enzimas digestivas para a obtenção dos nutrientes essenciais a sua atividade metabólica. Dentre as enzimas, as proteases são essenciais no metabolismo de aminoácidos, classificadas em serino, cisteino, aspártico e metalo proteases (Jongsma and Beekwilder 2011). As serino-proteases são um dos grupos mais estudados, atuamdo em uma reação de hidrólise de dois passos, onde um intermediário acil-enzima covalentemente ligado é formado. Esta acilação é seguida pela desacilação, processo no qual ocorre o ataque nucleofílico intermediado pela agua, resultando na hidrólise do peptídeo (Boldrini-França et al. 2019).

A ação dos herbívoros sobre as plantas tem efeitos negativos devido à redução da reprodução e sobrevivência das plantas atacadas, principalmente no início do ciclo de vida. No entanto, as plantas têm desenvolvido diferentes mecanismos de defesa, sendo constitutivos ou sintetizados em resposta ao ataque (induzidos) (Fürstenberg-Hägg et al. 2013). Um desses mecanismos é a produção de inibidores de proteases, proteínas que podem ser produzidas por órgãos reprodutivos, de armazenamento e tecidos vegetativos de algumas famílias de plantas; formam complexos estáveis com proteases alvo, bloqueando, alterando ou prevenindo o acesso ao sítio ativo da enzima (Meriño-Cabrera et al. 2019, Patarroyo et al. 2017, Macedo and Das Graças Machado Freire, 2011).

Os inibidores de proteases empregados neste estudo foram ApTI (do inglês Adananthera pavonina trypsin inhibitor) e ILTI (do inglês Inga laurina trypsin inhibitor). ApTI é um inibidor isolado a partir das sementes da árvore *Adananthera pavonina,* massa molecular aproximada de 21kDa, possui duas cadeias polipeptídicas ligadas por uma ligação dissulfeto, com alto nível de inibição de proteases, 3-16 vezes maior que o inibidor *Glycine max* (soja) (SKTI *Soybean Kunitz trypsin inhibitor*) (Rodrigues et al. 2018; Macedo et al. 2010; Migliolo et al. 2010;

Macedo et al. 2004); purificado pela primeira vez por Pranhu & Pattabiraman (1980). Por outro lado, ILTI é um inibidor de única sequência contendo 180 resíduos, contendo duas ligações dissulfeto, isolado da planta *Inga laurina* (Machado et al. 2017; Macedo et al. 2007; Macedo et al. 2011;).

A interação de inibidores de proteases de plantas e tripsinas de fitófagos Lepidoptera é um exemplo de reconhecimento molecular entre ligante-macromolécula. A compreensão desses mecanismos de reconhecimento é um dos aspectos centrais para o sucesso na descoberta de novos compostos inibidores. A obtenção de uma descrição do reconhecimento molecular receptor-ligante pode ser obtida a partir de ferramentas computacionais, ou seja, estudos *in silico*, devido à possibilidade de redução de tempo e custos. Especificamente, métodos como *docking* e dinâmica molecular entre receptor-ligante permitem identificar a conformação de ligação, objetivando uma otimização das moléculas promissoras selecionadas. Além disso, testes *in vitro* e *in vivo* são realizados para guiar e validar a otimização de compostos protótipos e são úteis para que outras caraterísticas, tal como toxidez, sejam analisadas (Salmaso & Moro 2018; Tsu et al. 1997).

Neste trabalho o modelo biológico de estudo foram as espécies fitófagas *Spodoptera cosmioides* and *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). *S. cosmioides* conhecida como lagarta preta ou lagarta da vagem, é uma praga em expansão devido à crescente incidência e intensidade de danos nos cultivos agrícolas. As lagartas desta espécie causam desfolhamento e perfuração das estruturas reprodutivas como botões florais, flores e vagens. Considerada praga nas culturas de algodão, soja, feijão, frutíferas, tais como morango e uva (Silva et al. 2016; Souza et al. 2013).

A lagarta-da-soja, *Anticarsia gemmatalis* é considerada a principal desfolhadora nas Américas e é uma das espécies mais comuns na cultura da soja. Sua época de ocorrência está correlacionada à latitude onde se encontra localizada a lavoura, tendo sido observado que os ataques ocorrem antes nas latitudes mais baixas, enquanto no sul do país os ataques são mais tardios. Atacam alfafa (Medicago sativa), algodão (Gossypium hirsutum), amendoim (Arachis hypogaea), arroz (Oryza sativa), batata(Solanum tuberosum), brócolis (Brassica oleracea L. var. itálica), cana-de-açúcar (Sacharum officinarum), couve (Brassica oleracea), couve-flor (Brassica oleracea var. Botrytis), ervilha (Pisum sativum), feijão (Phaseolus vulgaris), fumo (Nicotiana tabacum), mandioca (Manihot esculenta), maracujá (Passiflora edulis), milho (Zea mays), seringueira (Hevea brasiliensis), soja (Glycine max) (Dalazen et al. 2017).

Espécies pragas da ordem Lepidoptera têm sido controladas através da utilização de inseticidas organosintéticos. Entretanto, estes produtos químicos têm baixa eficácia devido à tolerância natural dessas pragas aos inseticidas (Carvalho et al. 2013). Além disso a dependência ao uso de inseticidas pode provocar prejuízos financeiros, desequilíbrio da cadeia alimentar, elevar pragas secundárias à categoria de pragas-chave, causar o surgimento de novas pragas e de pragas resistentes (Vianna et al. 2011). Essas consequências do uso de inseticidas têm incentivado a realização deste estudo, para aumentar a informação destas pragas e proporcionar métodos alternativos de controle.

Assim, o objetivo deste trabalho foi caraterizar a estrutura e cinética enzimática da interação entre inhibidores proteicos de plantas e tripsinas de intestino de insetos pragas, visando a derivação de peptídeos inibitórios a partir da interface destes complexos.

2. OBJETIVOS

Objetivo Geral

- Caraterizar a estrutura e cinética enzimática da interação entre inhibidores proteicos de plantas e tripsinas de intestino de insetos pragas, visando o desenvolvimento de peptídeos miméticos inibitórios a partir da interface destes complexos.

Objetivos específicos

- **Capitulo I**
  Determinar a atividade inibitória das proteínas *Inga laurina trypsin inhibitor* (ILTI) e *Adananthera pavonina trypsin inhibitor* (ApTI) sobre as enzimas tripsina-*like* de *Spodoptera cosmioides* mediante *docking* molecular e análises de cinética enzimática e sobrevivência
- **Capitulo II**
  Desenhar, sintetizar e caracterizar peptídeos extraidos da interface entre os complexos de *Inga laurina trypsin inhibitor* (ILTI) com tripsinas da espécie *Spodoptera cosmioides*, objetivando identificar compostos candidatos a biopesticidas.
- **Capitulo III**
  Avaliar mediante mapeamento molecular e cinética enzimática a capacidade de inibição da proteína *Adananthera pavonina trypsin inhibitor* (ApTI) sobre as enzimas tripsina-*like* da espécie *Anticarsia gemmatalis*

3. DELINEAMENTO EXPERIMENTAL

O efeito inibitorio das proteínas *Inga laurina trypsin inhibitor* (ILTI) e *Adananthera pavonina trypsin inhibitor* (ApTI) foi testado sobre as enzimas tripsina-*like* de *Spodoptera cosmioides* mediante *docking* molecular e análises de cinética enzimática e sobrevivência das larvas da espécie em estudo. O melhor inibidor, baseado nas análises anteriores, foi escolhido para a derivação de peptídeos a partir do seu sitio reativo. Os peptídeos foram sintetizados quimicamente, foram caraterizados e foi determinada sua funcionalidade para inibir tripsinas e sua toxicidade sobre as larvas da espécie *S. cosmioides*.

Além disso, o inhibidor proteico ApTI também foi testado sobre as enzimas tripsina-*like* da espécie *Anticarsia gemmatalis* mediante mapeamento molecular e cinética enzimática e análises biológicas.

4. REVISÃO DE LITERATURA

4.1 INIBIDORES DE PROTEASES

As proteases pertencem a um grupo de enzimas responsáveis por realizar a hidrólise de ligações peptídicas em proteínas, gerando assim peptídeos ou aminoácidos, dependendo do tipo de protease a ser utilizada. Essas enzimas podem ser subdivididas em dois grandes grupos, de acordo com o local onde ocorre a clivagem da proteína: endopeptidases ou exopeptidade. As endopeptidades atuam clivando a proteína em sítios específicos nas regiões internas da cadeia polipeptídica, enquanto que as exopeptidades atuam pelas extremidades removendo os resíduos de aminoácidos tanto pela região N ou C terminal da cadeia polipeptídica (Boldrini-França et al. 2019).

Além dessa subdivisão, as proteases geralmente são organizadas em classes, de acordo com a sua função, homologia na estrutura primária e a natureza do seu sítio ativo. Dentre as classes mais estudas têm-se as serino proteases, tendo como exemplo as enzimas tripsina e quimotripsina, além de serem as principais enzimas encontradas no trato digestivo de insetos da ordem lepidóptera e díptera (Mendoça et al. 2019, Da Silva Júnior et al. 2019, Meriño-Cabrera et al. 2018, Patarroyo et al. 2017, Faheem et al. 2016).

Grande parte dessas enzimas possuem sua atividade inibida por outro grupo de proteínas denominado inibidores de proteases. Essas proteínas formam complexos estequiométricos enzima-proteína reversíveis com enzimas proteolíticas, levando assim a uma inibição competitiva das suas funções catalíticas (Lira et al. 2019; Richardson, 1977). De uma forma geral, os inibidores de proteases possuem um papel importante na regulação da atividade proteolítica de uma protease específica, ou para sinalizar interações com receptores. Sendo assim, os inibidores de proteases acabam possuindo uma ampla aplicação biotecnológica. Estes já possuem um papel importante no desenvolvimento de diversos fármacos, sendo utilizados no tratamento de diversas doenças, como no caso de doenças cardiovasculares, tratamento e prevenção contra câncer, contra Síndrome de Imunodeficiência Adquirida (SIDA), em inglês: Acquired Immunodeficiency Syndrome (AIDS), Alzheimer, além de diversas doenças humanas (Shamsi et al. 2016). Além de possuir uma grande importância no desenvolvimento de fármacos, os inibidores de proteases também possuem um importante papel na agricultura, sendo utilizados como bioinseticidas, antifúngicos e também agentes antibacterianos (Meriño-Cabrera et al. 2019, Patarroyo et al. 2017, Brito et al. 2016; Kim et al., 2005).

Esses inibidores são encontrados, naturalmente, em uma grande diversidade de organismos. Dentre esses, podemos destacar as plantas (Macedo et al., 2011). Dessa forma, o estudo desses inibidores de proteases na defesa de plantas foi amplamente difundido, sendo verificado que essas moléculas evitam a herbivoria. Estudos iniciais demonstraram, inclusive, que as larvas de alguns insetos não se desenvolviam corretamente quando alimentadas com sementes de soja, justamente por serem ricas em inibidores de proteases. Assim, diversos outros estudos sobre inibidores de proteases isolados de plantas foram realizados, a fim de verificar se estes poderiam ser utilizados para o controle de pragas (Singh et al. 2018; Macedo et al., 2007). Os inibidores de proteases de origem vegetal são encontrados geralmente em sementes e em outros órgãos de armazenamento da planta. São moléculas extremamente estáveis, resistentes tanto a variações de temperatura quanto a variações no pH e à atividade proteolítica de outras proteases, devido, principalmente, à presença de ligações dissulfeto em sua estrutura (Swathi et al. 2016).

Assim como as proteases, os inibidores de proteases são classificados de acordo com a classe de protease em que o inibidor atua, podendo estes serem inibidores de serinoproteases, cisteínoproteases, metaloproteases e aspártil-proteases. As plantas possuem uma grande variedade de inibidores de serinoproteases, que são divididos em 16 famílias, sendo que as mais

estudadas e caracterizadas são as pertencentes às famílias dos inibidores do tipo Kunitz e do tipo Bowman-Birk (Clemente et al. 2019).

Os inibidores do tipo Kunitz são encontrados, principalmente, em sementes de leguminosas, e têm como função principal a de defesa dessas sementes, pois os mesmos atuam como inibidores de importantes enzimas digestivas, como a tripsina e quimotripsina, as principais enzimas digestivas presentes no trato digestivo de insetos. Inibidores dessa família, na maioria das vezes, no caso de inibidores do tipo Kunitz encontrados em plantas, apresentam uma cadeia polipeptídica composta por cerca de 60 residuos de aminoácido e massa molecular próxima de 20 kDa, geralmente, possuem quatro a seis cisteínas em sua estrutura primária, o que permite a formação de duas a três ligações dissulfeto (Clemente et al. 2019; Ranasinghe & McManus 2013).

Porém os inibidores Kunitz encontrados em plantas não possuem relação com os inibidores Kunitz encontrados em animais, divergindo muito em seu tamanho, composição de resíduos de aminoácido e número de ligações dissulfeto. O principal mecanismo de inibição dos inibidores de proteases do tipo Kunitz consiste em uma interação não covalente com uma serinoprotease, (Ranasinghe & McManus 2013), bloqueando o sítio ativo dessa proteína, por meio da formação de uma folha-β antiparalela entre a enzima e o inibidor, gerando um complexo enzima-inibidor, assim como o complexo enzima-substrato, previamente descrito por Michaelis-Menten (Clemente et al. 2019). Além de serem inibidores de serinoproteases os inibidores do tipo Kunitz também podem ser inibidores de cisteíno e aspártico-proteases (Singh et al. 2018). Porém a inibição dessas outras famílias de proteases é feita através da ligação de diferentes domínios da proteína à enzima, em relação ao modelo de interação observado entre inibidores Kunitz com as serinoproteases (Oliveira et al. 1993).

4.1.1 Inibidor de tripsina *Inga laurina* (ILTI)

Inga laurina, popularmente conhecido como ingá-branco ou ingá-mirim, é uma árvore nativa, não endêmica, pertencente à família Fabaceae, subfamília Mimosoideae, que possui uma ampla distribuição geográfica, indo no norte ao sul do Brasil, podendo ser encontrada nos biomas Amazônia, Caatinga, Cerrado e Mata Atlântica (Machado et al. 2017). Estudos anteriores relataram que as sementes de *I. laurina* possuem um inibidor de tripsina, denominado ILTI (do inglês Inga laurina Trypsin Inhibtor) (Carneiro et al. 2018; Macedo et al., 2007). O ILTI possui 180 residuos de aminoácido em sua cadeia polipeptídica única, massa molecular

próxima a 20kDa e sequência homóloga à dos inibidores de serinoprotease da família Kunitz (Carneiro et al. 2018; Macedo et al., 2007). Porém, foram observadas algumas diferenças em sua estrutura em relação aos modelos de inibidores da família Kunitz, como por exemplo a presença de somente uma cadeia polipeptídica, enquanto os membros dessa família geralmente apresentam duas cadeias e a presença de somente uma ligação dissulfeto em sua estrutura, enquanto que os inibidores dessa família são característicos por possuírem duas ligações ligando as duas cadeias polipeptídicas.

ILTI possui alta estabilidade, tolerando as variações de pH, tendo sua atividade residual próxima de 100% em uma faixa de pH 2,0 até 10,0, e extremamente resiste a variações de temperatura, tendo sua atividade residual diminuída somente em temperaturas acima de 70 ºC (Machado et al. 2017; Macedo et al., 2007). Apesar de possuir somente uma ligação dissulfeto em sua estrutura, foi verificado que a ausência da segunda ligação não interfere em sua estabilidade funcional e na sua atividade inibitória (Macedo et al., 2011). Após a caracterização de ILTI, estudos seguintes avaliaram o efeito inseticida deste inibidor. Tem sido verificado que ILTI possui um grande efeito inibidor em proteases extraídas do trato digestivo de insetos, reduzindo sua atividade residual em até 95% (Meriño-Cabrera et al. 2019). Além disso, foi verificado que o ILTI inibe o crescimento e desenvolvimento de insetos, como *Homalinotus coriaceus*, popularmente conhecido como broca do cacho de coqueiro, uma das principais pestes no Brasil em plantações de coqueiros, inibindo o seu desenvolvimento larval em 96% (Carneiro et al. 2018; Macedo et al., 2011).

Outra classe de insetos que obteve seu desenvolvimento prejudicado, devido à adição de ILTI às suas dietas, foram vários representantes da ordem Lepidóptera, dentre eles *Diatraea saccharalis*, popularmente conhecida como broca da cana-de-açúcar, ou somente broca da cana, a principal praga de cana-de açúcar no Brasil e em outros países da América do Sul (Ramos et al., 2012).

Por possuir sua estrutura bem definida, ser uma molécula extremamente estável e resistente e por inibir o desenvolvimento larval de vários insetos, isso faz do ILTI um candidato promissor a ser utilizado como um possível bioinseticida. Devido a essas características o ILTI foi produzido de forma heteróloga em *Escherichia coli*, e a molécula recombinante obtida mostrou atividade semelhante à molécula nativa em testes realizados *in vitro* (Ramos et al., 2012). Porém, não foi informado a quantidade de proteína produzida nesse trabalho, podendo assim considerar que a quantidade de proteína produzida tenha sido ínfima, o que mostra ser

necessário, portanto, testar um outro sistema de produção, a fim de verificar se este seria viável para a produção dessa molécula.

4.1.2 Inibidor de tripsina de *Adenanthera pavonina* (ApTI)

Essa espécie vegetal é encontrada naturalmente na Índia, porém foi introduzida em alguns países como: Porto Rico, Cuba, Jamaica, Venezuela, Costa Rica, Honduras, Estados Unidos e Brasil. Vulgarmente conhecida como Carolina, é uma planta decídua que alcança 6 - 15 metros de comprimento e 45 centímetros de diâmetro. É uma planta geralmente ereta, de cor marrom escuro e coroa espalhada. Suas flores são pequenas, de cor amarela com pontos marrom e perfumada. Cada flor possui forma de estrela com cinco pétalas. Suas sementes são duras de cor vermelha brilhante e geralmente usada como ornamento decorativo, historicamente a semente foi usada como medida de peso para jóias devido a sua pequena variação de peso (Melo et al. 2018).

O inibidor de *A. pavonina* (ApTI) é um inibidor do tipo Kunitz que inibe de forma competitiva enzimas do tipo tripsina, quimotripsina e papaína e apresenta massa molecular relativa de 19 kDa (Rodrigues et al. 2018; Macedo et al., 2009). ApTI foi isolado através de métodos clássicos de purificação de proteínas, cromatografia de exclusão molecular, cromatografia de troca iônica e cromatografia de afinidade em coluna de Sepharose – tripsina (Velmani et al. 2019).

4.2 MODELAGEM MOLECULAR

A modelagem molecular engloba todas as técnicas computacionais utilizadas para simular o comportamento de moléculas. Essas técnicas são amplamente utilizadas nos campos de química computacional e desenvolvimento de fármacos para estudar sistemas biológicos, podendo ser aplicado, portanto, na descoberta de inibidores de enzimas.

4.2.1 Modelagem comparativa de proteínas

A caracterização funcional de sequências proteicas é um problema frequente na área biológica. Atualmente é bem estabelecido que o conhecimento da estrutura molecular é uma ferramenta poderosa para entender, controlar e alterar funções de biomoléculas. Apesar de estruturas tridimensionais de proteínas poderem ser determinadas por cristalografia por raios-x

e ressonância magnética nuclear, esses experimentos demandam tempo e grandes quantidades de proteínas em auto grau de pureza, além de possuírem algumas limitações. A técnica de NMR é difícil de ser aplicada a grandes proteínas (maior que 250 residuos de aminoácido), ou proteínas muito flexíveis, enquanto a cristalografia por raios-x depende da obtenção de cristais com boa capacidade de difração, processo realizado por tentativa e erro, e da solução do problema de fase (Schneidman-Duhovny & Hammel 2018).

Entretanto, sequências de proteínas podem ser determinadas muito mais facilmente através da utilização de técnicas de biologia molecular e sequênciamento de proteínas. Portanto, em casos onde a estrutura não pode ser determinada experimentalmente, a modelagem por homologia pode frequentemente produzir um modelo tridimensional útil de uma sequência alvo baseado na sua similaridade a uma proteína com estrutura conhecida, utilizada como proteína molde (Ovchinnikov et al. 2014).

O principio da modelagem molecular por homologia se baseia no fato de que ao longo da evolução as estruturas das proteínas se encontram mais conservadas do que a sua sequência (Schneidman-Duhovny & Hammel 2018; Branden & Tooze 1991). A evolução biológica de proteínas obedece algumas regras como: homologia entre sequência de resíduos de aminoácido implica semelhança estrutural e funcional; proteínas homólogas apresentam regiões internas conservadas (principalmente constituídas de elementos de estrutura secundária: α-hélices e folhas-β); as alterações estruturais entre proteínas homólogas ocorrem nas regiões de alças (Branden & Tooze 1991). Além disso, as proteínas agrupam-se em um número limitado de famílias tridimensionais possibilitando modelar as proteínas de interesse se houver um membro da família que já possui sua estrutura determinada.

Um modelo construído por modelagem comparativa necessita que, pelo menos, uma estrutura 3D da família em questão tenha sido elucidada por técnicas experimentais. Outro ponto importante é a identidade entre as sequências (alvo e molde), esse valor deve ser acima de 25% para que o modelo gerado possa ser confiável (Schneidman-Duhovny & Hammel 2018; Cavasotto & Phatak 2009).

A modelagem molecular por homologia apresenta quatro etapas principais: a procura por sequências de proteínas homólogas, o alinhamento das sequências, a construção e otimização dos modelos e por fim a avaliação e validação das estruturas geradas (Cavasotto & Phatak 2009).

4.2.2 Phyre2 e Modelagem de proteínas

O sistema Phyre2 é uma combinação de softwares criados e escritos em vários idiomas por um grupo de pesquisadores em Londres, Inglaterra. O sistema é executado em programa Linux com aproximadamente CPU de 300 núcleos. O servidor Phyre2 pode ser usado de várias maneiras diferentes, dependendo do foco da pesquisa do usuário. A instalação mais usada é a previsão da estrutura 3D de uma única sequência de proteínas submetidas (Basyuni et al. 2018).

Os servidores Phyre e Phyre2 prevêem a estrutura tridimensional de uma sequência de proteínas usando os princípios e técnicas de modelagem de homologia. Como a estrutura de uma proteína é mais conservada na evolução do que sua sequência de resíduos de aminoácido, uma sequência de proteínas de interesse (o alvo) pode ser modelada com razoável precisão em uma sequência muito distante da estrutura conhecida (o modelo), desde que a relação entre o alvo e o modelo pode ser discernido através do alinhamento de sequência. Atualmente, os métodos mais poderosos e precisos para detectar e alinhar seqüências relacionadas remotamente dependem de perfis ou modelos de Markov ocultos (HMMs). Esses perfis / HMMs capturam a propensão mutacional de cada posição em uma sequência de resíduos de aminoácido com base nas mutações observadas em sequências relacionadas e podem ser pensadas como uma 'impressão digital evolutiva' de uma proteína específica (Basyuni et al. 2018).

Tipicamente, as sequências de resíduos de aminoácido de um conjunto representativo de todas as estruturas proteicas tridimensionais conhecidas são compiladas e essas sequências são processadas por varredura em um grande banco de dados de sequências de proteínas. O resultado é um banco de dados de perfis ou HMMs, um para cada estrutura 3D conhecida. Uma sequência de interesse do usuário é processada de maneira semelhante para formar um perfil / HMM. Esse perfil de usuário é verificado no banco de dados de perfis usando técnicas de alinhamento perfil-perfil ou HMM-HMM. Esses alinhamentos também podem levar em consideração padrões de elementos da estrutura secundária previstos ou conhecidos e podem ser pontuados usando vários modelos estatísticos (Basyuni et al. 2018).

4.2.3 Qualidade e Validação dos Modelos tridimensionais de proteínas

A qualidade dos modelos gerados depende principalmente da existência de moldes apropriados, ou seja, com boa resolução, alta cobertura e alta identidade. Para homólogos próximos, os programas mais utilizados na maioria dos casos geram modelos resolutivos, com

RMSD (*root mean square deviation* em inglês, ou desvio médio quadrático) de aproximadamente 2Å da estrutura experimental. Geralmente uma identidade de sequência acima de 35% é suficiente para produzir bons modelos para proteínas acima de aproximadamente 100 residuos de aminoácidos e a medida que a similaridade entre o alvo e molde diminui, o erro do modelo aumenta (Rayan 2009).

Entre os programas utilizados para validação das características estereoquímicas das estruturas de proteínas está o programa PROCHECK, que utiliza informações estereoquímicas selecionadas de estruturas de alta qualidade para providenciar uma avaliação geral da estrutura e para destacar regiões que necessitam refinamento. O programa pode ser utilizado independentemente dos dados experimentais e ser aplicado à estruturas já publicadas, ou para estruturas geradas por modelagem comparativa. O programa também analisa os ângulos de torção das cadeias principais (phi e psi) e laterais da molécula informando os maus contatos e a planaridade das ligações peptídicas (Brereton & Karplus 2015). Um dos arquivos de saída mais conhecido gerados pelo programa é o gráfico Ramachandran que apresenta uma correlação entre os ângulos torcionais da cadeia principal. A análise da rotação desses ângulos levou à identificação de regiões permitidas e não permitidas, onde ocorrem colisões entre os átomos (Brereton & Karplus 2015).

Outro programa amplamente utilizado para validação é o ProSa, uma ferramenta que se baseia na análise estatística de todas as estruturas de proteínas depositadas no PDB. Estruturas de proteínas solúveis cujos z-*scores*, uma pontuação utilizada pelo programa para avaliar a qualidade de modelos tridimensionais de proteínas, se afastam muito das médias obtidas para estruturas determinadas experimentalmente são incomuns e geralmente decorrentes de vários erros estruturais. Essa ferramenta utiliza uma função baseada em conhecimento, do tipo Potencial de Força Média (*Potential of Mean Force*), que descreve as geometrias preferenciais de determinada sequência de resíduos de aminoácidos através da análise estatística das geometrias de interação entre átomos de estruturas depositadas no PDB (Rasheed et al. 2015).

4.3 DOCKING MOLECULAR

A compreensão dos mecanismos de reconhecimento molecular proteína-ligante é um dos aspectos centrais para a descoberta e planejamento de novos compostos. Obter uma descrição acurada e automatizada do processo de reconhecimento molecular, utilizando

metodologias computacionais, pode permitir a redução do tempo e dos altos custos envolvidos no desenvolvimento de novos medicamentos (Meng et al. 2011).

Dentre estas metodologias, a de *docking* receptor-ligante tem contribuído significativamente para avanços no desenvolvimento de fármacos, sendo empregada no refinamento e otimização de compostos protótipos previamente identificados, na triagem virtual de bases de dados e na estimativa das afinidades de ligação proteína-ligante. A metodologia de *docking* molecular tem como objetivos predizer a orientação de ligação de duas moléculas que formam um complexo estável e estimar a afinidade de ligação entre elas. Portanto, o sucesso da técnica é medido através da comparação dos resultados preditos com modos de ligação determinados por cristalografia por raios-x dos complexos e medidas de afinidade determinadas em ensaios *in vitro* (Meng et al. 2011).

Para realizar o *docking* molecular são necessárias basicamente três etapas: definição da estrutura da molécula alvo, localização do sítio de ligação e predição do modo e da afinidade de ligação de um ligante utilizando algoritmos específicos. A estrutura pode ser obtida pelas técnicas de cristalografia por raios-x, NMR, ou predita computacionalmente por modelagem comparativa como foi descrito anteriormente. A aplicação destes modelos para *docking* já é bem estabelecida e representa uma importante alternativa quando estruturas experimentais ainda não estão disponíveis (Lionta et al. 2014).

A predição do modo e da afinidade de ligação é realizada através de algoritmos de busca e funções de avaliação, dois aspectos principais que diferenciam os programas de *docking*. Os algoritmos de busca são utilizados para amostrar as possíveis orientações dos ligantes ligados ao alvo proteico, considerando os graus de liberdade translacionais, rotacionais e conformacionais (que avaliam os ângulos diedrais associados às ligações covalentes simples).

As funções de avaliação podem ser divididas em três classes principais: funções baseadas em campos de força, funções empíricas e funções baseadas em conhecimento. As funções baseadas em campos de força utilizam um campo de força para calcular a energia de ligação entre o ligante e o alvo proteico. A funções empíricas utilizam métodos empíricos e semi-empíricos cujos coeficientes foram pré-otimizados tomando-se como base resultados experimentais de estruturas receptor-ligante e suas respectivas constantes de inibição. As funções baseadas em conhecimento também utilizam dados experimentais, mas utilizam estruturas cristalográficas para descrever as geometrias de interação receptor-ligante, ao invés

de usar dados das constantes de inibição. Através de análises estatísticas dessas geometrias são derivados "Potenciais de Força Média", que avaliam a variação de energia-livre em função das coordenadas interatômicas. Por causa dos erros associados a essas funções de avaliação das três classes, os programas mais recentes estão utilizando combinações dessas funções para produzirem funções consenso, que parece aumentar a qualidade dos resultados (Han et al. 2018).

4.3.1 *Docking* Proteína-proteína

O *docking* proteína-proteína tem imensas aplicações. É particularmente importante na previsão de vias metabólicas, interacções macromoleculares e *assemblies* macromoleculares. Devido à dificuldade em determinar *assemblies* macromoleculares experimentalmente, a previsão computacional dos possíveis modos de ligação é um dos principais objetivos deste tipo de *docking* (Macalino et al. 2018).

O *docking* proteína-proteína simula o reconhecimento molecular e é a tarefa mais complexa do *docking*. Isto porque o número de graus de liberdade é enorme, não sendo uma possibilidade fazer uma pesquisa exaustiva do espaço conformacional. É por esta razão que muitos algoritmos de *docking* tratam as proteínas como corpos rígidos (London et al. 2010).

Neste trabalho, foi empregado o servidor Cluspro 2.0 para os *dockings* proteína-proteina. Este servidor realiza o *docking* de corpo rígido e gera 109 complexos, realizando movimentos de rotação e translação de uma proteína (“ligante”) em relação a outra (“receptor”, mantido fixo). As conformações de *docking* são classificadas de acordo com as propriedades dos seus clusters. Para classificar as conformações de *docking*, o programa considera o potencial de interação entre pares, a energia de solvatação, além das contribuições de van der Waals (atração e repulsão) e eletrostática. Os 1000 complexos energeticamente mais favoráveis são então agrupados (10 a 30 grupos). Para agrupar as conformações de *docking* e encontrar o maior cluster representativo dos complexo o programa utiliza um método de agrupamento baseado em RMSD (Vajda et al. 2017).

4.4 PEPTÍDEOS

A prospecção e caracterização de peptídeos e proteínas bioativos oriundos da biodiversidade, contribui para o desenvolvimento da biotecnologia tanto na área de novas moléculas terapêuticas como na produção de plantas transgênicas (Brand et al., 2012).

A diversidade química e biológica presente nessas moléculas constitui um potencial exploratório promissor para o desenho de novas moléculas sintéticas. Atualmente mais de 7000 peptídeos bioativos foram identificados no organismo humano, esses exercem diversas funções biológicas que incluem: hormônios, neurotransmissores, fatores de crescimentos entre outros. Esses peptídeos são altamente seletivos e específicos na interação com o seu respectivo alvo molecular (receptores, canais iônicos entre outros) (Fosgerau & Hoffmann, 2015).

Além disso, possuem algumas propriedades intrínsecas a sua atividade, que incluem: alta especificidade, potência elevada e pouca toxicidade. Alguns peptídeos, como os peptídeos endógenos, estão presentes em sua forma inativa na proteína parental e são liberados por meio da clivagem proteolítica de polipeptídeos. Outros peptídeos podem ser obtidos por meio da alimentação (componentes de natureza proteica), durante a digestão enzimática que ocorre no trato gastro intestinal ou ainda, por meio de alimentos processados (Kaiser et al. 2016).

A estrutura primária dos peptídeos é determinante no processo de desencadeamento da atividade biológica no organismo. Eles podem exercer diversas ações associadas a modulação fisiológica do sistema endócrino, imune, cardiovascular e sistema nervoso (Wada & Lonnerdal, 2014).

Na literatura já foram caracterizados peptídeos que apresentam diversas atividades biológicas, como antimicrobianas, opióides, hipotensoras, antitrombóticas, entre outras. Devido a essas propriedades, os peptídeos bioativos destacam-se atualmente como um ponto de partida interessante no desenho de novos fármacos e de aplicação na indústria de alimentos (Fosgerau & Hoffmann, 2015).

Peptídeos, desempenham papéis cruciais em quase todo processo fisiológico, são cadeias de resíduos de aminoácidos ligados por ligações peptídicas. Vincent Du Vigneaud sintetizou a primeira droga peptídica oxitocina em 1953, que ganhou o Prêmio Nobel em 1955.

Desde então, os peptídeos se desenvolveram rapidamente, e mais de 80 medicamentos peptídicos foram aprovados para mercado mundial. Vantagens das drogas peptídicas incluem sua boa bioatividade e maior estabilidade comparado com drogas protéicas. O desenvolvimento de síntese química e expressão biológica tecnologia para peptídeos reduziu consideravelmente o custo dos medicamentos peptídicos, que geralmente são muito mais baratos do que os medicamentos de proteína / anticorpo com funções semelhantes. Essas vantagens únicas tornaram os medicamentos peptídicos um importante campo de pesquisa e desenvolvimento de novas drogas.

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Capítulo I:

Inibição de tripsinas digestivas mediante proteínas de planta tipo Kunitz reduz a viabilidade das larvas de *Spodoptera cosmioides* (Lepidoptera:Noctuidae)

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Inhibition of digestive trypsins by plant Kunitz proteins reduces the viability of *Spodoptera cosmioides* larvae

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Abstract

The acquired resistance by insects and the harmful environmental effects of chemical pesticides has encouraged the search of new tools for the proper pest management. Among them, the use of protease inhibitors (PIs) obtained from plants gains strength, because they are a natural system agaist herbivorism, organic molecules with higher specificity, and have potential to cause less nature damage. The aim of this work was to characterize the inhibitory potential of the proteins ApTI (*Adenanthera pavonina* trypsin inhibitor) and ILGA (*Inga laurina* trypsin inhibitor) on the digestive trypsins of *S. cosmioides* through molecular docking, enzymatic kinetics and biological survival analyzes. The docking between trypsins and inhibitors was performed using the program CLUSPRO; the inhibitory constant ki and the inhibition type was determined through chromogenic assays; in order to the survival analyses, was tested on neonatal larvae several concentrations of ApTI and ILTI inhibitorsd in the artificial diet. In this study was determined that ILTI binds to the active site of the trypsins with a specificity similar to its natural substrate. While, ApTI showed that the inhibitor site reactive is not in contact with the trypsins catalytic site. ILTI and ApTI inhibitors were characterized as competitive and uncompetitive tight-binding inhibitors. The survival curves obtained using Kaplan-Meier estimators indicated that the lowest percent survival (20%) for all inhibitors tested were obtained using 1.0% doses at a development time of less than 20 days. We concluded that ILTI and ApTI present biotechnological potential as agents against phytophagous Lepidoptera insects, inhibiting trypsins by tight binding inhibition, with characeristic competitive and non-competitive, respectively. The action of ApTI and ILTI on the development of *S. cosmioides* larvae shows toxic effects.

Keywords: docking, enzymology, insect, plant inhibitors, proteases.

Introduction

Spodoptera cosmioides (Walker, 1858) (Lepidoptera: Noctuidae) is a polyphagous insect, belongs to family Noctuidae and genus Spodoptera. In this group are included several pests of the American continent, that cause reductions in agricultural yields (Pogue 2002). *S. cosmioides* is included among a Neotropical species complex with select distribution at South-America. *S. cosmioides* is recognized as key pest in Brazil with a high tolerance to the Cry1Ac protein, which is expressed in some Bt crops. Larvae are defoliators and feed gregariously between the first and third instars, often skeletonizing leaves (Bernardi *et al*. 2014). In Brazil, this specie is pest of tomato, cotton, cowpea, rice, soybeans, among others causing significant yield reduction and economic losses approximately US$602.1 million per year for the control of genus Spodoptera pests (Da Silva *et al*. 2017; Pogue 2002). It also has the capacity to produce successive generations throughout the year, which emphasizes their relevance for integrated pest management (IPM) (Da Silva *et al*. 2017).

Mainly the chemical pesticides carry out the control of this pest (Diez-Rodriguez & Omoto, 2001). The acquired resistance by insects and the harmful environmental effects of these products are reasons that drive research new tools for the proper pest management. Among them, the use of protease inhibitors (PIs) obtained from plants gains strength, because they are a natural system against herbivorism, organic molecules with higher specificity, and have potential to cause less nature damage (Dias *et al*., 2012).

PIs suppress *in vivo* and *in vitro* proteolytic activity by forming stable stoichiometric complexes with the enzyme (Radisky *et al*., 2004). Inhibitors of serine proteases (ISP) show high potential to control pests due to high concentration of enzymes such as trypsin-like proteins in the insect gut, important for food digestion (Meulenbroek *et al*., 2012). This trypsins are targets for insect pest management because catalyse amino acid release, providing essential nutrients for normal growth and insect development.

Several studies corroborate that PIs can reduce insect growth and survival when added to the diet. In addition, deleterious effects such as reduced fecundity and oviposition, weight decrease, increased mortality and severe deformations had been observed (Saadati & Bandani, 2011). The ISP ability to interfere with insect development is attributed to their capability to bind and to inhibit digestive proteases.

The interaction between PIs and insect proteases is an example of ligand-macromolecule recognition, required in the plant defense process. The understanding of these recognition mechanisms is one of the central aspects for the success in the discovery of new promissory compounds. The characterization of binding mode between inhibitor-protease could be obtained from several methods, among them the *in silico* studies that allow the reduction of time and costs, specifically docking-molecular analyzes allow to identify the binding conformation and affinity quantification (Pagadala *et al*., 2017).

In this study, the ability of compounds ApTI (*Adenanthera pavonina* trypsin inhibitor) and ILTI (*Inga laurina* trypsin inhibitor) of inhibit trypisin-*like* proteins from *S. cosmioides* were mensured by molecular docking and enzymatic assay. ApTI is a natural peptide, extracted from plant *Adenanthera pavonina* L. (Fabaceae), subfamily Mimosoidae, composed by two polypeptide conected by one disulfide bond with total molecular weight of 21 KDa and with inhibitory activity against serine proteases (trypsins and chymotrypsins) and cysteine proteases (papain) (Richardson *et al*., 1986; Macedo *et al*., 2004; Migliolo *et al*., 2010). On the other hand, the peptide ILTI is serine protease inhibitor composed of a single chain containing 180 residues and with high stability due to the presence of two disulfide bridges. This peptide was isolated from the *Inga laurina* (Fabaceae) from the subfamily Mimosoidae (Faculdade de Educação, UNICAMP, Campinas-SP- Brazil) (Macedo *et al*., 2007; Macedo *et al*., 2011; Machado *et al*., 2017). Futhermore, the potential of proteins for pest control due to inhibition of digestive trypsins was also determined using larvae of *S. cosmioides* fed with the compounds.

Materials and methods.

In silico Assay

Obtaining the sequences

Due to the unavailability of trypsin sequences or genome of *S. cosmioides*, the sequences used in *in silico* analysis were retrived from genome (NCBI ID ASM221328v1 (Kakumani *et al*., 2014)) of *Spodoptera frugiperda* (J. E. Smith, 1797) thath belong the same genus of interested organism. In order to identify potential trypsins encoded genes in *S. frugiperda* genome, the tBLASTn algorithm (Altschul, 1990) version 2.8.0 implemented in the NCBI website were performed using as query a well-caracterized trypsin protein sequence from *Apis*

mellifera considering significant hits with E-value < 10^{-5}, identity > 30% and query cover > 70%. The selected sequences are showed in the Table 1.

Since proteins sequences from *S. frugiperda* could have polymorphisms compared with sequences from *S. cosmioides* and these variations can affect the interaction of a protein with an inhibitor, the trypsin protein sequences of others phylogenetically related species from the same Lepidoptera order were also retrived from NCBI to evaluate if the predicted molecular mechamism is conserved within the order. For this *Plutella xylostella*, *Manduca sexta*, and *Bombyx mori*, were selected because they have the best hits to trypsin proteins from *S. frugiperda* sequence using the BLASTp algorithm version 2.8.0 implemented in NCBI website (Altschul, 1990). The access numbers of trypsin-*like* enzymes in the NCBI for the different insect genus are: NP_001306954.1 (*Plutella xylostella*), NCBI: AAA29340.1 (*Manduca sexta*), and NCBI: XP_012549169.1 (*Bombyx mori*). Analysis of conservation of catalytic amino acids in selected tripsins were carried after global aligment of protein sequences by the Clustal Omega software (Li *et al*., 2015).

Inhibitors sequences were obtained from the UniProt database (Uniprot, 2017), the ILTI inhibitor access code is UniProt : J9PZR2, and ApTI inhibitor are UniProt: P09941 and UniProt: P09942 for the α and β chain, respectively. These inhibitors have been chosen due to the potential previously characterized to inhibit trypsin from other insects of different order (Macedo et al., 2004; Macedo et al., 2007).

Molecular modeling and docking

The three-dimensional structures were predicted for nine trypsin-*like* sequences and the two inhibitor proteins using the Phyre2 server (Kelley *et al*., 2015), suite of tools available on the web for the prediction of the 3D structure of a single submitted protein sequence. This technique is approximately 80% accurate if the backbone is correct (Kelley *et al*., 2015).

The models quality were mensured by evalution of geometry and stereochemistry, energy distribution, and other characteristics of the 3D models using the web server PRoSA (Protein Structucture Analysis) analysis package (https://prosa.services.came.sbg.ac.at/prosa.php), ERRAT (http://services.mbi.ucla.edu/ERRAT/) analyzes Ramachandran Plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) and Verify3D (http: / /

/services.mbi.ucla.edu/Verify_3D/). The results of this analysis are showed in the suplementary table 1.

Furthemore, a functional classification of protein sequences was carried out by the dominioins identification of trypsin enzymes using Pfam (Finn *et al*., 2016).

The docking between trypsins and inhibitors was performed using the program CLUSPRO (Kozakov *et al*., 2013; Kozakov *et al*., 2017), obtained in total 18 docked system. The binding mode between inhibitors and proteins were visualizated and analyzed using the program PyMOL (Molecular Graphics System, Version 2.0 Schrodinger, LLC). The conservation of amino acids from inhibitors involved in potential interactions with the trypsins was carried out using the webserver WebLogo (Crooks *et al*., 2004).

In vitro assay

Trypsins purification of *Spodoptera cosmioides*

A total of 50 instar larvae of *S. cosmioides* were dissected, and their midgut transferred to 1 mM HCl at 4°C at a ratio of five intestines per 1 mL of solution following the maceration with the presence of liquid nitrogen. The samples were centrifuged at 10.000 g for 30 min at 4 °C and was filtered using 22 μm membrane, the supernatant was stored at -20 °C.

The total sample volume/chromatography was 4ml of enzyme extract containing the equivalent of 20 midugts.

Enriched trypsin fractions were obtained by use HiTrap Benzamidine (high sub) 5ml (GE Healthcare) column equilibrated in buffer Tris-HCl 0,05 mol.L^{-1} NaCl 0,5 mol.L-1, pH 7,5. Protein elution was performed with buffer Glycine 0,05 mol.L-1, pH 3,0, with continuous flow of 1 mL/min and collected in 2.0 mL fractions. The trypsin-*like* activity in the eluted fractions was determined using the L-BApNA as substrate (Erlanger *et al*., 1961). Fractions corresponding to the elution peak were pooled and stored at -20°C for further use in enzyme kinetics assays. The procedure was performed in Fast Protein Liquid Chromatography (FPLC). Samples from the purification were submitted to electrophoresis unidimensional (Laemmli, 1970) with 12.5% polyacrylamide gel in the presence of SDS (0.1%). The gel was revealed using Commassie Blue solution.

Inhibition kinetic

The determination of inhibition constants was performed in buffer Tris-HCl 0,1mol.L-1, $CaCl_2$ 20 mmol.L-1, pH 8,2, in the presence of L-BApNA chromogen substrate and the ApTI and ILTI inhibitors.

The purified inhibitors were supplied by the research group Protein Purification and Biological Functions Laboratory (LPPFB), Universidade Federal de Mato Grosso do Sul, Campo Grande, Brazil and the purification was perfomed and analyzed according to Macedo *et al.* 2007 and Macedo *et al*. 2004, with 98% of purity.

The concentrations employed for the *S. cosmioides* trypsins-*like* inhibition study were: L-BApNA substrate: 1.0 mM; 2.0 mM; 4.0 mM; 8.0 mM; 16.0 mM and 32.0 mM. Inhibitors were 2,38 µM/L; 4,76 µM/L; 7,14 µM/L; 9,52 µM/L and 11,9 µM/L using as control the reaction without inhibitor. The total concentrarion of enrriched trypsin-*like* was 3 µg/µl.

The initial velocities were determined by the p-nitroanilide product formation by absorption measure at 410 nm during 120 seconds of incubation using the specific molar extinction coefficient of 8800 $M^{-1}.cm^{-1}$ for the product (Erlanger *et al*., 1961). The experiments were performed in triplicate. The resulting velocities were then fitted to the Morrison equation. The initial velocity (V0), enzyme concentration [E] and inhibition constant (Ki) were estimated by non-linear regression (Morrison, 1969). All analyzes were performed using GraphPad Prism 5.0 software (Motulsky, 2007).

In vivo assay

Biological material

Eggs: *S. cosmioides* eggs were kept indoors the Insect Laboratory of the Biochemistry and Molecular Biology Department from University Federal of Viçosa (UFV) at 25 °C ± 2 °C and 70 ± 10% relative humidity. The *S. cosmioides* egg masses were laid, usually after three days on the paper surface that internally lined the cage. These were removed and cut into 2.5 cm wide x 10 cm long strips, placed in plastic cups (500 mL) with a circular hole with about 2 cm in a tulle fabric cover. These cages were transferred to a 25 °C chamber with relative humidity of 60 ± 10% and a 14 hour photoperiod.

Larvae: Newly hatched larvae were fed on 15 x 15 x 15 mm artificial diet cubes every two days (Bavaresco *et al*., 2004).

Pupae: pupae of this insect were placed inside Petri dishes in cages (50 x 50cm) coated internally with A4 bond paper.

Adults: after emergence of the pupae, adults of this insect were fed a nutrient solution with honey (10.5 g), beer (350 mL), sucrose (60 g), ascorbic acid (1.05 g), nipagin (1.05 g) and water (1050 mL) in cotton balls placed in Petri dishes (100 x 15 mm) (Bavaresco *et al*., 2004).

ApTI and ILTI inhibitors effect on development and survival of *Anticarsia gemmatalis*

Neonatal larvae were fed an artificial diet (Bavaresco *et al*., 2004) containing the ApTI and ILTI inhibitors separately, at concentrations 0.7%, 1.0% and 1.2% w inhibitor/v diet. Each inhibitors concentration in the artificial diet was considered like a treatment, containing the experiment three total treatments and one negative control (diet without inhibitor). For each treatment 30 neonatal larvae were placed individually in transparent plastic containers (20ml). Each larvae was considered a treatment repetition. All subjects were maintained at mean temperature of 26 °C and 65-70% relative humidity. Survival analyzes were performed every two days during the period from neonates to the final-instar larva; this stage was identified because is characterized by changes in behavior and appearance larvae such as turning a cocoon, or leave the food to wander in search of a pupation site (Danks 2000). The mortality was analyzed through mechanical stimulation with tweezers or brush and direct observation of the movement of the larvae.

Results

Molecular Docking

Molecular docking performed by ClusPro generated a total of 10 models with predicted bind mode between lepidopteran trypsins and ILTI and ApTI inhibitors. The best model was chosen according to the results of the PROSA, Ramachandran, Verify3D and WHATCHECK validation analyzes. After trypsin sequences alignment, the amino acids from catalytic site were identified and were verified its participation in the interaction with each inhibitor (Fig 1-4).

The graphical representation of the standard residues from the alignment of the trypsin-bounded inhibitors sequences was performed using WEBLOGO; the participants residues in the binding to trypsins was shown like the fragment of the complete sequence with the largest

number of continuous residues (Fig. 5-6). ILTI inhibitor residues binding to all selected trypsins were Ile35, Hys37, Gly38, Asp39, Hys40, Leu41, Cys42, Arg50, Tyr51 and Thr52 (Fig 5A). The ApTI inhibitor residues binding to all selected trypsins were Pro62, Pro63, Arg64, Ile65, Arg66, Tyr67, Glu71, Phe72 and Tyr73 (Fig. 6A).

In the alignment of the selected trypsins, the residues that are predicted in the interface trypsin/inhibitor complex were labeled (Fig. 5 and 6). In the trypsin sequences alignment bounded to ILTI inhibitor, were observed a Histidine (Hys70) and Serine (Ser206) like participants, being part of the trypsins catalytic triad (labeled with upper arrow) and Glycine (Gly223), residues conserved for all the nine representative trypsins (Fig. 5B).

In the trypsin sequences alignment bounded with the ApTI inhibitor, the residues participation at the positions 105, 108, 222 and 224 was observed in all the trypsins analyzed, the residues type in these positions are not conserved in the nine trypsin representatives, but the physical and chemical propieties of these residues are highly conserved (Fig. 6). At position 105 the located residue varies depending on the species, being able to find Ala, Arg, Pro, Gly and Ser; at position 108 for the trypsin *B. mory* species is found the residue Phe, whereas in the rest of the trypsins a Leu is positioned; at 222, the Phe or Trp residues can be found depending on the species; at position 224 exist variotion residues, finding both Val, Ile, Phe, Lys, Glu and Met (Fig. 6B). In the predicted structures the catalytic residues are also localized in the same protein pocket suggesting that the catalytic site of trypsins from the order Lepidoptera are higly conserved.

Trypsins purification of *Spodoptera cosmioides*

In order to evaluate the potential inhibitory of the ILTI and ApTI peptides, the trypsin-*like* intestinal proteases of *S. cosmioides* was used. First, chromatographic profile of this enzymes family using the HiTrap Benzamidine column showed that pH changes from 8.5 (equilibration buffer) to 3.0 (elution buffer) allowed the enzymes elution bounded to the resin of the column (Fig. 7). In the chromatogram, the presence of two peaks with trypsinolytic activity, detected using L-BApNA as substrate, was observed (Fig. 7A). The first peak (fractions 5-10) is associated with non-specific proteolysis of the L-BApNA substrate by other enzymes with trypsin-*like* activity, present in the insect gut extract. The second peak (fractions 40-46) represents the trypsin-*like* serine proteases contained in the soluble extract having affinity to the column and with proteolytic capacity against the L-BApNA substrate (Fig. 7A); in this peak

activities were observed between 2.0 and 3.2 μM/s. In addition, the protein profile (Abs 595 nm) was found to be coincident with the activity profile (Abs 410 nm) (Fig. 7B).

In a single purification step using the HiTrap Benzamidine column, the specific trypsin activity increased from 0.115 to 1.083 μmol.s-1/μg of protein, getting a purification factor of 10 (Table 2). In addition, there was no loss of total activity, on the contrary it increased from 1.54 to 3.35 μM.s-1 getting a yield prification of 211% (Table 2).

The enriched trypsin-*like* fractions obtained after affinity chromatography were evaluated by SDS-PAGE. Comparative analysis between crude intestinal extract and purified samples revealed, after staining by Comossie, several bands of molecular weight in trypsin-*like* proteins range between 20 and 35 KDa (Fig 7C) different of non-purified extract that have a range between of lower than 15 a 75 KDa related to total protein of crude extract.

Inhibition kinetic

The inhibitors ApTI and ILTI were active against trypsins of *S. cosmioides* in chromogenic assays. The type of inhibition found for both inhibitors was tight-binding, verified from the linear variation between the IC50 value of ILTI (R2 = 0.88 p = 0.01) and ApTI (R2 = 0.78 p = 0.04) in function of the enzyme concentration [E] (Fig. 8A and 8C).

The ILTI inhibitor was characterized as being of competitive tight-binding because was verified that the IC50 value of this inhibitor varies linearly as a function of substrate concentration (R^2=0.96, p=0.01) (Fig. 8B). The ApTI inhibitor presented uncompetitive tight-binding characteristics due to the absence of linear variation between the IC50 value in function of the substrate concentration (R^2=0.65, p=0.19) (Fig. 8D). The Ki value for ILTI and ApTI was 0.597 and 1.004, respectively.

Survival Assay

The survival plots for *S. cosmioides* larvae reared on diet containing ApTI, ILTI or SKTI inhibitor at different doses are shown in Fig. 9. The survival curves obtained using Kaplan-Meier estimators indicated significant differences in percent survival among the different inhibitors doses (ILTI Log-Rank χ^2= 58.82 P<0.0001; ApTI Log-Rank χ^2= 32.07 P<0.0001; SKTI Log-Rank χ^2= 24.18 P<0.0001).

For the inhibitors studied the 1% dose was the most efficient to kill and prevent the development of *S. cosmioides*. Comparing protein inhibitors with SKTI control, both protein inhibitors reduced developmental time and increased larval mortality. The lower dose of the protein inhibitors reduced the population to less than 50% in a maximum of 30 days, whereas SKIT can not reach the same efficiency even after 40 days of experimentation. This difference was more pronounced at lower doses and the ILTI inhibitor was more efficient than the ApTI inhibitor.

Discussion

Several studies have shown that protease inhibitors are active against the enzymes of different insect species both *in vitro* and *in vivo* bioassays (Macedo *et al*., 2010; Patarroyo *et al*., 2017). In this work, the interaction *in silico* of two inhibitors against trypsins of different Lepidoptera were compared, and the mode of action in *S. cosmioides* larvae was examined.

From the formed complexes *in silico* among the nine serine proteases trypsin-*like* and the two protein inhibitors isolated from plants, ILTI and ApTI, were determined that of the 178 amino acids that form the inhibitor ILTI, 10 amino acid residues are close the activite site with distance lower than 4 angstroms including the Asp 39 and Hys 40 that interact with the trypsins of Lepidóptera by hydrogen bounds and Tyr 51, Leu 41 and Ile 25 by hydrophobic interactions. Experimental studies using x-ray crystallography technique, analyse the residues Kunitz inhibitors in contact with pancreatic trypsin, demonstrate that residues such as Asp, Tyr, Arg, and Ile are important for the links with these proteases (Huang *et al*., 2010), In this study, Arg of the inhibitor ILTI interact also with the trypsins of Lepidóptera. The residue Arg may be favored in the interaction because it has two nitrogen atoms, not loaded, offering two electrons pairs at the end of the side chain, favoring the electrostatic interaction. In addition, it has been reported that Arg when present in protease inhibitors in position P1 keep in touch with Asp trypsins residue, through its guanidine group by ionic interactions (Huang *et al*., 2010). On the other hand, the presence of Tyr in ILTI inhibitor, has been reported in other studies. This residue at position P2, interact with Leu residues in trypsins by its bulky lateral chain (phenol ring) (Kelsey *et al*., 2014).

In the complex ILTI-trypsins is observed several residues interacting with the catalytic site (Hys-Asp-Ser). In addition, the residue Gly, adjacent to Ser catalytic, was another amino acid of trypsins kept in interaction with ILTI, and it has been reported that Gly allows entry of

bulky side-chains of aminoacids in the trypsins hydrophobic site (Craik *et al*., 1985); increasing the contact points of hydrophobic interactions and the affinity to the enzyme active center.
ILTI is an inhibitor with competitive inhibitory mechanism as described in the *in silico* analyzes, where an inhibitor fraction is inserted at the active site of the target trypsins with similar specificity to substrate (Farady & Craik, 2010).

The analyzes of the interaction between the ApTI inhibitor and the trypsins of Lepidóptera showed the site reactive of the inhibitor is not in contact with the catalytic cavity of the trypsins, in other words, it does not block the access of the substrate to the active site. Of the 176 amino acid residues that are part of ApTI inhibitor, 9 amino acid residues interact with the trypsins of Lepidóptera at a distance of four angstroms. A greater number of hydrophobic residues are those who participate in the interaction space beteween trypsins and ApTI, whereby the hydrophobic interactions are the most common. Then, there are ioninc binding between the residue Glu of the inhibitor and the Arg residues of trypsins, it has been reported that may form salt bridges between its side-chains (Yang *et al*., 2012). The *in silico* assay results show that ApTI is an inhibitor with non-competitive inhibitory mechanism.

In the chromatogram, the first peak (fractions 5-10) is associated with the proteolysis not specific to the substrate L-BApNA by other enzymes with similar activity to trypsins, present in the intestine extract of insects (Terra & Ferreira, 1994). The second and larger peak (fractions 40-46) represents the serine proteases trypsin-*like* contained in the extract soluble that have affinity for column and proteolytic capacity to the substrate L-BApNA. Affinity chromatography by column HiTrap Benzamidine is an efficient method for partial purification of proteases trypsin-*like* and has been used in other studies getting similar results (Patarroyo *et al*., 2017; Pilon *et al*., 2017). The efficiency of the method may be due to the fact that the benzamidine is a potent competitive inhibitor of trypsin-*like* that occupies the subsítio S1 (site of specificity) of the enzyme; benzamidine is stabilized by hydrophobic interactions in its hydrophobic pocket and electrostatic interactions between its the amidina group and carboxyla group belonging to the residue aspartic acid present in the bottom of the S1 site (Oliveira *et al*., 1993; Pilon *et al*., 2017).

The efficiency of the purification method was also confirmed by the results of SDS-PAGE with reduction of protein species in the purified sample compared to the crude extract was observed in the gel. The bands with molecular weight between 20 and 35 KDa were determined, result that is in agreement with the cited data in the literature, since in enzymes

intestinal insects as trypsins has been reported with molecular weights ranging in size between 20 and 35 kDa (Patarroyo *et al*., 2017; Pilon *et al*., 2017) and 18 and 25 kDa (Saboia-Vahia *et al*., 2013). The fact that *S. cosmioides* present trypsins of molecular weight reduced is related to aspects of production and recycling of these enzymes in the digestive process in this insect; it has been reported in other studies that trypsins of reduced mass is related to its proteolytic action, with the ability to overcome the peritrophic membrane and gain the ectoperitrophic space by means of a countercurrent mechanism (Pytelkova *et al*., 2009). Once present in the ectoperitrophic space, the digested nutrients are absorbed by the microvilli of the intestinal epithelium and the trypsins can diffuse back into the endoperitrophic space to be subsequently reused (Fazito do Vale *et al*., 2007; Telleria *et al*., 2010).

In all treatments of enzymatic interactions with inhibitory molecules it is assumed that the inhibitor concentration required to achieve 50% of inhibition is much greater than the enzyme concentration of in the reaction. The inhibitor concentration that is kidnapped in the formation of the complex EI is, therefore, a fraction too small of the total concentration of inhibitor added to the reaction (Copeland, 2002). In the case of ILTI and ApTI inhibitor, the previous idea is not valid. In the inhibition kinetics analysis was determined that ILTI and ApTI are inhibitors to the type tight binding, which indicates that bind to the trypsin-*like* enzymes with high affinity and the free inhibitors is significantly deplet by formation of the enzyme-inhibitor or enzyme-substrate-inhibitor complex, these inhibitors have a decoupling rate low (Copeland, 2013). ILTI was characterized as an inhibitor tight binding competitive and ApTI like non-competitive in the concentration range of inhibitors and substrates analyzed. These results guide and support the *in silico* analysis performed.

For the inhibitor ILTI was given a value of Ki lower (0.5) that the value determined for ApTI, (1.0) which indicates that the inhibitor ILTI can have higher inhibitory potency compared with ApTI. The predominance of polar reactive site of ILTI demonstrating in the analysis *in silico* may be favoring its interaction with the active site of the enzyme.

In survival analyzes, the ILTI inhibitor was the most potent inhibitor followed by ApTI. This result agrees with other studies conducted with these inhibitors. When ILTI was incorporated into the artificial diet to a level of 0.05%, ILTI decreased weight gain by 60% and thus reduced the *Homalinotus coriaceus* larval survival to 10% (Macedo *et al*., 2011). In our study ILTI decreased survival by a higher percentage (35%) at the 0.1% level when compared

to the previously mentioned study. Confirming its efficiency as a trypsin inhibitor in lepidopteran insects.

The inhibitory activity inefficient of ApTI has been demonstrated against *Sitotroga cerealella* (Oliver) (Macedo *et al*., 2010), but in this study ApTI inhibitor showed inhibitory activity against trypsins of *S. cosmioides*, decreasing the number of individuals by 50% in the lowest concentration (0.1%) and 30% in the concentration 1.0%. The effect of these inhibitors on the development of *S. cosmioides* can be explained because proteinase inhibitors inhibit insect gut proteinases by binding tightly to the binding site. The inability to utilize ingested protein and to recycle digestive enzymes results in a critical amino acid deficiency, which affects the growth, development and survival of the herbivore (Nanasahe *et al*., 2008).

In the lower proportion of inhibitors in the diet (0.1%) a sublethal effect was observed, where low doses of inhibitor increased the survival time of *S. cosmioides* (>35 days). The extended median survival time also led to slower larval development, coupled with a proportional decrease in pupal weigh (data not shown). These results are in agree with some results of stimulatory sublethal effects of toxic compounds, phenomenon also known as hormesis, where such stimulatory effect may lead to higher fitness and progeny production (Guedes *et al*., 2009). However, lack of fitness impact is the more frequent expectation (Guedes *et al*., 2010), as seems to be the case with IlTI and ApTI inhibitor.

The increasing of the survival time of *S. cosmioides* could be explained by the inhibited protein digestion, avoiding to assimilate the necessary amino acids for its development in the period of accustomed time. Besides that, adaptive mechanisms including overproduction of existing digestives enzymes, synthesis of inhibitor-insensitive protease isoforms, and activation of specific proteases that detoxify the PIs are some of the responses that insects have to plant defensive protease inhibitors (Zhu-Salzman & Zeng, 2015), and *S. cosmioides* could also express front the ingest of ILTI and ApTI inhibitors.

Concluding, ILTI and ApTI present high biotechnological potential as agents against phytophagous Lepidoptera insects, inhibiting trypsins by tight binding inhibition, with characeristic competitive and non-competitive, respectively. The action of ApTI and ILTI on the development of *S. cosmioides* larvae shows that this inhibitors influences the larval survival, indicating that this proteins may have great toxic potential.

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Tables

Table 1. Trypsins sequences of *Spodoptera frugiperda* genome

GenBank	Genome annotation
AIR09768.1	Trypsin-like serine protease precursor
AIR09767.1	Trypsin-like serine protease precursor
ACR25157.1	Trypsin
XP_013190449.1	Trypsin-1-like
ABU96714.1	Trypsin-like serine protease
ADI32887.1	Serine protease

Table 2. Trypsin-like purification from the soluble extract and gut purificated of *Spodoptera cosmioides* (Lepidoptera: Noctuidae).

Material	Total protein (µg/ µl)	Total activity ($\mu M.s^{-1}$)	Specific activity ($\mu M.s^{-1}$/ µg)	Fold purification	Enzyme Yield (%)
Extract	13.37	1.54	0.115	1.0	100
Purificated	3.0	3.25	1.083	10.0	211

Figures

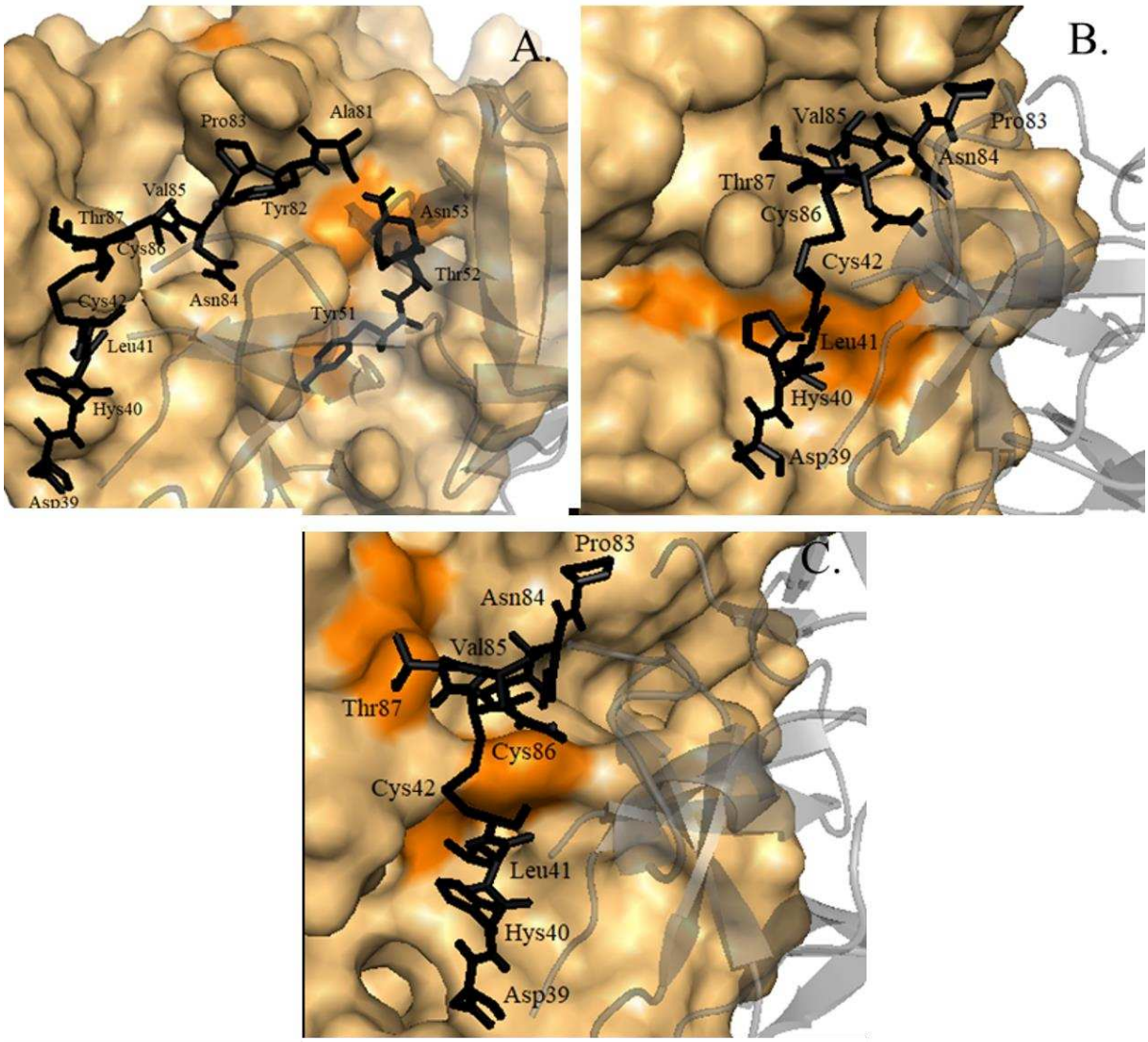

Figure 1. Structural binding mode between Inga laurina trypsin inhibitor (ILTI) and trypsin of *Bombyx mori* (A), *Manduca sexta* (B) and *Plutella xyllostella* (C). Barrel shape representing the trypsins in beige, catalytic aminoacids in larange and the ILTI residues as black cartoon involved in the trypsin-ILTI interaction.

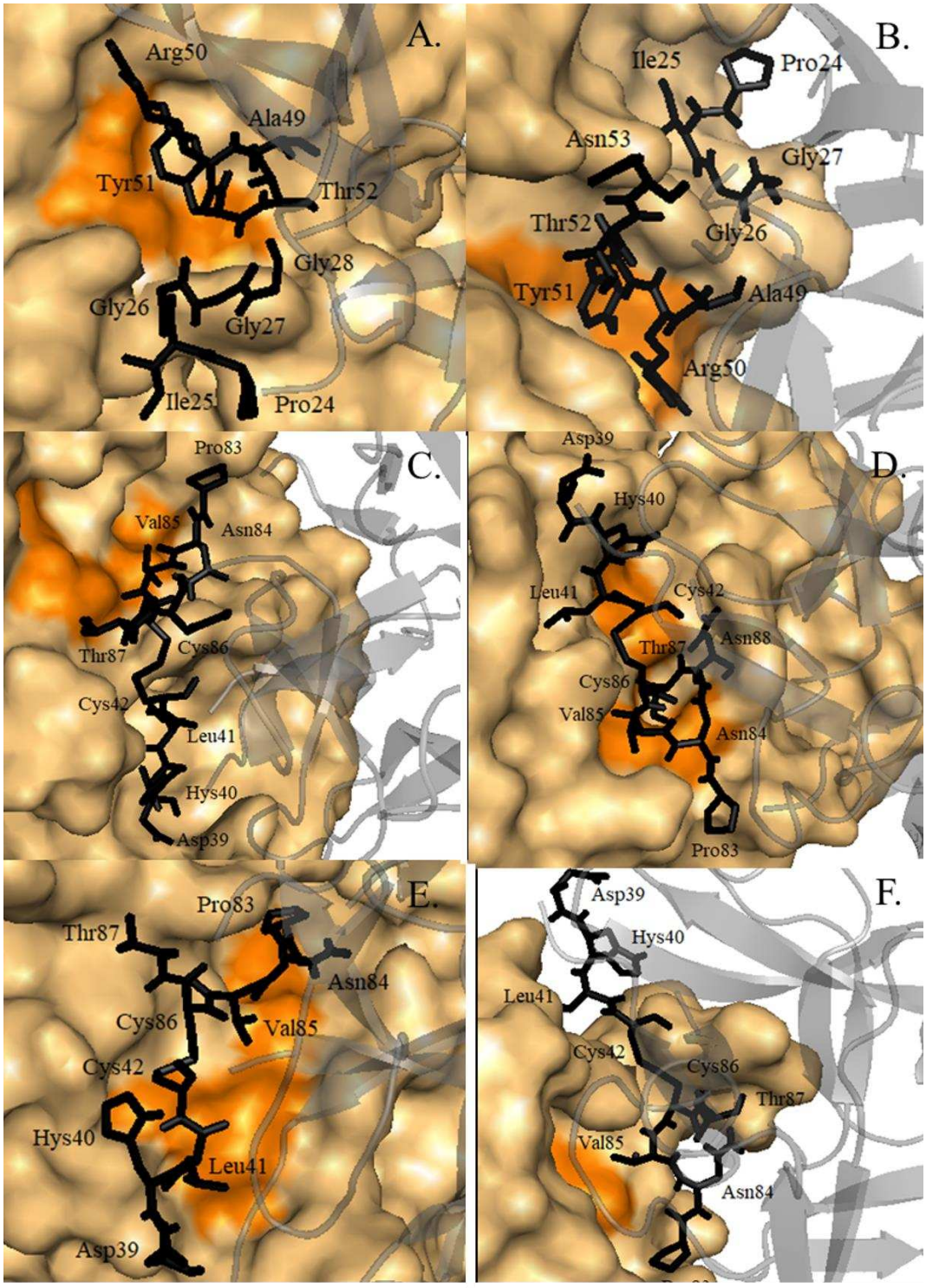

Figure 2. Structural binding mode between Inga laurina trypsin inhibitor (ILTI) and trypsin of *S. frugiperda 1 (A), S. frugiperda 2 (B) S. frugiperda 3 (C) S. frugiperda 4 (D) S. frugiperda 5* (E) and *S. frugiperda* 6 (F). Barrel shape representing the trypsins in beige, catalytic aminoacids in larange and the ILTI residues as black cartoon involved in the trypsin-ILTI interaction.

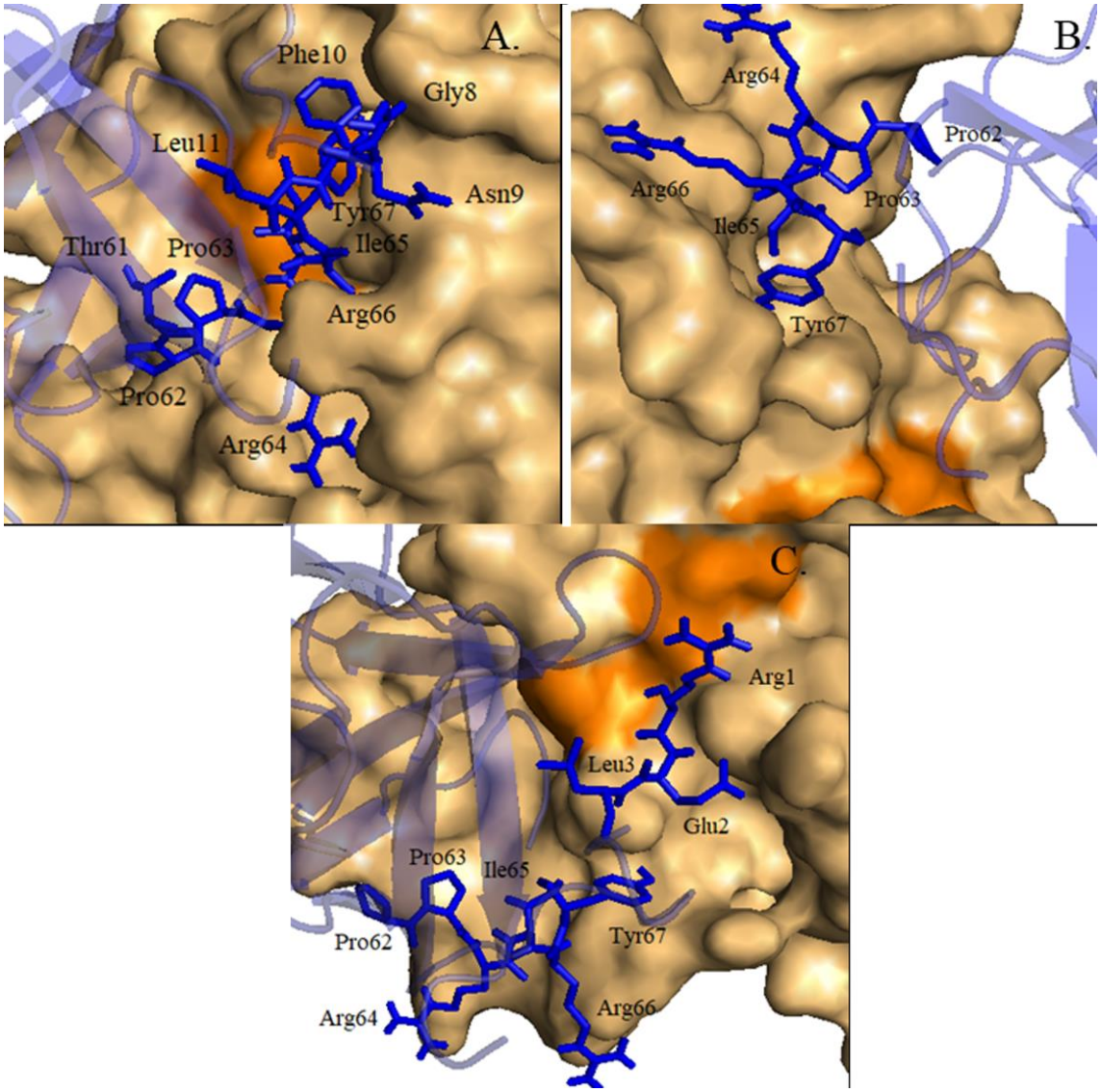

Figure 3. Structural binding mode between *Adenanthera pavonina* trypsin inhibitor (ApTI) and trypsin of *Bombyx mori* (A), *Manduca sexta* (B) and *Plutella xyllostella* (C). Barrel shape representing the trypsins in beige, catalytic aminoacids in larange and the ApTI residues as blue cartoon involved in the trypsin-ApTI interaction.

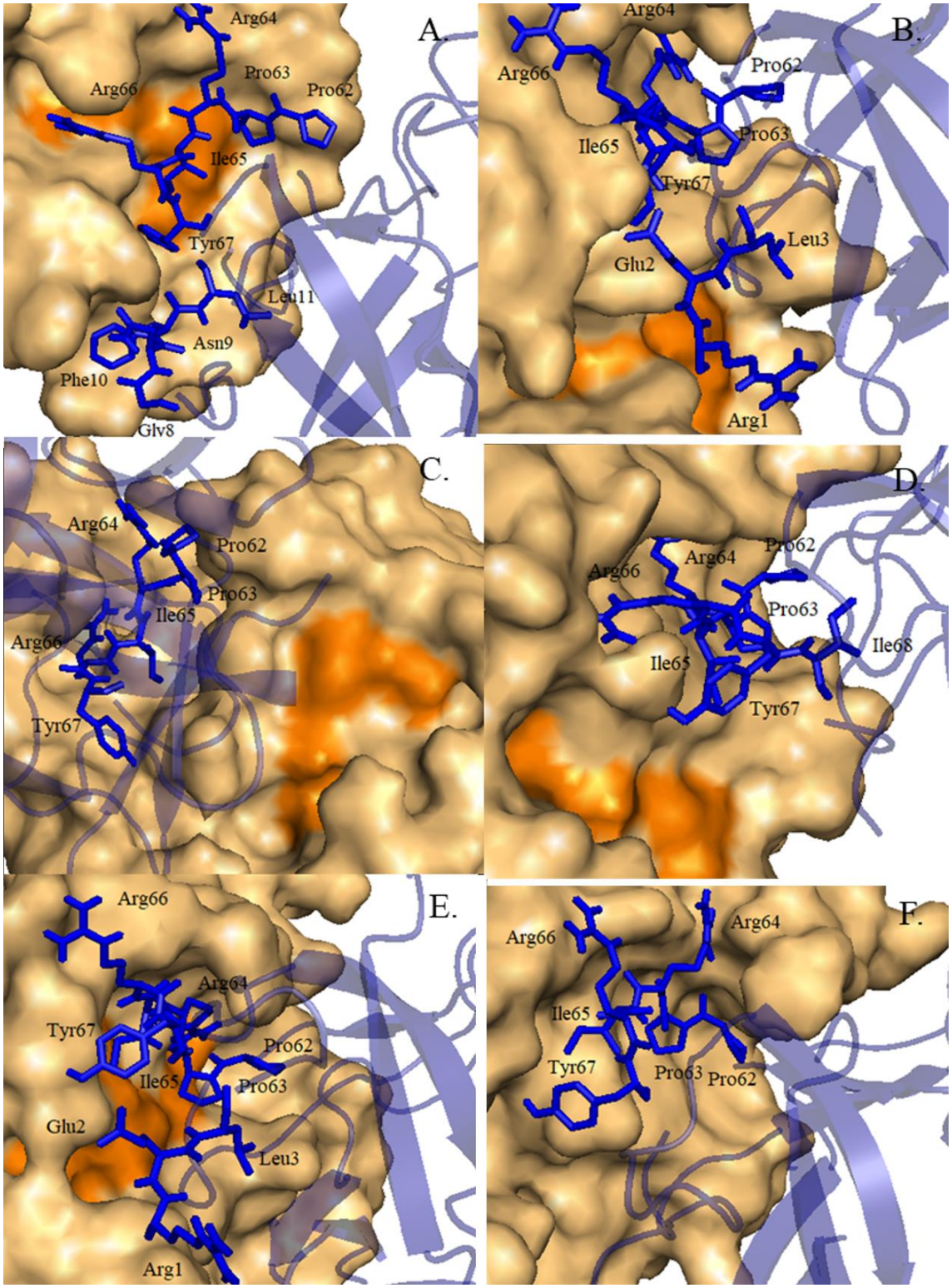

Figure 4 Structural binding mode between *Adenanthera pavonina* trypsin inhibitor (APTI) and trypsin of *S. frugiperda* 1 (A), *S. frugiperda* 2 (B) *S. frugiperda* 3 (C) *S. frugiperda* 4 (D) *S. frugiperda* 5 (E) and *S. frugiperda* 6 (F). Barrel shape representing the trypsins in beige, catalytic aminoacids in larange and the ApTI residues as blue cartoon involved in the trypsin-ApTI interaction.

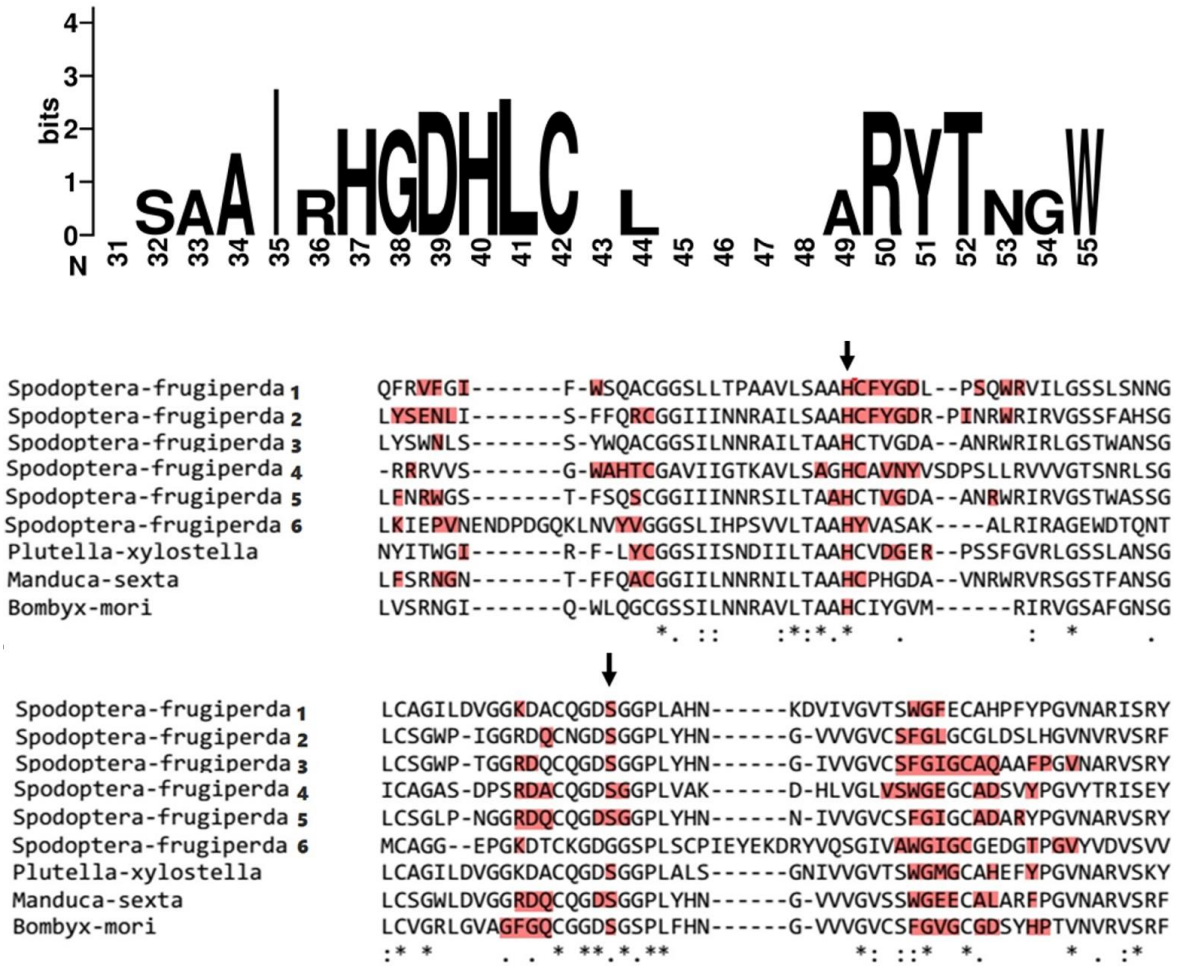

Figure 5. Conserved ILTI residues participants in the interaction with the trypsins represented in the WEBLOGO graph and Multiple sequence alignment of Lepidoptera trypsin-like enzymes. Conserved residues of trypsin digestives participing in the interaction with ILTI inhibitor are highlighted in pink. Arrows indicate catalytic residues.

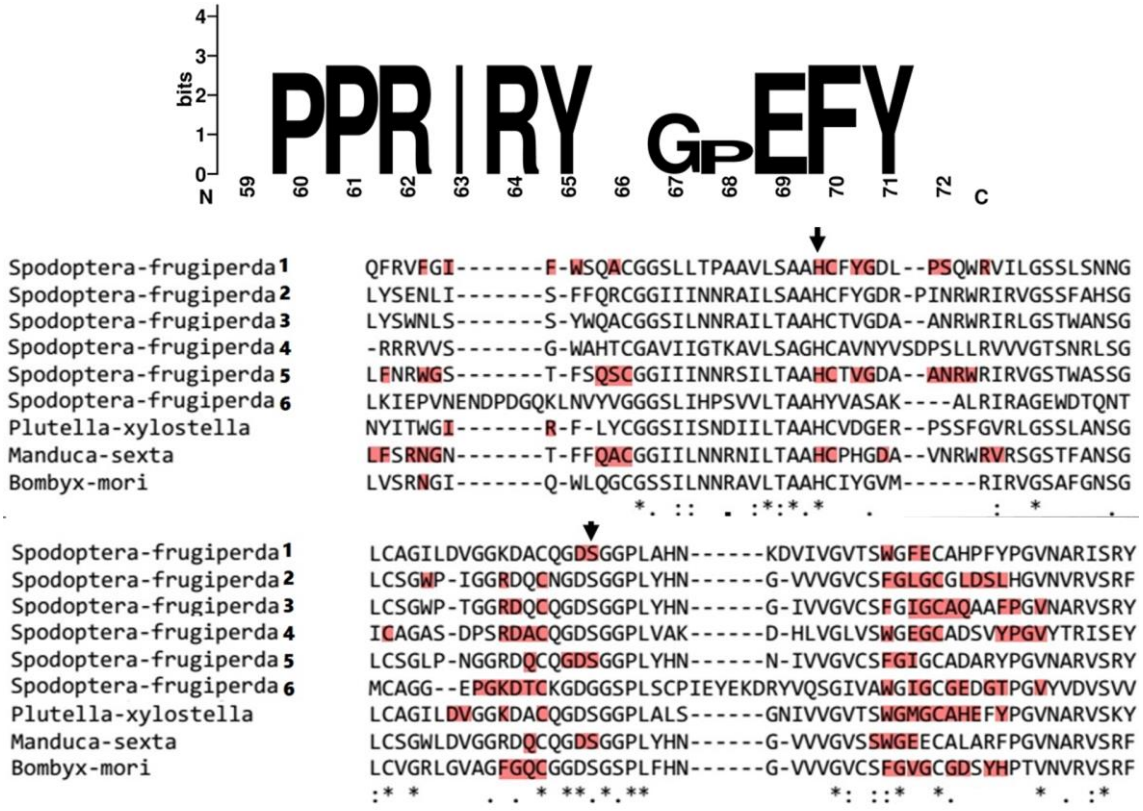

Figure 6. Conserved ApTI residues participants in the interaction with the trypsins represented in the WEBLOGO graph and Multiple sequence alignment of Lepidoptera trypsin-like enzymes. Conserved residues of trypsin digestives participing in the interaction with ApTI inhibitor are highlighted in pink. Arrows indicate catalytic residues.

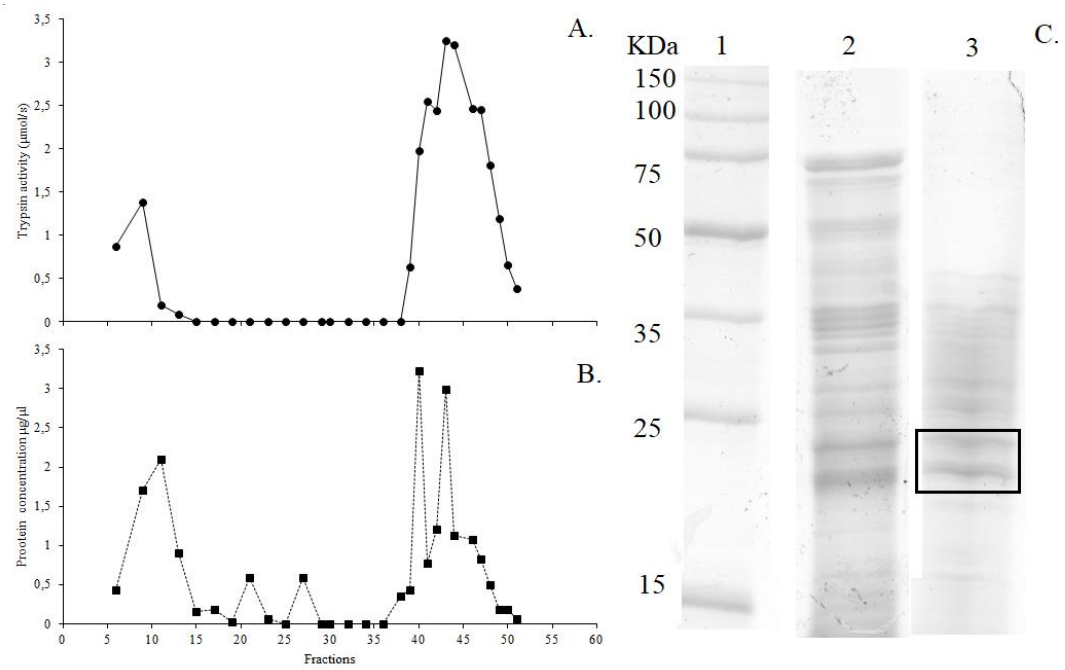

Figure 7. Chromatographic profile of gut soluble extract *Spodoptera cosmioides* (Lepidoptera: Noctuidae) in HiTrap Benzamidine (high sub) (GE) column. Tripsyn activity (µM.s-1) using the substrate L-BApNA (A). Protein concentration (µg/µL) (B). Electrophoretic profile in SDS-PAGE (12%) gut-soluble serine protease of *Spodoptera cosmioides* (Lepidoptera: Noctuidae) (C). Standard molecular mass (Promega®) (1). Crude extract soluble fraction (2). Enzimatic fraction corresponding to the peak with activity after purification in HiTrap Benzamidine (high sub) (GE) column (3).

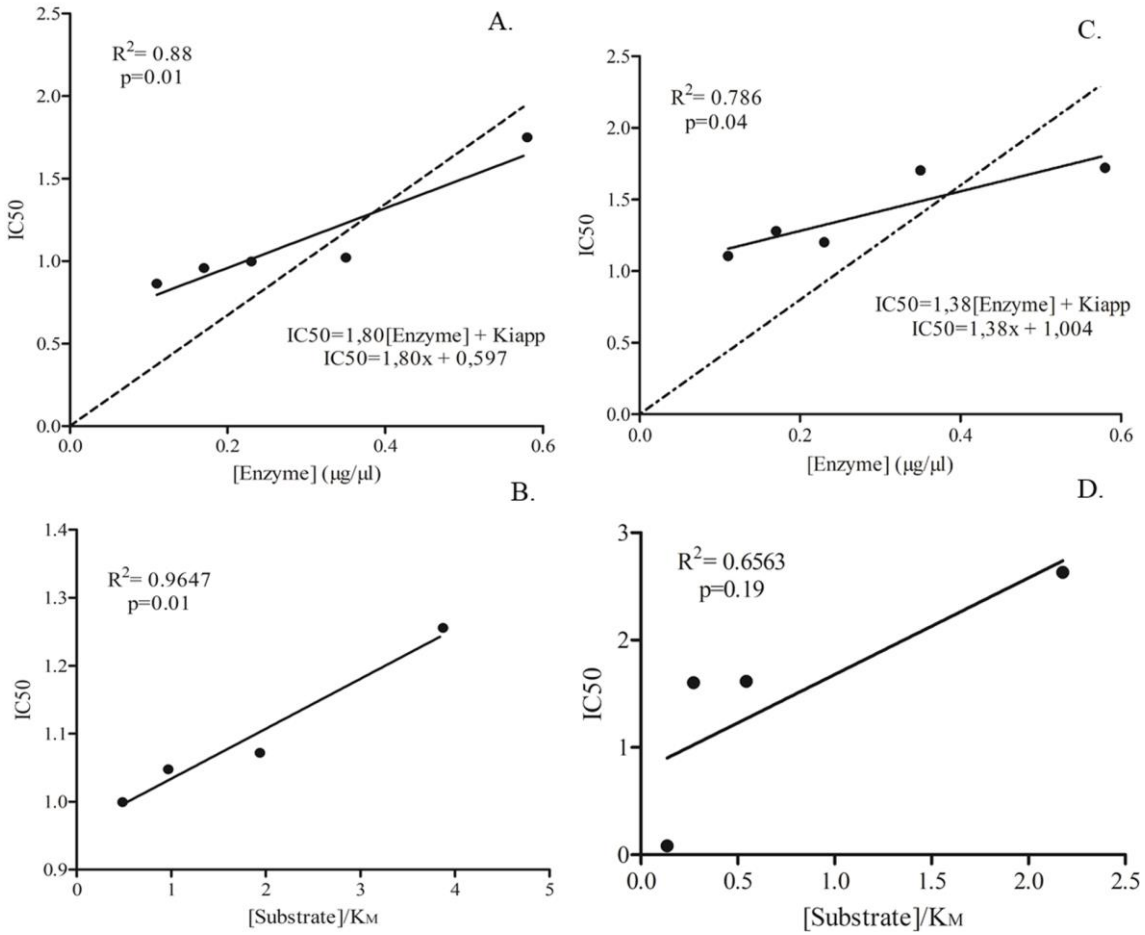

Figure 8. Thigt binding inhibition plot (A) and competitive kinetic (B) of the *Inga laurina* trypsin inhibitor (ILTI) and Thigt binding inhibition plot (C) and non-competitive kinetic (D) of the *Adananthera pavonina* trypsin inhibitor (ApTI) on the trypsins-*like* of *Spodoptera cosmioides* (Lepidoptera: Noctuidae).

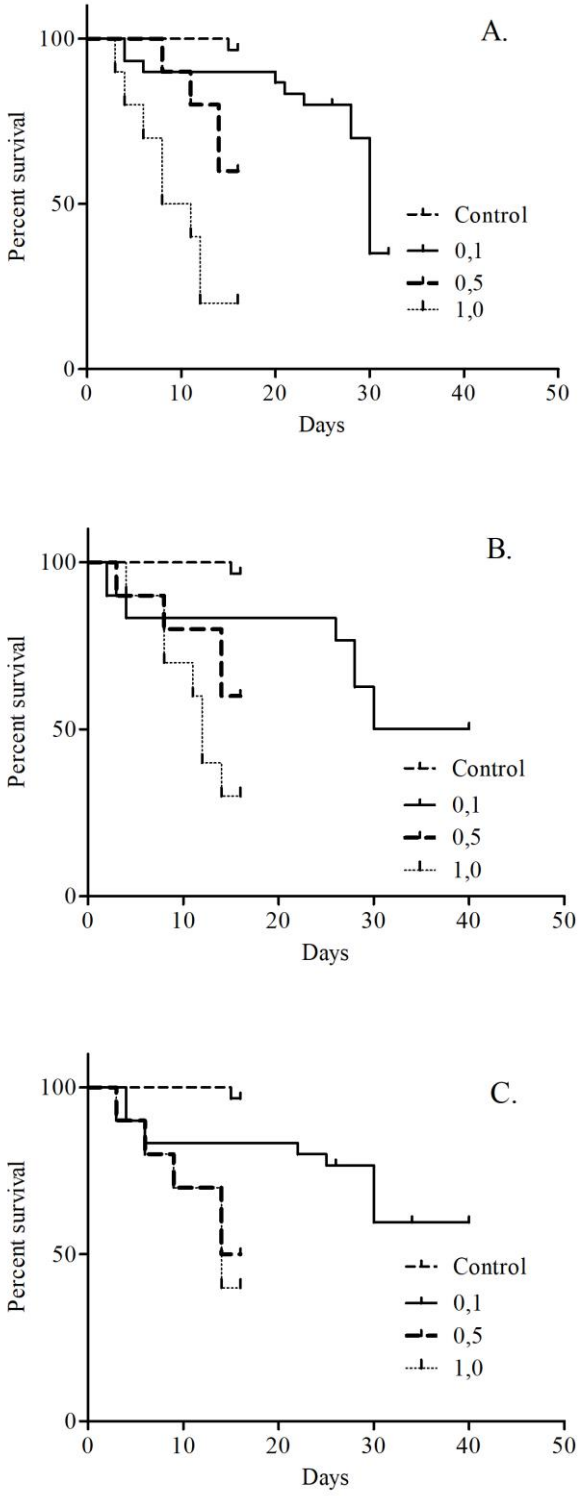

Figure 9. Survival plots of the *Spodoptera cosmioides* (Lepidoptera: Noctuidae) larvae reared on diet containing ILTI (A), ApTI (B) and SKTI (positive controle) (C) inhibitors at concentrations 0.1%, 0.5% and 1.0% w inhibitor/v diet. The last point of each line represent the change of larvae to pupal stage.

Capítulo II:

Desenho racional de peptídeos miméticos baseados na interação entre *Inga laurina trypsin inhibitor* e tripsinas para o controle da praga*Spodoptera cosmioides*

Peptideos desenhados sob deposito de Patente (**ANEXO 1**).

Artigo sob normas da revista *International Journal of Biological Macromolecules*

Rational design of mimetic peptides based on the interaction between Inga laurina inhibitor and trypsins for *Spodoptera cosmioides* pest control

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Abstract

The interaction of *Inga laurina* Kunitz inhibitor with insect trypsins is a example of protein-protein interaction with potential application for the pest control. However, the crop field application of proteins as inhibitors is limited due to high production cost, the large molecular size and low environmental stability. The use of mimetic peptides that have molecular features associated with the protein inhibitor can result in a product with lower cost and higher efficiency for the agricultural application. The rational design of the mimetic peptides may be assisted by computational analysis of mechanism of interaction between protein inhibitors and their targets. Here, we designed mimetic peptides deriving from globular domains of ILTI inhibitor that are predicted to interact with trypsin enzymes of Lepidoptera pest. Thereby, molecular docking and dynamic simulation technique were used for the *in silico* analyzes (Free energy of binding, RMSD, RMSF and pharmacophoric profile), K_i constant determination by simultaneous nonlinear regression method let to determinate of affinity binding of the derived peptides and *in vivo* survival and toxicity analyzes of the *Spodoptera cosmioides* larvae exposed to the peptides included in the diet. Thus, two linear peptides were identified and synthetized from the interaction trypsin-ILTI complexes, this peptides were derived due to its high energy contribution in the interface to the affinity of binding between the enzyme-protein inhibitor. The peptides has structural stability, propensity to adopt the bound conformation also without the context of the protein, inhibitory activity and toxic effects on the *S. cosmioides* trypsin, indicating that can be used as potential inhibitors and optimization targets to bind with higher affinity to their trypsin pest receptors, higher bio-availability and resistance to proteolysis.

Key-words: enzymes, inhibition competitive, docking, dymanic molecular, kinetic.

1. Introduction

A doubling of current food production will be required to sustain the future population at projected levels. However, an estimated 10–20% of major crops worth billions of dollars are lost to herbivorous insects, representing a major constraint to achieving this goal (Bonning & Chougule, 2014). In addition, post-harvest losses resulting from insect and mite-associated damage of stored food, cause estimated losses of 30%, valued globally at >100 billion US dollars (Boyer et al. 2012). A pest that has increased its harmful effects on crops in Brazil is *Spodoptera cosmioides*, the larvae of this species are defoliators and feed gregariously between the first and third instars, often skeletonizing leaves (Bernardi *et al*. 2014). In Brazil, this specie is pest of tomato, cotton, cowpea, rice, soybeans, among others causing significant yield reduction and economic losses approximately US$602.1 million per year for the control of genus Spodoptera pests (Da Silva *et al*. 2017).

Driven mainly by the significant deleterious impact of this insect on food production and associated economic losses, several research entities focus on pest management and crop protection solutions. However, the management of arthropod pests to protect agriculture and public health remains largely dependent on the application of chemical insecticides. There are a number of disadvantages associated with their use, including the development of resistance by pest populations, deleterious impacts on non-target organisms, environmental pollution and potential effects on human health (Bonning & Chougule, 2014; Casida & Quistad, 1998). Thereby, one of the environmentally appropriate alternatives by the pest manage are the proteases inhibitors (PIs).

The PIs are molecules that exert their action by regulating peptidase activity. In plant development, peptidase inhibitors are involved in the same physiological processes than the peptidases they control (Martinez et al. 2012; Volpicella et al. 2011). The defense proteins, they are inhibiting peptidases from the pests and pathogens that attack the plant (Hörger & Van der Hoorn, 2013). Inga Laurina Trypsin inhibitor (ILTI) is one of these inhibitory proteins. It is a serine protease inhibitor composed of a single chain containing 180 residues, with molecular weight approximately 20 kDa and it has high stability due to the presence of two disulfide bridges. This protein was isolated from the *Inga laurina*

FE, Unicamp, Campinas, SP- Brazil. (Fabaceae) from the subfamily Mimosoidae (Macedo *et al*., 2007; Macedo *et al*., 2011; Machado *et al*., 2017).

The interaction of ILTI with insects trypsin is a potential example of protein-protein interaction by the pest control, but the agricultural application *ex vivo* of this inhibitor is limited due to high production cost, the large molecular size and low environmental stability. The identification of potential hits computer-aided for a biopesticides target before delving into extensive experimental assays, lends cost- and labor-efficiency (Macalino et al. 2018). Nevertheless, like any other approaches, *in silico* methods are not infallible and are more valuable when they are employed in combination with other tools and analysis (Macalino et al. 2018).

In this study, we examine on the possibility of deriving inhibitory peptides from globular domains of ILTI inhibitor as a general mechanism to inhibit trypsin enzymes of Lepidoptera pest. Such peptides could serve as promising leads for the rational design of inhibitory compost for the pest control. Thereby, the aim of this paper was design mimetic peptides from the reactive site of ILTI and analyze its inhibition potential by *in vitro* tests on purified trypsins of *S. cosmioides* and toxicity analyzes of the larvae exposed to the peptides included in the diet.

2. Materials and Methods

2.1 *In silico strategies for the peptides design*

From the study inhibition of digestive trypsins by plant Kunitz proteins (Meriño-Cabrera et al. 2019), was determinated that ILTI inhibitor has high potential reducing the viability of *S. cosmioides* larvae. ILTI was selected like the protein model by the inhibirory peptides discovery (Fig. 1).

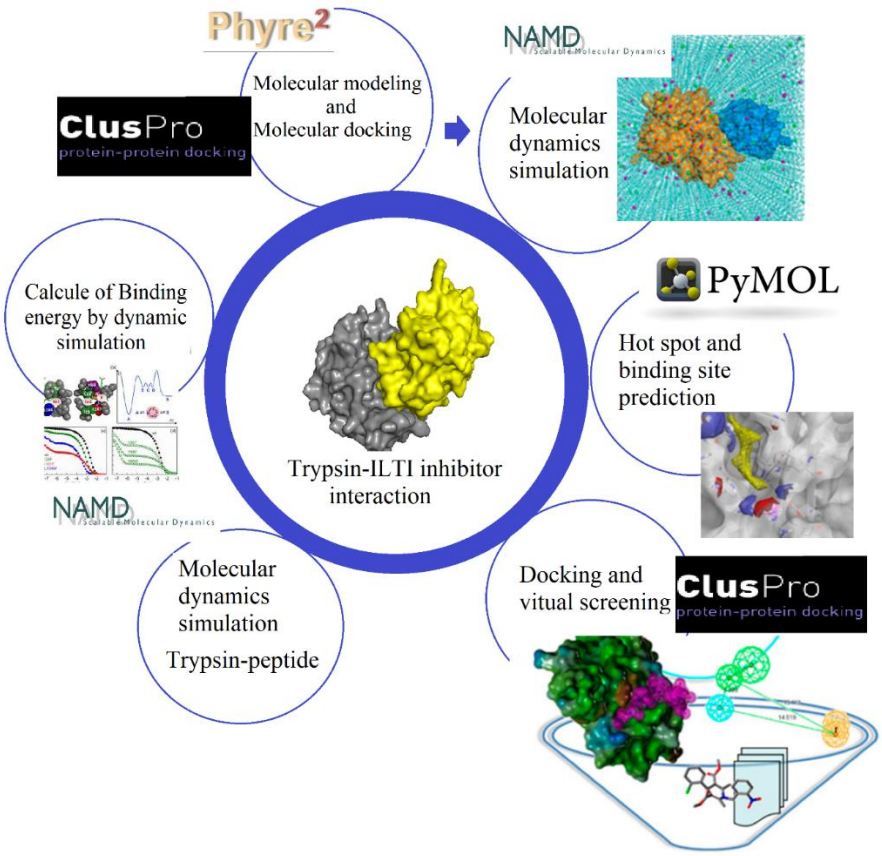

Figure 1. Diverse computational tools encompass the peptides discovery, including elucidation of complex interactions and dynamics, the characterization of interface and hot spots, optimization and binding energy calculation of peptides. Edited and taken of Macalino et al. 2018.

The docked complex between *S. cosmioides* trypsin and ILTI inhibitor was chosen, this complexes were obtained using the online tool Cluspro (Kozakov *et al*., 2017) (Fig. 1). This tool allows made docking protein-protein and protein-peptide, first perform a rigid-body docking, followed cluster retained conformation and refine by CHARMM minimization obtained 10 models like output (Kozakov *et al*., 2017).

A molecular dynamic simulation was done by the minimization and interaction analysis between trypsin-ILTI over time. We used the molecular dynamics simulation package NAMD 2.12, using the force field CHARMM36, which can be used for proteins (Fig. 1). The solvation of the system was done using the TIP3P water model, following the consideration that the distance between the end of the protein and the limit of the simulation box was >15 Å in each direction. To maintain the neutrality of the total charge of the system molecules were used as ions sodium chloride and calcium chloride. The

insertion of the ions was performed using the tool *Autoionize* of VMD. After 1000 minimization steps, the system was simulated at 310 ºK and pressure of 1 atm by 10 ns in the constant pressure ensemble (NPT). The temperature was controlled using Langevin dynamics (1.0) and pressure using the Langevin piston method, with an oscillation period of 2fs. For the unbound interactions, the cutoff was adjusted to 12 Å and for the long range the cutoff was modeled using the Particle Mesh Ewald method (PME).

From the energetic analysis of the residues of the interface protein-protein with proximity less than 4 Å. This pocket was visualized and analysed using the program PyMOL (Molecular Graphics System, Version 2.0 Schrodinger, LLC).

2.2 Analysis of the inhibitory potential of the designed peptides

2.2.1 Binding energy and Pharmacophoric profile

Molecular docking, pharmacophoric profile and molecular dynamic were performed between the determined peptides and 3D trypsin model of *S. cosmioides*. The docking results help to obtain the pharmacophoric profile between the complexes using the program Discovey Studio (Dassault Systèmes BIOVIA 2017).

The binding free energy calculation was performed by the linear interaction energy method (Hansson et al. 1998), it provides approximate estimates to the absolute binding and hydration free energies with low computational effort, from molecular dynamics simulations of the initial state (peptides solution) and final state (enzyme-peptide complex in solution). Within this approximation, the ligand–protein binding free energy is given by

$$\Delta G_{Bind} = \beta\left(\langle V_{l-s}^{el}\rangle_{bound} - \langle V_{l-s}^{el}\rangle_{free}\right) + \alpha\left(\langle V_{l-s}^{vdw}\rangle_{bound} - \langle V_{l-s}^{vdw}\rangle_{free}\right) + \gamma \qquad (1)$$

where $\langle V_{l-s}^{el}\rangle_Y$ and $\langle V_{l-s}^{vdw}\rangle_Y$ are the average values of the electrostatic and van der Waals interactions between the ligand (l) and its surroundings (s) in the ligand state Y, which can be either free in solution or bound to the protein. The α and β parameters are, respectively, dispersion and electrostatic adjustable energy scale factors and γ is a constant term, They are dependent of chemical nature of dispersion and electrostatic adjustable energy scale factors ligand.

2.2.2 Theretical inhibition constant

The enzyme-ligand molecular recognition process is driven by a combination of enthalpy and entropic effects. These effects can be stimated by Gibbs-free energy from enzyme-ligand interaction. (ΔG_{Bind}) which in turn is directly related to the inhibition constant Ki, which was theoretically calculated by the following equation (2):

$$\Delta G_{Bind} = RTln\, K_i \qquad (2)$$

2.2.3 Analysis in vitro of the inhibitory potential

2.2.3.1 Peptides synthesis

The peptides were design based on the primary structure of the ILTI inhibitor, from the reactive site specifically. Chemically synthesized by GenOne company and the synthesis and purification methodology adopted allowed the obtaining of the peptides with 95% purity grade.

2.2.3.2 Inhibition kinetic

Concentration-dependent reduction in activity of *S. cosmioides* trypsin was estimated through specific chromogenic substrate BApNA (Benzoyl-L-arginyl-p-nitroanilide). The purification of the gut trypsins was performed by affinity chromatography using HiTrap Benzamidine (high sub) 5 mL (GE Healthcare) column as previously described (Meriño-Cabrera et al. 2019). The assays were performed in buffer of Tris–HCl 0.1 mol/L, CaCl2 20 mmol/L pH 8.2, using concentrations of L-BApNA substrate: 1.0, 2.0, 16.0 and 32.0 mM. The Inhibitors concentration were, PN inhibitor: 0.23, 0.47, 0.98 1.98 mM/L, and DH inhibitor: 0.35, 0.71, 1.42, 2.84 mM/L, reactions without inhibitor were used like enzyme control. The total concentration of enriched trypsin-like was 1.2 µg/µL.

The velocities observed versus substrate concentration data (in the presence and absence of inhibitors) were fit simultaneously according to nonlinear regression fitting using Graphpad Prims 5.0 program (Motulsky, 2007). Two approaches were applied to this analysis. In one instance all of the data were analyzed simultaneously (Vmax, [E] Km, and Ki). In the other instance, the control data (no inhibitor) were first analyzed to obtain estimates of Kcat and Km. Those values were then fixed in fitting the average rate

data obtained from the inhibition experiments for estimation of Ki (Copeland 2013), the equation describing this relationship was:

$$\frac{K_M}{K_{cat}} = \frac{K_M \,.\, [I]}{K_i \,.\, K_{cat}} + \frac{K_M}{K_{cat}} \qquad (3)$$

2.2.4 Effects of peptides on the development and nutritional parameters of Spodoptera cosmioides

Bioassays were conducted by feeding *S. cosmioides* larvae on an artificial diet containing 2.0% w inhibitor/v diet. The added inhibitors were the synthetic peptides and SKTI (Soybean Kunitz trypsin inhibitor) as positive control. Three independent sets of n= 30 first instar larvae were used for analysis. Each larvae was placed in a separate plastic container containing 3g of diet. Insects were maintained at 25 ± 1°C, 16:8 h (light/dark). On alternate days, the larvae were weighed; the amount of fecal pellets produced and diet remaining was recorded. The assay was performed during five days and the moratlity were observed.

The mortality was analysed through mechanical stimulation with tweezers or brush and direct observation of the movement of the larvae. Nutritional parameters, namely efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD) and approximate digestibility (AD) were calculated (Joshi et al., 2014).

Statistical analysis of growth reduction pattern was performed by two-way ANOVA. Asterisks indicate significant differences (*$p < 0.05$; **$p< 0.001$). The survival curves were obtained using Kaplan–Meier estimators (Borgan, 2014) to determinate significant differences in percentage survival among the different inhibitors dose.

3. Results

As a first step, we examine the data previosly obtained (Meriño-Cabrera et al. 2019) of protein interactions for the prevalence of high affinity linear peptide segments that participate in the Lepidoptera trypsin-ILTI inhibitor interface. This results were reviewed and the binding partners were studied, determining that 90% of the protein

complex structures of Lepidoptera trypsins-ILTI, two peptide derived from a two linear segments at the interface (Fig. 2A).

To confirm this results in this study was used molecular simulations to best analyse of the interface trypsin-ILTI. In order to identify the dynamic behavior of ILTI inhibitor when associated a trypsins of *S. cosmioides*, RMSD values were calculated for the inhibitor, this values fluctuate between 0.5 to 2.5 Å for the ILTI ligand associated to trypsin receptor (Fig. 3A).

The forces derived from the (potential) energy induce movements both in the protein and ligand, these are commonly measured using the Root Mean Square Fluctuation (or RMSF). This quantity measures the average fluctuation of a residue's atom around a reference position. In this case, the reactive site of ILTI inhibitor fragmented in two regions Asp39 to Cys42 (Fig. 3B) and Pro83 to Asn88 (Fig. 3B) presented a fluctuation between 3.5 to 2.0 Å and 2.5 to 1.2 Å, respectively.

Moreover, was performed binding free energy (ΔG_{Bind}) contribution of each residue in the interface trypsin-ILTI (distance less than 4Å) to identify the residues that are important for the interaction in the environment (physiological medium) (Fig. 3C). The residues name and number with the highest energy contribution are showed (Fig. 3C); The residues Leu41 had the highest energetic contribution (-17.14 Kcal/mol) (Fig. 3C), followed of Cys42, Pro83, Asn84, Val85, Thr87, Asn88 with a free energy of binding less than -5.0Kcal/mol and were estimated to contribute over half of the interaction energy of the entire complex (Fig. 3C).

The interaction between ILTI inhibitor and trypsins of *S. cosmioides* is mediated by two reactive site like confirmed in the molecular docking and dynamic simulation (Fig. 2A), and the linear segments identified from the reactive site of ILTI were the peptide 1: Asp-Hys-Leu-Cys (D-H-L-C) and the peptide 2: Pro-Asn-Val-Cys-Thr-Asn (P-N-V-C-T-N) (Fig. 2A). The binding validation of the peptides to *S. cosmiodes* trypsin performed by molecular docking using Swissdock showed the peptide DH has lower affinity (-6.93 Kcal/mol) compared to peptide PN (-8.90Kcal/mol) (Table 1).

The docking analyses pointed that for the complex trypsin- PN peptide, the ligand block the S1 pocket trypsin and interacts with residues therein, this S1 pocket was characterized by the presence of the chatalityc triad Hys70, Asp111 and Ser221 and at the top in the site of Asp206 residue (Fig. 2B). On the other hand, the DH peptide form

a complex with trypsin by the bonds in the S2 site included the Hys70, Ala106, Phe108 and Asp111 residues (Fig 2C).

3.1 Binding energy and Pharmacophoric profile

Pharmacophoric profile was performed for the peptides in the binding site to trypsin after docking and molecular dynamic simulation. The ligand regions intimately associated to the receptor were identified, as well as the receptor residues involved in each binding (Fig. 4). Several type binding like conventional Hydrogen bond, Carbon Hydrogen bond, and alkyl were found in the pharmacophoric analyses (Fig. 4).

The Pharmacophoric profile for the trypsin-peptide DH after docking showed that the ligand interact with the trypsin receptor only with one type of carbon hydrogem connection with Ala106 in the site S2 (Fig. 4A), and most ligand structure has its solvent-accessible area. After molecular dynamics simulation, the temporal analysis showed a decrease of solvent-accessible area when the peptidic DH ligand is mostly stabilized by the Hydrogen interactions with the residues side chain of trypsin like Arg34, Arg35 and Asn214 in the S2' site (Fig. 4C).

The Pharmacophoric profile for the trypsin-peptide PN complex showed that the interactions measured, which takes into account the amino acid angle and distance, approximately 80% of the interactions are Hydrogen bonds with Gln202, Gly198, Gly202, Gly204, Ser29, Hys166 and Thr234 of the S1 pocket (Fig 4B). In addition, alkyl interactions were observed between the ligand PN and the trypsin residues Val146, Val197, Cys221 and Cys203 (Fig. 4B). After molecular dynamics simulation, the temporal analysis showed that the peptide PN ligand is mostly stabilized by the Hydrogen interactions with the residues side chain of trypsin, corroborating the molecular docking results (Fig. 4D).

The binding affinity (K_D) was calculated for the complexes trypsin-DH and trypsin-PN from the molecular dynamic simulations, founding values of 61.24 and 33.49, respectively (Table 1). The Ki theoretical for each peptide was determinate using the K_D value, obtaining a Ki equal to 2.7 for DH peptide and 1.7 for the PN peptide (Table 1).

The stability of the individual peptides and the residues that conform them were analyzed from the RMSD and RMSF analyzes. The RMSD values of DH peptide fluctuate between 0-3.8 Å and for the PN peptide was between 0-2.5 Å (Fig. 5A).

The RMSF values in the residues of DH peptide were lower than 2.5 Å and the residues of PN peptide presented values lower than 1.5 Å, with exception of the Asn88 residue, which had an RMSF value greater than 2.5 Å (Fig. 5B)

3.2 Inhibition kinetic

The simultaneous nonlinear regression method was used to determinate of peptides Ki, the control data (without inhibitor) were analyzed by nonlinear regression to obtain the best fitting values for K_{cat} and K_M (Fig. 6A and 6B). Those values were then fixed in the subsequent analysis of all of the data (control and inhibition data) for the Ki calculate. The Ki value was 3.31 mM for the DH inhibitor (Fig. 6C) and 1.48 mM for the PN inhibitor (Fig. 6D).

3.3 Effects of peptides on the development and nutritional parameters of Spodoptera cosmioides

The survival curve obtained using Kaplan–Meier estimator indicated significant differences in percentage survival among the different inhibitors analyzed (log-rank χ^2 = 9.46, $p < 0.05$). Comparing peptides inhibitors with SKTI control, both peptides reduced the survial of the *S. cosmioides* larvae, but the peptide PN reduced 35% the survival compared with of 30% caused by DH peptide (Fig. 7).

Nutritional analyses revealed that PN and DH peptides presented a toxic effect when ingested by *S. cosmioides* larvae. PN and DH, when incorporated in an artifitial diet at 2.0% reduced ECI and ECD and increased AD and metabolic cost (CM) (Fig. 8) for *S. cosmioides* larvae when compared with the control. ECI and ECD showed significant decreases of 20.3% and 11.0%, respectively, and CM was increased by 30% when compared with larvae of *S. cosmioides* reared with control diets.

4. Discussion

The design and generation of molecules capable of mimicking the binding and/or functional sites of proteins represents a promising strategy for the exploration and

modulation of protein function through controlled interference with the underlying molecular interactions. Synthetic peptides have proven an excellent type of molecule for the mimicry of protein sites because such peptides can be generated as exact copies of protein fragments, as well as in diverse chemical modifications (Groß et al. 2016). Thus, interaction complexes between *S. cosmioides* trypsins-ILTI analyzed previously (Meriño-Cabrera et al. 2019) together with the molecular dynamics simulations performed in this study, served as a base to determinate the hot spot regions that stablish the binding affinity for the stabilization of the complex.

The binding free energy is a global property of a system. As such the free energy is dependent on the total volume of configurational or phase space available to the system. Thus, the total free energy of the system can be only expressed in terms of a sum of components as far as the total system can be separated into a series of independent subsystems. In other words, the change in free energy associated with altering a specific interaction or residue will depend on the conrrelation between that interaction or residue and all other interactions/residues in the system (Mark & van Gunsteren 1994). However, the energy contribution that the residue or interaction can make on the binding free energy if it can be calculated.

In this study, the binding free energy contribution of each residue in the vicinity of *S. cosmioides* trypsin site was performed (distance less than 4Å) to identify the residues that are important in the complex formation Trypsin-ILTI inhibitor. Approximately eleven amino acid residues have the highest energy contribution at the trypsin- ILTI inhibitor interface, this aminoacid number is expected, because previous studies that have investigated the structural nature of globular protein-protein interfaces and characterized the atomic details of the interactions in terms like size and shape, showed that a small number of so-called hotspot residues in this interface contribute the majority of the binding energy (London et al. 2010; Clackson & Wells, 1995).

The energy analysis *via* docking and molecular dynamic experiments indicates this high-affinity segments indeed favor their original conformation within the context of the entire protein within some considerably large basin of attractions. This makes them worthy candidates for inhibitory peptides, or at least promising leads for designing small inhibitory molecules and peptides with increased affinity, stability and bio-availability. Thus, two linear sequences of inhibitory peptides

were derived from interface trypsin-ILTI: Asp-Hys-Leu-Cys and Pro-Asn-Val-Cys-Thr-Asn. Each one of this aminoacid residues had a energy contribution different, these indicate that although all can be classified as hot spots, each residue contributes distinctive functions in a complex (Macalino et al. 2018; Kimura et al. 2001).

Generally, a interface of protein-protein interaction is split into a core and a rim region (David & Sternberg 2015). The core region is buried, and consists of residues with higher hydrophobicity and conservation, whereas the rim region is in the adjacent solvent-accessible area with more polar and flexible residues (Macalino et al. 2018; Yan et al. 2008). The aminoacids residue of the derived peptides seem to be part of the interface rim region of Trypsin-ILTI, because the majority are polar aminoacid residues uncharged (Cys, Pro, Asn and Thr), indicating that they can be form hydrogens bonds with water and other residues by them functional groups; and the rest of residue are either positively (Hys) or negatively (Asp) charged or hydrophobic (Leu and Val).

The pharmacophoric profile after molecular docking and dynamic let to identify of the binding site and the kind of interaction between the peptides and trypsin receptor, revealing that the peptide PN has characteristics of a competitive inhibitor, occupying the S1 site of the enzyme, this results can be explained to the fact that smaller size of the peptide compared to the protein confers advantage in competitive binding thanks to increased effective peptide concentration at the interface, resulting in increased effective binding ability compared to ILTI protein (Wells & McClendon 2007). The results suggest that PN peptide adopted a similar binding position even when cut out of their protein context (ILTI inhibitor), result observed in other studies of peptide design (Saikhedkar et al. 2018; Vlieghe et al. 2010).

On the other hand, DH peptide bind in the site S2´ of the trypsin enzyme; the protease interface for this site in *S. cosmioides* trypsin is comprised of the side chains of the residues Arg24, Arg35, Tyr37 in the top of the pockect and Asn214, the number and characteristic of the residue is agree with trypsin in another insects (Meriño-Cabrera et al. 2019) and bovine trypsin (Scheidig et al. 1997). The S2´ site is inhibited by hydrophobic binding with inhibitor residue 34 in bovine trypsin, in this study the Leu present in the DH peptide substitute the residue 34, but is always hydrophobic (Gopal et al. 2017; Ma et al. 2005).

A molecular docking and dynamic study revealed that the trypsin inhibitory activities of the DH and PN peptides were mainly attributable to the hydrogen bond, this

result coincides with another data reported, indication that hydrogen bond interactions play an irreplaceable role in stabilizing the structure of the complex enzyme-inhibitor and is related to inhibitory potency of the peptides (Sun et al. 2019; Ni et al. 2017; Coombs et al. 1999).

The tridimensional model of *S. cosmioides* trypsin complexed with PN and DH peptides were used in the calculation of the binding energy of these inhibitors. The calculated Ki derived from the free energy change upon binding of these inhibitors to trypsin were compared with the experimental values, showing that the calculated binding constants Ki values for the peptides are consistent with the calculated experimental Ki values of approximately 1.7mM in PN peptide and 3.5mM DH peptide.

The PN peptide has higher trypsin affinity and inhibitory activity, this result may be related to the fact that the PN inhibitor creates higher number of hydrogen bonds, stabilizing the ligand at site S1 and increasing its inhibitory potency.

Although the DH peptide formed fewer hydrogen bonds with the receptor, the length of its sequence may have a positive effect on its inhibitory capacity, due has been reported that shorter length/low molecular weight peptide can be easily absorbed. from the intestines as well as easily binds to the active site of the enzyme resulting in an almost complete inhibition of the enzyme activity (Lee and Hur 2019).

The Ki values of PN and DH peptides were compared to the Ki value reported in vitro analyzes for a protein ILTI when complexed to *S. cosmioides* trypsin (0.597µg/µL) (Meriño-Cabrera et al. 2019), demonstring that smaller size of peptides may increase its effective binding ability, but the peptide binding affinity is dreceased due to the loss of entropy upon binding (London et al. 2010). However, the Ki values and the binding stability demostred by RMSD and RMSF analyses indicate that derived peptides of ILTI can be used as potential inhibitors and optimization targets to bind with higher affinity to their trypsin pest receptors, higher bio-availability and resistance to proteolysis.

The survival of *S. cosmioides* larvae was reduced and index of dietary utilization showed that ECI and ECD decreased when a 2.0% of PN and DH peptides in diet were employed. In the present study, the AD value for larvae of *S. cosmioides* was increased throughout the feeding period of the analyse. This results suggests that, during the treatments, the food remained for a greater time in the insects gut to allow the detoxification of the peptides.

This increased the exposure of the food to digestive enzymes, probably allowing an increased digestibility of the food in comparison to that seen in the control. This behavior was also observed in other Lepidopetera insect like *Anagasta kuehniella* (Da Silveira et al. 2009). ECI is an overall measurement of an insect ability to utilize the food that it ingests for growth. A drop in ECI indicates that more food is being metabolized for energy and less is being converted to body mass and growth of insect (Koul et al. 2004). ECD also decreases as the proportion of digested food metabolized for energy increases (Wheeler and Isman, 2001).

Reduction of ECI and ECD lead to delay in larval growth and formation of smaller pupae which have a direct relationship to fecundity and longevity of the adult insect and make them susceptible to diseases and natural enemies. The ability of an organism to convert nutrients, especially protein, will positively influence its growth and development (Teimouri et al. 2015).

5. Conclussion

Two linear peptides were identified and synthetized from the interaction trypsin-ILTI complexes, these peptides were derived due to its high-energy contribution in the interface to the affinity of binding between the enzyme-protein inhibitor. The peptides has structural stability, propensity to adopt the bound conformation also without the context of the protein, inhibitory activity and toxic effects on the S. cosmioides trypsin, indicating that can be used as potential inhibitors and optimization targets to bind with higher affinity to their trypsin pest receptors, higher bio-availability and resistance to proteolysis.

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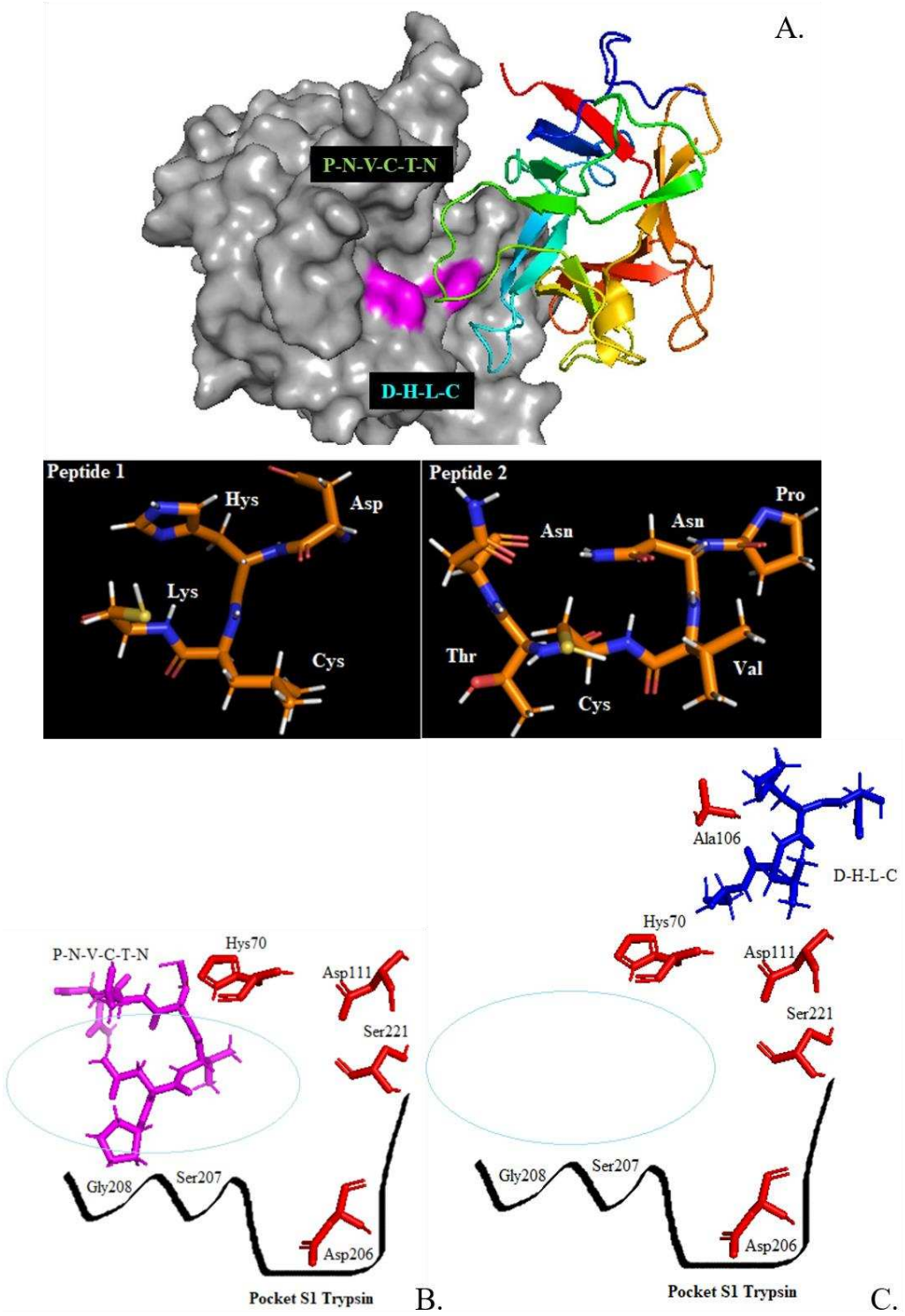

Figure 2. (A) Structural binding mode between Inga laurina trypsin inhibitor (ILTI) and trypsin of *Spodoptera cosmioides,* barrel shape represents tre trypsin in gray color, catalytic triad in pink color, ILTI inhibior in mosaic cartoon. The green and blue loops of ILTI represent the location of the fragments chosen for peptide synthesis, below the peptides and amino acid residues that make them up. (B) Interaction of the P-N-V-C-T-N peptide with the trypsin S1 pockect of *S. cosmiodes* trypsin. (C) Interaction of the D-H-L-C peptide with the trypsin S1 pockect of *S. cosmiodes* trypsin.

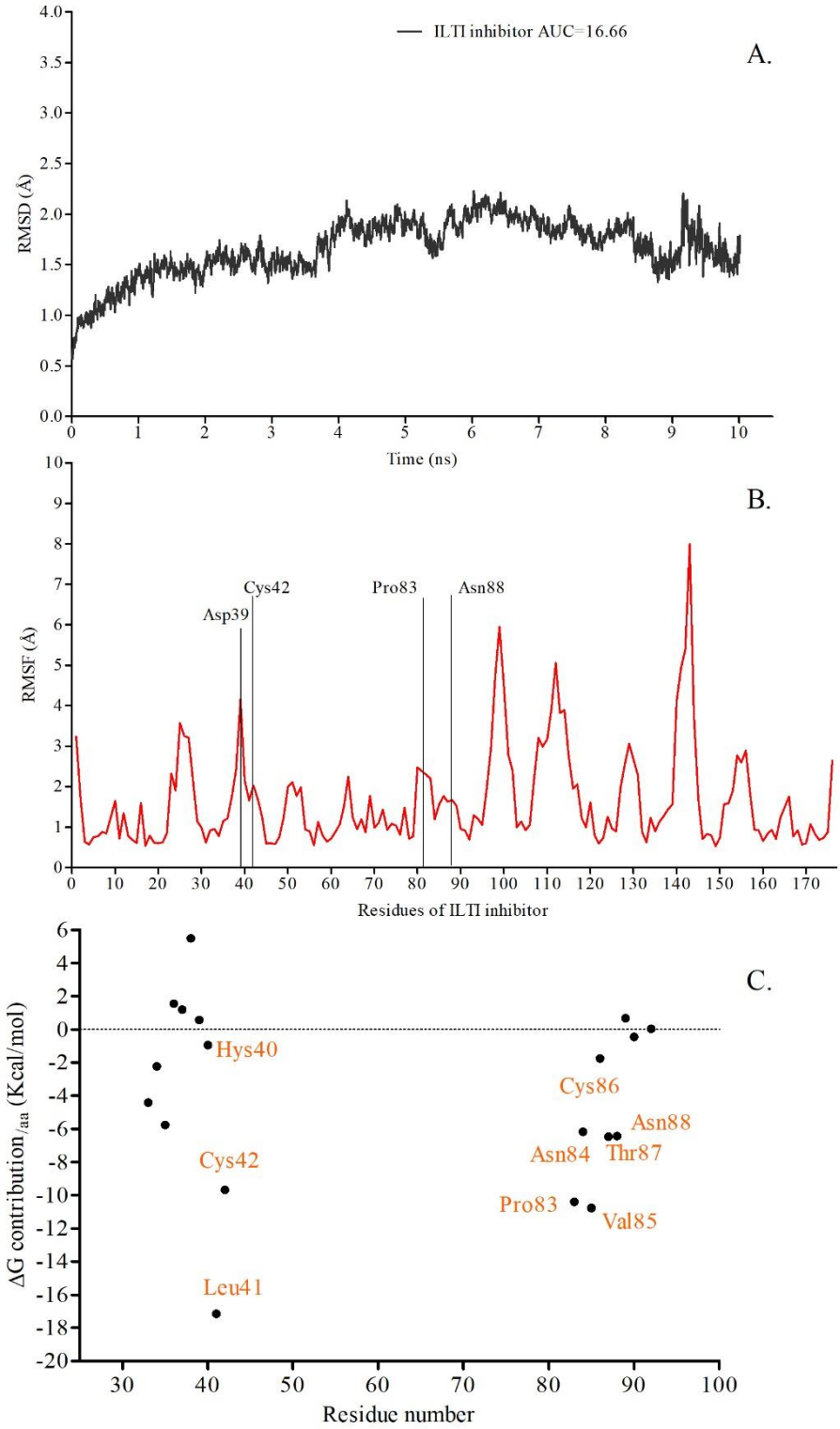

Figure 3. Root mean square deviation (RMSD) (A) and Root mean square fluctuation (RMSF) values of ILTI inhibitor when associated with *S. cosmioides* trypsin. (C) Per-residue binding free energy contribution of *S. cosmioides* trypsin-ILTI inhibitor, calculated from molecular dynamic simulation of the complexe in water box. Residues with high-energy contribution (the energy contribution ≤ -1.0Kcal/mol) were labeled.

Table 1. Afinity binding energy values ($Kcal/mol$) calculated by molecular docking and dissociation potencial by molecular dynamic simulation (MD) and inhibition constant experimental and theoretical Ki for the interaction between and D-H-L-C and P-N-V-C-T-N peptides and *S. cosmioides* trypsin protein.

Peptide	ΔG_{Bind} (Kcal/mol)(Docking)	K_D (Dissociation Potencial) (MD)	Ki experimental	Ki theoretical
D-H-L-C	-6.93	61.24	3.31	2.7
P-N-V-C-T-N	-8.90	33.49	1.48	1.7

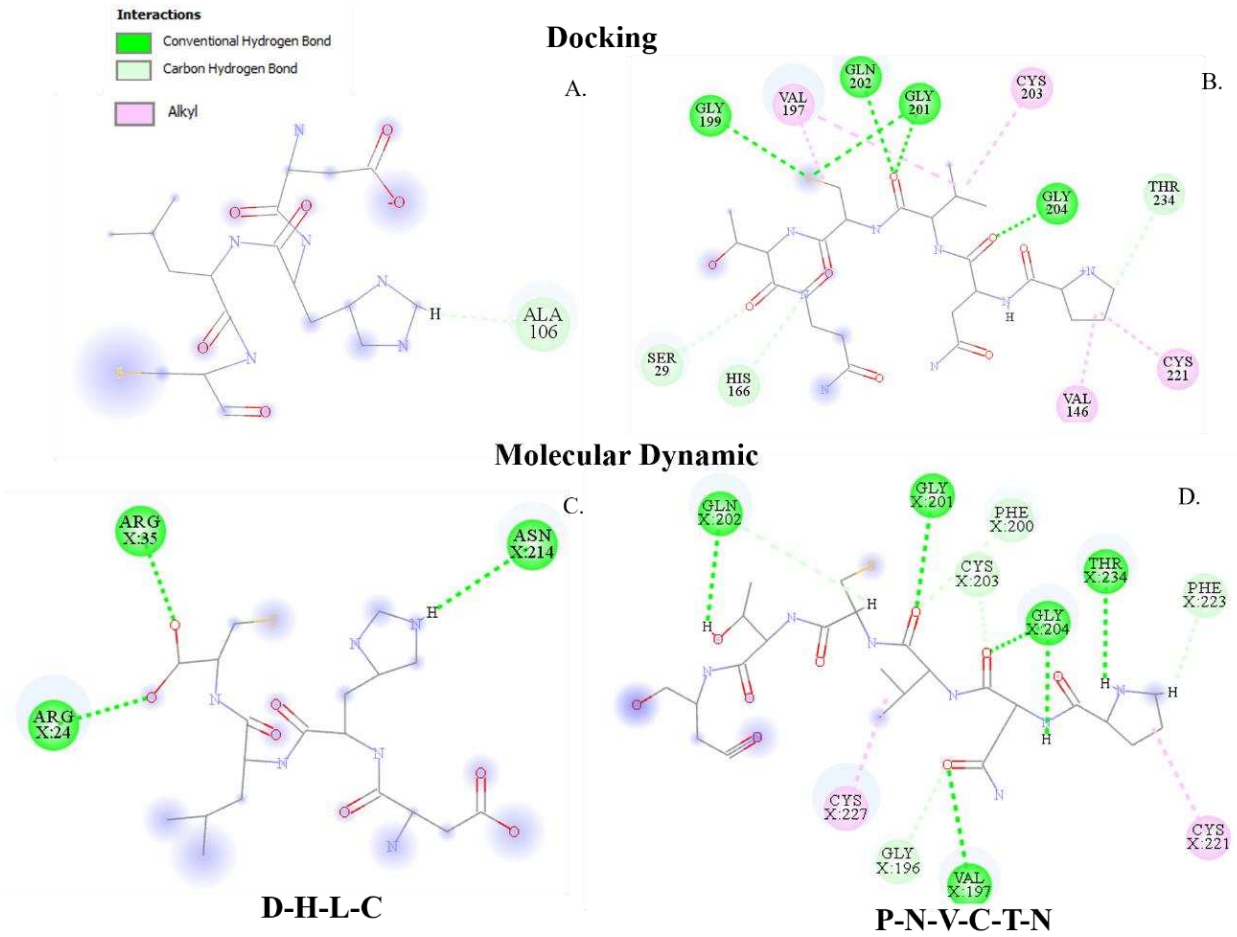

Figure 4. Pharmacophoric profile of the inhibitor peptides-trypsin complexes after docking molecular (A and B) and dynamic simulation (C and D). D-H-L-C bound to *S. cosmioide* trypsin (A and C) and P-N-V-C-T-N bound to *S. cosmioide* trypsin (B and D). Color ball and pointed line indicate the interaction type between peptides and residues side chain of trypsin.

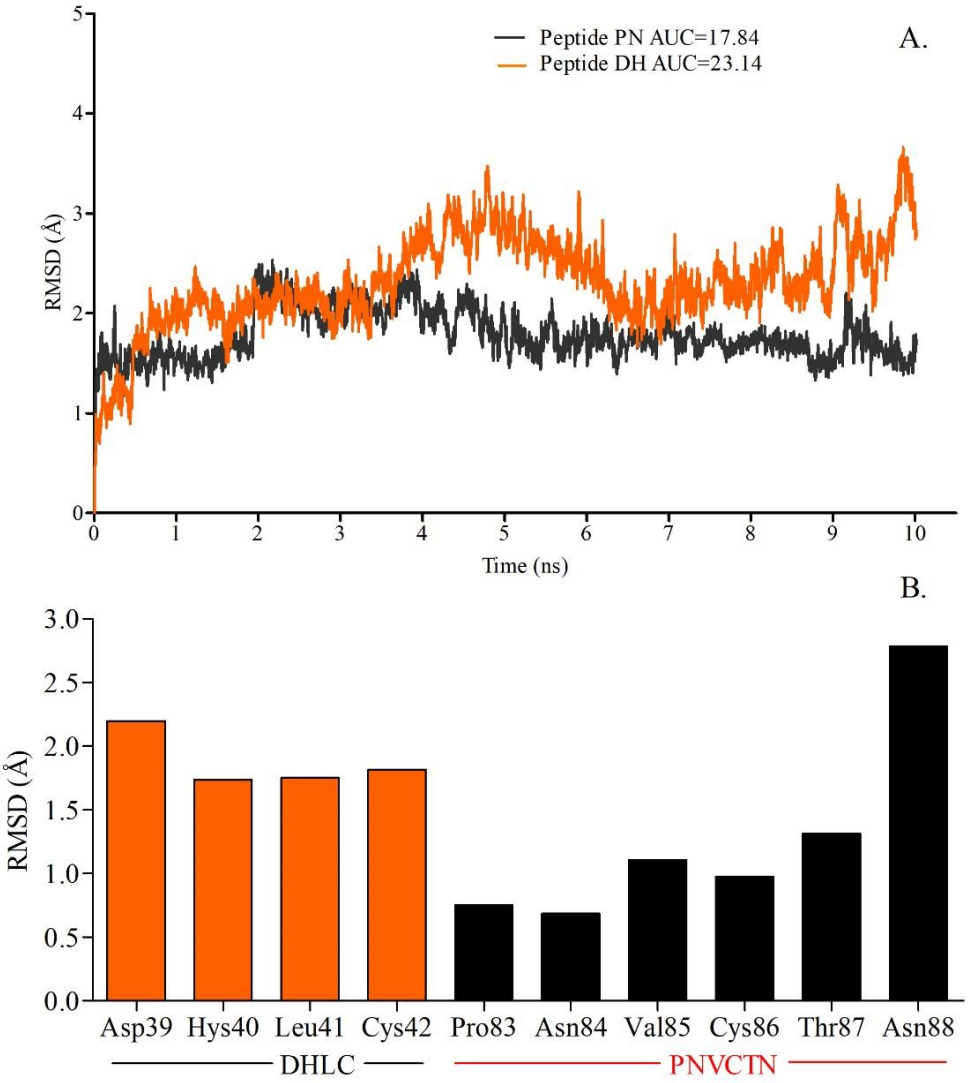

Figure 5. Analyzes from Dynamic simulation in water box, including root mean square deviation (RMSD) (A) and root mean square fluctuation (RMSF) (B) values of peptides inhibitor when associated with *S. cosmioides* trypsin. Orange line and bars represent D-H-L-C peptide data and black line and bars represent P-N-V-C-T-N peptide data.

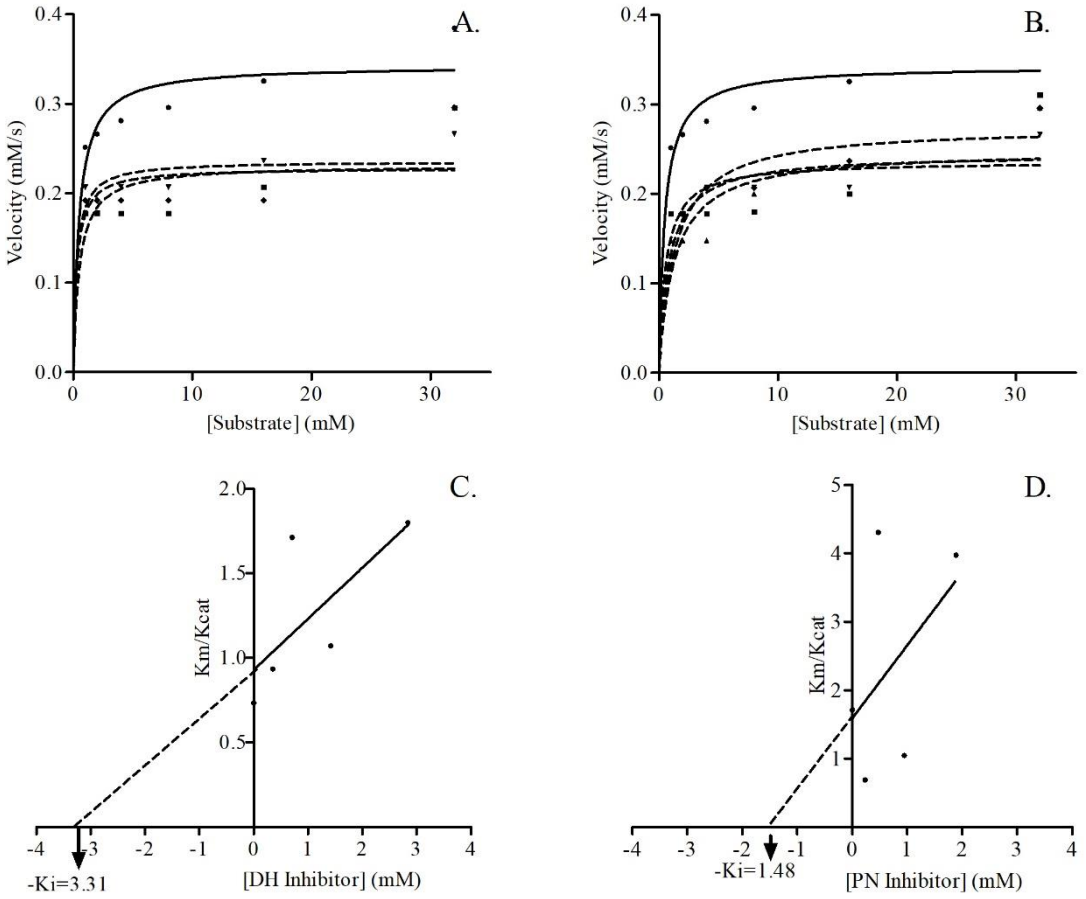

Figure 6. SNLR (Simultaneous nonlinear regression) method for analysis of inhibitory activity of the peptides D-H-L-C (A and C) and P-N-V-C-T-N (B and D), on the trypsins-*like* of *Spodoptera cosmioides* (Lepidoptera: Noctuidae).

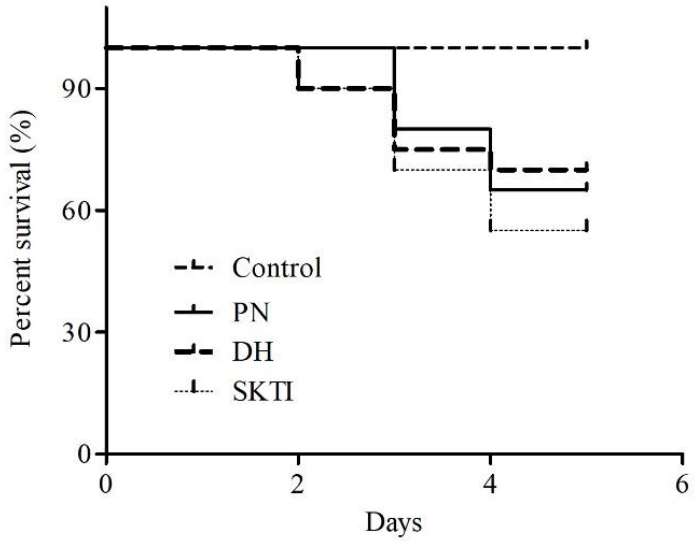

Figure 7. Survival plots of the *Spodoptera cosmioides* (Lepidoptera: Noctuidae) larvae reared on diet containing P-N-V-C-T-N peptide(PN), D-H-L-C peptide (DH) and SKTI (positive controle) inhibitors at concentration 2.0% w inhibitor/v diet.

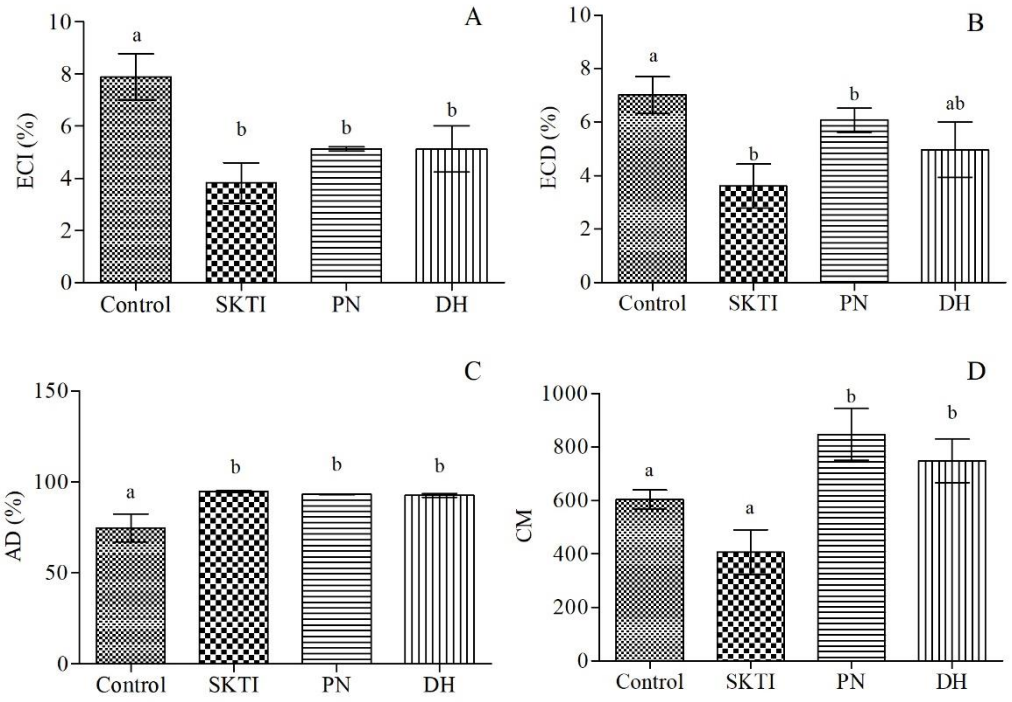

Figure 8. Nutritional indices of *Spodoptera cosmioides* larvae on 2.0% peptide- and SKTI(positive control)-treated and control diets (no inhibitor). A. Efficiency of conversion of ingested food (ECI); B. efficiency of conversion of digested food (ECD); C. approximate digestibility (AD) and D. Cost Metabolic (CM).

Capítulo III:

Inibidor de protease extraído de sementes da planta *Adenanthera pavonina*: efeitos sobre tripsinas digestivas de *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)

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Proteinase inhibitor from *Adenanthera pavonina* seeds: effects on the digestive trypsin in *Anticarsia gemmatalis*

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Abstract

The economic loss in soybean crops caused by the Lepidoptera insects has encouraged the search for several strategies to control this pest but It is mainly controlled using synthetic insecticides. Therefore in this paper was evaluated the ApTI (Adenanthera pavonina trypsin inhibitor) ability to inhibit trypisin-*like* proteins from *Anticarsia gemmatalis* by molecular docking, enzymatic and survival assay. The docking between trypsin and ApTI was performed using the program CLUSPRO; the inhibitory constant ki and the inhibition type was determined through chromogenic assays; in order to the survival analyses, was tested on neonatal larvae several concentrations of ApTI on the artificial diet. In this study was determined that the ApTI reactive site is able to block substrate access due to four interactions with a enzyme region, forming a complex with trypsin with a surface area of 1183.7 $Å^2$ and was characterized as uncompetitive tight-binding inhibitors by the kinetics analysis. The survival curves obtained using Kaplan-Meier estimators indicated that the highest mortality was 60% using 1.2% doses of ApTI inhibitor. The *in vitro* and *in silico* studies demonstrated that ApTI was a strong non-competitive inhibitor of trypsin and present biotechnological potential as agents against *A. gemmatalis* insect, inhibiting trypsins by tight binding inhibition.

Keywords: Binding, Kunitz inhibitor, insects, molecular docking, non-competitive, trypsin enzyme.

1. INTRODUCTION

In the course of evolution, plants have evolved adaptive mechanisms that provide them significant resistance against diverse kinds of unfavorable conditions including insects and phytopathogens (Krishnan & Murugan, 2015). Plant protease inhibitors (PIs) constitute one of the most important plant defensive traits against insect pests (War et al., 2018; Zhu-Salzman & Zeng, 2015). They are highly effective against lepidopteran, hemipteran and coleopteran insects (Tanpure et al., 2017; Gazara et al., 2017).

The chronic ingestion of protease inhibitors by insects leads to the inhibition of proteolytic digestion, consequently interfering with the bioavailability of essential amino acids required for insect growth, development and reproduction (Macedo et al., 2010), Previous workers (Xavier-Filho et al., 1976; Prabhu and Pattabiraman, 1980) have reported that seeds of *Adenanthera pavonina* contain very high levels of proteinase inhibitors which are 3-16-times more abundant in this species than in the inhibitor-rich seeds of soybean. The Adenanthera pavonina Trypsin Inhibitor (ApTI) used in this study was isolated from seeds of the Brazilian Carolina tree (*A. pavonina* L., Indian red sandalwood or bead tree), has two polypeptide chain conected by one disulfide bond with total molecular weight of 21 KDa (Richardson et al., 1986; Macedo et al., 2004; Migliolo et al., 2010).

On the other hand, the economic loss in soybean crops caused by the Lepidoptera insect *Anticarsia gemmatalis* Hübner (Lepidoptera: Eribidae) has encouraged the search for several strategies to control this pest but It is mainly controlled using synthetic insecticides (Krinski et al., 2018; Bueno et al., 2017). However, new approaches are needed to reduce risks to the environment and natural enemies and to avoid or delay the onset of insecticide resistance. The use of plant-based insecticides is an alternative for the control of lepidopteran pests primarily by having low toxicity and short persistence in the environment (Krinski et al., 2018; Gupta & Dikshit, 2010). In this context, ApTI may be a promising alternative for the control of *A. gemmatalis*, due to its inhibitory activity agaist serine proteases (trypsins and chymotrypsins) and cysteine proteases (papain) in insects; compromising the insect normal physiology and affecting its development due that the reactive site of the PIs interacts with the its target enzyme, forming a stable complex and consequently blocking the enzyme's activity (Bezerra et al., 2017; Jamal et al., 2015). When the trypsins activity is blocked decrease amino acid release and essential nutrients availability for normal growth insects.

In this paper was evaluated the ApTI (Adenanthera pavonina trypsin inhibitor) ability to inhibit trypisin-*like* proteins from *A. gemmatalis* by molecular docking, enzymatic and survival assay.

2. MATERIALS AND METHOD

2.1 *In silico* docking of ApTI inhibitor and trypsin

2.1.1 Sequence and structure retrieval

Trypsin partial sequence of *A. gemmatalis* was obtained of NCBI website with GenBank code: AWL83215.1; the sequence were first submitted to the Pfam database (http://pfam.sanger.ac.uk) (Finn et al., 2016) to confirm the domain architecture of the trypsins family.

Inhibitor sequence were obtained from the UniProt database (Uniprot, 2017), UniProt: P09941 and UniProt: P09942 for the α and β chain of the Adenanthera pavonina trypsin inhibitor (ApTI) (Macedo et al., 2004; Macedo et al., 2007).

The Phyre2 server was used to predict the three-dimensional (3-D) structures of the trypsin-*like* sequence of *A. gemmatalis* and the ApTI inhibitor protein (Kelley et al., 2015). This server predict the 3-D structure of a protein using the principles and techniques of homology modelling, secondary structure prediction and domain analysis (Kelley et al., 2015). The Protease of *A. gemmatalis* and the ApTI inhibitor were modelled with 100.0% confidence by the single highest scoring template.

After the construction of the models, its quality was assessed considering both geometric and energetic aspects using PROCHECK (Laskowski et al. 1993), ERRAT, PROSA and VERIFY 3D (Lutty et al. 1992) for internal consistency and reliability. The Ramachandran plot was used to provide the residue position in particular segment based on the dihedral angles.

2.1.2 Protein-protein Docking

A docking study was conducted to evaluate the predictive ability of the ApTI inhibitor of bind to the *A. gemmatalis* trypsin. In order to perform protein-protein docking between the models, were submitted the PDB files separately to online software ClusPro 2.0, (Kozakov et al., 2017; Kozakov et al., 2013). The software Pymol (http://pymol.source-forge.net/) is very flexible, extensible package for molecular visualization and was used to generate clear, informative and attractive representation of atomic data.

2.2 *In vitro* inhibitory activity assay

2.2.1 Trypsin enzyme extraction

2.2.1.1 Biological material

Anticarsia gemmatalis eggs were kept in the Insect Laboratory of Biochemistry and Molecular Biology Department in the UFV at 25 °C ± 2 °C and 70 ± 10% relative humidity. Pupae of this insect were placed in Petri dishes in cages (500 x 500 mm) coated internally with A4 bond paper. After emergence, adults of this insect were fed a nutrient solution with honey (10.5 g), beer (350 mL), sucrose (60 g), ascorbic acid (1.05 g), nipagin (1.05 g) and water (1050 mL) in cotton balls placed in Petri dishes (100 x 15 mm). The *A. gemmatalis* egg masses were laid, usually after three days on the paper surface that internally lined the cage. These were removed and cut into 2.5 cm wide x 10 cm long strips, placed in plastic cups (500 mL) with a circular hole with about 20 mm in a tulle fabric cover. These cages were transferred to a 25 °C chamber with relative humidity of 60 ± 10% and a 14 h photoperiod. Newly hatched larvae were fed on 15 x 15 x 15 mm artificial diet cubes every two days (Hoffman-Campo et al., 1985).

2.2.1.2 Preparation of gut extracts

Midguts of *A. gemmatalis* larvae in fifth instar were dissected and transferred to ice-cold (4 °C) buffer 1 mM HCl at a ratio of five intestines per 1 mL of buffer. Were used fifty midgusts by the extract. The maceration process was performed by liquid nitrogen. The extract was centrifuged at 10000 g for 30 min at 4 °C and the supernatant

stored at -20 °C. The protein content was determined with BSA by Bradford method (Bradford, 1976).

2.2.1.3 Chromatography and SDS-PAGE

The trypsin enzymes were purified by affinity chromatography on a HiTrap Benzamidine (high sub) 5ml (GE Healthcare) column equilibrated with buffer Tris-HCl 0.05 M, NaCl 0.5 M, pH 7.5. The extract of the *A. gemmatalis* midgut was used and the trypsin were eluted with glycine buffer 0.05 M, pH 3.0. The flow was 1 mL/min and the collected fractions volume was 1.5 mL. The eluted fractions were analyzed through trypsin-like activity with L-BApNA substrate and protein concentration determination.

The fractions corresponding to trypsin activity peak were pooled and stored at -20 °C for the enzyme kinetics assays. The procedure was performed in FPLC equipment.

Fractions from the affinity chromatography were submitted to electrophoresis unidimensional (Laemmli, 1970) with 12.5% polyacrylamide gel in the presence of SDS (0.1%). The gel was revealed using Commassie Blue solution.

2.2.2 Adenanthera pavonina Trypsin Inhibitor obtainment

The purified ApTI inhibitor were supplied by the research group Laboratorio de Purificação de proteínas e suas funções biologicas (LPPFB), Universidade Federal de Mato Grosso do Sul, Campo Grande, Brazil and the purification was perfomed and analyzed according to Macedo et al., 2007 and Macedo et al., 2004, with 98% of purity. This inhibitor was chosen by the ability of inhibition reported in previous studies (Meriño-Cabrera et al. 2019)

2.2.3 Enzyme inhibitory assays

The inhibitory activity was determined using L-BApNA chromogen substrate, trypsin-like enriched sample of *A. gemmatalis* gut and the ApTI inhibitor. The assays were performed in Tris-HCl 0,1 mol.L-1, $CaCl_2$ 20 mmol.L-1, pH 8,2 buffer. Stoichiometric proportions between inhibitor and enzyme activities was obtained by construction of a dose response curve. The concentrations employed for the *A.*

gemmatalis trypsins-*like* inhibition study were: L-BApNA substrate: 1.0 mM; 2.0 mM; 4.0 mM; 8.0 mM; 16.0 mM and 32.0 mM. Inhibitors were 2,38 µM/L; 4,76 µM/L; 7,14 µM/L; 9,52 µM/L and 11,9 µM/L using as control the reaction without inhibitor. The total concentrarion of enrriched trypsin-*like* was 1.5 µg/µl.

The molar extinction coefficient used was 8800 $M^{-1}.cm^{-1}$ by the p-nitroanilide product and the wavelength for the analyzes was 410 nm (Erlanger et al., 1961). All the assays were performed in triplicate.

The resulting velocities were then fitted to the Morrison equation. The initial velocity (V0), enzyme concentration [E] and inhibition constant (Ki) were estimated by non-linear regression (Morrison, 1969). GraphPad Prism 5.0 software was used to statistic analyzes (Motulsky, 2007).

2.3 ApTI inhibitor effect on survival of *Anticarsia gemmatalis*

The inhibitory effect of ApTI was analyzed on the neonatal larvae of *A. gemmatalis.* The larvae were fed an artificial diet (Bavaresco et al., 2004) containing the ApTI inhibitor at concentrations 0.7%, 1.0% and 1.2% w inhibitor/v diet. Was considred a treatment each inhibitor concentration in the artificial diet, so the experiment had three total treatments and one negative control (diet without inhibitor). 30 neonatal larvae were used in each treatment, using 120 total individuals for the experiment. Each larvae was considered a treatment repetition. All subjects were maintained at mean temperature of 26 °C and 65-70% relative humidity. During the period from neonates to pupae survival analyzes were performed every two days.

3. RESULTS

3.1 Sequence and structure retrieval

The first validation was carried out using Ramachandran plot analysis computed with PROCHECK by checking residue-by-residue stereochemical quality of the protein structure. The analysis showed that residues of *A. gemmatalis* trypsin in the most favorable region was 81.8%, in the allowed region was 15.9% and outlier region was 2.3%.

The interaction energy per residue was also calculated by the PROSA program. The PROSA Z-Score indicates overall model quality. Global analysis of the model trypsin with PROSA showed a Z-Score of −1.31, indicating no significant deviation from typical native structures of similar size as the template.

The final evaluation of the *A. gemmatalis* trypsin structure was checked by VERIFY 3D. The VERIFY 3D analysis indicated a reasonably good sequence-to-structure agreement because none of the amino acids had a negative score (average 3D score=0.2). It is to be noted that compatibility scores above zero correspond to acceptable side chain environment.

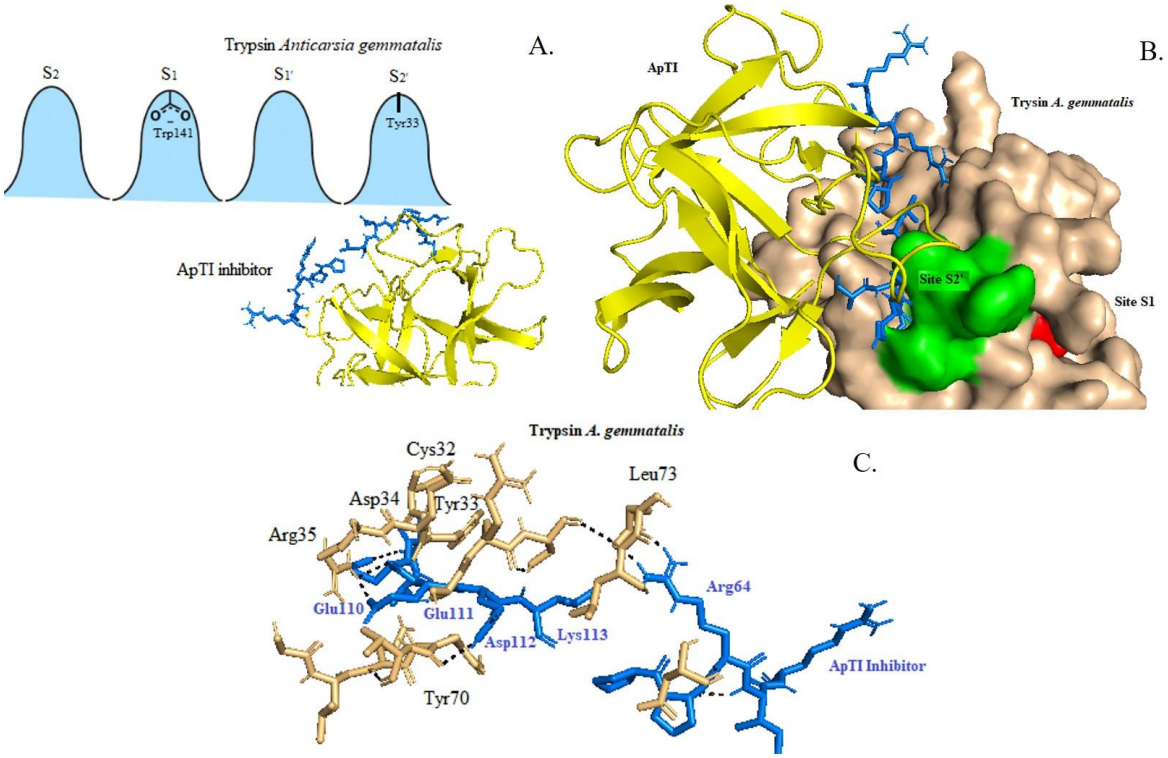

Figure 1 General and local overview of the best ranked docking pose of Adananthera pavonina trypsin inhibitor binding with digestive trypsin *Anticarsia gemmatalis*. Barrel shape representing the trypsins in beige, catalytic aminoacids in red, aminoacids of the S2´ site in green, the ApTI residues as blue cartoon involved in the trypsin-ApTI interaction and dotted lines black representing Hidrogens bonds.

3.2 Protein-protein docking

The complex between ApTI and trypsin structure was used for the study of the enzyme–inhibitor interaction (Fig. 1). The model of interaction showed a mechanism of inhibition of the non-competitive. The inhibitor reactive site is able to block substrate

access due to four interactions with a enzyme region, forming a complex with trypsin with a surface area of 1183.7 $Å^2$ (Fig. 1). Those contacts prevent substrate access to enzyme, although no direct reaction with the catalytic site (S1) was observed. Ligations were observed between the reactive site and the S2´ site of trypsin, which pocket is characterized by the presence of a tyrosine residue at the top (Fig 1A and 1B).

The inhibitor residues involved in the interaction were Arg64, Gln111, Glu109 and Glu110; The NH+ atom positive charge of Arg64 residue interacted with the OH− atoms of Ser27 and Glu71 residue in the trypsin receptor, forming a hydrogen bond. Others polar contacts were observed between Glu109 and the Tyr33 (residue receptor) and Glu110 and Arg35 (residue receptor) (Fig. 1C). All inhibitor residues participating in the interaction form part of his alpha chain.

Table 1. Trypsin-like purification from the soluble extract and gut purificated of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae).

Material	**Total protein (µg/ µl)**	**Total activity ($\mu M.s^{-1}$)**	**Specific activity ($\mu M.s^{-1}$/ µg)**	**Fold purification**	**Enzyme Yield (%)**
Extract	12.21	5.07	0.41	-	-
Purificated	2.67	4.33	1.62	4	117

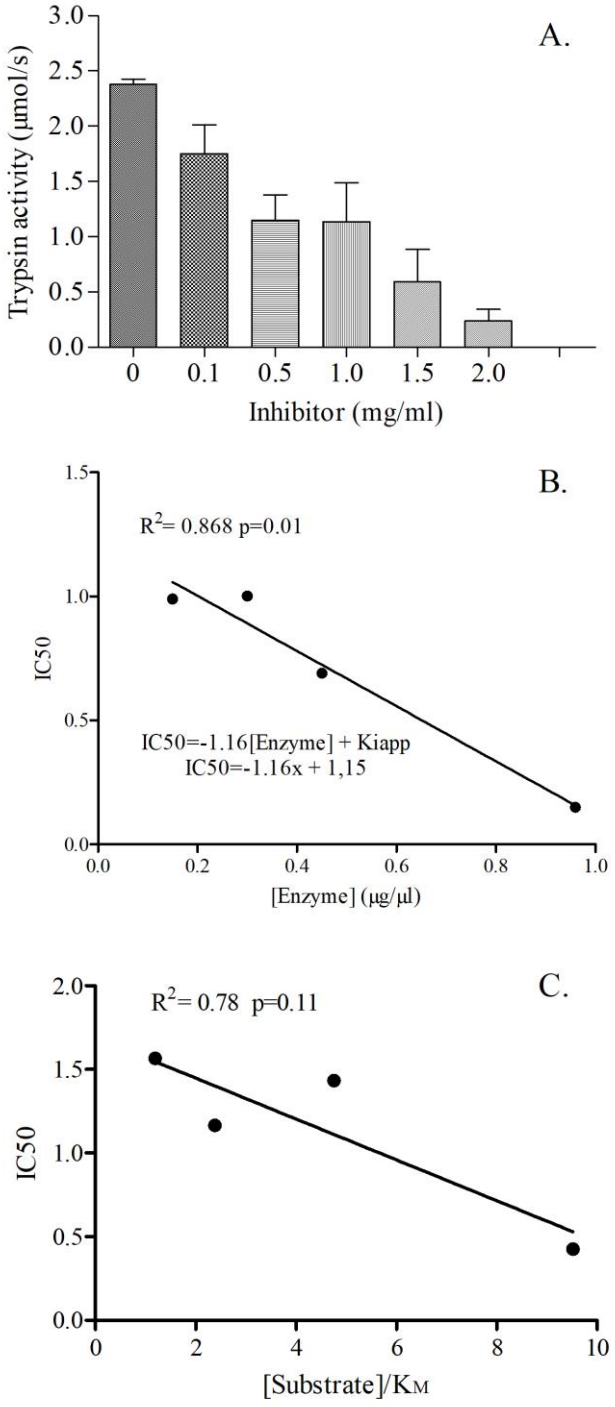

Figure 3 Effect of different concentrations of the inhibitor ApTi on trypsin enzymes of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) (A). Thigt binding inhibition plot (B) and non-competitive kinetic (C) of the Adananthera pavonina trypsin inhibitor (ApTI) on the trypsins-*like* of *A. gemmatalis*.

3.3 *In vitro* inhibitory activity assay

In order to analyze the possibility of ApTI inhibit the trypsin-*like* intestinal activity of *A. gemmatalis* was used enriched trypsin fractions and the purified inhibitor. In the chromatogram, the presence of two peaks with trypsinolytic activity, detected using L-BApNA as substrate, was observed (Fig. 2A). The first peak (fractions 5-15) is associated with non-specific proteolysis of the L-BApNA substrate by other enzymes with trypsin-*like* activity, present in the insect gut extract. The second peak (fractions 37-47) represents the trypsin-*like* serine proteases contained in the soluble extract having affinity to the column and with proteolytic capacity against the L-BApNA substrate (Fig. 2A); in this peak the highest activity was 4.39 μM/s. In addition, the protein profile (Abs 595 nm) was found to be coincident with the activity profile (Abs 410 nm) (Fig. 2A). In a single purification step using the HiTrap Benzamidine column, the specific trypsin activity increased from 0.41 to 1.62 μmol.s-1/μg of protein, getting a purification factor of 4 AND getting a yield prification of 117% (Table 1).

The enriched trypsin-*like* fractions obtained after affinity chromatography were evaluated by SDS-PAGE (Fig. 2B). Comparative analysis between crude intestinal extract and purified samples revealed, after staining by Comassie, several bands of molecular weight in trypsin-*like* proteins range between 20-25 KDa, 30-35KDa and a band with weight close to 60KDa (Fig. 2B) different of non-purified extract that have a range between of lower than 15 a 75 KDa related to total protein of crude extract (Fig. 2B).

The inhibitor ApTI was active against trypsins of *A. gemmatalis* in chromogenic assays (Fig. 3A). The type of inhibition found was tight-binding, verified from the linear variation between the IC50 value of ApTI ($R2 = 0.868$ $p = 0.01$) in function of the enzyme concentration [E] (Fig. 3B). The ApTI inhibitor presented uncompetitive tight-binding characteristics due to the absence of linear variation between the IC50 value in function of the substrate concentration (R^2=0.78, p=0.11) (Fig. 3C). The Ki value was 1.15.

3.3 ApTI inhibitor effect on survival of *Anticarsia gemmatalis*

The survival plots for *A. gemmatalis* larvae reared on diet containing ApTI inhibitor at different doses are shown in Fig. 4. The survival curves obtained using Kaplan-Meier estimators indicated significant differences in survival among larvae exposed to the different doses of ApTI (Log-Rank X^2 =17.24; df = 3; P= 0.0006) (Fig.

4). These differences were reflected in the median survival times (LT50's) observed for caterpillars at each dose of proteinase inhibitor, which exhibited linear increases with dose up. The highest mortality was 60% using 1.2% doses of ApTI inhibitor (Fig. 4).

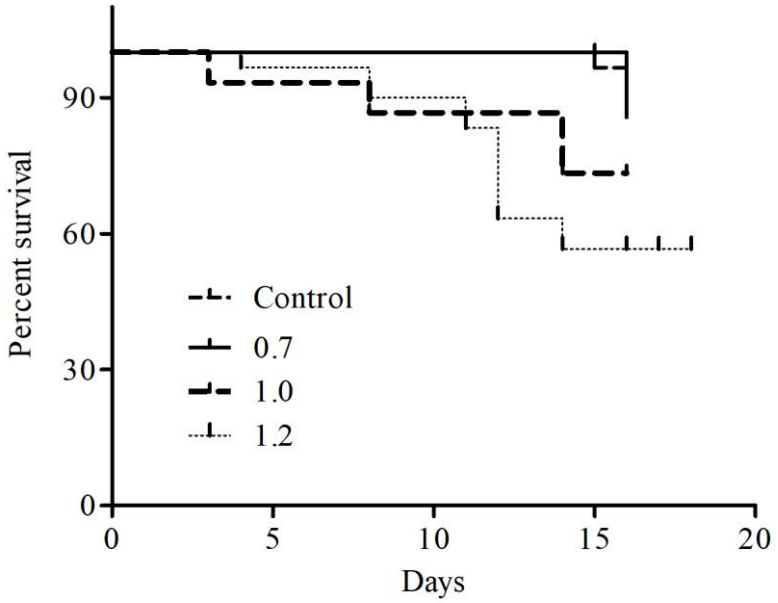

Figure 4 Survival plots of the *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae reared on diet containing ApTI inhibitor at concentrations 0.7%, 1.0% and 1.2% w inhibitor/v diet**.**

DISCUSSION

Protease inhibitors (PIs) are molecules that block the activity of proteases, and typically function on classes of proteases with similar mechanisms of action. Protease inhibitors can either be in the form of proteins, peptides, or small molecules (Rustgi et al. 2018). They can be found in all kingdoms of cellular life including viral genomes, thus exhibiting its wide distribution in nature. PIs are classified based on the protease they inhibit, which comprises of serine, cysteine, aspartic and metalloproteases. On the basis of conserved functional motifs they can be partitioned into many classes, being the Kunitz-type inhibitors the best characterized of them, probably due to their copiousness in several organisms (Rustgi et al. 2017).

The three-dimensional inhibitor model of ApTI analysed in this study present two polypeptide chains with twelve antiparallel –sheets connected for long loops forming a beta barrel. This structural fold is commonly found in beta family Kunitz-type inhibitors resolved by X-ray diffraction, such as *Glycine max*, *Drosera regia* and *Copaifera langsdorffii* (Vajravijayan et al. 2018; Migliolo et al. 2010). The two polypeptide chains (alpha and beta) linked by disulfide bonds, found in ApTI, are also observed in other Kunitz-type trypsin inhibitors found in the Mimosoideae subfamily (Vajravijayan et al. 2018; Migliolo et al. 2010).

The non-competitive mechanism found for the inhibition of ApTI on the trypsin-*like* enzymes of *A. gemmatalis* agrees with other *in silico* studies carried out with trypsins from other species of the order Lepidoptera (Ramalho et al. 2018; Migliolo et al. 2010) and the reactive (inhibitory) peptide bond was identified in the alpha chain with the participating of the Arg64 residue. This bond is exactly homologous position to the reactive sites identified in the soybean inhibitor at Arg-63-Ile-64 (McPherson et al. 2019) in *Albizzia* at Arg-66-I1e-67 (Sharma et al. 2012) and in *Psophocarpus* at Arg64-Ser-65 (Yamamoto et al. 1983).

The binding number observed between ApTI and *A. gemmatalis* trypsin is similar to the numbers of interactions have also been reported in previous works using crystallographic methods (Song and Suh 1998). When comparing these data with those from previous works it is possible to set the relevance of some amino acid residues for the right recognition and activity of Kunitz inhibitors toward serine proteases. The geometry of the carbonyl group at P1 residue, for example, has been described as being of great importance in comprising the interactions between inhibitor and protease during the catalytic mechanism (Sasaki et al. 2015; Sweet et al. 1974). In this context, has been experimentally described the relevance conserved arginine residue located at the positions P1 of the reactive site loop of a soybean Kunitz trypsin inhibitor (SKTI) in the formation of hydrogen bonds with a porcine trypsin. Interestingly, similar results were here obtained by in silico methods, where the NH1 and NH2 atoms of the Arg64, from ApTI was able to interact with the oxygen atoms of the Ser23 and Glu71 from trypsin by hydrogen bonds.

The proteins between 25-30 kDa finding in SDS-PAGE correspond to trypsin-like enzymes of the *A. gemmatalis* midgut with a similar molecular weight reported for most insects (Muhlia-Almazán et al. 2008), such as 27 and 24 kDa for *Sesamia nonagroides* Lefebvre (Lepidoptera: Noctuidae) (Lopes et al. 2006); and 26 and 29 kDa for

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) . The 60 kDa protein is similar to the 67 and 70 kDa of *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae) (Brito et al. 2001). The presence of Trypsin 60 kDa in *A. gemmatalis* midgut may be explained by insect proteases associated with the peritrophic matrix (Telleria et al. 2010), causing the anchoring proteases and other digestive enzymes to reduce enzyme loss and greatly improve digestive efficiency (Hegedus et al. 2015). This enzymes of high molecular weight found in the *A. gemmatalis* larval gut and/or peritrophic matrix can be lack one or more of the critical serine, histidine, and aspartic acid residues required for catalytic activity and are likely to be inactive, like reported in others studies with Lepidoptera. The abundance of non-catalytic forms suggests that they contribute something to insect digestive biochemistry, possibly sequestering protease inhibitors in the diet (Hegedus et al. 2015).

ApTI was characterized as an inhibitor tight binding non-competitive in the concentration range of inhibitors and substrates analyzed. These results guide and support the *in silico* analysis performed. This tight binding mechanism mean that exist a variation of the IC50 value observed for ApTI inhibitor with total enzyme concentration at a fixed substrate concentration, like was observed in the results and It is the more defining feature of tight binding inhibitors (TBI). This is true because a TBI interacts with the enzyme in nearly stoichiometric fashion. Hence, the higher the concentration of enzyme present, the higher the concentration of inhibitor required to reach half-maximal saturation of the inhibitor binding sites (Copeland 2013).

Many herbivorous lepidopterous pests obtain essential amino acids by using trypsin-like proteases secreted into the midgut lumen to digest food proteins (Zhu-Salzman & Zeng 2015). For this reason, the effect of ApTI inhibitor was tested on survival of *A. gemmatalis*, finding that this PI work as an important part of the plant defense system by inhibiting digestive proteases in *A. gemmatalis* midgut, leading to inadequate digestion and absorption of essential amino acids, evidenced by the arrested growth and eventually death by starvation (Kaur et al. 2017).

However, the mortality of the larvae was not 100%, this can be explained because the pests can produce serine proteases and/or alter expression of alternative gut proteases to circumvent the negative effects of PIs produced by those host plants during evolutionary process (Pilon et al. 2017; Meriño-Cabrera et al. 2018).

In this study, differences between doses of the inhibitor and an approximate mortality of 60% were found, making ApTI a candidate with a better inhibitory effect compared to

other inhibitors such as Benzamidine, since in other studies it was found that mortality of *A. gemmatalis* larvae was affected by the presence of benzamidine sprayed on the plant; however, we found no difference between the doses and any of the doses of benzamidine caused approximately 50 % of larval mortality (Pilon et al. 2018).

Concluding, the *in vitro, in vivo* and *in silico* studies demonstrated that ApTI was a strong non-competitive inhibitor of trypsin and present biotechnological potential as agents against *A. gemmatalis* insect, inhibiting trypsins by tight binding inhibition.

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CONCLUSÕES GERAIS

- Os inibidores *Inga laurina trypsin inhibitor* (ILTI) e *Adanhantera pavonina trypsin inhibitor* (ApTI) apresentam alto potencial biotecnológico como agentes contra insetos fitófagos Lepidoptera, inibindo tripsinas por inibição *tight-binding*, com características competitivas e não competitivas, respectivamente.
- A ação de ApTI e ILTI no desenvolvimento de larvas de *S. cosmioides* e *A. gemmatalis* mostra que estes inibidores influenciam a sobrevivência larval, indicando seu potencial tóxico
- A predição das interações resíduo-resíduo através da interface entre o inibidor ILTI e tripsinas de *S. cosmioides* mostrou que pequenas sequências lineares de aminoácidos são conservadas em manter o contato e estabilizar o complexo do inibidor com a estrutura tridimensional das tripsinas analisadas.
- As análises de docking molecular e contribuição energética, RMSD e RMSF realizadas a partir dos resultados de dinâmica molecular permitiram o desenho racional de dois peptídeos a partir da interface ILTI-tripsina.
- Os peptídeos derivados do sitio reativo do inibidor ILTI apresentam afinidade pelas enzimas tripsinas de *S. cosmioides* e reduzem sua atividade biológica, confirmando o potencial inibitório destes compostos.
- A exposição das larvas de *S. cosmiodes* aos peptídeos sintéticos na dieta reduz a viabilidade e sobrevivência destes insetos, indicando o potencial tóxico e biopesticida dos peptídeos.

ANEXO 1

Peptídeos sintetizados a partir de proteínas de plantas com ação bioinseticida para controle de insetos praga da ordem Lepidoptera

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Campo Técnico da invenção

1. Os peptídeos sintetizados são compostos por vários resíduos de aminoácidos e são produto dos estudos de minimização molecular de uma proteína da planta *Inga laurina* com capacidade inibitória de tripsinas do intestino de insetos pragas, ou seja, são molécula orgânicas que podem ter a mesma eficácia que produtos químicos inseticidas mas reduzindo o impacto negativo no ambiente, com aplicação no setor agrícola.

Estado da Técnica

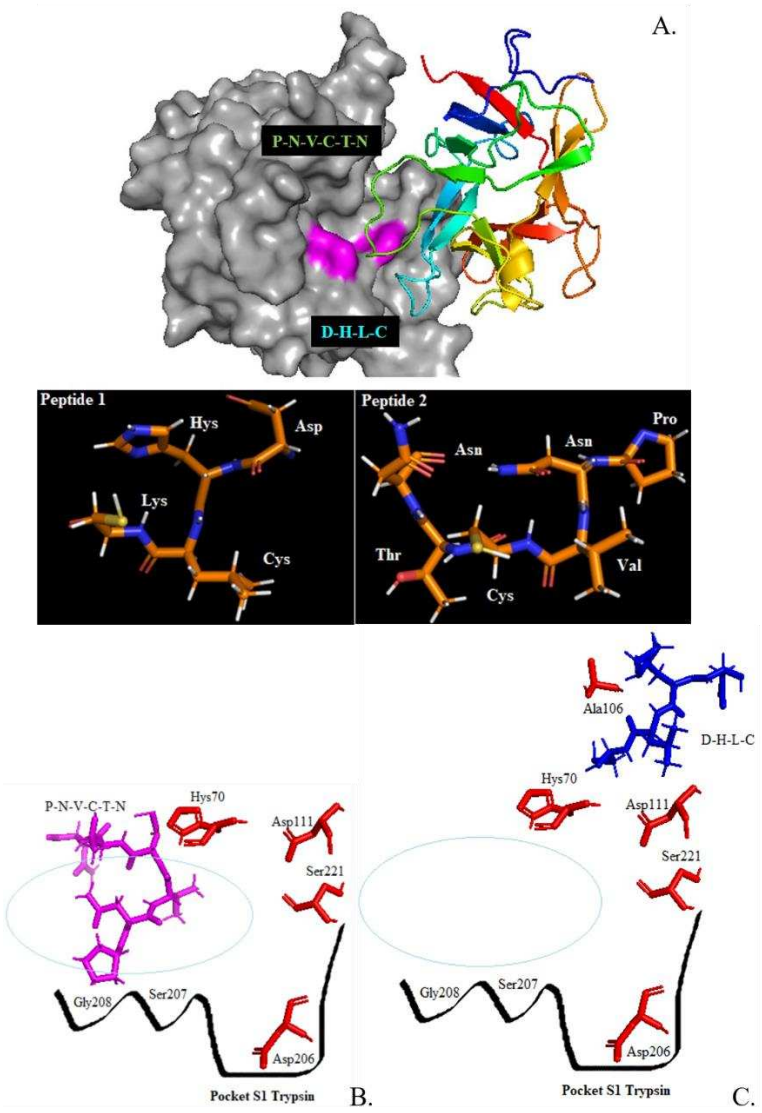
2. No curso da evolução, as plantas desenvolveram mecanismos adaptativos que lhes proporcionam uma resistência significativa contra diversos tipos de condições desfavoráveis, incluindo insetos e fito-patógenos. Os inibidores de proteases de plantas (PIs) constituem uma das mais importantes características defensivas de plantas contra pragas de insetos. Eles são altamente eficazes contra insetos lepidópteros, hemípteros e coleópteros (Tanpure, R. S., Barbole, R. S., Dawkar, V. V., Waichal, Y. A., Joshi, R. S., Giri, A. P., & Gupta, V. S. (2017). Improved tolerance against Helicoverpa armigera in transgenic tomato over-expressing multi-domain proteinase inhibitor gene from Capsicum annuum. Physiology and molecular biology of plants, 23(3), 597-604).

3. A ingestão crônica de inibidores de proteases por insetos leva à inibição da digestão proteolítica, consequentemente interferindo na biodisponibilidade de aminoácidos essenciais necessários para o crescimento, desenvolvimento e reprodução de insetos-pragas. Pesquisas relatam que sementes de diversas plantas arboreas contêm níveis muito altos de inibidores de proteinase que são 3-16 vezes mais abundantes nestas espécie do que em plantas menores (Gazara, R. K., Cardoso, C., Bellieny-Rabelo, D., Ferreira, C., Terra, W. R., & Venancio, T. M. (2017). De novo transcriptome sequencing and comparative analysis of midgut tissues of four non-model insects pertaining to Hemiptera, Coleoptera, Diptera and Lepidoptera. Gene, 627, 85-93).

4. Por outro lado, a perda econômica em lavouras de soja, café, tomate, milho, algodão entre outras, causada por insetos tais como: *Anticarsia gemmatalis, Leucoptera coffeella*, *Spodoptera frugiperda* e *Spodoptera cosmioides* tem incentivado a busca de diversas estratégias para o controle destas pragas (Krinski, D., Foerster, L. A., & Deschamps, C. (2018). Ovicidal effect of the essential oils from 18 Brazilian Piper species: controlling Anticarsia gemmatalis (Lepidoptera, Erebidae) at the initial stage of development. Acta Scientiarum. Agronomy, 40; Bueno et al. 2017; Bueno, A. D. F., Carvalho, G. A., Santos, A. C. D., Sosa-Gómez, D. R., & Silva, D. M. D. (2017). Pesticide selectivity to natural enemies: challenges and constraints for research and field recommendation. Ciência Rural, 47(6)).

5. O inibidor da Inga Laurina Trypsin (ILTI) é uma dessas proteínas inibidoras promissoras para o controle de pragas. É um inibidor de serino-protease composto por uma única cadeia contendo 180 resíduos de aminoacidos, com massa molecular de aproximadamente 20 kDa e possui alta estabilidade devido à presença de duas ligações dissulfeto. Esta proteína foi isolada da planta *Inga laurina* FE, Unicamp, Campinas, SP- Brasil. (Fabaceae) da subfamília Mimosoidae (Macedo M.L.R., Machado M.F., Franco O.L., Migliolo L., Ramalho C.F.O. (2011) Practical and theoretical characterization of Inga laurina Kunitz inhibitor on the control Homalinotus coriaceus. Comparative Biochemistry and Physiology - Part B, 158, 164-172; Machado S., de Oliveira C., Zério N., Parra J., Macedo M. (2017) Inga laurina trypsin inhibitor (ILTI) obstructs Spodoptera frugiperda trypsins expressed during adaptive mechanisms against plant protease inhibitors. Archives of Insect Biochemistry and Physiology, 95, 21393).

6. No entanto, sua aplicação agrícola *ex vivo* é limitada devido ao grande tamanho molecular e à instabilidade ambiental, que poderiam ser superados por pequenos peptídeos. Assim, neste estudo foram construidos dois peptideos (D-H-L-C e P-N-V-C-T-N) a partir do sitio reativo do inhibidor Inga Laurina Trypsin inhibitor (ILTI) (**Ilustração parte A**) com capacidade inibitoria sobre tripsinas intestinais de *Spodoptera cosmioides* e outros insetos lepidoteros. A ligação *in vitro* foi testada mediante docking e dinâmica molecular com tripsinas-*like*; ensaios *in vitro* foram realizados testando a atividade inhibitoria sobre extratos de tripsinas purificadas do intestino de *S. cosmioides* e os efeitos *in vivo* foram analisados atraves da aplicação dos peptideos sobre a dieta dos insetos *S. cosmioides*.



7. A justificativa desta invenção está baseada no fato de que, atualmente, o uso de produtos químicos sintéticos para o controle de pragas agrícolas suscita várias preocupações relacionadas ao ambiente e à saúde humana e animal. Uma alternativa é a utilização de produtos naturais eficientes e ecologicamente corretos. Devido aos grandes males causados pela utilização em larga escala de agrotóxicos e inseticidas sintéticos, existe a necessidade de desenvolver novas tecnologias a fim de minimizar esta utilização. Além disso, há crescente interesse por produtos livres de agrotóxicos, bem como a conscientização de produtores e consumidores levando-os a atitudes ecologicamente corretas. Todos estes aspectos impulsionam a busca por produtos alternativos que não agridam o ambiente como é o caso dos peptídeos.

8. Os peptídeos sintetizados D-H-L-C e P-N-V-C-T-N devido a sua estabilidade estrutural, capacidade inibitória e efeitos tóxicos analisados a partir do estudo sobre tripsinas de *S. cosmioides*, são candidatos para seu uso como bio-pesticidas e além disso, alvos de otimização para se ligar com maior afinidade aos seus receptores (tripsinas de insetos-pragas), maior biodisponibilidade e resistência à proteólise no ambiente.

9. Esta nova tecnologia pode ser comparada com outros peptídeos sintetizados (Bakail, M., Gaubert, A., Andreani, J., Moal, G., Pinna, G., Boyarchuk, E., ... & Guichard, B. (2019). Design on a Rational Basis of High-Affinity Peptides Inhibiting the Histone Chaperone ASF1. Cell chemical biology; Rismani, E., Rahimi, H., Arab, S. S., Azadmanesh, K., Karimipoor,

M., & Teimoori-Toolabi, L. (2018). Computationally design of inhibitory peptides against Wnt signaling pathway: in silico insight on complex of DKK1 and LRP6. International Journal of Peptide Research and Therapeutics, 24(1), 49-60; Zhang, W., Zhang, C., Luo, C., Zhan, Y., & Zhong, B. (2019). Design, cyclization, and optimization of MMP13–TIMP1 interaction-derived self-inhibitory peptides against chondrocyte senescence in osteoarthritis. International journal of biological macromolecules, 121, 921-929), mas nossos peptídeos diferem em vários aspectos a os outros achados na literatura, primeiro o alvo de interesse, em vários estudos de desenho racional de peptideos o objetivo final é o tratamento de várias doenças humanas, em este projeto o foco de inibição são tripsinas de insetos pragas, objetivando que os peptídeos desenhados inibam estas enzimas e diminuam o desenvolvimento ou causem a morte de pragas agrícolas; segundo, a composição e estrutura, na literatura ainda não existem peptídeos com a composição de aminoácidos e ordem de posicionamento dos resíduos nos fragmentos peptídicos desenhados, assim sendo outro aspecto inovador desta invenção.

Descrição detalhada da invenção

10. Os peptídeos sintetizados foram identificados de duas alças da proteína ILTI que formaram interações importantes durante as análises de docking e dinâmica molecular com tripsinas de *S. cosmioides*. Os peptideos estão formados pelos resíduos de aminoácidos, Aspartado (D), Histidina (H), Leucina (L) e Cisteina (C) para o D-H-L-C, e por Prolina (P), Asparagina (N), Valina (V), Cisteina (C), Treonina (T) e Asparagina (N) formando o P-N-V-C-T-N. São fragmentos lineares com estabilidade estrutural e boa afinidade pelas tripsinas desta praga como demonstrado nas simulações computacionais a partir do cálculo da energia livre de ligação (-6.93 Kcal/mol e -8.90 Kcal/mol para o peptídeo DH e PN, respectivamente). São estabilizados no sitio ativo das tripsinas de insetos mediante ligações de hidrogênio principalmente (**Ilustração parte B e C**); foram sintetizados quimicamente pela empresa GenOne, e foi testado o potencial inibitório, encontrando constante de inibição de 3.31 e 1.48 mM para o peptídeo DH e PN, respectivamente (**ANEXO-Figura 1**) e efeito toxico dos peptídeos diminuindo a sobrevivência das lagartas da praga *S. cosmioides* (**ANEXO-Figura 2**).

Conclusão

11. Os peptídeos indicados para sínteses e objeto da invenção, são uma alternativa de utilização de produtos naturais eficientes e ecologicamente corretos, dentro do processo de defesa de plantas, e alvos biotecnológico para o controle de insetos-praga e com potencial para a construção de plantas transgênicas ou produto bio-pesticida.

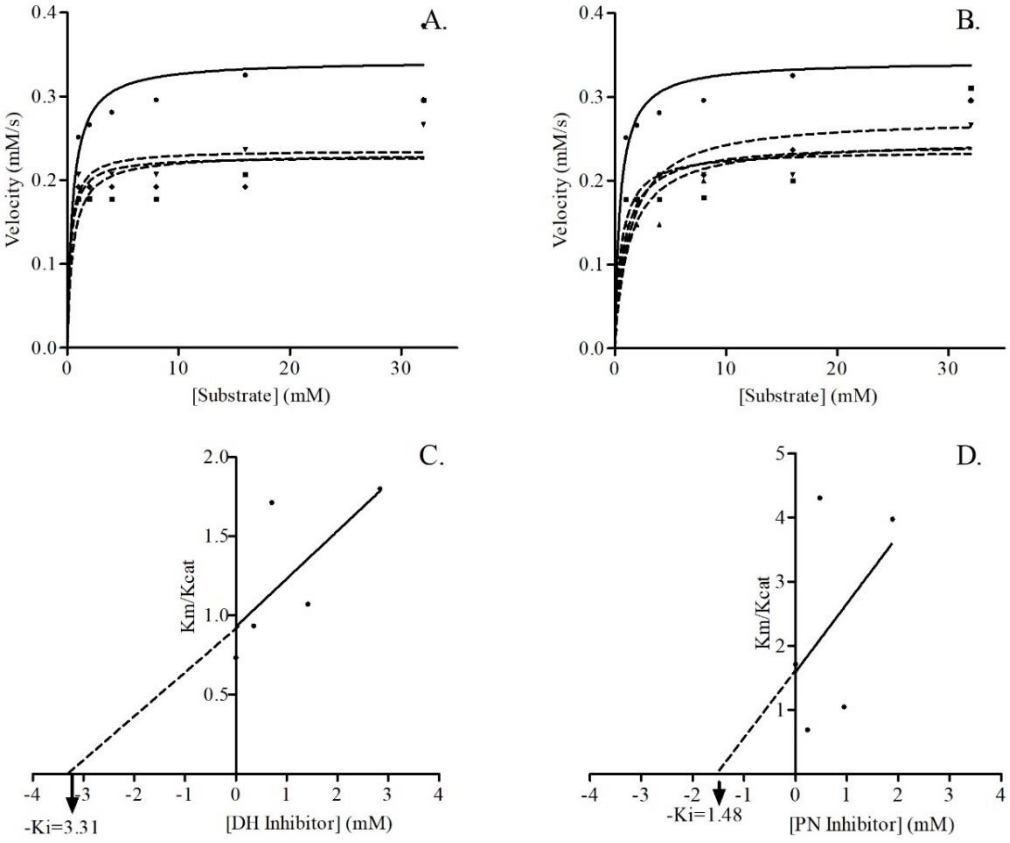


Figura 1. Método SNLR (regressão não linear simultânea) para análise da atividade inibitória dos peptídeos D-H-L-C (A e C) e P-N-V-C-T-N (B e D), nas tripsinas da praga *Spodoptera cosmioides* (Lepidoptera: Noctuidae).

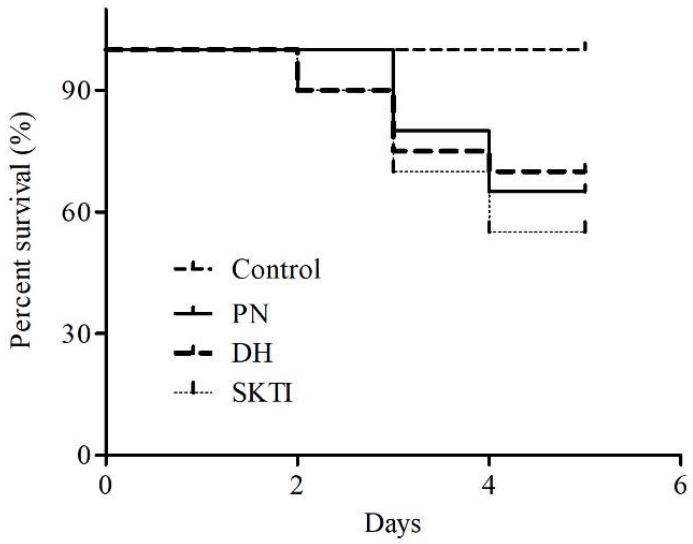


Figure 2. Análises de sobrevivência das larvas de *Spodoptera cosmioides* (Lepidoptera: Noctuidae) criadas em dieta contendo peptídeo P-N-V-C-T-N (PN), peptídeo D-H-L-C (DH) e inibidor SKTI (controle positivo) na concentração 2,0% w inibidor/v dieta.