UNIVERSIDADE FEDERAL DO CEARÁ
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

WALLACE TEIXEIRA DA CRUZ

ENZYMATIC AND STRUCTURAL STUDIES OF PERUVIANIN-I: THE FIRST GERMIN-LIKE PROTEIN WITH PROTEOLYTIC ACTIVITY

FORTALEZA

2019

WALLACE TEIXEIRA DA CRUZ

ENZYMATIC AND STRUCTURAL STUDIES OF PERUVIANIN-I: THE FIRST GERMIN-LIKE PROTEIN WITH PROTEOLYTIC ACTIVITY

Tese apresentada ao programa de Pós graduação em Bioquímica da Universidade Federal do Ceará, como parte dos requisitos para obtenção de título de Doutor em Bioquímica. Áres de concentração: Bioquímica vegetal

Orientador: Prof. Dr. Cleverson Diniz Teixeira de Freitas.

FORTALEZA

2019

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Sistema de Bibliotecas
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

C965e Cruz, Wallace Teixeira da.
Enzymatic and structural studies of peruvianin-I: the first germin-like protein with proteolytic activity / Wallace Teixeira da Cruz. – 2019.
116 f. : il. color.

Tese (doutorado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica , Fortaleza, 2019.
Orientação: Prof. Dr. Cleverson Diniz Teixeira de Freitas.

1. Laticifer. 2. Germin-like proteins. 3. Crystal structure. I. Título.

CDD 572

WALLACE TEIXEIRA DA CRUZ

ENZYMATIC AND STRUCTURAL STUDIES OF PERUVIANIN-I: THE FIRST GERMIN-LIKE PROTEIN WITH PROTEOLYTIC ACTIVITY

Tese apresentada ao programa de Pós graduação em Bioquímica da Universidade Federal do Ceará, como parte dos requisitos para obtenção de título de Doutor em Bioquímica.

Aprovada em ___/___/_____

BANCA EXAMINADORA

_____

Prof. Dr. Cleverson Diniz Teixeira de Freitas (Orientador)

Universidade Federal do Ceará (UFC)

_____

Prof. Dr. Márcio Viana Ramos

Universidade Federal do Ceará (UFC)

_____

Prof. Dr. Bruno Anderson Matias da Rocha

Universidade Federal do Ceará (UFC)

_____

Prof. Dr. Bruno Lopes de Sousa

Universidade Estadual do Ceará (UECE)

_____

Dr. Felipe Domingos de Sousa

Universidade Federal do Ceará (UFC)

À minha família...

AGRADECIMENTOS

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro com a manutenção da bolsa de auxílio.

Ao Prof. Dr. Cleverson Diniz Teixeira da Freitas, por representar de forma muito digna um profissional muito além do papel de orientador, demonstrando compreensão e paciência durante todos os 6 anos de trabalhos compartilhados. A sua incesante vontade de enriquecer o trabalho foi um dos fatores primordiais por todas as nossas conquistas. Mesmo com algumas pendências experimentais, conseguimos alcançar os principais objetivos do nosso estudo. Enfim, foi muito gratificante compartilhar todos estes momentos com você ao longo destes anos.

Ao Prof. Dr. Márcio Viana Ramos, pelo excelente acolhimento em seu laboratório. Por representar a figura de um grande pesquisador, íntegro, sincero e impressionantemente talentoso na arte da escrita científica. Tenho certeza que me apropriarei de todos os seus ensinamentos para os decorrentes anos da minha vida.

Ao Professor Dr. Bruno e o Dr. Eduardo Salviano, pela disponibilidade e contribuição em todas as etapas de análises estruturais do presente trabalho.

A todos os amigos do laboratório de plantas laticíferas: Zelândia, por contribuir em grande parte dos resultados; Isabel, Daniel Brito, Deborah, Camila, João Pedro, Bia, Ayrles, Larissa. De forma direta ou indireta vocês contribuíram para a construção deste trabalho.

Aos meus queridos amigos, Francimauro, Sandro, Raphael Coelho, Eilton, Júnior, Antonio Neto, Antônio Edson e Ana Luiza. Por todos os maravilhosos momentos que vocês me proporcionaram, as palhaçadas, as cervejas e cafés compatilhados, que sem dúvida enriqueceram mais ainda o nosso pensamento científico. Enfim, sou muito grato pelo companheirismo de vocês..

À minha família: meus pais, pelos sagrados ensinamentos; pelo amor incondicional e demonstração valiosa de confiança e liberdade nas minhas escolhas; meu irmão, Wilami Teixeira, pelo apoio, rigidez, colaboração e acima de tudo representatividade no que concerne todas as conquistas profissionais que almejo.

RESUMO

A proteína semelhante à germina (GLP) purificada de *Thevetia peruviana*, Peruvianina-I, é a única descrita como possuindo atividade proteolítica. Portanto, o objetivo deste estudo foi investigar as características estruturais responsáveis pela sua atividade enzimática. Embora a sequência de aminoácidos da Peruvianina-I apresentasse alta identidade com outras GLPs, ela exibiu mutações pontuais, responsáveis pela ausência da atividade de oxalato oxidase. A análise filogenética mostrou que a Peruvianina-I não pertence a nenhuma classificação de subfamílias GLP. Além disso, a Peruvianina-I contém uma tríade catalítica encontrada em todas as peptidases cisteínicas vegetais. Simulações de acoplamento molecular confirmaram o papel da tríade catalítica na sua atividade proteolítica. Ensaios de dicroísmo circular de radiação síncrotron confirmaram que a Peruvianina-I era estável em pH variando de 5,0 a 8,0 e que apresentava alterações estruturais significativas apenas acima de 60 °C. A adição de iodoacetamida causou alterações na sua conformação nativa, mas apenas um leve efeito foi observado após a adição de um agente redutor. A estrutura cristalina de raios X da Peruvianina-I foi resolvida para uma resolução nominal de 2,15 Å. A estrutura geral da peruvianina-I mostra um arranjo composto por monômeros presos em um homohexâmero (um trímero de dímeros). Os monômeros de peruvianina-I adotam a dobra típica do barril β das proteínas semelhantes a Germina (GLPs). Ao contrário das Germins e GLPs, o Peruviana-I não apresenta íons dentro dos monômeros. Nossos resultados mostraram através do mapa de densidade eletrônica a presença de dois sítios de ligação de glicanos localizados em ASN55 e ASN144. Este estudo relata uma proteína incomum com estrutura semelhante a um germe, sem atividade típica de oxalato oxidase. Em vez disso, a atividade proteolítica observada sugere que a proteína é uma peptidase cisteínica. Detalhes do sítio ativo foram usados para apresentar e discutir um mecanismo de ação plausível para a Peruvianina-I. Essas peculiaridades estruturais fazem da Peruvianina I um modelo interessante para maior compreensão da ação dos fluidos laticíferos na defesa das plantas.

Palavras-chave: laticíferos; proteína semelhante à germina; estrutura cristalina.

ABSTRACT

The germin-like protein (GLP) purified from *Thevetia peruviana*, Peruvianin-I, is the only one described as having proteolytic activity. Therefore, the goal of this study was to investigate the structural features responsible for its enzymatic activity. Although the amino acid sequence of Peruvianin-I showed high identity with other GLPs, it exhibited punctual mutations, which were responsible for the absence of oxalate oxidase activity. The phylogenetic analysis showed that Peruvianin-I does not belong to any classification of GLP subfamilies. Moreover, Peruvianin-I contains a catalytic triad found in all plant cysteine peptidases. Molecular docking simulations confirmed the role of the catalytic triad in its proteolytic activity. Synchrotron radiation circular dichroism assays confirmed that Peruvianin-I was stable at pH ranging from 5.0 to 8.0 and that it presented significant structural changes only above 60 °C. The addition of iodoacetamide caused changes in its native conformation, but only a slight effect was observed after adding a reducing agent. The x-ray crystal structure of Peruvianin-I was solved to a nominal resolution of 2.15 Å. The overall structure of peruvianin-I shows an arrangement composed by monomers locked into a homohexamer (a trimer of dimers). The peruvianin-I monomers adopts the typical β-barrel fold of the Germin-like proteins (GLPs). In contrast to Germins and GLPs, Peruvian-I does not present ions inside the monomers. Our results showed through of electron density map the presence of two glycan-binding sites located at ASN55 and ASN144. This study reports an unusual protein with germin-like structure, lacking typical oxalate oxidase activity. Instead, the proteolytic activity observed suggests that the protein is a cysteine peptidase. Details of the active site were used to present and discuss a plausible mechanism of action for Peruvianin-I. These structural peculiarities make Peruvianin-I an interesting model for further understanding of the action of laticifer fluids in plant defense.

Keywords: laticifer; germin-like protein; crystal structure.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

SUMÁRIO

1 INTRODUÇÃO GERAL

Látex é um líquido de aspecto leitoso sintetizado e acumulado sob pressão em canais especializados denominados de laticíferos, que podem ser compostos por várias células ou por uma única célula que se alonga entre os tecidos. Cerca de 10% de todas as angiospermas produzem látex, que é liberado mediante alguma injúria causada por insetos ou patógenos. Os laticíferos estão amplamente disseminados no reino vegetal, achando-se presentes em mais de 20.000 espécies, distribuídas em mais de 40 famílias (AGRAWAL; KONNO, 2009; KEKWICK, 2001; KONNO, 2011).

Diversas partes da planta podem conter látex, contudo, ele é mais abundante nos tecidos verdes, como as folhas, frutos e caule (AGRAWAL; KONNO, 2009). O látex, na maioria das vezes, apresenta-se esbranquiçado, como em *Calotropis procera* e *Thevetia peruviana,* no entanto, este pode exibir uma coloração amarelada ou alaranjada entre plantas pertencentes à família Papaveraceae; esverdeado ou ciano na espécie *Sebertia acuminata;* avermelhado em *Cannabis* spp. ou pode ser límpido como em *Nerium oleander*. (BANDARA *et al*., 2010; CALLAHAN *et al*., 2008; KEKWICK, 2001; KONNO, 2011).

O látex tem sido observado, descrito e usado por seres humanos há milhares de anos. Dentre as inúmeras espécies laticíferas, a *Hevea brasiliensis* (seringueira) tem o látex mais estudado. Isto se deve, principalmente, a sua importância econômica, destacando-se a produção de borracha e seus derivados. A produção global de borracha natural alcançou em 2011 mais de 10 milhões de toneladas por ano, sendo a Tailândia e a Indonésia os principais produtores (HO, 2014). Também vale salientar a importância científica de espécies como *C. procera*, *Cryptostegia grandiflora e Plumeria rubra,* no que concerne o potencial biotecnológico de moléculas oriundas de seus látices (FREITAS *et al*., 2011; LIMA *et al*., 2012; RAMOS *et al*., 2014).

2 REVISÃO DE LITERATURA

Látex: Aspectos Químicos

A composição química de fluidos laticíferos é bastante complexa, incluindo uma grande variedade de metabólitos secundários (alcalóides, glicosídeos cardíacos, terpenóides, canabinóides, taninos) e de proteínas, assim como substâncias similares a açúcares e muitos outros componentes (Konno, 2011). A maioria desses compostos proporciona resistência aos mais variados fitopatógenos e insetos (AGRAWAL; KONNO, 2009; EVERT, 2006; MITHÖFER; BOLAND, 2012). Dentre as moléculas citadas, as proteínas destacam-se devido a sua abundância e frequente ocorrência nos látices. Entre as proteínas, as enzimas são as mais comuns, tais como peptidases, glicosidades, amilases, fosfatases ácidas, quitinases e β-1,3-glucanases (DOMSALLA; MELZIG, 2008; FREITAS *et al*., 2007; RAMOS *et al*., 2013). A grande maioria destas proteínas tem sido relacionada à defesa da planta (KONNO, 2011).

Polifenol oxidases (PPO) e peroxidases (POD) são oxidases de plantas comumente encontradas em látices (Sethi et al., 2009). Estas também são consideradas proteínas de defesa, uma vez que convertem mono e di-hidroxifenol em espécies moleculares reativas, como as quinonas, que em seguida ligam-se covalentemente a nucleófilos de aminoácidos, tornando-os inacessíveis, o que diminui o valor nutritivo das proteínas (KONNO, 2011; ZHU-SALZMAN; LUTHE; FELTON, 2008).

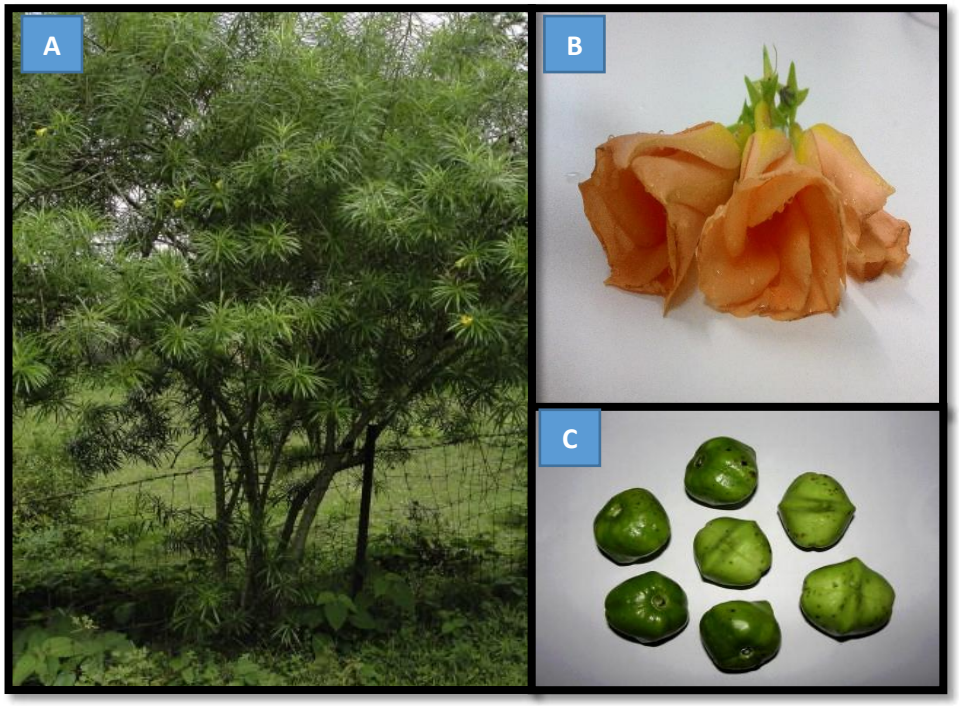
A borracha representa o constituinte de maior proporção do látex, sendo formada por cis-1,4-poliisopreno (SWIEZEWSKA; DANIKIEWICZ, 2005). Mais de 80 % da massa seca do látex de *Calotropis procera* corresponde a borracha, enquanto que a fração restante, livre de borracha, compreende menos do que 20 %. Nesta fração, o conteúdo de proteínas solúveis pode

chegar a 8 mg/mL, representando, aproximadamente, 17 % do peso seco do látex. O conteúdo proteico desta fração é representado, em sua maior parte, por peptidases cisteínicas, inibidores de peptidases, quitinases etc (RAMOS *et al*., 2007).

Thevetia peruviana

Thevetia peruviana (Pers.) K. Schum, também conhecida como yellow oleander, é um arbusto que pertence à família Apocynanceae, sendo originária das regiões tropicais da américa central (Shepherd, 2004). A espécie tem sido utilizada como arbusto ornamental em todas as partes tropicais e subtropicais do mundo (Figura 1) (Bandara et al., 2010).

Figura 1. Planta Chapéu-de-Napoleão (*Thevetia peruviana* Schum). Imagem do arbusto (A); Partes florais (B) e Frutos (C) da espécie *Thevetia peruviana.* **Fonte**: próprio autor.



No Brasil é conhecida popularmente como Chapéu de Napoleão ou Aguaí. Na medicina popular a espécie *T. peruviana* é utilizada devido às suas propriedades antipiréticas, moluscicidas, rodenticida e antibacteriana, assim como é introduzida em tratamentos gastrointestinal, doenças inflamatórias, insufuciência cardíaca e tumores de pele (Langford and Boor, 1996; Ramos-Silva et al., 2017). Todas as partes da planta são venenosas devido à presença de toxinas cardíacas, mas o fruto de *T. peruviana* caracteriza-se por ser a parte mais tóxica, uma vez que possui o maior e mais diverso conteúdo de glicosídeos cardíacos. Existem muitos casos relatados de envenenamento intencional e acidental em humanos através da ingestão de frutas e folhas (Tian et al., 2016).

Os dados disponíveis na literatura provenientes da *T. peruviana* restringem-se basicamente ao estudo dos glicosídeos cardíacos presentes em frações metanólicas de diferentes partes da planta. Para tanto, a composição proteica do látex da referida espécie foi aprofundada

apenas a partir do trabalho de Freitas e colaboradores (2016), o que evidenciou a presença de proteínas de defesa vegetal, como peroxidases, osmotinas, proteases etc.

Peptidases: Papéis Fisiológicos e Aplicações Biotecnológicas

As peptidases, também chamadas de proteases ou proteinases, são enzimas, encontradas em todos os organismos vivos, capazes de hidrolizar ligações peptídicas. Em organismos superiores, ou multicelulares, cerca de 2 % do código genético é representado por essas enzimas (RAWLINGS; BARRETT; BATEMAN, 2010). As peptidases podem ser divididas em exopeptidases e endopeptidases, baseando-se na porção da ligação peptídica na qual ela vai atuar.

Quando a clivagem ocorre entre aminoácidos da porção amino ou carboxiterminal, as peptidases são ditas exopeptidases. Estas ainda subdividem-se em aminopeptidases e carboxipeptidases, baseado nos seus sítios de atuação catalítica N- ou C- terminal, respectivamente. As endopeptidases (EC 3.4) atuam internamente nas cadeias polipeptídicas, porém não hidrolisam ligações peptídicas entre os aminoácidos das porções terminais (LEWINSOHN, 1991). Geralmente, estas biomoléculas são sintetizadas como pró-enzimas inativas para impedir uma ação em locais ou momentos indesejados. Tais segmentos peptídicos que mantêm a enzima inativa estão, predominantemente, localizados na extremidade N-terminal do precursor (KARDOS *et al*., 1999).

As endopeptidases podem ser classificadas de acordo com os resíduos de aminoácidos presentes no sítio ativo da enzima, bem como seu mecanismo de catálise. Peptidases serínicas (EC 3.4.21) apresentam um resíduo de serina no sítio ativo; as que pertencem a subclasse das peptidases cisteínicas (EC 3.4.22) têm resíduo de cisteína; aquelas pertencentes a EC 3.4.23 (peptidases aspárticas) dependem do resíduo de aspartato para sua atividade catalítica; metalopeptidases (EC

3.4.24) utilizam um metal, normalmente o Zn^{2+}, no seu mecanismo catalítico (BARRETT *et al*., 2013).

As treonino e as glutamil peptidases são um distinto, e recentemente reclassificado grupo de enzimas, que não haviam sido descritas até 1995 e 2004, respectivamente. As peptidases do tipo treonina são caracterizadas pela presença de um resíduo de treonina em seu sítio ativo. Estas são encontradas em fungos, plantas, vírus, animais e bactérias (BARRETT *et al*., 2013; RAWLINGS *et al*., 2010). As glutamil peptidases possuem um resíduo de ácido glutâmico no sítio ativo, sendo descritas apenas em fungos *(*SIMS *et al*., 2004). Em geral, as peptidases, dependendo do pH ótimo de atuação, são também nomeadas de: peptidases ácidas, ativas em pH entre 2,0-3,5; peptidases neutras, ativas em pH entre 6,5-7,5 e; peptidases alcalinas, ativas em pH 7,5-10,0 (MAHAJAN; BADGUJAR, 2010).

As peptidases são fundamentais em várias etapas bioquímicas durante o ciclo de vida das plantas, que incluem participação da germinação, senescência, apoptose, turnover e mobilização proteica, além de outros processos (DOMSALLA; MELZIG, 2008). Em praticamente todas as partes da planta é possível encontrar peptidases (BADGUJAR; MAHAJAN, 2014).

Além dos papéis fisiológicos desempenhados pelas peptidases, suas aplicações comerciais são de extrema importância, uma vez que estas representam um dos três maiores grupos de enzimas industriais, constituindo cerca de 60% da totalidade mundial de vendas de enzimas (BADGUJAR *et al*., 2014). As peptidases são empregadas no desenvolvimento de tecnologias chamadas “amigas do ambiente”, incluindo o tratamento de couro e vários processos de biorremediação, bem como na formulação de detergentes (CHARNOCK; MCCLEARY, 1998; DOMSALLA; MELZIG, 2008; LÓPEZ *et al*., 2017). Em termos farmacológicos, elas podem atuar na preparação de medicamentos, como pomadas para desbridamento de feridas, assim como na

ciência e tecnologia de alimentos, incluindo a hidrólise de grandes polipeptídeos em peptídeos menores e aminoácidos, o que facilita a digestão e absorção de proteínas (FEIJOO-SIOTA; VILLA, 2010; MORCELLE *et al*., 2004).

Diversos trabalhos atribuem ações farmacológicas às peptidases cisteínicas de plantas. Extratos enzimáticos de *Ananas comosus* (Bromeliaceae), *Azadirachta indica* (Meliaceae), *Caesalpinia crista* (Caesalpiniaceae), *Vernonia anthelmintica* (Asteraceae), *Fumaria parviflora* (Papaveraceae) e *Embelia ribes* (Myrsinaceae) mostraram eficácia contra helmintos que parasitam o trato gastro intestinal de ruminantes (HÖRDEGEN *et al*., 2003; THOMSON *et al*., 2001). A bromelaína apresenta atividade anti-inflamatória, *in vivo*, sem desenvolver efeitos adversos (HALE *et al*., 2005).

A estabilidade e atividade das peptidases cisteínicas são fatores importantes para determinar a sua viabilidade econômica em processos industriais. Uma alta estabilidade frente a variações de pH e temperatura é considerado uma vantagem econômica, o que promove uma atenção especial por parte de indústrias alimentícias e biotecnológicas (PATEL; JAGANNADHAM, 2003). Em plantas, estas moléculas representam um grupo enzimático com propriedades similares, tais como peso molecular de 20 a 30 kDa e pH ótimo entre 3,5 e 8,0. Essas propriedades podem ser cruciais para a manutenção de sua atividade em condições extremas (VIEILLE; ZEIKUS, 2001).

Peptidases em Flúidos Laticíferos

As plantas laticíferas secretam de forma imediata o látex quando suas folhas, caules ou frutos são injuriados (AGRAWAL; KONNO, 2009). A coagulação do látex é essencial, pois permite a formação de uma barreira física que impede, na maioria das vezes, o ataque de patógenos.

Neste caso, as enzimas proteolíticas presentes no látex podem participar deste processo de coagulação. Alguns trabalhos já demonstraram que, durante a coagulação do látex, um grande número de peptídeos são proteoliticamente processados de uma maneira não aleatória (BAUER *et al*., 2014; SILVA *et al*., 1997).

As enzimas proteolíticas dos tipos serínicas e cisteínicas são as mais abundantes em látices (MORCELLE; CAFFINI; PRIOLO, 2004). Algumas plantas laticíferas destacam-se devido as propriedades e aplicabilidade de suas peptidases. Destas, podemos citar: *Ficus carica*, que tem sido usada na medicina tradicional indiana para o tratamento de algumas doenças (BADGUJAR *et al*., 2014; JOSEPH; RAJ, 2011); as peptidases do látex de *Carica papaya,* que apresentam eficácia antihelmíntica frente a vários parasitas de animais (HÖRDEGEN *et al*., 2003; MANSUR *et al*., 2014); Frações proteicas, ricas em peptidases cisteínicas, oriundas do látex das espécies *Calotropis procera* e *Crypstostegia grandiflora* mostraram-se atuantes no processo de coagulação do leite, o que lhe dá uma prospecção ao desenvolvimento de produtos lácteos diversificados (Freitas et al., 2016). O trabalho de Souza e colaboradores (2011) evidenciou que frações proteicas do látex, exibindo atividade proteolítica do tipo cisteínica, bem como duas peptidases cisteínicas purificadas de diferentes látices, foram capazes de inibir a germinação de esporos e o crescimento das hifas de diferentes fungos fitopatogênicos. Recentemente, Ramos e colaboradores (2014), identificaram uma peptidase cisteínica (Cg24-I), pertencente ao látex de *Cryptostegia grandiflora,* com atividade antifúngica.

Além do papel desempenhado na defesa, peptidases têm sido associadas a diversos papéis fisiológicos da planta, por exemplo: atuando como peptídeo sinal na microsporogênese; desenvolvendo respostas hipersensitivas (HR); na diferenciação de tecidos especializados, como o estomático etc (ANTÃO *et al*., 2005).

O foco deste trabalho está relacionado à classe das peptidases cisteínicas (EC 3.4.22). Essas enzimas atuam via ataque nucleofílico de um ânion sulfeto, presente no sítio ativo, sobre a ligação peptídica (KUNAKBAEVA *et al*., 2003). Para estas enzimas, o resíduo de cisteína é fundamental na atividade proteolítica. As peptidases cisteínicas estão divididas em onze clãs (CA, CD, CE, CF, CL, CM, CN, CO, CP, CQ, CR), onde cada um desses distribuem-se em famílias e subfamílias (BAH *et al*., 2006). A maioria das peptidases cisteínicas pertencem a família *Papain* (C1), que está inclusa no clã CA. Muitas dessas têm sido isoladas de látices, frutas e sementes (VIERSTRA, 1996).

As peptidases cisteínicas podem intervir no reconhecimento de agentes patogênicos e parasitas, corroborando com a defesa da planta (SOUZA *et al*., 2011). Adicionalmente, as peptidases cisteínicas oriundas de látex, também têm a capacidade de desempenhar atividade trombolítica, degradando componentes da cascata de coagulação como fibrinogênio e fibrina, o que cientificamente valida o uso de látex de plantas na interrupção do sangramento e na cicatrização de feridas pelos adeptos da medicina popular em algumas partes do mundo (SHIVAPRASAD *et al*., 2009; RAMOS *et al*., 2013).

A peptidase cisteínica vegetal mais amplamente estudada é a papaína (EC 3.4.22.2), pertencente ao látex de *Carica papaya,* o qual apresenta outras peptidases em sua composição, como a quimopapaína (EC 3.4.22.6), caricaína (EC 3.4.22.30) e glicil endopeptidase (EC 3.4.22.25), também chamada de proteinase papaia IV (BARRETT; WOESSNER, 2013; OTTO; SCHIRMEISTER, 1997). A papaína foi a primeira enzima da família a ter sua estrutura tridimensional determinada por cristalografia de raios-X (MONTI *et al*., 2000).

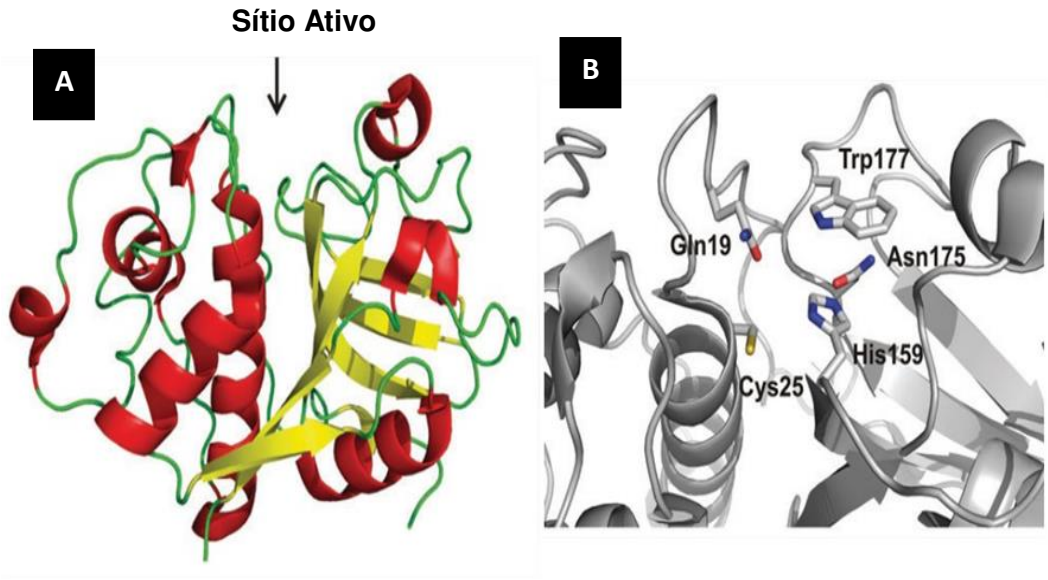
Peptidases Cisteínicas: Uma Abordagem Estrutural

As peptidases cisteínicas estão presentes em todos os organismos vivos. Atualmente, cerca de 121 famílias estão reportadas na literatura, de acordo com o banco de dados MEROPS. Estas enzimas apresentam uma massa molecular entre 21-30 kDa, exibindo uma melhor atividade proteolítica em intervalos de pH 4 – 6.5. Devido à alta tendência do grupo tiol à oxidação, o ambiente destas peptidases deve conter um componente redutor. A glutationa serve como um agente redutor nas células, enquanto a adição de beta-mercaptoetanol ou ditiotreitol (DTT) é necessário para experimentos *in vitro* (GRZONKA *et al*., 2001; BUTTS *et al*., 2016).

A família de peptidase cisteínica mais detalhadamente estudada é o da Papaína (C1A). Todos os membros inclusos nessa família, mesmo que de organismos diferentes, estão relacionados estruturalmente com a papaína (*Carica papaya*), como por exemplo as catepsinas, que são categorizadas proteínas similares à papaína (papain like peptidases) (GRZONKA *et al*., 2001; NOVINEC and LENARCIC, 2013).

As papaínas-like maduras são proteínas globulares monoméricas composta de duas metades de tamanhos aproximadamente iguais, denominadas domínios L e R. O sítio ativo está localizado na interface de ambos os domínios e no topo da molécula, posicionado em uma fenda em forma de V (Figura 2A) (NOVINEC and LENARCIC, 2013). Neste sítio ativo está inserido a díade catalítica formada pelo par de íons Cys^{-} - H^{+} . Para tanto, vários resíduos adicionais são necessários para a eficiência catalítica destas enzimas. Os resíduos Asn175, Gln19 e Trp 177 são necessários para o posicionamento adequado da díade catalítica, gerando o seu caráter nucleofílico e consequentemente possibilitando a hidrólise do substrato proteico (Figura 2B) (BUTTS *et al*., 2016).

Figura 2. Estrutura tridimensional da papaína evidenciando os dois domínios de sua composição, bem como a fenda onde está inserido o sítio catalítico da enzima (código de acesso ao PDB: 1PPN). (A); (B) Resíduos do sítio catalítico da papaína. Cys25 e His159 formam a díade catalítica, e Gln19, Asn175 e Trp177 estão envolvidos na manutenção do caráter nucleofílico destes pares de resíduos.



Germinas e *Germin-like Proteins* (GLPs)

As germinas constituem um grupo de proteínas homólogas encontradas, principalmente, em cereais, incluindo arroz, aveia, trigo, cevada, milho e centeio (LANE, 2002). A germina foi identificada, inicialmente, como um marcador específico relacionado ao início da germinação em embriões de trigo; a partir desta função foi dado o nome "Germina" (THOMPSON; LANE, 1980). Alguns anos depois ela foi caracterizada como uma glicoproteína homopentamérica (FAYE; CHRISPEELS, 1988; JAIKARAN *et al*., 1990), com atividade de oxalato oxidase (LANE, 1984). Essa atividade enzimática gera como produtos finais o gás carbônico e peróxido de hidrogênio (H_2O_2), que pode intervir diretamente contra a invasão de fitopatógenos (LANE, 2000;

SCHWEIZER; CHRISTOFFEL; DUDLER, 1999). Em contraste com o que já havia sido proposto, Woo e colaboradores (1998, 2000) determinaram que a estrutura da germina é composta por seis monômeros contidos dentro de um hexâmero, formando um trímero de dímeros.

Em termos de sequência, todas as germinas compartilham um segmento peptídico característico, PHIHPRATEI, localizado na porção N-terminal (LANE *et al*., 1991). Proteínas que têm em média de 50% de identidade com a germina do trigo, e que também possuem a referida sequência citada acima, foram encontradas em diversas plantas, além dos cereais. Estas foram designadas como proteínas similares a germina ou *germin-like proteins* (GLPs). Tanto as germinas como as GLPs estão incluídas na superfamília das cupinas, uma vez que estas conservam a estrutura em β-barril (DUNWELL *et al*., 2008).

Diferente das germinas verdadeiras, as GLPs são codificadas por um grupo heterogêneo de genes presentes em muitas plantas terrestres, incluindo as monocotiledôneas, dicotiledôneas, gimnospermas e musgos. Além da presença de sequências características destas proteínas, as GLPs são conhecidas por, geralmente, não apresentarem atividade de oxalato oxidase, o qual está incluso em "germinas verdadeiras", também chamadas de Oxalato Oxidases (OXO) (DUNWELL *et al*., 2008).

Características Estruturais de Germinas e GLPs

Ambas as Germinas e Germin-likes (GLPs) são compostas de três regiões conservadas de aminoácidos, denominadas "Box", as quais são representadas pelas letras A, B e C (BERNIER; BERNA, 2001). A sequência da caixa A contém um dos dois resíduos de cisteína responsável por formar uma ponte de dissulfeto interna que estabiliza a conformação N-terminal. As caixas B e C, contêm um Glutamato e três resíduos de Histidina, que proporciona ligação a metais, formando a

estrutura em β-barril, característico da superfamília das cupinas, onde Germinas e GLPs estão inclusas.

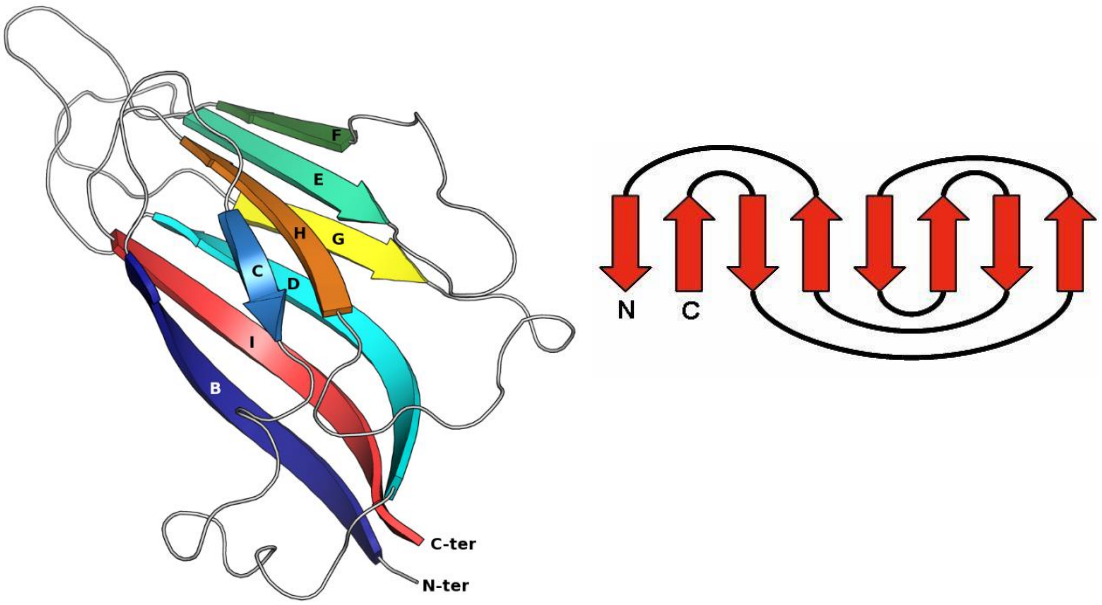
De acordo com o banco de dados de classificação estrutural de proteínas (SCOP-Structural Classification of Proteins), o termo Cupin ou RmlC-Like cupins refere-se a proteínas que apresentam o motivo double stranded β-helix (DSBH), o qual é representado por dois pares de fitas antiparalelas ligadas através de curvas curtas (Rajavel et al., 2008). Estes motivos geram uma aparência de um sanduíche na estrutura proteica, daí é designado o termo "β-sandwich" (Uberto and Moomaw, 2013). As proteínas inclusas nesta superfamília apresentam de forma majoritária uma topologia do tipo Jelly-Roll, composta de oito fitas betas (β-strand), que é distribuída em duas folhas beta (β-sheet) de quatro fitas betas antiparalelas (Figura 3) (SKERN, 2018).

Os membros ancestrais pertencentes à superfamília das "cupinas" eram, basicamente, representados por moléculas simples e pequenas, que apresentavam domínios de ligação a açúcares e nucleotideos cíclicos (DUNWELL et al., 2001). Atualmente a superfamília das cupinas (clã cl09118) abrange milhares de proteínas presentes em seres tanto procarióticos como eucarióticos, incluindo os seguintes membros exemplares: proteínas ligantes a sacarose (PIROVANI *et al*., 2002), auxina (GRANDITS and OOSTENBRINK, 2014), proteínas de reserva (DUNWELL *et al*., 2004) e até mesmo enzimas com atividade proteolítica (FREITAS *et a*l., 2016).

O acesso ao banco de dados de estruturas proteicas (PDB- protein data bank) aponta uma totalidade de aproximadamente 300 moleculas estruturalmente resolvidas pertencentes à superfamília das Cupinas. Quando trata-se de Germinas e GLPs esse número drasticamente é reduzido para apenas quatro estruturas, sendo estas pertencentes ao mesmo organismo*, Hordeum vulgare.* A partir do estudo detalhado destas estruturas tridimensionais foi possível descrever os mecanismos enzimáticos responsáveis pela atividade de oxalato oxidase (OXO) e Superóxido

Dismutase (SOD) da Germina. No entanto, ainda é inexistente na literatura, estruturas de Germinas ou GLPs que desempenham outras atividades enzimáticas.

Figura 3. Topologia em Jelly roll de uma proteína do capsídeo viral do tabaco. A estrutura supersecundária é composta de oito fitas beta distribuídas em duas folhas beta com quatro fitas antiparalelas em cada segmento. (BAN and MCPHERSON, 1995)



***Peruvianina-I*: uma germin-like com atividade proteolítica**

As etapas inicias de caracterização e prospecção de biomoléculas do látex de *Thevetia peruviana*, provenientes do trabalho de Freitas e colaboradores (2016), permitiram identificar toda uma diversidade de proteínas, até então não descritas na literatura. Na fração proteica do látex de *T. peruviana* destacou-se a presença, principalmente, de proteínas de defesa vegetal, como: osmotinas, peroxidases, quitinases e majoritariamente peptidases cisteínicas. A riqueza de enzimas proteolíticas do tipo cisteínica têm sido claramente associado ao látex de diversas espécies vegetais

(Ramos et al., 2019). Diante desta diversidade de enzimas uma glicoproteína de peso molecular de aproximadamente 100 kDa foi purificada e parcialmente caracterizada através de análises enzimáticas, o que foi possível associar a esta uma atividade peptidásica do tipo cisteínica (Figura 4). À proteína em destaque foi dado o nome de peruvianina-I, tendo por referência o nome científico da espécie. Adicionalmente às análises enzimáticas, o sequenciamento da porção N-terminal da peruvianina-I revelou, de forma inesperada, uma identidade de 64% a uma germin-like (GLP) da espécie *Oryza sativa*. As atividades características de germinas e GLPs, como oxalato oxidase (OXO) e superóxido dismutase (SOD) mostraram-se ausentes na peruvianina-I. Assim como as evidências da sequência N-terminal, as imagens provenientes da microscopia de força atômica reforçaram a similaridade estrutural da peptidase em estudo com uma germina da espécie *Hordeum vulgare* (código de acesso ao PDB: 1FI2) (Figura 5). Contudo, estes resultados evidenciaram de forma inédita, uma GLP com atividade proteolítica.

Todas essas evidências foram fatores centrais em objetivar o presente trabalho de tese à uma descrição estrutural detalhada da peruvianina-I, a fim de compreender as nuances moleculares envolvidas nesta nova função enzimática, propondo por final um mecanismo enzimático plausível para tal atividade.

Figura 4. (A) 1D SDS-Page (12%) da proteína peruvianina-I; (B) Zimograma para detecção de atividade proteolítica; (C) Gel para detecção de glicoproteína utilizando reagente de Schiff's.

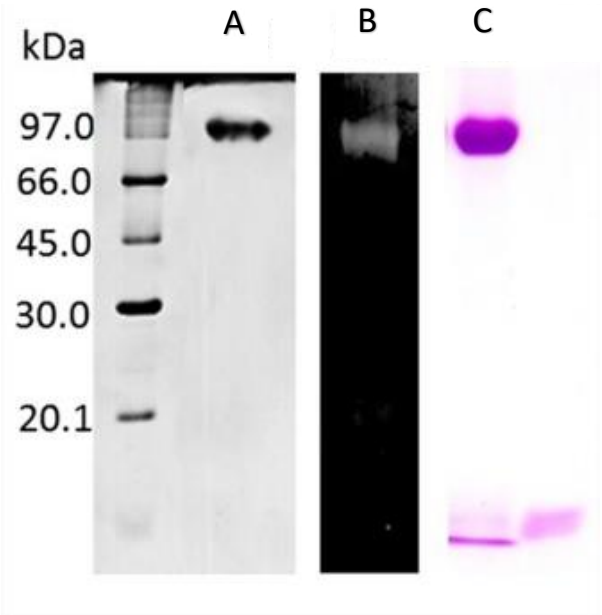
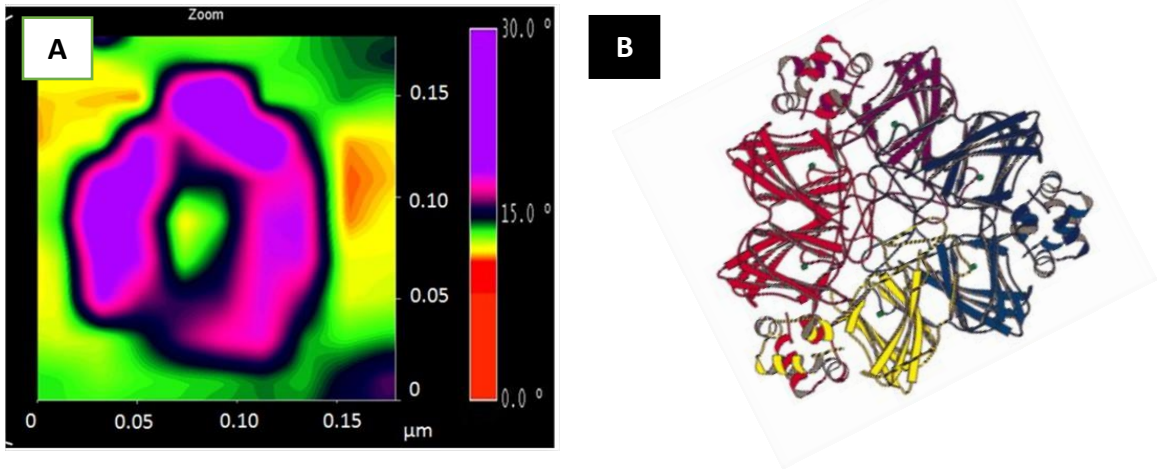


Figura 5. (A) Análise estrutural da peruvianina-I por microscopia de força atômica; (B) Estrutural tridimensional da germina (código de acesso ao PDB: 1FI2); A disposição estrutural de ambas as proteínas enfatizam a similaridade entre as mesmas. É possível deduzir que a peruvianina-I apresenta uma organização estrutural composta por um trímero de dímeros formando um anel central



.

REFERÊNCIAS

Agrawal, A.A., Konno, K., 2009. Latex: A Model for Understanding Mechanisms, Ecology, and Evolution of Plant Defense Against Herbivory. Annu. Rev. Ecol. Evol. Syst. 40, 311–331. https://doi.org/10.1146/annurev.ecolsys.110308.120307

Antão, C.M., Malcata, F.X., 2005. Plant serine proteases: biochemical, physiological and molecular features. Plant Physiol. Biochem. 43, 637–50. https://doi.org/10.1016/j.plaphy.2005.05.001

Badgujar, S.B., Mahajan, R.T., 2014. Identification and characterization of Euphorbia nivulia latex proteins. Int. J. Biol. Macromol. 64, 193–201. https://doi.org/10.1016/j.ijbiomac.2013.12.006

Badgujar, S.B., Patel, V. V, Bandivdekar, A.H., Mahajan, R.T., 2014. Traditional uses, phytochemistry and pharmacology of Ficus carica: a review. Pharm. Biol. 52, 1487–503. https://doi.org/10.3109/13880209.2014.892515

Bah, S., Paulsen, B.S., Diallo, D., Johansen, H.T., 2006. Characterization of cysteine proteases in Malian medicinal plants. J. Ethnopharmacol. 107, 189–98. https://doi.org/10.1016/j.jep.2006.03.008

Ban, N., McPherson, A., 1995. The structure of satellite panicum mosaic virus at 1.9 Å resolution. Nat. Struct. Biol. 2, 882–890. https://doi.org/10.1038/nsb1095-882

Bandara, V., Weinstein, S. a, White, J., Eddleston, M., 2010. A review of the natural history, toxinology, diagnosis and clinical management of Nerium oleander (common oleander) and Thevetia peruviana (yellow oleander) poisoning. Toxicon 56, 273–81. https://doi.org/10.1016/j.toxicon.2010.03.026

Barman, A.R., Banerjee, J., 2015. Versatility of germin-like proteins in their sequences, expressions, and functions. Funct. Integr. Genomics. https://doi.org/10.1007/s10142-015-0454-z

Barrett, A.J., Fred Woessner, J., 2013. Handbook of Proteolytic Enzymes, Handbook of Proteolytic Enzymes. Elsevier. https://doi.org/10.1016/B978-0-12-382219-2.00838-3

Bauer, G., Gorb, S.N., Klein, M.-C., Nellesen, A., von Tapavicza, M., Speck, T., 2014. Comparative study on plant latex particles and latex coagulation in Ficus benjamina, Campanula glomerata and three Euphorbia species. PLoS One 9, e113336. https://doi.org/10.1371/journal.pone.0113336

Bernier, F., Berna, A., 2001. Germins and germin-like proteins: Plant do-all proteins. But what do they do exactly? Plant Physiol. Biochem. https://doi.org/10.1016/S0981-9428(01)01285-2

Butts, C.T., Zhang, X., Kelly, J.E., Roskamp, K.W., Unhelkar, M.H., Freites, J.A., Tahir, S., Martin, R.W., 2016. Sequence comparison, molecular modeling, and network analysis predict

structural diversity in cysteine proteases from the Cape sundew, Drosera capensis. Comput. Struct. Biotechnol. J. 14, 271–282. https://doi.org/10.1016/j.csbj.2016.05.003

Callahan, D.L., Roessner, U., Dumontet, V., Perrier, N., Wedd, A.G., O'Hair, R. a J., Baker, A.J.M., Kolev, S.D., 2008. LC-MS and GC-MS metabolite profiling of nickel(II) complexes in the latex of the nickel-hyperaccumulating tree Sebertia acuminata and identification of methylated aldaric acid as a new nickel(II) ligand. Phytochemistry 69, 240–51. https://doi.org/10.1016/j.phytochem.2007.07.001

Charnock, S.., McCleary, B.., 1998. Enzymes: Industrial and Analytical Applications, in: Tecchnique.

Chruszcz, M., Maleki, S.J., Majorek, K.A., Demas, M., Bublin, M., Solberg, R., Hurlburt, B.K., Ruan, S., Mattisohn, C.P., Breiteneder, H., Minor, W., 2011. Structural and Immunologic Characterization of Ara h 1 , a Major Peanut Allergen * □ 286, 39318–39327. https://doi.org/10.1074/jbc.M111.270132

Cruz, W.T., Bezerra, E.H.S., Grangeiro, T.B., Lopes, J.L.S., Silva, M.Z.R., Ramos, M. V, Rocha, B.A.M., Oliveira, J.S., Freitas, D.C., Freitas, C.D.T., 2019. Structural and enzymatic characterization of Peruvianin - I , the fi rst germin-like protein with proteolytic activity. Int. J. Biol. Macromol. 126, 1167–1176. https://doi.org/10.1016/j.ijbiomac.2019.01.023

de Freitas, C.D.T., Nogueira, F.C.S., Vasconcelos, I.M., Oliveira, J.T.A., Domont, G.B., Ramos,

M.V., 2011. Osmotin purified from the latex of Calotropis procera: biochemical characterization, biological activity and role in plant defense. Plant Physiol. Biochem. 49, 738–43. https://doi.org/10.1016/j.plaphy.2011.01.027

Domsalla, A., Melzig, M.F., 2008. Occurrence and properties of proteases in plant latices. Planta Med. https://doi.org/10.1055/s-2008-1074530

Dunwell, J.M., Culham, A., Carter, C.E., Sosa-Aguirre, C.R., Goodenough, P.W., 2001. Evolution of functional diversity in the cupin superfamily. Trends Biochem. Sci. 26, 740–746. https://doi.org/10.1016/S0968-0004(01)01981-8

Dunwell, J.M., Gibbings, J.G., Mahmood, T., Naqvi, S.M.S., 2008. Germin and germin-like proteins: Evolution, structure, and function.

Dunwell, J.M., Purvis, A., Khuri, S., 2004. Cupins: the most functionally diverse protein superfamily? Phytochemistry 65, 7–17. https://doi.org/10.1016/j.phytochem.2003.08.016

Evert, R.F., 2006. Esau's Plant Anatomy, Esau's Plant Anatomy: Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function, and Development: Third Edition. John Wiley & Sons, Inc.

Faye, L., Chrispeels, M.J., 1988. Common antigenic determinants in the glycoproteins of plants, molluscs and insects. Glycoconj. J. 5, 245–256. https://doi.org/10.1007/BF01049085

Feijoo-Siota, L., Villa, T.G., 2010. Native and Biotechnologically Engineered Plant Proteases with Industrial Applications. Food Bioprocess Technol. 4, 1066–1088. https://doi.org/10.1007/s11947-010-0431-4

Freitas, C.D.T., Leite, H.B., Oliveira, J.P.B., Amaral, J.L., Egito, A.S., Vairo-Cavalli, S., Lobo, M.D.P., Monteiro-Moreira, A.C.O., Ramos, M. V., 2016. Insights into milk-clotting activity of latex peptidases from Calotropis procera and Cryptostegia grandiflora. Food Res. Int. 87, 50–59. https://doi.org/10.1016/J.FOODRES.2016.06.020

Freitas, C.D.T., Oliveira, J.S., Miranda, M.R. a, Macedo, N.M.R., Sales, M.P., Villas-Boas, L. a, Ramos, M.V., 2007. Enzymatic activities and protein profile of latex from Calotropis procera. Plant Physiol. Biochem. 45, 781–9. https://doi.org/10.1016/j.plaphy.2007.07.020

Freitas, C.D.T. De, Wallace, T., Maria, C., 2016a. Proteomic analysis and purification of an unusual germin-like protein with proteolytic activity in the latex of Thevetia peruviana. https://doi.org/10.1007/s00425-016-2468-8

Freitas, C.D.T. De, Wallace, T., Maria, C., 2016b. Proteomic analysis and purification of an unusual germin-like protein with proteolytic activity in the latex of Thevetia peruviana 1115–1128. https://doi.org/10.1007/s00425-016-2468-8

Gomes, M.T.R., Ribeiro, H.A., Lopes, M.T.P., Guzman, F., Salas, C.E., 2010. Biochemical comparison of two proteolytic enzymes from Carica candamarcensis: Structural motifs underlying resistance to cystatin inhibition. Phytochemistry 71, 524–530. https://doi.org/10.1016/j.phytochem.2009.12.018

Grandits, M., Oostenbrink, C., 2014. Molecular dynamics simulations of the auxin-binding protein 1 in complex with indole-3-acetic acid and naphthalen-1-acetic acid. Proteins 82, 2744–2755. https://doi.org/10.1002/prot.24639

Grzelczak, Z.F., Lane, B.G., 1984. Signal resistance of a soluble protein to enzymic proteolysis. An unorthodox approach to the isolation and purification of germin, a rare growth-related protein. Can. J. Biochem. Cell Biol. 62, 1351–1353. https://doi.org/10.1139/o84-172

Grzonka, Z., Jankowska, E., Kasprzykowski, F., Kasprzykowska, R., Łankiewicz, L., Wiczk, W., Wieczerzak, E., Ciarkowski, J., Drabik, P., Janowski, R., Kozak, M., Jaskólski, M., Grubb, A., 2001. Structural studies of cysteine proteases and their inhibitors. Acta Biochim. Pol. 48, 1–20.

Hale, L.P., Greer, P.K., Trinh, C.T., Gottfried, M.R., 2005. Treatment with oral bromelain decreases colonic inflammation in the IL-10-deficient murine model of inflammatory bowel disease. Clin. Immunol. 116, 135–42. https://doi.org/10.1016/j.clim.2005.04.011

Ho, C.C., 2014. Chapter 4 - The Production of Natural Rubber from Hevea brasiliensis Latex: Colloidal Properties, Preservation, Purification and Processing, RSC Polymer Chemistry Series. Royal Society of Chemistry, Cambridge. https://doi.org/10.1039/9781849737647

Hördegen, P., Hertzberg, H., Heilmann, J., Langhans, W., Maurer, V., 2003. The anthelmintic efficacy of five plant products against gastrointestinal trichostrongylids in artificially infected lambs. Vet. Parasitol. 117, 51–60.

Jaikaran, A.S.I., Kennedy, T.D., Dratewka-kos, E., Lane, B.G., Glcnac-fuc, X.G., 1990. Covalently Bonded and Adventitious Glycans in Germin * 265, 12503–12512.
Joseph, B., Raj, S.J., 2011. Pharmacognostic and phytochemical properties of Ficus carica Linn – An overview. Int. J. PharmaTech Res. 3, 8–12.

Kardos, J., Bódi, A., Závodszky, P., Venekei, I., Gráf, L., 1999. Disulfide-linked propeptides stabilize the structure of zymogen and mature pancreatic serine proteases. Biochemistry 38, 12248–57.
Kekwick, R., 2001. Latex and laticifers. Encycl. Life Sci. 1–6.

Konno, K., 2011. Plant latex and other exudates as plant defense systems: Roles of various defense chemicals and proteins contained therein. Phytochemistry. https://doi.org/10.1016/j.phytochem.2011.02.016

Kunakbaeva, Z., Carrasco, R., Rozas, I., 2003. An approximation to the mechanism of inhibition of cystein proteases: nucleophilic sulphur addition to Michael acceptors type compounds. J. Mol. Struct. THEOCHEM 626, 209–216. https://doi.org/10.1016/S0166-1280(03)00086-1

Lane, B.G., 2002. Oxalate , Germins , and Higher-Plant Pathogens 67–75. https://doi.org/10.1080/10399710290038954
Lane, B.G., 2000. Oxalate oxidases and differentiating surface structure in wheat : germins 321, 309–321.

Lane, B.G., Bernierp, F., Dratewka-kos, E., Shafai, R., Kennedy, D., Pyne, C., Munro, J.R., Vaughan, T., Walters, D., Altomare, F., Gene, G., 1991. Homologies between Members of the Germin Gene Family in Hexaploid Wheat and Similarities between These Wheat Germins and Certain. J. Biol. Chem. 266, 10461–10469.

Langford, S.D., Boor, P.J., 1996. Oleander toxicity: an examination of human and animal toxic exposures. Toxicology 109, 1–13. https://doi.org/10.1016/0300-483X(95)03296-R

Lewinsohn, T.M., 1991. The geographical distribution of plant latex. Chemoecology 2, 64–68. https://doi.org/10.1007/BF01240668

Lima, R.C. de S., Silva, M.C.C., Aguiar, C.C.T., Chaves, E.M.C., Dias, K.C.F., Macêdo, D.S., de Sousa, F.C.F., Carvalho, K. de M., Ramos, M.V., Vasconcelos, S.M.M., 2012. Anticonvulsant action of Calotropis procera latex proteins. Epilepsy Behav. 23, 123–6. https://doi.org/10.1016/j.yebeh.2011.11.008

López, L.M.I., Viana, C.A., Errasti, M.E., Garro, M.L., Martegani, J.E., 2017. Latex peptidases of Calotropis procera for dehairing of leather as an alternative to environmentally toxic sodium sulfide treatment, Bioprocess and Biosystems Engineering. https://doi.org/10.1007/s00449-017-1796-9

Mahajan, R.T., Badgujar, S.B., 2010. Biological aspects of proteolytic enzymes : A Review, Journal of Pharmacy Research.

Mansilla, A.Y., Segarra, C.I., 2012. Structural and Functional Features of a Wheat Germin-Like Protein that Inhibits Trypsin 624–632. https://doi.org/10.1007/s11105-011-0372-8

Mansur, F., Luoga, W., Buttle, D.J., Duce, I.R., Lowe, A., Behnke, J.M., 2014. The anthelmintic efficacy of natural plant cysteine proteinases against two rodent cestodes Hymenolepis diminuta and Hymenolepis microstoma in vitro. Vet. Parasitol. 201, 48–58. https://doi.org/10.1016/j.vetpar.2013.12.018

Mithöfer, A., Boland, W., 2012. Plant defense against herbivores: chemical aspects. Annu. Rev. Plant Biol. 63, 431–50. https://doi.org/10.1146/annurev-arplant-042110-103854

Mnif, I.H., Siala, R., Nasri, R., Mhamdi, S., Nasri, M., Kamoun, A.S., 2015. A cysteine protease isolated from the latex of Ficus microcarpa: purification and biochemical characterization. Appl. Biochem. Biotechnol. 175, 1732–44. https://doi.org/10.1007/s12010-014-1376-2

Monti, R., Basilio, C.A., Trevisan, H.C., Contiero, J., 2000. Purification of Papain from Fresh Latex of Carica papaya. Brazilian Arch. Biol. Technol. 43, 501–507.

Morcelle, S.R., Caffini, N.O., Priolo, N., 2004. Proteolytic properties of Funastrum clausum latex. Fitoterapia 75, 480–93. https://doi.org/10.1016/j.fitote.2004.04.006

Novinec, M., Lenarcic, B., 2013. Papain-like peptidases : structure , function , and evolution. BioMol Concepts 4, 287–308. https://doi.org/10.1515/bmc-2012-0054

Otto, H.-H., Schirmeister, T., 1997. Cysteine Proteases and Their Inhibitors. Chem. Rev. 97, 133–172. https://doi.org/10.1021/cr950025u

Patel, B.K., Jagannadham, M. V, 2003. A high cysteine containing thiol proteinase from the latex of Ervatamia heyneana: purification and comparison with ervatamin B and C from Ervatamia coronaria. J. Agric. Food Chem. 51, 6326–34. https://doi.org/10.1021/jf026184d

Pirovani, C.P., Mace, J.N.A., Loureiro, M.E., Fontes, E.P.B., 2002. A sucrose binding protein homologue from soybean exhibits GTP-binding activity that functions independently of sucrose transport activity 4008, 3998–4008. https://doi.org/10.1046/j.1432-1033.2002.03089.x

Rajavel, M., Kulkarni, N.N., Gopal, B., 2008. Conformational Studies Suggest That the Double Stranded Helix Scaf- fold Provides an Optimal Balance Between Protein Stability and Function 244–249.

Ramos-Silva, A., Tavares-Carreón, F., Figueroa, M., De la Torre-Zavala, S., Gastelum-Arellanez, A., Rodríguez-García, A., Galán-Wong, L.J., Avilés-Arnaut, H., 2017. Anticancer potential of Thevetia peruviana fruit methanolic extract. BMC Complement. Altern. Med. 17, 1–11. https://doi.org/10.1186/s12906-017-1727-y

Ramos, M.V., Demarco, D., da Costa Souza, I.C., de Freitas, C.D.T., 2019. Laticifers, Latex, and Their Role in Plant Defense. Trends Plant Sci. xx, 1–15. https://doi.org/10.1016/j.tplants.2019.03.006

Ramos, M. V., Freitas, C.D.T., Stanisçuaski, F., Macedo, L.L.P., Sales, M.P., Sousa, D.P., Carlini, C.R., 2007. Performance of distinct crop pests reared on diets enriched with latex proteins from Calotropis procera: Role of laticifer proteins in plant defense. Plant Sci. 173, 349–357. https://doi.org/10.1016/j.plantsci.2007.06.008

Ramos, M. V, Araújo, E.S., Jucá, T.L., Monteiro-Moreira, a C.O., Vasconcelos, I.M., Moreira, R. a, Viana, C. a, Beltramini, L.M., Pereira, D. a, Moreno, F.B., 2013. New insights into the complex mixture of latex cysteine peptidases in Calotropis procera. Int. J. Biol. Macromol. 58, 211–9. https://doi.org/10.1016/j.ijbiomac.2013.04.001

Ramos, M. V, Souza, D.P., Gomes, M.T.R., Freitas, C.D.T., Carvalho, C.P.S., Júnior, P. a V.R., Salas, C.E., 2014. A phytopathogenic cysteine peptidase from latex of wild rubber vine Cryptostegia grandiflora. Protein J. 33, 199–209. https://doi.org/10.1007/s10930-014-9551-4

Rawlings, N.D., 2016. Biochimie Peptidase specificity from the substrate cleavage collection in the MEROPS database and a tool to measure cleavage site conservation. Biochimie 122, 5–30. https://doi.org/10.1016/j.biochi.2015.10.003

Rawlings, N.D., Barrett, A.J., Bateman, A., 2010. MEROPS: the peptidase database. Nucleic Acids Res. 38, 227–33. https://doi.org/10.1093/nar/gkp971

Rzychon, M., Commission, E., Selvita, S.A., 2015. Modes of inhibition of cysteine proteases Circled white star. Acta Biochim. Pol. 51, 861–873.

Schweizer, P., Christoffel, A., Dudler, R., 1999. Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance. Plant J. 20, 541–52.

Segarra, Â., Anahõ, C., Pinedo, M.L., Ronchi, V.P., Ine, C., Ciencias, F. De, Mar, U.N. De, 2003. RESEARCH PAPER A germin-like protein of wheat leaf apoplast inhibits serine proteases 54, 1335–1341. https://doi.org/10.1093/jxb/erg139

Sethi, A., McAuslane, H.J., Rathinasabapathi, B., Nuessly, G.S., Nagata, R.T., 2009. Enzyme induction as a possible mechanism for latex-mediated insect resistance in romaine lettuce. J. Chem. Ecol. 35, 190–200. https://doi.org/10.1007/s10886-009-9596-6

Shepherd, R.C.H., 2004. Pretty but poisonous : plants poisonous to people : an illustrated guide for Australia. R. G; F. J Richardson 202p.

Shivaprasad, H. V, Riyaz, M., Venkatesh Kumar, R., Dharmappa, K.K., Tarannum, S., Siddesha, J.M., Rajesh, R., Vishwanath, B.S., 2009. Cysteine proteases from the Asclepiadaceae plants latex exhibited thrombin and plasmin like activities. J. Thromb. Thrombolysis 28, 304–8. https://doi.org/10.1007/s11239-008-0290-2

Silva, L.G., Garcia, O., Lopes, M.T., Salas, C.E., 1997. Changes in protein profile during coagulation of latex from Carica papaya. Braz. J. Med. Biol. Res. 30, 615–9.

Sims, A.H., Dunn-Coleman, N.S., Robson, G.D., Oliver, S.G., 2004. Glutamic protease distribution is limited to filamentous fungi. FEMS Microbiol. Lett. 239, 95–101. https://doi.org/10.1016/j.femsle.2004.08.023

Skern, T., 2018. Investigating Proteins with β-Sheets 197–226. https://doi.org/10.1007/978-3-319-76858-8

Souza, D.P., Freitas, C.D.T., Pereira, D. a, Nogueira, F.C., Silva, F.D. a, Salas, C.E., Ramos, M. V, 2011. Laticifer proteins play a defensive role against hemibiotrophic and necrotrophic phytopathogens. Planta 234, 183–93. https://doi.org/10.1007/s00425-011-1392-1

Swiezewska, E., Danikiewicz, W., 2005. Polyisoprenoids: structure, biosynthesis and function. Prog. Lipid Res. 44, 235–58. https://doi.org/10.1016/j.plipres.2005.05.002

Thompson, E.W., Lane, B.G., 1980. Relation of protein synthesis in imbibing wheat embryos to the cell-free translational capacities of bulk mRNA from dry and imbibing embryos. J. Biol. Chem. 255, 5965–70.

Thomson, A.B., Keelan, M., Thiesen, A., Clandinin, M.T., Ropeleski, M., Wild, G.E., 2001. Small bowel review: normal physiology part 1. Dig. Dis. Sci. 46, 2567–87.

Tian, D.M., Cheng, H.Y., Jiang, M.M., Shen, W.Z., Tang, J.S., Yao, X.S., 2016. Cardiac Glycosides from the Seeds of Thevetia peruviana. J. Nat. Prod. 79, 38–50. https://doi.org/10.1021/acs.jnatprod.5b00611

Uberto, R., Moomaw, E.W., 2013. Protein Similarity Networks Reveal Relationships among Sequence , Structure , and Function within the Cupin Superfamily 8. https://doi.org/10.1371/journal.pone.0074477

Viana, C.A., Oliveira, J.S., Freitas, C.D.T., Alencar, N.M.N., Carvalho, C.P.S., Nishi, B.C., Ramos, M. V, 2013. Thrombin and plasmin-like activities in the latices of Cryptostegia grandiflora and Plumeria rubra. Blood Coagul. Fibrinolysis 24, 386–92. https://doi.org/10.1097/MBC.0b013e32835d540b

Vieille, C., Zeikus, G.J., 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol. Mol. Biol. Rev. 65, 1–43. https://doi.org/10.1128/MMBR.65.1.1-43.2001

Vierstra, R.D., 1996. Proteolysis in plants: mechanisms and functions. Plant Mol. Biol. 32, 275–302. https://doi.org/10.1007/BF00039386

Woo, E.-J., Dunwell, J.M., Goodenough, P.W., Pickersgill, R.W., 1998. Barley oxalate oxidase is a hexameric protein related to seed storage proteins: evidence from X-ray crystallography. FEBS Lett. 437, 87–90. https://doi.org/10.1016/S0014-5793(98)01203-4

Woo, E., Dunwell, J.M., Goodenough, P.W., Marvier, A.C., Pickersgill, R.W., 2000. Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. Nat. Struct. Biol. 7.

Woo, E., Marshall, J., Bauly, J., Chen, J., Venis, M., Napier, R.M., Pickersgill, R.W., 2002. Crystal structure of auxin-binding protein 1 in complex with auxin 21.

Zhu-Salzman, K., Luthe, D.S., Felton, G.W., 2008. Arthropod-inducible proteins: broad spectrum defenses against multiple herbivores. Plant Physiol. 146, 852–8. https://doi.org/10.1104/pp.107.112177

Structural and enzymatic characterization of Peruvianin-I, the first germin-like protein with proteolytic activity

ABSTRACT

The germin-like protein (GLP) purified from *Thevetia peruviana*, Peruvianin-I, is the only one described as having proteolytic activity. Therefore, the goal of this study was to investigate the structural features responsible for its enzymatic activity. Although the amino acid sequence of Peruvianin-I showed high identity with other GLPs, it exhibited punctual mutations, which were responsible for the absence of oxalate oxidase activity. The phylogenetic analysis showed that Peruvianin-I does not belong to any classification of GLP subfamilies. Moreover, Peruvianin-I contains a catalytic triad found in all plant cysteine peptidases. Molecular docking simulations confirmed the role of the catalytic triad in its proteolytic activity. Synchrotron radiation circular dichroism assays confirmed that Peruvianin-I was stable at pH ranging from 5.0 to 8.0 and that it presented significant structural changes only above 60 °C. The addition of iodoacetamide caused changes in its native conformation, but only a slight effect was observed after adding a reducing agent. This study reports an unusual protein with germin-like structure, lacking typical oxalate oxidase activity. Instead, the proteolytic activity observed suggests that the protein is a cysteine peptidase. These structural peculiarities make Peruvianin-I an interesting model for further understanding of the action of laticifer fluids in plant defense.

Keywords: glp; laticifer; oxalate oxidase

INTRODUCTION

Germins are highly conserved plant proteins that exhibit oxalate oxidase (OxO) activity, responsible for producing H_2O_2, a mediator of oxidative burst and cellular signaling, which suggests their defensive roles against biotic and abiotic stresses [1,2]. The term germins comes from the fact they were first identified from germinated wheat grains. To date, they have been described only in cereals [3]. However, proteins similar to germins have been identified, called germin-like proteins (GLPs).

GLPs have been reported in monocots, dicots, gymnosperms, and mosses [4,5]. They are very heterogeneous in amino acid sequences and this diversity can explain, at least in part, their different biochemical properties, including serine protease inhibition, ADP- glucose pyrophosphatase/phosphodiesterase, and polyphenol oxidase activity [6–8]. Recently, a new member of the germin-like protein (GLP) group, named Peruvianin-I, was identified in *Thevetia peruviana* latex. This protein was purified and characterized as a GLP due to the high identity shared with other GLPs in its N-terminus region. Interestingly, Peruvianin-I presented striking proteolytic activity, which had never been described before for any other GLP [9].

Peptidases are a group of proteins that are able to catalyze the hydrolytic cleavage of peptide bonds in proteins and peptides [10]. In plants, the biological roles of peptidases are related to senescence, initiation of cell death, protein mobilization, and seed germination [11]. In addition, peptidases can also participate in defense mechanisms against aggressors such as fungi and insects [12,13].

Considering that the GLP from *T. peruviana* latex exhibits proteolytic properties, but lacks oxalate oxidase activity, the purpose of the study was to investigate in detail its three-dimensional structure, in an at- tempt to understand this peculiar enzymatic activity. Peruvianin-I

cDNA was cloned, sequenced, and its three-dimensional model was predicted by homology modeling, for comparison with other germins and GLPs. The active site of Peruvianin-I was analyzed by molecular docking using oxalate oxidase substrate or peptidase inhibitor. Finally, the protein was purified and its secondary structure was characterized by synchrotron radiation circular dichroism spectroscopy and its enzymatic kinetics was compared to another standard peptidase.

MATERIAL AND METHODS

RNA extraction, cDNA synthesis and 3′RACE PCR

Thevetia peruviana leaves were collected, washed with distilled water and ground in liquid nitrogen to yield a fine powder. Then, the total RNA was isolated using the RNeasy Mini kit according to the manufacturer's instructions (Qiagen, Germany). The RNA was analyzed by agarose gel electrophoresis and quantified by absorbance at 260 nm. The cDNAs were synthesized from the DNA-free total RNA previously extracted by using the ImProm-II Reverse Transcription System (Promega, USA) and the 3′ RACE adapter (5′-GCGAGCACAGAATTAATACGACTCACTATAGG(T)12VN-3′), as described by the manufacturer (ThermoFisher Scientific, USA).

The PCR assays were performed by using the 3′ RACE outer primer (5′-GCGAGCACAGAATTAATACGACT-3′) and the specific primer designed for Peruvianin-I, the GLP from *T. peruviana* latex (5′-CCGG GCYGATCCWGGTCCHTTRCARGA-3′), called PeruvF. The N-terminus amino acid sequence of Peruvianin-I (ADPGPLQDF) and other GLPs were used to construct PeruvF (forward primer). The amplification re- actions were performed in a final volume of 25 µl containing the first strand cDNA (900 ng), 200 µM dNTP, 1.5 mM MgCl2, 0.5 Mm primer, 2 U Taq DNA Polymerase (GE Healthcare Life Sciences, USA) and 10× the

reaction buffer (GE Healthcare Life Sciences, USA). The PCR cycles were performed under the following conditions: an initial denaturation step of 2 min at 95 °C and, sequentially, 32 cycles of 45 s at 95 °C, 45 s at different temperatures (45–65 °C), and 1.5min at 72 °C. Finally, after the last cycle the reaction mixture was incubated for 5 min at 72 °C and cooled to 4 °C. The reaction products were visualized by 1% (v/v) agarose gel electrophoresis stained with ethidium bromide (0.8 mg/ml).

Cloning and sequence analysis

PCR products were excised from the agarose gels, purified using the DNA Gel Extraction Column kit (Promega, USA), ligated into the pGEMT Easy vector usingT4DNA ligase (Promega, USA), and then used to transform electrocompetent DH5α Escherichia coli cells by electroporation. The plasmid DNAs were isolated from the antibiotic resistant colonies using the NucleoSpin Plasmid kit (Macherey Nagel, Germany) and sequenced by Macrogen Inc. (Seoul, South Korea) using the primers T7 promoter and SP6. The software package Phred-Phrap-Consed-Polyphred (PPCP) was used to produce a unique consensus sequence encoding one distinct polypeptide chain [14–16].

The amino acid sequences obtained were analyzed by multiple alignments with the Clustal-W software [17] and the similarities with other proteins were determined using BLASTp [18]. The theoretical molecular mass and isoelectric point (pI) were evaluated using the ExPASy ProtParam Proteomics Server [19] and the presence of disulfide bonds was predicted by the DIANNA web server [20]. Finally, the prediction of N-glycosylation sites was evaluated using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/).

Phylogenetic tree and 3D models

Amino acid sequences were aligned using ClustalW with the following parameters: gap opening penalty 10 and gap extension penalty 0.2 [17]. These sequences were used to construct the phylogenetic tree employing the neighbor joining method by the MEGA 7.0 program [21]. Several plant GLP sequences were used to compare the different germin groups (Supplementary Table 1).

The protein models were formulated using different platforms: Swiss Model (https://swissmodel.expasy.org/), GalaxyWeb (http:// galaxy.seoklab.org/) and M4T server v. 3.0 (manaslu. aecom.yu.edu/ M4T/) [22–24]. The crystal structure of Hordeum vulgare germin (PDB number: 1FI2) was selected as a template [25] to build the three- dimensional models of Peruvianin-I, because both proteins share 41% sequence identity. The models were analyzed from their geometric and stereochemical quality using the PROCHECK [26] and WHAT IF [27] programs. The PyMOL software was used to analyze and visualize the three-dimensional models generated (http://pymol.org/).

Molecular docking

AutoDock 4.2 and AutoDockVina were utilized to perform the molecular docking analysis [28]. The gridmapsof40 Å × 40 Å × 40Å were centered on the possible oxalate oxidase or proteolytic activity sites of Peruvianin-I and calculated with the AutoGrid software using germin (1FI2) and papain (1PPN) structures as templates. The molecular structures of iodoacetamide (IAA, a specific cysteine peptidase inhibitor) and oxalate (a specific substrate for oxalate oxidase activity) were obtained from the Pubchem Substance Database and used for docking calculations. Iodoacetamide and oxalate exhibited free rotation, while the protein was held rigid. The ten best

structures were analyzed and ranked according to the predicted binding affinity (expressed in kcal/mol). Three-dimensional images of the interactions between ligands and the proteins were prepared using the PyMOL software.

Synchrotron radiation circular dichroism (SRCD) spectroscopy analysis

All spectrometric analyses were performed with the native Peruvianin-I, which was purified, and its proteolytic activity was con- firmed by enzymatic assays using 1% azocasein as substrate at pH 6.0 [9,29]. SRCD spectroscopy was employed to investigate the structure of Peruvianin-I (instead of the conventional circular dichroism method), be- cause of the ability to measure lower wavelength data and the improved signal-to-noise ratio of the technique. These data allow higher accuracy in determining the secondary structure of proteins with low content of helix and high content of beta conformation [30]. The SRCD spectra of Peruvianin-I (0.66 mM) in aqueous solutions were collected at the AUCD beamline of the ASTRID2 synchrotron (Aarhus, Denmark), taking three successive scans over the wavelength range from 170 to 270 nm, in 1 nm intervals, using a 98.6 μm path length Suprasil quartz cuvette at 25 °C.

Additionally, a dehydrated film of Peruvianin-I (0.7 nM) was obtained on the surface of a quartz plate, by depositing the protein solution on the plate and keeping it under vacuum overnight. The SRCD spectra of the dehydrated films were obtained from 280 to 155 nm, at 25 °C, taking four different rotations on the plate (0°, 90°, 180°, and 270°) in order to avoid any linear dichroism effect. Protein stability was investigated at different pH levels by incubating protein for 30 min in buffers: 20mM sodium acetate (pH4.0 and 5.0) or 20 mM sodium phosphate (pH 6.0, 7.0, and 8.0) in the presence of 1mM dithiothreitol (DTT), a reducing agent and activator of

cysteine peptidases, and taking the respective SRCD spectra. Protein at pH 6.0 was also incubated at temperatures ranging from 20 to 90 °C, in 10 °C steps, allowing 5 min equilibration at each temperature, and taking three scans at each point.

All SRCD spectra were processed using the CDTools software [31] and consisted of averaging the individual scans, subtracting the respective averaged baseline (solution containing all components of the sample, except the protein), smoothing with the Savitzky-Golay filter, zeroing at 263–270 nm, and expressing the final SRCD spectra in delta epsilon units, using a mean residual weight of 110.9. Estimation of Peruvianin-I secondary structure content was performed with the Dichroweb server [32].

To assess the role of the disulfide bridge in maintaining the secondary structure of Peruvianin-I, different concentrations of DTT (1–10 mM) were incubated at pH 6.0 with the protein at 25 °C. The influence of iodoacetamide (IAA), a cysteine peptidase inhibitor, on Peruvianin-I's structural conformation was also investigated under the same conditions. In this case, Peruvianin-I was evaluated for its structural behavior, either in its native (free) form or complexed with IAA (10mM) under reducing conditions (1 mM DTT) at pH 6.0.

Histochemical detection of oxalate oxidase (OxO) activity OxO

OxO activity in situ was detected using the procedure of Dumas et al. [33]. Leaves of *T. peruviana*, approximately 8×2 cm, were incubated in a bleach solution (0.15% TCA, 75% ethanol, 25% chloroform) for 60 min, washed with distilled water, and immersed in an activation solution (40 mM succinate buffer, pH 4.0, containing 60% ethanol, 3 mM oxalic acid, 0.1 mg/ml 4-chloro-1-naphthol and 3 mM EDTA) for 60 min at 37 °C. Oryza sativa leaves were used as

positive control for oxalate oxidase activity [34]. The appearance of blue spots in the plant tissue evidenced the oxalate oxidase activity, which was easily observed using a light microscope.

Kinetic parameters for proteolytic activity

The kinetic parameters of Peruvianin-I peptidase activity were determined at 37 °C, using Nα-Benzoyl-DL-arginine β-naphthylamide hydrochloride (BANA)(Sigma, Brazil) as specific substrate for cysteine peptidases. The reaction mixture consisted of 20 µg of Peruvianin-I (20 µl, 1mg/ml in 50mMsodiumphosphate buffer, pH 6.0) and a final con- centration of BANA ranging from 0.1 to 0.5 mM, in 50 mM sodium phosphate buffer (pH 6.0), containing 1 mM DTT. The final volume of the reaction was 500 µl. After 30 min at 37 °C, the reaction was stopped by adding 500 µl of 2% HCl in ethanol and 500 µl of 0.06% 4-(dimethyl-amino) cinnamaldehyde (Sigma, Brazil). After 40 min, the resulting yellow color was measured by absorbance at 540 nm [29]. All assays were performed in triplicate. Vm, Km and kcat were calculated using linear regression analysis based on the Lineweaver–Burk plot. Purified papain (a cysteine peptidase from Carica papaya latex) was used as control [35]. Statistical significance was calculated by the paired t-test ($p < 0.05$) using the GraphPad Prism 6 program.

RESULTS AND DISCUSSION

Sequence analysis of Peruvianin-I

All 10 cDNA clones obtained by 3′ RACE PCR encoded only one polypeptide sequence of 202 amino acids without signal peptide, because the forward primer used was based on the N-terminus of the mature Peruvianin-I (Fig. 1). The sequences of other germin-like proteins that share the highest identity with Peruvianin-I are shown in Fig. 1. Similar to other GLPs,

Peruvianin-I has two cysteine residues located close to its N-terminal, which are involved in the formation of a disulfide bond [36,37]. Interestingly, another cysteine residue (Cys77) is present solely in the Peruvianin-I sequence, while all the other GLPs have predominantly a leucine or valine at the same position (Fig. 1).

Most of the germins and GLPs have highly conserved amino acid residues, located in regions denominated germin boxes A, B and C [38]. As shown in Fig. 1, Peruvianin-I exhibited these three conserved se- quences. However, two His and one Glu, which are highly conserved in germin box B, are replaced in Peruvianin-I by Arg, Asn and Ser, respectively. On the other hand, other amino acids in box C, including His, were conserved in Peruvianin-I (Fig. 1). The His and Glu in box B and the His in box C are fundamental for oxalate oxidase activity of germins [39].

Two putative N-glycosylated sites were predicted in Peruvianin-I, at positions Asn55 and Asn144, using in silico analysis (Fig. 1). Similarly, two N-glycosylation sites were predicted in *Calotropis procera* GLPs (Asn41, Asn57) and wheat germin (Asn47, Asn52)[40,41]. This in silico analysis was confirmed by previous results, since Peruvianin-I has been described as a glycoprotein with a carbohydrate content of almost 22% [9]. Asparagine N-linked glycosylation is the best known co- and post- translational modification of secretory proteins [42], and N-glycosylation is essential for many biological processes, including expression and folding of glycoprotein, glycan-dependent quality control processes in the endoplasmic reticulum (ER), and protein–protein interactions [43]. However, the specific role played by glycosylation in GLPs is still unknown.

Peruvianin-I presented a mature sequence of 202 amino acids, predicted molecular mass of 21.85 kDa and isoelectric point (pI) of 5.58 (Supplementary Table 1). These values are in concordance with those described previously [9], since analysis by SDS-PAGE showed that

Peruvianin-I had an apparent molecular mass of20kDa, which was confirmed by mass spectrometry, which only detected peaks of approximately 20,522 Da [9]. Two-dimensional gel electrophoresis of Peruvianin-I indicated the presence of spots with molecular masses around 20 kDa and pI values between 4.0 and 5.0 [9]. These biochemical characteristics were quite similar to several other GLPs, in which the mature amino acid sequences (without signal peptides) ranged from 189 to 207 amino acids, molecular masses from 18.2 to 22.7 kDa and pI from 5.45 to 8.03 (Supplementary Table 1).

Structural analysis of several germins and GLPs permitted grouping them into ten different clades/subfamilies, namely GER 1, GER 2, GER 3, GER 4, GER 5, GER 6, GER 7, GER 8 and bryophyte subfamilies 1 and 2 [38]. The phylogenetic analysis of Peruvianin-I and several germins and GLPs (Supplementary Table 2), representing all clades/subfamilies, showed that Peruvianin-I did not belong to any classification previously described (Fig. 2). When the three amino acids from box B in Peruvianin- I (Arg, Asn and Ser) were replaced by two His and one Glu (amino acids conserved in germin and GLPs), Peruvianin-I was included in clade GER 2, similar to GLPs from *C. procera* latex [40]. These results show that Peruvianin-I should be classified in a new clade/subfamily of GLPs. Therefore, we suggest a new clade/subfamily named GER 9, in which GLPs exhibiting activities other than oxalate oxidase should be gathered, such as serine protease inhibition, ADP-glucose pyrophosphatase/phosphodiesterase and polyphenol oxidase activity, besides proteolytic activity [6–9].

Prediction of the active site for oxalate oxidase activity in Peruvianin-I

The three-dimensional models of Peruvianin-I were obtained by homology modeling using *H. vulgare* germin (PDB number: 1FI2) as a template. The Ramachandran plot and the analysis performed by the PROCHECK and WHAT IF servers showed that the best models were those generated by the GalaxyWeb platform (Supplementary Table 3). Structural comparisons between the overall structure of Peruvianin-I and the template revealed a RMSD of 3.428 Å. Crystallographic studies of barley germin showed that oxalate oxidase activity requires a manganese center buried in its β-barrel jellyroll domain which is bound by the side chains of three histidines and one glutamate residue (His88, His90, Glu95 and His137) (Fig. 3A), forming a trigonal bipyramidal geometry [25]. Specifically, for the active site the Peruvianin-I three-dimensional model and germin crystal structure showed RSMD of 0.504 Å. The manganese ion was not bound in the Peruvianin-I β-barrel jellyroll domain, because it has the residues Arg88, Asn90, Ser95 and His135 instead of His88, His90, Glu95 and His135 (Fig. 3B). In another study, in silico analysis showed that the exchange of one histidine (His102) by one tyrosine, in germin box B of SlGLP (*Solanum lycopersicum*), modified the architecture of the active site for Mn2+. Consequently, the protein did not exhibit oxalate oxidase activity [44]. These results provide further evidence that the three histidines and glutamate are essential for oxalate oxidase activity, being responsible for correct interaction of the active site of the oxalate substrate [36]. These findings corroborate our previous results that showed Peruvianin-I does not have oxalate oxidase activity *in vitro* [9].

Molecular docking calculations using the oxalate substrate were also performed to better understand the function of four conserved amino acids in germins (His, His, Glu and His) for oxalate oxidase activity. A careful inspection of the active site of barley germin (1FI2) indicated

that oxalate substrate adapted itself in a cage surrounded by His88, His90, Glu95 and His137, with calculated interaction energy of −4.1 kcal·mol^{-1} (Fig. 3C). As expected, the oxalate ligand interacted strongly with residues responsible for manganese ion binding. The two histidines (His88 and His90) present in the central cores of the ß- barrel of barley germin formed two hydrogen bonds with oxalate. The bonds were between the OH group of oxalate and the N atoms from imidazole rings of His88 (2.0 Å) and His90 (2.6 Å) (Fig. 3C). By comparison, the oxalate substrate was docked only at an α-helical C-terminus domain of Peruvianin-I (Fig. 3D). As pointed out before, three residues present in the active site of barley germin were not conserved in Peruvianin-I (Fig. 1), and these changes undoubtedly influenced the binding pattern with the oxalate substrate. The hydrophobic surrounding of the active site of germin is also very important for oxalate oxidase activity [45]. Substitution of the Val77 and Phe153 residues present in barley germin (1FI2) by less hydrophobic residues (Cys77 and Leu151) in the Peruvianin-I also negatively influenced the interaction with the oxalate. Accordingly, it was shown that the decrease of nonpolar residues in the active site of Arabidopsis GLP significantly decreased its interaction with the oxalate [45].

Thevetia peruviana leaves were used to detect possible in situ activity of oxalate oxidase, since purified Peruvianin-I can lose its activity during the purification process [9]. No oxalate oxidase activity was detected in *T. peruviana* leaves, in contrast to *Oryza sativa* leaves, used as positive control (Fig. 4). This result corroborates those from in silico analysis and supports that Peruvianin-I does not have oxalate oxidase activity.

Characterization of the active site for proteolytic activity of Peruvianin-I

Although previous results showed that Peruvianin-I is a cysteine peptidase, its structural characterization was not performed [9]. Based on the primary and tertiary structures of Peruvianin-I, it was possible to observe that it contains the same catalytic triad (Cys77, Asn90 and His135) present in the structure of several cysteine peptidases, including papain (Cys25, His159 and Asn175) (Fig. 5). Interestingly, these results show that Peruvianin-I underwent punctual mutations, which were responsible for the loss of oxalate oxidase activity while at the same time exhibiting proteolytic activity (Fig. 1). The structural overlap shows that the active-site architecture and the spatial arrangement of amino acid residues involved in enzyme catalysis of papain are reasonably similar to Peruvianin-I residues (Fig. 5C), which are located within its β-barrel jellyroll domain (Fig. 5B).

Most plant cysteine peptidases belong to the papain (C1) and legumain (C13) families. All these peptidases have a nucleophilic cysteine thiol in their catalytic triad (Cys, His, and Asn) [46]. Interestingly, Peruvianin-I presents a similar spatial arrangement of the catalytic triad responsible for this proteolytic activity, despite having completely different structural domains than proteins belonging to the papain family. The molecular docking analysis between cysteine peptidase inhibitor (iodoacetamide, IAA) and papain (1PPP) showed that IAA forms hydrogen bonds of 2.2 Å and 2.9Å with Gln19 andHis159 residues, respectively, as well as having interaction energy of −3.5 kcal·mol−1 (Fig. 6A). In Peruvianin-I, there was one hydrogen bond between the nitrogen of the imidazole ring of His135 and IAA, with a mean distance of 2.6 Å, and another one between IAA and the residue of Cis77,with total energy of −2.9 kcal·mol−1 (Fig. 6B). This interaction was confirmed when the proteolytic activity of Peruvianin-I was totally and specifically inhibited by IAA [9]. The interaction between IAA and cysteine peptidases generates an alkylation

reaction between the iodine group of the inhibitor and the sulfur group of the cysteine residue of the catalytic triad, forming a stable thioether bond [47]. This reaction results in a very stable enzyme–inhibitor complex, which is responsible for irreversible inhibition of peptidases [9].

The proteolytic activity of Peruvianin-I and papain followed Michaelis-Menten kinetics (Fig. 7). The km of Peruvianin-I, using BANA as substrate, was 0.237 ± 0.05 mM, almost four times smaller than papain, 0.809 ± 0.08 mM (p b 0.05). The Vmax and Kcat values were 0.54 × 10−9 M·s−1 and 0.1 ± 0.02 s−1 and 9.2 × 10−9 M·s−1 and 2 ± 0.05 s−1 for Peruvianin-I and papain, respectively (p b 0.05). The catalytic efficiency of papain (Kcat/Km =2.4 ×104) was about 60- fold higher than that of Peruvianin-I (Kcat/Km = 4.2 × 102). Although Peruvianin-I has the same catalytic triad, its overall and active site structures are very different from most plant cysteine peptidases, including papain. In addition, the presence of polar residues such as Ser95 and Arg88 possibly decreased the hydrophobic interaction between the catalytic site of Peruvianin-I and the hydrophobic rings of BANA. Accordingly, replacing some amino acids at the Ervatamin C active site drastically altered its catalytic efficiency [48,49]. These results clearly demonstrate that the kinetic performance of an enzyme is not strictly related to the amino acid composition of its catalytic clefts, but is closely related to the protein's adjacent residues and overall structure.

A previous study showed that Peruvianin-I exhibited no antifungal activity on different phytopathogens and that the lack of antifungal effect could be correlated to its low proteolytic activity compared to other (latex) antifungal cysteine peptidases, such as papain [9]. As pointed out before, the low proteolytic activity may be associated with its unusual germin-like tridimensional structure, which is far from the typical structure of cysteine peptidases so far described. Latex cysteine peptidases have also been related to plant defense against insects [50].

Therefore, the possible role played by Peruvianin-I in latex physiology is unclear and deserves more research.

Effect of pH, temperature, reducing agent and peptidase inhibitor on structure of Peruvianin-I

The SRCD spectrum of Peruvianin-I in aqueous solution (pH6.0) was typical of a β-rich protein, displaying a broad negative band with small magnitude at 217 nm, and a large positive maximum at 197 nm. Since the SRCD method was employed, an additional large negative band was observed at 175 nm, which is also characteristic of the β-strand structure. These spectral features are commonly observed in proteins with elevated concentrations of β-sheet segments [51,52]. Similar SRCD spectra were observed for native Peruvianin-I independent of pH conditions (Fig. 8A). Although at pH 4.0 a small increase in the peak at 197 nm was observed, the protein's structural content was un- changed from pH 5.0 to 8.0.

The estimation of the secondary structural content of Peruvianin-I from its SRCD spectrum at 25 °C was 35% organized in β-strands, 40% disordered, plus a small content of α-helices (~9%). These results are similar to previous results of molecular modeling (Fig. 3), and are in agreement with the structural content observed in germins and GLPs [25,36,53], which are usually characterized by a beta-barrel core structure.

The SRCD spectra of the Peruvianin-I subjected to the temperature denaturation assay showed only slight changes in the range from 20 to 50 °C (Fig. 8B), revealing protein thermal stability within these temperatures. But band shifts and a significant signal reduction were seen between 60 and 70 °C, and the protein assumed a completely unfolded state at 80 and 90 °C. These

results corroborate those from in vitro as- says, where Peruvianin-I was 100% active until 45 °C and then was completely inactive at 65 °C [9].

The presence of the reducing agent DTT at concentrations of 1–10 mM (Fig. 8C) produced the same discrete change in the SRCD spectra of Peruvianin-I, with an increase of the peak at 197 nm and narrowing of the negative band at 217 nm, without changing the spectrum's typical profile. These changes can be associated with the disruption of the disulfide bridge located in the protein's N-terminus portion (Fig. 1). Because the disulfide bond is located far from the active site, its disruption did not significantly change the protein structure and its proteolytic activity remained stable [9].

In addition to analysis of SRCD in solution, the deposition of Peruvianin-I on partially dehydrated films allowed collecting data at even smaller wavelengths, close to 155 nm, adding more transitions to be monitored and further converted into structural information. The SRCD spectra of the Peruvianin-I on the film kept all the spectral bands seen in the solution and showed the full negative peak at 175 nm and a positive peak at 160 nm, both in the presence of the beta-strands. The SRCD spectra of Peruvianin-I on the film in the presence of DTT or DTT and IAA are also shown in Fig. 8D. Once again, it can be seen that DTT alone did not cause any significant structural change in Peruvianin-I. However, when IAA was added, a drastic reduction of the peaks attributed to the beta-content was seen, revealing a loss of its native conformation that affected its enzymatic activity. Similar spectroscopic studies performed with papain revealed that the alkylation of the thiol group in its active site caused changes in its secondary structure [54]. In another study, the use of iodoacetamide (IAA) modified the structure of ficin, a cysteine peptidase, and also inhibited its activity irreversibly, generating large amounts of protein aggregate [55].

Several studies have shown that iodoacetamide can cause rapid aggregation of cellular proteins [56].

Conclusion

Peruvianin-I is the only germin-like protein (GLP) described as having proteolytic activity. We showed that it has primary, secondary and tertiary structures very similar to other germins and GLPS. However, it underwent punctual mutations in three amino acid residues, which were responsible for the absence of the oxalate oxidase activity. On the other hand, some of these mutations together with the presence of a single free cysteine residue were responsible for forming a catalytic triad, which was highly conserved in papain-like cysteine peptidases. SRCD results confirmed the native state of Peruvianin-I and its classification as a β-rich protein, and agreed with previous proteolytic assays, in which Peruvianin-I exhibited thermostability until 50 °C and an optimal pH stability ranging from 5.0 to 7.0. Furthermore, the protein interacted with and had its secondary structure significantly changed in the presence of a cysteine peptidase inhibitor. The involvement of Peruvianin-I in latex functionalities still deserves more investigation. The structure of the protein reveled highly punctual replacements of key amino acid residues, suggesting shift of protein activity. Whether these new features are involved in new physiological roles remains to be answered.

Conflict of interest

The authors confirm that the contents of this article pose no conflicts of interest.

Contributions

WTC, MVR, BAMR, EHSB and CDTF performed the main research work, including peptidase purification, enzymatic assays and bioinfor-matic analyses. WTC, MZRS, JSO, DCF and TBG performed RNA isolation, cDNA synthesis, amplification and cloning. JLSL performed all the SRCD assays. All authors contributed to data analysis, discussion and writing of the manuscript.

Funding

This work was supported by grants from the following Brazilian agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq): grants 303513/2016-0 and 406429/2016-2 to JLSL; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP). We are grateful for the access to the AU-CD beamline on ASTRID2 at ISA Synchrotron (Aarhus, Denmark) (to JLSL). This study is part of the consortium “Molecular Biotechnology of Plant Latex”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.01.023.

REFERENCES

[1] V.C. Beracochea, N.I. Almasia, L. Peluffo, V. Nahirñak, E.H. Hopp, Sunflower germin- like protein HaGLP1 promotes ROS accumulation and enhances protection against fungal pathogens in transgenic Arabidopsis thaliana, Plant Cell Rep. 34 (2015) 1717–1733, https://doi.org/10.1007/s00299-015-1819-4.

[2] Y. Zhang, X. Wang, X. Chang, M. Sun, Y. Zhang, W. Li, Y. Li, Overexpression of germin-like protein GmGLP10 enhances resistance to Sclerotinia sclerotiorum in transgenic tobacco, Biochem. Biophys. Res. Commun. 497 (2018) 160–166, https://doi.org/10.1016/j.bbrc.2018.02.046.

[3] R.M. Davidson, P.A. Reeves, P.M. Manosalva, J.E. Leach, Germins: a diverse protein family important for crop improvement, Plant Sci. 177 (2009) 499–510, https:// doi.org/10.1016/j.plantsci.2009.08.012.

[4] J. Breen, M. Bellgard, Germin-like proteins (GLPs) in cereal genomes: gene cluster- ing and dynamic roles in plant defence, Funct. Integr. Genomics 10 (2010) 463–476, https://doi.org/10.1007/s10142-010-0184-1.

[5] M. Ilyas, A. Rasheed, Functional characterization of germin and germin-like protein genes in various plant species using transgenic approaches, Biotechnol. Lett. 38 (2016) 1405–1421, https://doi.org/10.1007/s10529-016-2129-9.

[6] A.Y. Mansilla, C.I. Segarra, R.D. Conde, Structural and functional features of a wheat germin-like protein that inhibits trypsin, Plant Mol. Biol. Report. 30 (2012) 624–632, https://doi.org/10.1007/s11105-011-0372-8.

[7] M. Rodriguez-Lopez, E. Baroja-Fernandez, A. Zandueta-Criado, B. Moreno-Bruna, F.J. Munoz, T. Akazawa, J. Pozueta-Romero, Two isoforms of a nucleotide-sugar pyrophosphatase/phosphodiesterase from barley leaves (Hordeum vulgare L.) are distinct oligomers of HvGLP1, a germin-like protein, FEBS Lett. 490 (2001) 44–48, https://doi.org/10.1016/S0014-5793(01)02135-4.

[8] X. Cheng, X. Huang, S. Liu, M. Tang, W. Hu, S. Pan, Characterization of germin-like protein with polyphenol oxidase activity from Satsuma mandarine, Biochem. Biophys. Res. Commun. 449 (2014) 313–318, https://doi.org/10.1016/j.bbrc.2014. 05.027.

[9] C.D.T. Freitas, W.T. Cruz, M.Z.R. Silva, I.M. Vasconcelos, F.B.M.B. Moreno, R.A. Moreira, A.C.O. Monteiro-Moreira, L.M.R. Alencar, J.S. Sousa, B.A.M. Rocha, M.V. Ramos, Prote- omic analysis and purification of an unusual germin-like protein with proteolytic activity in the latex of Thevetia peruviana, Planta 243 (2016) 1115–1128, https:// doi.org/10.1007/s00425-016-2468-8.

[10] N.D. Rawlings, Biochimie peptidase specificity from the substrate cleavage collection in theMEROPS database and a tool tomeasure cleavage site conservation, Biochimie 122 (2016) 5–30, https://doi.org/10.1016/j.biochi.2015.10.003.

[11] A. Schaller, A cut above the rest: the regulatory function of plant proteases, Planta 220 (2004) 183–197, https://doi.org/10.1007/s00425-004-1407-2.

[12] K. Ekchaweng, E. Evangelisti, S. Schornack, M. Tian, N. Churngchow, The plant de- fense and pathogen counter defense mediated by Hevea brasiliensis serine protease HbSPA and Phytophthora palmivora extracellular protease inhibitor PpEPI10, PLoS One 12 (2017), e0175795. https://doi.org/10.1371/journal.pone.0175795.

[13] M.K. Jashni, R. Mehrabi, J. Collemare, C.H. Mesarich, P.J.G.M. deWit, The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant – pathogen, Front. Plant 6 (2015) 1–7, https://doi.org/10.3389/fpls.2015.00584.

[14] B. Ewing, P. Green, Base-calling ofautomated sequencer traces using Phred. II. Error probabilities, Genome Res. 8 (1998) 186–194, https://doi.org/10.1101/gr.8.3.186.

[15] B. Ewing, L. Hillier, M.C. Wendl, P. Green, Base-calling ofautomated sequencer traces using Phred. I. Accuracy assessment, Genome Res. 8 (1998) 175–185, https://doi. org/10.1101/gr.8.3.175.

[16] D. Gordon, C. Abajian, P. Green, Consed: a graphical tool for sequence finishing, Ge- nome Res. 8 (1998) 195–202, https://doi.org/10.1101/gr.8.3.195.

[17] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position- specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.

[18] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410, https://doi.org/10.1016/S0022-2836 (05)80360-2.

[19] M.R. Wilkins, E. Gasteiger, A. Bairoch, J.C. Sanchez, K.L. Williams, R.D. Appel, D.F. Hochstrasser, Protein identification and analysis tools in the ExPASy server, Methods Mol. Biol. 112 (1999) 531–552.

[20] F. Ferrè, P. Clote, DiANNA: a web server for disulfide connectivity prediction, Nucleic Acids Res. 33 (2005) W230–W232, https://doi.org/10.1093/nar/gki412.

[21] S. Kumar, G. Stecher, K. Tamura, MEGA: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2017) 1870–1874, https://doi. org/10.1093/molbev/msw054.

[22] L. Bordoli, F. Kiefer, K. Arnold, P. Benkert, J. Battey, T. Schwede, Protein structure homology modeling using SWISS-MODEL workspace, Nat. Protoc. 4 (2009) 1–13, https://doi.org/10.1038/nprot.2008.197.

[23] J. Ko, H. Park, L. Heo, C. Seok, GalaxyWEB server for protein structure prediction and refinement, Nucleic Acids Res. 40 (2012) 294–297, https://doi.org/10.1093/nar/ gks493.

[24] N. Fernandez-fuentes, C.J. Madrid-aliste, B.K. Rai, J.E. Fajardo, A. Fiser, M4T: a comparative protein structure modeling server, Nucleic Acids Res. 35 (2007) 363–368, https://doi.org/10.1093/nar/gkm341.

[25] O. Opaleye, R.S. Rose, M.M. Whittaker, E.J. Woo, J.W. Whittaker, R.W. Pickersgill, Structural and spectroscopic studies shed light on themechanismofoxalate oxidase, J. Biol. Chem. 281 (2006) 6428–6433, https://doi.org/10.1074/jbc.M510256200.

[26] R.A. Laskowski, M.W. Macarthur, D.S. Moss, J.M. Thornton, PROCHECK: a program to check the stereochemical quality ofprotein structures, J. Appl. Crystallogr. 26 (1993) 283–291.

[27] G. Vriend, WHAT IF: a molecular modeling and drug design program, J. Mol. Graph. 8 (1990) 52–56.

[28] O. Trott, A.J. Olson, AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading, J. Comput. Chem. 31 (2011) 455–461, https://doi.org/10.1002/jcc.21334.AutoDock.

[29] C.D.T. Freitas, J.S. Oliveira, M.R.A. Miranda, N.M.R. Macedo, M.P. Sales, L.A. Villas- Boas, M.V. Ramos, Enzymatic activities and protein profile of latex from Calotropis procera, Plant Physiol. Biochem. 45 (2007) 781–789, https://doi.org/10.1016/j. plaphy.2007.07.020.

[30] P.S. Kumagai, R. Demarco, J.L.S. Lopes, Advantages of synchrotron radiation circular dichroism spectroscopy to study intrinsically disordered proteins, Eur. Biophys. Soc. Assoc. 46 (2017) 599–606, https://doi.org/10.1007/s00249-017-1202-1.

[31] J.G. Lees, B.R. Smith, F. Wien, A.J. Miles, B.A. Wallace, CDtool — an integrated soft- ware package for circular dichroism spectroscopic data processing, analysis, and ar- chiving, Anal. Biochem. 332 (2004) 285–289, https://doi.org/10.1016/j.ab.2004.06. 002.

[32] L. Whitmore, B.A. Wallace, Protein secondary structure analyses from circular di- chroism spectroscopy: methods and reference databases, Biopolymers 89 (2008) 392–400, https://doi.org/10.1002/bip.20853.

[33] B. Dumas, G. Freyssinet, K.E. Pallett, Tissue-specific expression ofgermin-like oxalate oxidase during development and fungal infection of barley seedlings, Plant Physiol. 107 (1995) 1091–1096, https://doi.org/10.1104/pp.107.4.1091.

[34] X.Y. Zhang, Z.H. Nie, W.J. Wang, D.W. Leung, D.G. Xu, B.L. Chen, Z. Chen, L.X. Zeng, E.E. Liu, Relationship between disease resistance and rice oxalate oxidases in trans- genic rice, PLoS One 24 (2013), e78348. https://doi.org/10.1371/journal.pone. 0078348.

[35] M. Azarkan, A. Moussaoui, D. Wuytswinkel, G. Dehon, Y. Looze, Fractionation and purification of the enzymes stored in the latex of Carica papaya, J. Chromatogr. B 790 (2003) 229–238, https://doi.org/10.1016/S1570-0232(03)00084-9.

[36] E. Woo, J.M. Dunwell, P.W. Goodenough, A.C. Marvier, R.W. Pickersgill, Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismut- ase activities, Nat. Struct, Biology (2000) 7, https://doi.org/10.1038/80954.

[37] X. Chen, M.L. Wang, C. Holbrook, A. Culbreath, X. Liang, Identification and character- ization of a multigene family encoding germin-like proteins in cultivated peanut (Arachis hypogaea L.), Plant Mol. Biol. Report. 29 (2011) 389–403, https://doi.org/ 10.1007/s11105-010-0237-6.

[38] A.R. Barman, J. Banerjee, Versatility of germin-like proteins in their sequences, ex- pressions, and functions, Funct. Integr. Genomics 15 (2015) 533–548, https://doi. org/10.1007/s10142-015-0454-z.

[39] A. Sakamoto, T. Nishimura, Y. Miyaki, S. Watanabe, H. Takagi, S. Izumi, H. Shimada, In vitro and in vivo evidence for oxalate oxidase activity of a germin-like protein from azalea, Biochem. Biophys. Res. Commun. 458 (2015) 536–542, https://doi. org/10.1016/j.bbrc.2015.02.002.

[40] C.D.T. Freitas, D.C. Freitas, W.T. Cruz, C.T.M.N. Porfírio, M.Z.R. Silva, J.S. Oliveira, C. Paiva, S. Carvalho, M.V. Ramos, Identification and characterization of two germin- like proteins with oxalate oxidase activity from Calotropis procera latex, Int. J. Biol. Macromol. 105 (2017) 1051–1061, https://doi.org/10.1016/j.ijbiomac.2017.07.133.

[41] H.Y. Pan, M.N. Whittaker, R. Bouveret, A. Berna, F. Bernier, J.W.Whittaker, Characterization of wheat germin (oxalate oxidase) expressed by Pichia pastoris, Biochem. Biophys. Res. Commun. 356 (2007) 925–929, https://doi.org/10.1016/j.bbrc.2007. 03.097.

[42] R. Strasser, Plant protein glycosylation, Glycobiology 26 (2016) 926–939, https:// doi.org/10.1093/glycob/cww023.

[43] D.N. Hebert, L. Lamriben, E.T. Powers, J.W. Kelly, The intrinsic and extrinsic effects of N-linked glycans on glycoproteostasis, Nat. Chem. Biol. 10 (2014) 902, https://doi. org/10.1038/nchembio.1651.

[44] T. Chattopadhyay, In silico analysis of the germin like protein multigene family members of tomato with predicted oxalate oxidase activity, Int. J. Agric. Environ. Biotechnol 7 (2014) 669–678, https://doi.org/10.5958/2230-732X.2014.01374.6.

[45] C.N. Khobragade, S.D. Beedkar, R.G. Bodade, A.S. Vinchurkar, Comparative structural modeling and docking studies of oxalate oxidase: possible implication in enzyme supplementation therapy for urolithiasis, Int. J. Biol. Macromol. 48 (2011) 466–473, https://doi.org/10.1016/j.ijbiomac.2011.01.007.

[46] M. Díaz-Mendoza, B. Velasco-Arroyo, P. González-Melendi, M. Martínez, I. Díaz, C1A cysteine protease-cystatin interactions in leaf senescence, J. Exp. Bot. 65 (2014) 3825–3833, https://doi.org/10.1093/jxb/eru043.

[47] M.T.R. Gomes, H.A. Ribeiro, M.T.P. Lopes, F. Guzman, C.E. Salas, Biochemical comparison of two proteolytic enzymes from Carica candamarcensis:structural motifsun- derlying resistance to cystatin inhibition, Phytochemistry 71 (2010) 524–530, https://doi.org/10.1016/j.phytochem.2009.12.018.

[48] R. Ghosh, S. Chakraborty, C. Chakrabarti, J.K. Dattagupta, S. Biswas, Structural in- sights into the substrate specificity and activity ofervatamins, the papain-like cyste- ine proteases from a tropical plant, Ervatamia coronaria, FEBS J. 275 (2008) 421–434, https://doi.org/10.1111/j.1742-4658.2007.06211.x.

[49] S. Dutta, J.K. Dattagupta, S. Biswas, Enhancement of proteolytic activity of a thermo- stable papain-like protease by structure-based rational design, PLoS One 8 (2013), e62619. https://doi.org/10.1371/journal.pone.0062619.

[50] K. Konno, Plant latex and other exudates as plant defense systems: roles of various defense chemicals and proteins contained therein, Phytochemistry 72 (2011) 1510–1530, https://doi.org/10.1016/j.phytochem.2011.02.016.

[51] J.T. Pelton, L.R. Mclean, Spectroscopic methods for analysis of protein secondary structure, Anal. Biochem. 176 (2000) 167–176, https://doi.org/10.1006/abio.1999. 4320.

[52] L. Whitmore, B.A. Wallace, Protein secondary structure analyses from circular di- chroism spectroscopy: methods and reference databases, Biopolymers 89 (2008) 392–400, https://doi.org/10.1002/bip.20853.

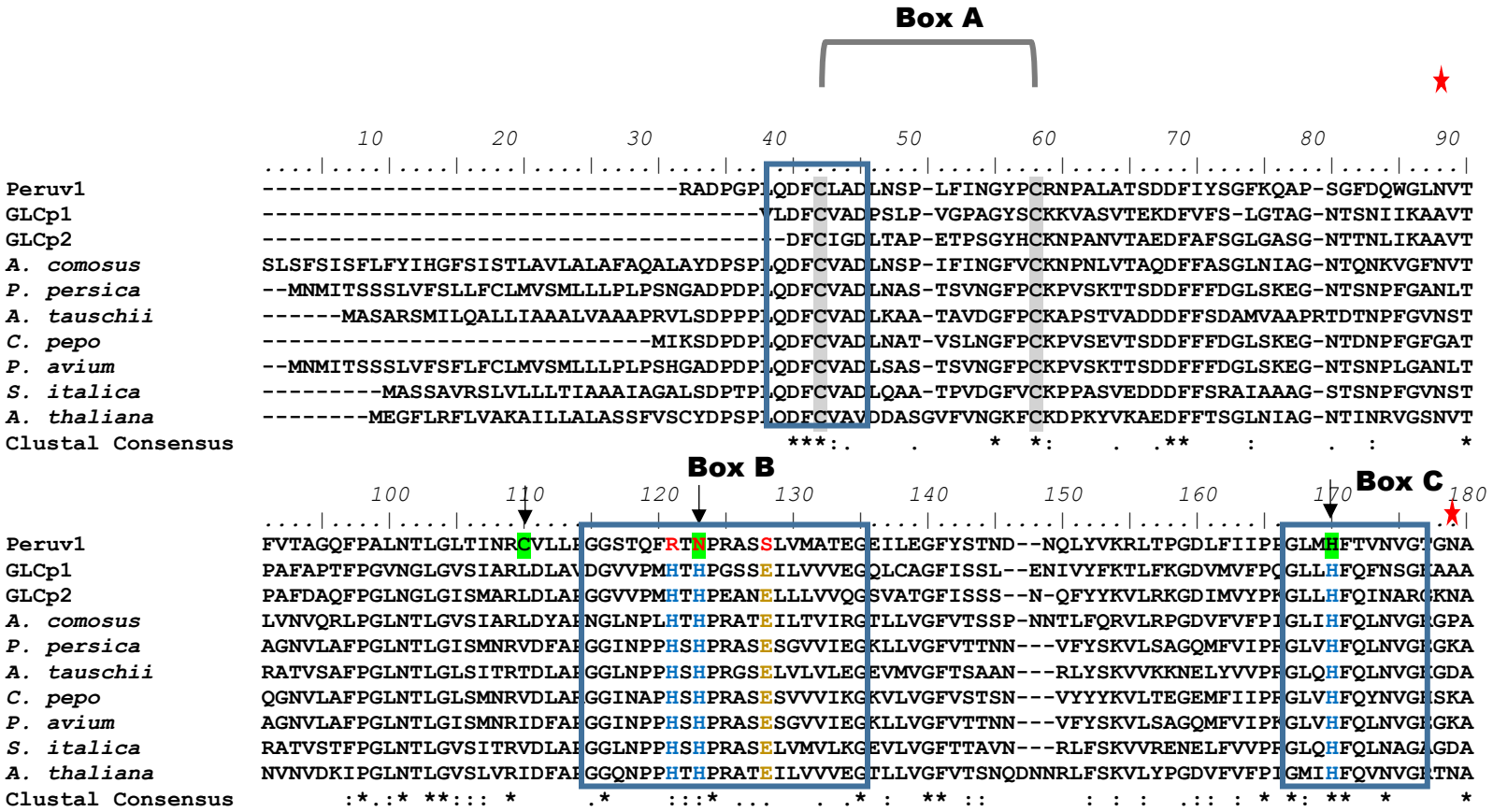
[53] C. Carter, R.W. Thombur, Germin-like proteins: structure, phylogeny, and function, J. Plant Biol. 42 (1999) 97–108.

[54] A.O. Barel, A.N. Glazer, Spectroscopic studies on papain and some inactive deriva- tives spectroscopic studies on papain and some inactive derivatives, J. Biol. Chem. 25 (1969) 268–273.

[55] H. Zare, A.A. Moosavi-Movahedi, M. Salami, N. Sheibani, K. Khajeh, M. Habibi-Rezaei, Autolysis control and structural changes of purified ficin from Iranian fig latex with synthetic inhibitors, Int. J. Biol. Macromol. 84 (2016) 464–471, https://doi.org/10. 1016/j.ijbiomac.2015.12.009.

[56] A.E. Kabakov, V.L. Gabai, Protein aggregation as primary and characteristic cell reac- tion to various stresses, Experientia 49 (1993) 706–710, https://doi.org/10.1007/ BF01923956.

Figure 6. Multiple sequence alignment among Peruvianin-I (Peruv1) and different germin-like proteins (GLPs). The two cysteine residues responsible for formation of a disulfide bond, which is highly conserved in all GLPs, are highlighted in gray. Putative N-glycosylation sites are marked with a red asterisk. The three histidine residues, colored in blue (box B and C), and the glutamate, in orange (box B), represent the amino acids known to be involved in oxalate oxidase activity. The amino acid residues of Peruvianin-I that were different than the amino acids of the catalytic site for oxalate oxidase in GLPs are indicated in red. The amino acids present in Peruvianin-I responsible for its proteolytic activity are highlighted in green and by arrows.



```
                        190       200       210       220       230
                    ....|....|....|....|....|....|....|....|....|....|...
Peruv1              TFYASLNSQNPGGQIVGLMDETVIAHLKAQYPNVVSQMLPSVNDRINSKIPVIKLPL-
GLCp1               VAYASFGSSYPGLQITDFALFKN--DLPTEIVVKTTFLDVAQVKKLKGVLGGKN----
GLCp2               IAFVSFSSSNPGLQILDFALFGN--NIPTSLLQKSTFLDAAQIKMLKGVLGGTN----
A. comosus          VAISGFNSQNPGLIVIADAVFGSNPPIPVQVLEKSFQVGQNVIQELQAQFK-------
P. persica          LAFTAFNSQLPGAVVLPLTLFASMPSIPDQVLTKALQVDKDVINTIRSKFGF------
A. tauschii         VFMAMFDAQSPGLVTPTLGLFAAKPAMSMEVLTKTFLMGEDEVSAMKSKFAGF-----
C. pepo             ILLTAFNSQLPGAVIVSRTLFASNPPIPLEILTKTFQVDDGVINSIKSKFA-------
P. avium            LAFTAFNSQLPGAVVLPLTLFASTPSIPDQVLTKALRVDKDVINTIRSKFGF------
S. italica          VFVAMFDSQSPGLVTPTFAMFATKPAMPMEVLTKTFLMGEDEVSAMKSKFAAF-----
A. thaliana         VAFAGLGSQNPGTITIADAVFGSKPSIMPEILAKAFQLDVNVVKYLEARFSSNYDRHY
Clustal Consensus       : **              :  .       :     . :.. :
```

Figure 7. Phylogenetic analyses of Peruvianin-I and other plant GLPs. The phylogenetic tree was constructed with the neighbor-joining algorithm using MEGA7 software. The clades/subfamilies were organized into different symbols and colors. Peruvianin-I, a member of a newclade/subfamily, is highlighted by the red arrow. The GenBank accession numbers of the sequences analyzed are available in Supplementary Table 2.

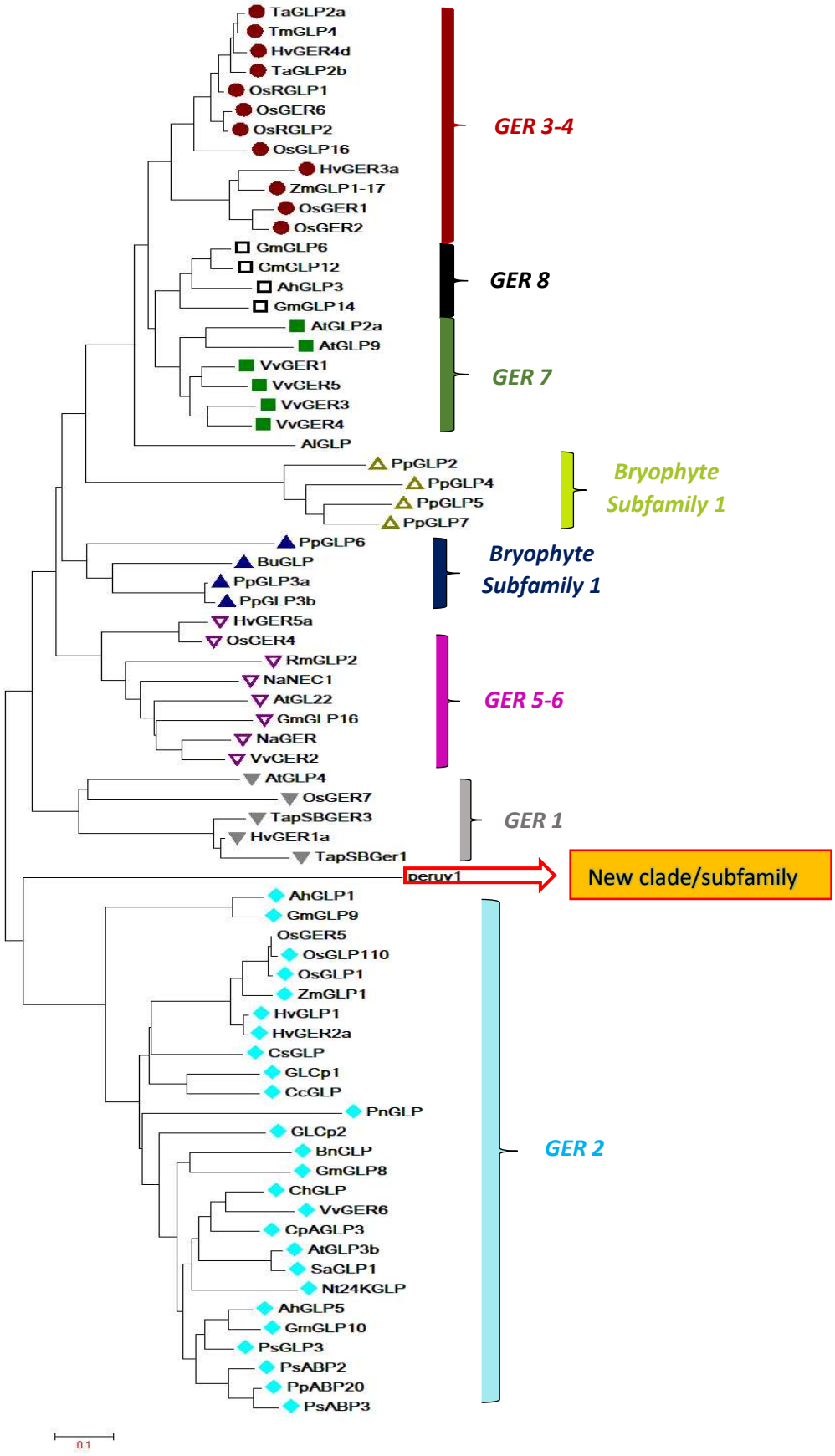
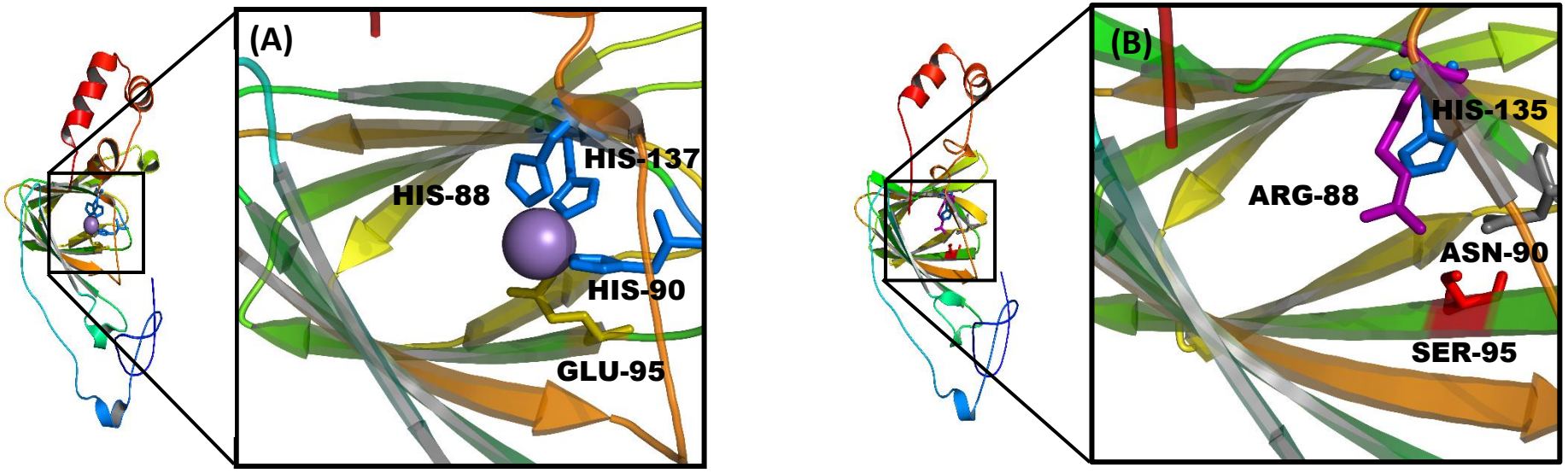
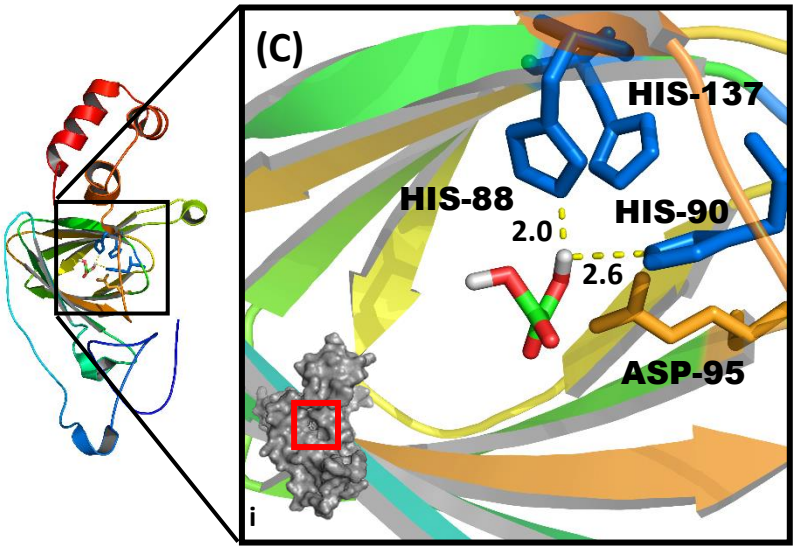

TaGLP2a
TmGLP4
HvGER4d
TaGLP2b
OsRGLP1
OsGER6
OsRGLP2
OsGLP16
HvGER3a
ZmGLP1-17
OsGER1
OsGER2
GER 3-4
GmGLP6
GmGLP12
AhGLP3
GmGLP14
GER 8
AtGLP2a
AtGLP9
VvGER1
VvGER5
VvGER3
VvGER4
GER 7
AtGLP
PpGLP2
PpGLP4
PpGLP5
PpGLP7
Bryophyte Subfamily 1
PpGLP6
BuGLP
PpGLP3a
PpGLP3b
Bryophyte Subfamily 1
HvGER5a
OsGER4
RmGLP2
NaNEC1
AtGL22
GmGLP16
NaGER
VvGER2
GER 5-6
AtGLP4
OsGER7
TapSBGER3
HvGER1a
TapSBGer1
GER 1
peruv1
New clade/subfamily
AhGLP1
GmGLP9
OsGER5
OsGLP110
OsGLP1
ZmGLP1
HvGLP1
HvGER2a
CsGLP
GLCp1
CcGLP
PnGLP
GLCp2
BnGLP
GmGLP8
ChGLP
VvGER6
CpAGLP3
AtGLP3b
SaGLP1
Nt24KGLP
AhGLP5
GmGLP10
PsGLP3
PsABP2
PpABP20
PsABP3
GER 2
0.1

Figure 8. Comparison of the active site of the barley germin (1FI2) and ofthe three-dimensional model of Peruvianin-I related to the oxalate oxidase activity, as well as the molecular docking of these structures against the ligand oxalate (substrate for oxalate oxidase activity). (A) Four amino acids involved in the oxalate oxidase activity of the barley germin and their interaction with Mn2+ ion. (B) In these same positions, different residues (Arg88, Asn90,Ser95) are present in the structure of Peruvianin-I. (C) Molecular docking simulation of the interactions between oxalate and barley germin. (D) The lack of residues responsible for oxalate oxidase activity in Peruvianin-I generated no coupling of the ligand oxalate. Interaction distances between oxalate and residues are given in Å units.




(C)
HIS-137
HIS-88
HIS-90
2.0
2.6
ASP-95
i

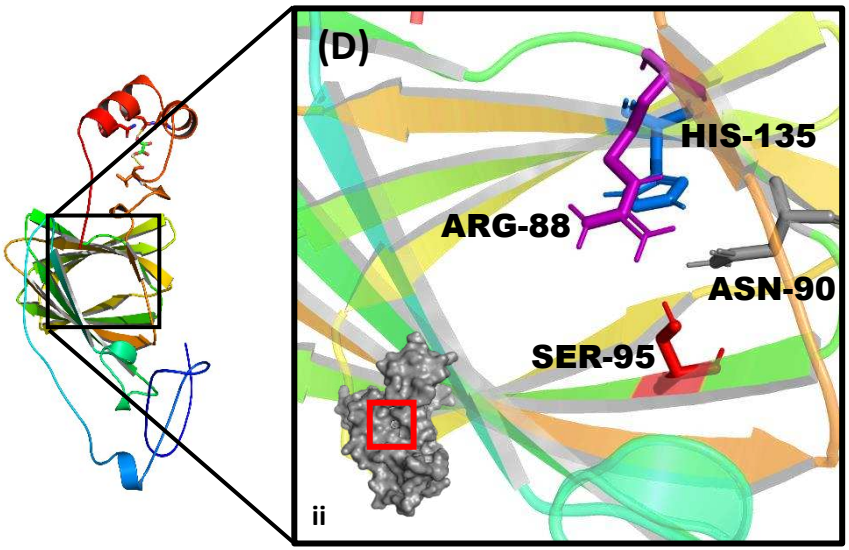

(D)
HIS-135
ARG-88
ASN-90
SER-95
ii

Figure 9. In situ location of oxalate oxidase activity in *Thevetia peruviana* leaves. Presence (+) and absence (−) of substrate oxalate. The leaves were incubated for 2h in the activation solution at 25 °C (see Material and Methods). The blue dots, indicated by some black arrows, show the presence of activity. Leaves of *Oryza sativa* were used as positive control. The analyses were performed under a light microscope (40× magnification)

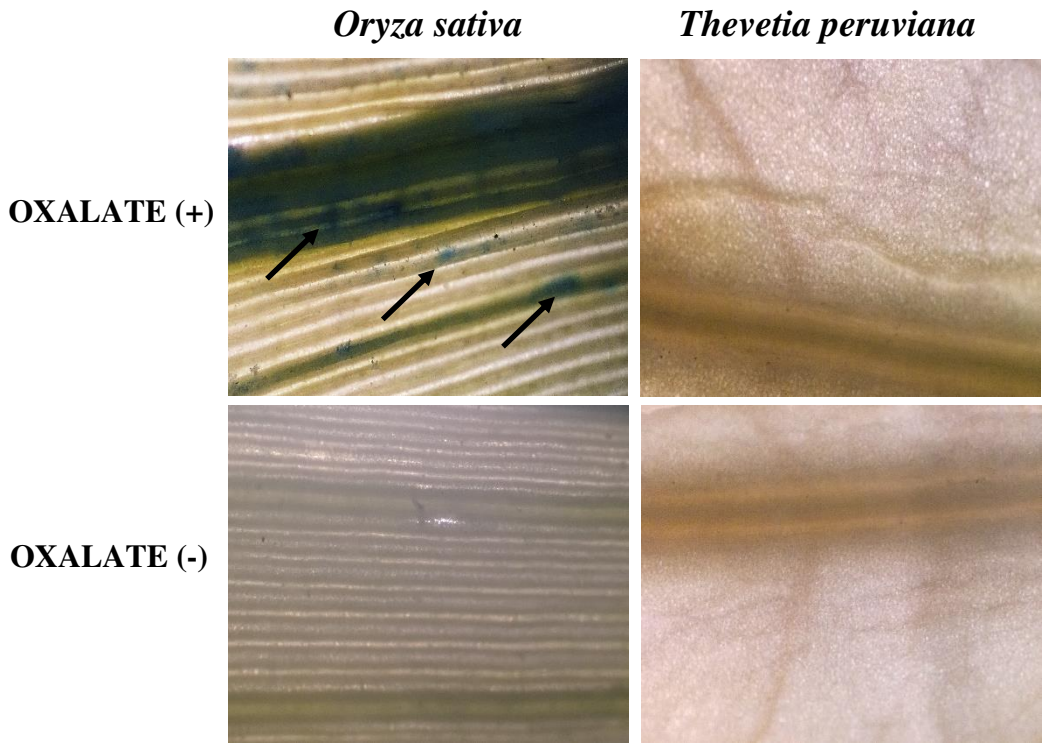
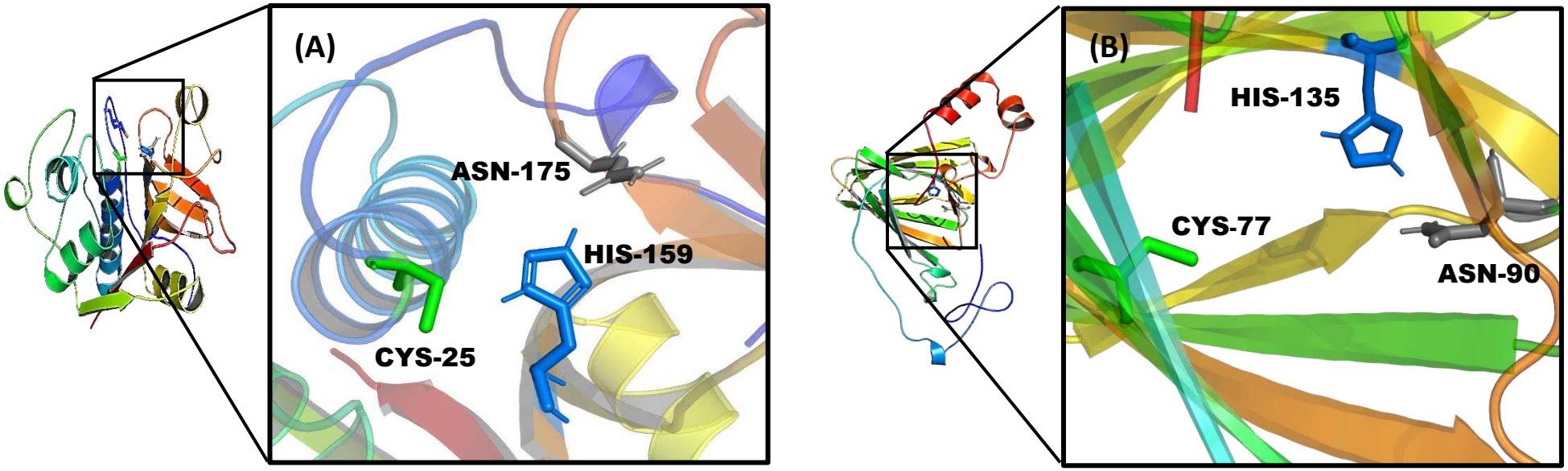


Figure 10. Comparison of the catalytic site for cysteine proteolytic activity of papain (1PPP) and Peruvianin-I. (A) Amino acids (histidine, asparagine and cysteine) involved in the proteolytic activity of papain (A) and Peruvianin-I (B). (C) Structural overlap between the residues of the catalytic site of papain and Peruvianin-I.



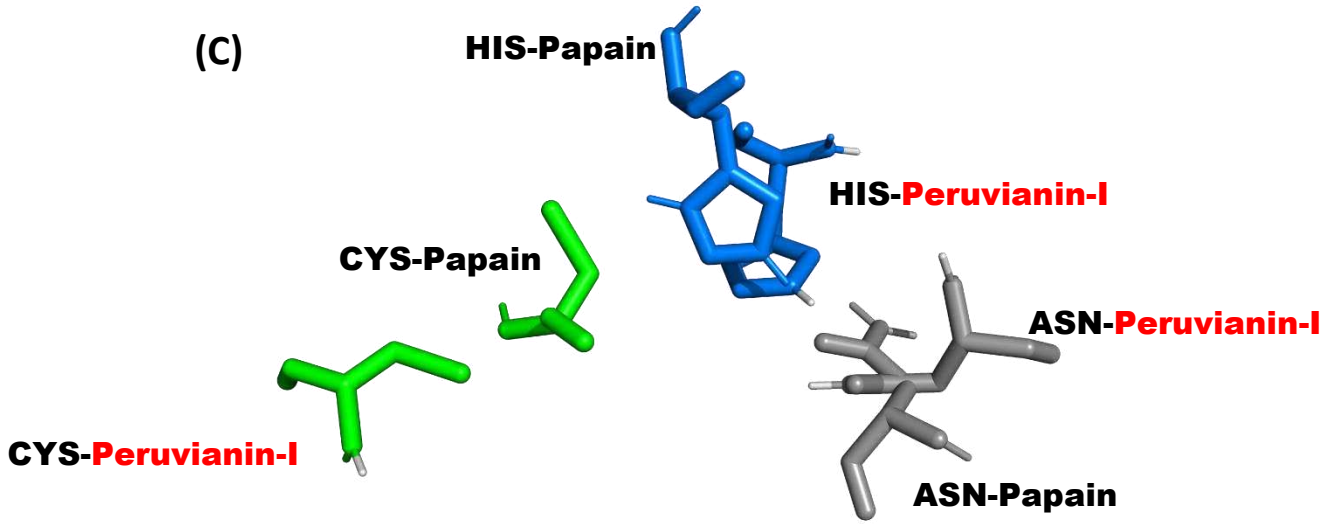

(C)
HIS-Papain
HIS-Peruvianin-I
CYS-Papain
ASN-Peruvianin-I
CYS-Peruvianin-I
ASN-Papain

Figure 11. Molecular docking evidencing the amino acid residues involved in the cysteine proteolytic activity of papain (A) and Peruvianin-I (B) and their interaction with the cysteine peptidase inhibitor iodoacetamide (IAA).

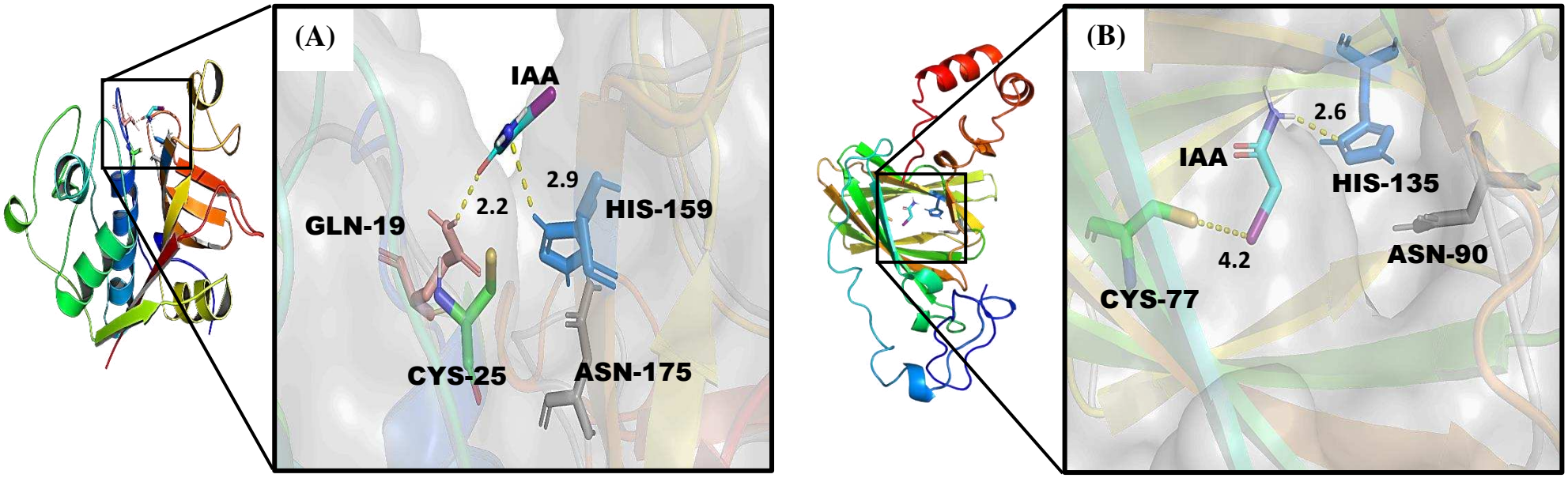


Figure 12. Effect of substrate concentration (BANA) on proteolytic activity of papain and Peruvianin-I at pH 6.0 and 37 °C. Inset: Lineweaver-Burk plot. Km and Vmax were calculated from the Lineweaver-Burk plot.

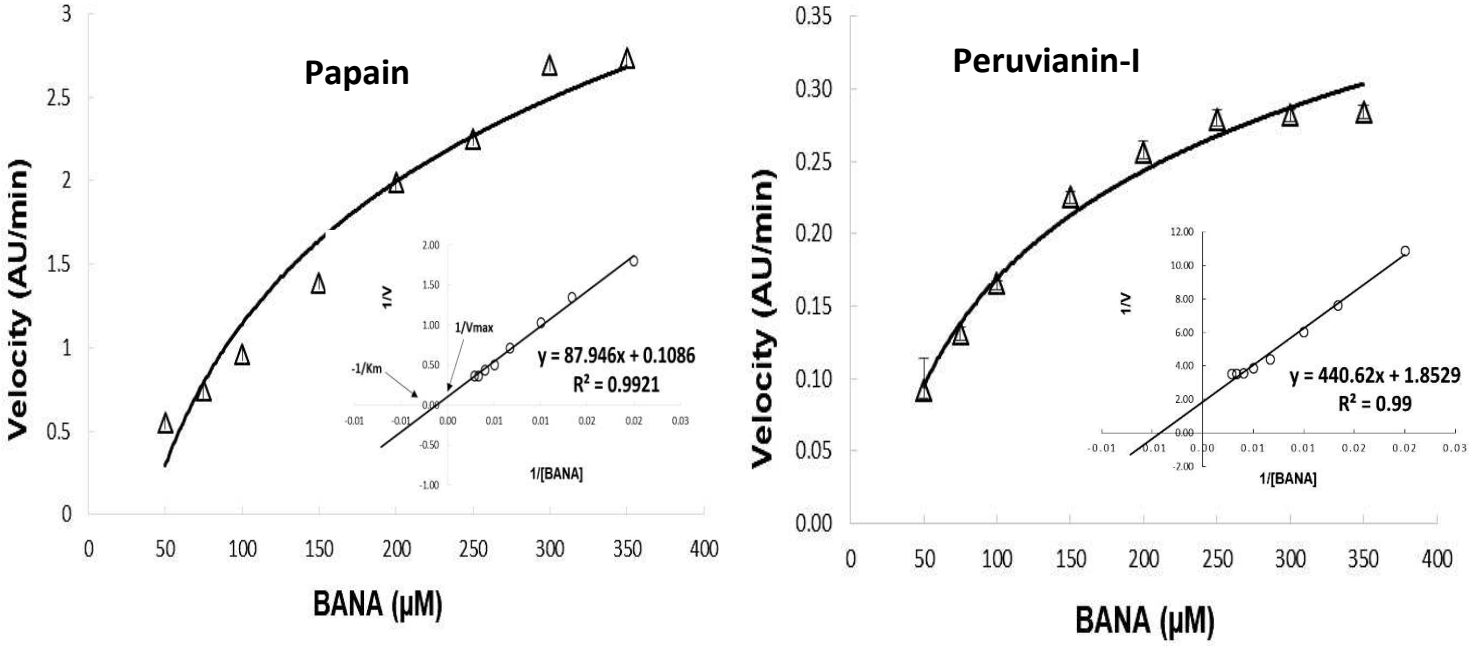


Figure 13. Synchrotron radiation circular dichroism (SRCD) spectra of Peruvianin-I at pH ranging from 4.0 to 8.0 (A); effect of temperature (from 20 to 90 °C) on the conformation of Peruvianin-I (B); SRCD spectra of the protein in the presence of the reducing agent DTT (at 1, 5, or 10 mM) (C); and SRCD spectra of partially dehydrated films of Peruvianin-I deposited on quartz-glass plates (D) in the absence or presence of DTT (1 mM), and the influence of a cysteine peptidase inhibitor iodoacetamide (IAA, 10 mM).

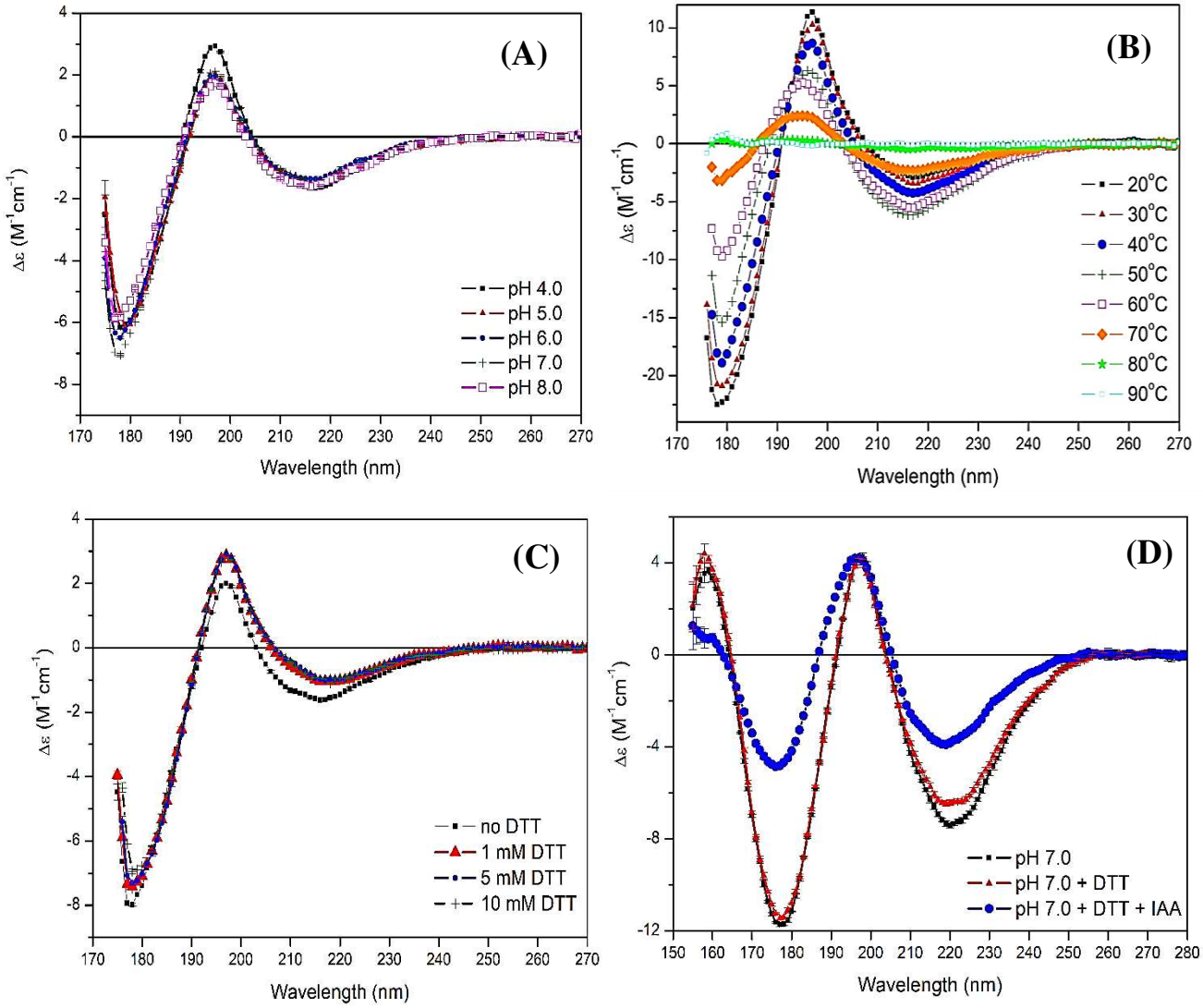


Tabela 1. Comparison of some biochemical properties of Peruvianin-I with other germins and germin-like proteins.

Plant	Protein	Sequence Lenght [a]	Molecular mass (Da)	pI	NCBI number	% Identity Peruvianin-I
Thevetia peruviana	Peruvianin-I	202	21851.95	5.58	-	100
Calotropis procera	CpGLP1	190	19889.06	6.98	-	38
Calotropis procera	CpGLP2	206	19792.66	7.08	-	40
Ananas comosus	Germin-like	195	21089.23	8.03	XP_020109888.1	47
Arabidopsis thaliana	Germin-like	177	18962.58	5.61	U75206	38
Camelia sinensis	Germin-like	191	19976.92	5.45	ARQ80482.1	36
Coffea canephora	Germin-like	188	19859.88	6.30	CDP03095.1	37
Glycine max	Germin-like	200	21875.93	6.46	ACG69478.1	42
Nicotiana tabacum	Germin-like	191	19855.02	6.31	BAC77634.1	38
Oryza sativa	Germin-like	189	19366.96	5.67	BAA74702.1	36
Triticum aestivum	Germin	209	22725.94	6.07	CAA71050.1	41
Zea mays	Germin-like	189	19541.25	5.69	AAQ95582.1	37
Hordeum vulgare	Germin	200	21216.24	5.77	ABG46232.1	41

*Sequences analyzed without the signal peptide.

Tabela 2. List of all sequences used for the alignment and construction of the phylogenetic tree of Fig. 7.

Sl.	Plant	Accession	GLP name
1	*Arabidopsis thaliana*	NP_001119334.1	AtGLP2a
		AAB51573.1	AtGLP3b
		NP_564067.2	AtGLP4
		NP_193199.1	AtGLP9
		NM_001083981	AtGL22
2	*Arachis hypogaea*	ADD71875.1	AhGLP1
		GU457419	AhGLP3
		GU457421	AhGLP5
3	*Atriplex lentiformis*	AB024338	AlGLP
4	*Brassica napus*	AAA86365.1	BnGLP
5	*Burbula unguiculata*	BAC53790.1	BuGLP
6	*Camelia sinensis*	ARQ80482.1	CsGLP
7	*Calotropis procera*	-	GLCp1
		-	GLCp2
8	*Chimonanthus praecox*	ABV03161.1	ChGLP
9	*Coffea canephora*	CDP03095.1	CcGLP
10	*Glycine max*	ACG69482.1	GmGLP6
		ACG69484.1	GmGLP8
		ACG69485.1	GmGLP9
		ACG69486.1	GmGLP10
		ACG69488.1	GmGLP12
		ACG69490.1	GmGLP14
		ACG69492.1	GmGLP16

11	*Hordeum vulgare*	Y15962	HvGLP1
		ABG46232.1	HvGER1a
		ABG46233.1	HvGER2a
		ABG46234.1	HvGER3a
		ABG46236.1	HvGER4d
		ABG46237.1	HvGER5a
12	*Ipomoea nil* (*Pharbitis nil*)	BAA08266.1	PnGLP
13	*Nicotiana attenuate*	AAR97545.1	NaGER
14	*Nicotiana plumbaginifolia*	AAF03355.1	NaNEC1
15	*Nicotiana tabacum*	BAC77634.1	Nt24KGLP
16	*Oryza sativa*	BAA74702.1	OsGLP1
		AAC04832.1	OsGER1
		AAC04833.1	OsGER2
		AAC04835.1	OsGER4
		AAC04836.1	OsGER5
		Q6YZZ2.1	OsGER6
		AAC25777.1	OsGER7
		AAD43973.1	OsRGLP1
		AAD43972.1	OsRGLP2
		AAB97470.1	OsGLP16
		AAC05682.1	OsGLP110
17	*Physcomitrella patens*	BAD86507.1	PpGLP2
		BAD86499.1	PpGLP3a
		BAD86504.1	PpGLP3b
		BAD86500.1	PpGLP4
		BAD86501.1	PpGLP5
		BAD86502.1	PpGLP6

		BAD86503.1	PpGLP7
18	*Pisum sativum*	CAC34417.1	PsGLP3
19	*Prunus persica*	AAB51240.1	PpABP20
20	*Prunus salicina*	ACA03785.1	PsABP2
		ACA03784.1	PsABP3
21	*Rhododendron mucronatum*	BAG75123.1	RmGLP2
22	*Sinapis alba*	CAA59257.1	SaGLP1
23	*Triticum aestivum*	CAA71050.1	TapSBGer1
		CAB55558.1	TaGLP2a
		CAB55559.1	TaGLP2b
		CAA71052.1	TapSBGER3
24	*Triticum monococcum*	AAT67049.1	TmGLP4
25	*Vitis vinifera*	ABL60872.1	VvGER1
		ABH09468.1	VvGER2
		AAQ63185.2	VvGER3
		ABL60873.1	VvGER4
		ABL60874.1	VvGER5
		ABL60875.1	VvGER6
26	*Zea mays*	AAQ95582.1	ZmGLP1
		ACG41245.1	ZmGLP1-17

Tabela 3. Quality evaluation of the three-dimensional model of Peruvianin-I built using different platforms.

Protein	Platform	ProSA[1] (z-score)	ERRAT2[2] (Global quality factor)	Verify 3D[3] (%)	Ramachandran graphic (Molprobity)[4]
Peruvianin-I	GalaxyWeb	-4.38	74.346	67,82	0
	M4T	-4.32	58.247	63,37	0
	Phyre 2	-4.43	71.354	67.89	1 (LEU_{184})

[1]Calculates local quality for each amino acid; [2]Verifies the global quality of the modeled structures; [3]Evaluates the chemical environment for each amino acid showing the reliability of the folding of the model; [4]Amino acids in disallowed regions.

Crystal structure of Peruvianin-I, a germin-like protein with proteolytic activity

Wallace T. da Cruz[a], Eduardo H.S. Bezerra[a], Márcio V. Ramos[a], Bruno A. M. Rocha[a], Cleverson D.T. Freitas[a*]

[a] Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, Campus do Pici, CEP 60451-970, Fortaleza, Ceará, Brasil.

*Corresponding author: Cleverson D.T. Freitas (cleversondiniz@ufc.com). Phone number: +55 85 3366 9816.

ABSTRACT

Peruvianin-I is a cysteine peptidase (EC 3.4.22) purified from *Thevetia peruviana* latex. Previous studies have shown that it is the unique germin-like protein (GLP) with proteolytic activity described so far. In this work, the x-ray crystal structure of peruvianin-I was resolved to a nominal resolution of 2.15 Å. Its overall structure shows an arrangement composed by a homohexamer (a trimer of dimers) and each monomer exhibiting a typical β-barrel fold of the GLPs and two glycan-binding sites located at ASN55 and ASN144. Analysis of its active site confirmed the absence of essential amino acids for enzymatic activities of GLPs, such as oxalate oxidase (EC 1.2.3.4) and superoxide dismutase (EC 1.15.1.1). Molecular docking studies using the cysteine peptidase inhibitor (iodoacetamide) demonstrated strong interaction with the key residues of its catalytic site responsible by peptidase activity. Details of the active site were used to present and discuss a plausible mechanism for proteolytic activity of peruvianin-I.

Keywords: cysteine protease; glp; latex; *Thevetia peruviana*;

Introduction

Peptidases (EC 3.4) belong to group of proteins that catalyze the cleavage of other proteins/peptides. These enzymes can be classified according to the amino acid involved in their catalytic mechanisms, such as cysteine, serine, and aspartic peptidases. Additionally, the MEROPS database categorizes theses enzymes in families, based on the similarities of their amino acid sequences, and then the homologous families are grouped in clans (http://merops.sanger.ac.uk) (Rawlings, 2016).

Several proteolytic enzymes have been studied from different plant latex fluids, including species from Euphorbiaceae, Moraceae, Asclepiadaceae, Apocynaceae, and Caricaceae family (Mnif et al., 2015; Ramos et al., 2019). Interestingly, the predominant peptidase class belongs to cysteine group (Domsalla and Melzig, 2008; Ramos et al., 2013). In general, these enzymes are monomers of approximately 20-30 kDa and have globular structures with two domains that interact with each another, forming a cleft on the surface of the enzyme, where the substrate binds (Novinec and Lenarcic, 2013; Rawlings et al., 2010).

On the other hand, an unusual cysteine peptidase, named peruvianin-I, was purified and partially characterized from the *Thevetia peruviana* latex (Freitas et al., 2016). This enzyme is a glycoprotein with estimated molecular mass of 120 kDa. Atomic force microscopy suggested that it is arranged as trimer of dimers, very similar to proteins belonging to germin-like group (Woo et al., 2000; Freitas et al., 2016). Therefore, Freitas and collaborators (2016) were the first to report a cysteine peptidase with structural characteristics of germin-like proteins (GLPs).

GLPs exhibit a double stranded β-helix motif, which is represented by two pairs of antiparallel strands linked with short turns (Rajavel et al., 2008). This motif resembles the appearance of a sandwich in the protein structure, hence the term β-sandwich. These proteins exhibit a Jelly-Roll topology composed of eight β-strands distributed in two antiparallel β-

sheets (Uberto and Moomaw, 2013). Recently, the complete amino acid sequence and predicted three-dimensional model of peruvianin-I were reported (Cruz et al., 2019). The model suggested a β-barrel structure (Jerry-roll topology) similar to that of GLPs (Woo et al., 2000). Interestingly, some differences in active site led the authors to suggest peruvianin-I as a new GLP group (Cruz et al., 2019).

To date, there are only three crystal structures of proteins belonging to the GLP family deposited in protein databank (PDB), which limits a greater understanding of the structural and functional characteristics of these proteins. Moreover, peruvianin-I is a new GLP member with unique enzymatic and structural properties, deserving thus, more studies at structural level. Here, the crystal structure of peruvianin-I was determined and characterized at 2.15 Å resolution.

Material and methods

Purification, protein content, and proteolytic activity of peruvianin-I

Peruvianin-I was purified according to the Freitas et al. (2016), with slight modifications. Briefly, *T. peruviana* latex protein fraction (TpLP) was subjected to gel filtration chromatography using a Superdex-75 (10/300 GL) column, equilibrated with 25 mM Tris-HCl buffer (pH 8.0), coupled to a high-performance liquid chromatographic system (AKTA Purifier, GE Healthcare) at 0.5 mL/min flow rate. Dissimilar to previously described methodology, peruvianin-I was not lyophilized. It was concentrated using a 10 kDa cut-off Vivaspins® (GE Healthcare), frozen and used in all further assays. The protein content was estimated using Bradford method and bovine serum albumin as standard (Bradford, 1976). The native structure of peruvianin-I was confirmed by enzymatic assays using azocasein as substrate (Freitas et al., 2016).

Crystallization

Different concentrations of peruvianin-I were assayed and the appropriate concentration for crystallization screening was determined to be 20 mg/mL, using the Pre-Crystallization Test (Hampton Research). Hanging drop crystallization experiments were conducted with peruvianin-I in 50 mM Tris-HCl (pH 8.0) and crystal screen 1 and 2 kits (Hampton Research). Crystals were screened at 25 ºC by the hanging-drop method in vapor-diffusion 24-well Linbro cell-culture plates. The protein solutions (1 µL) were mixed with same volume of well solution and the mixture was equilibrated against 300 µL of well solution. At crystallization condition was added 25% glycerol as cryoprotectant to avoid ice formation.

Data collection

The data were collected at 1.42 Å wavelength at the MX21 beamline of the Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil) beamline MX2, using a PILATUS 2M placed 150 mm from the crystal. The crystals were rotated through 360° with a 0.2° oscillation range per frame using a fine ϕ-slicing strategy, collecting a total of 1800 frames. All data were collected at 100 K to avoid radiation damage (Jardim et al., 2016)

Data processing and structural determination

X-ray diffraction data were processed with XDS program (Kabsch, 2010) and scaled using Scala in the CCP4 Program (Winn et al., 2011). Crystal belonged to the space group P41212 and the Matthews coefficient (Matthews, 1968) of 2.69 Å3 Da^{-1} indicated the presence of three molecules in the asymmetric unit. The crystal structure was determined by molecular replacement using Phaser program (McCoy, Grosse-Kunstleve et al. 2007). The atomic coordinate used as a model was obtained from germin structure (PDB code: 1FI2) (Woo et al. 2000).

The initial generated structure was refined using Phenix program (Adams et al. 2010, Afonine et al. 2012, Echols et al. 2012) and then modeled using COOT program (Emsley and Cowtan 2004). After addition of 235 water molecules and two N-linked glycans, a second restrained refinement was performed resulting in a R_{factor} of 18.4% and a R_{free} of 21%. Finally, the stereochemistry of the structure was assessed using a Ramachandran plot analysis with Molprobity software (Chen, Arendall et al. 2010). The figures were generated using PyMOL program (DeLano and Lam 2005).

2.5. Molecular docking

The active site of peruvianin-I was assessed using iodoacetamide (a specific cysteine peptidase inhibitor) and oxalate (a substrate for oxalate oxidase activity) using Autodock Vina and Autodock Tools 1.5.6 (Morris et al. 2008; Trott and Olson 2010) as described by Cruz et al. (2019). A grid 40x40x40 Å was used at the same coordinates centered on the possible oxalate oxidase or proteolytic activity of peruvianin-I (Cruz et al., 2019).

Results and discussion

Crystallization and overall structure

The best crystals of peruvianin-I were obtained at 298 K using 0.1 M sodium acetate trihydrate at pH 4.6, containing 2.0 M sodium formate. Bipyramidal crystals grown after 7 days are shown in Figure 1. A complete data set was collected at a resolution of 2.07 Å and scaled to 2.15 Å for suitable crystallographic statistics, using barley germin as template (PDB code: 1FI2) because it is the closest structural homologue of peruvianin-1 (41% identity). The crystallographic and refinement data are shown in Table 1. The asymmetric unit of peruvianin-I contained three monomers, being two composing a dimer and a third molecule from an adjacent dimer.

The structure of a monomer was composed of a jelly-roll/β-barrel motif, which is formed of ten β-strands, with a β-barrel composed by two five-stranded β-sheets (Fig. 2). The β-sheets are connected through loops and a short α-helice, very similar to other germin proteins (Woo et al., 2000; Opaleye et al., 2006) The biological assemble was obtained by symmetry operation, presenting a trimer of dimers, in a three-fold symmetry axis, forming a central channel (Fig. 2A). Remarkably, the crystal structure of peruvianin-I monomers lacked an expected α-helical C-terminal domain (Fig. 2B), which is present in germin proteins (Fig. 2C) and was modeled using the entire sequence of peruvianin-I (Cruz et al., 2019). This C-terminal domain comprises the amino acids 161-202 of peruvianin-I and it is believed to be fundamental for the stabilization of trimers, clasping together these regions that are stabilized by hydrophobic interactions (Chruszcz et al., 2011). The absence of electron density for this domain can be related to the dynamic of this region, which has been reported to be very flexible (Maruyama et al., 2001; Chruszcz et al., 2011). Despite the non-visualization of the interaction between the trimers through the α-helical C-terminal domain, it was observed that monomers interact to form the dimers through the β-sheets loops mediated by polar interactions (THR111-GLY158, THR111-GLN159, ASN114-GLN159, ASN114-ILE160 and MET134- GLN159). Similar to peruvianin-I, the role of the β-barrel and N-terminal extensions in germins, GLPs and auxin binding proteins is to stabilize the trimer of dimers filling the center of hexamer (Woo et al., 2000)

Glycosylation

Although germin and GLP are glycoproteins (Barman and Banerjee, 2015), there is a few studies describing glycosylation models of these proteins due to the high flexibility of these glycans. A pioneer study described by Woo and collaborators (2000) showed only a poor

electron density map for N-glycosylation of ASN47 from barley germin. In contrast, peruvianin-I crystals showed high densities for asparagine residues at two different sites (ASN55 and ASN144) (Fig. 3). Both N-glycans present as main chains Man →β(1,4) GlcNAc →β (2,4) GlcNAc1 → ASN. In addition, there is a Fuc bond to GlcNAc (α→ 1,3) in ASN55 (Fig. 3). These results confirm those obtained previously, where two putative N-glycosylated sites were predicted in peruvianin-I, at positions ASN55 and ASN144, using in silico analysis (Cruz et al., 2019). This glycosylation is positioned at the protein surface and does not contribute for the interaction among the dimers or monomers at all (Fig. 1). The role played by these glycans in germin and GLP was not described yet. Likely, the N-glycosylation in peruvianin-I can be involved in its enzymatic stability or protection against proteolysis, since *T. peruviana* latex has several peptidase isoforms (Hebert et al., 2014; Freitas et al., 2016).

Reactive center for cysteine peptidase activity of peruvianin-I

The enzymatic functionality of germins and GLPs have been correlated with the conservation of one glutamate and three histidine residues internalized in their β-barrel structures, which are directly involved in the coordination of the metal ions present in oxalate oxidase (OxO), superoxide dismutase (SOD) and oxalate decarboxylase activities (Dunwell et al., 2008; Uberto and Moomaw, 2013). The figure 4 shows the differences in the composition of the active site by the structural overlap between the germin (1FI2) and peruvianin-I. Previous studies showed that peruvianin-I does not exhibit activities such as OxO and SOD (Freitas et al., 2016). Accordingly, the structure described herein did not present any type of metal ion. Besides, peruvianin-I has a SER, ASN and ARG instead of GLU and two HIS found in germin proteins (Fig. 4). Considering these results, it is possible to reinforce the importance of three histidine and glutamate residues in the coordination of manganese ion and consequently enzymatic activity of germins and GLPs.

Although there are different functionalities described for gemins and GLPs, such as oxalate oxidase, superoxide dismutase and serine protease inhibitor (Mansilla, 2012; Segarra et al., 2003; Woo et al., 2000), peruvianin-I is the unique GLP with cysteine peptidase activity described so far. Moreover, peruvianin-I does not exhibit any sequence or structural homology with any another cysteine peptidase studied (Cruz et al., 2019). Our crystallographic results show that the active site of peruvianin-I is basically formed by the catalytic triad (CYS77, ASN90 and HIS135), which are buried into β-barrel structure located in a hydrophobic environment (Supplementary Fig. 1). In comparison with other cysteine peptidases, papain-like enzymes contain the same catalytic triad (Novinec and Lenarcic, 2013). To explore the active site and understand the catalytic mechanism of peruvianin-I, it was realized a protein-ligand analysis using a specific cysteine peptidase inhibitor (Iodoacetamide, IAA) (Supplementary Fig. 1). Our results showed that IAA forms hydrogen bonds of 3.1 Å and 3.3 Å with GLN19 and HIS135 residues, respectively. In addition, an interaction (4.2 Å) of the iodoacetyl group should allow a nucleophilic substitution of iodine with a sulfur atom from the sulfhydryl group (CYS77), resulting in a stable thioether linkage (Gomes et al., 2010). This reaction results in a very stable enzyme–inhibitor complex, which is responsible for irreversible inhibition of peruvianin-I (Freitas et al., 2016). Similarly, the molecular docking analysis of peruvianin-I model and IAA showed interactions among the inhibitor and the nitrogen of the imidazole ring of HIS135, concomitantly with the CYS77 residue, which confirms a strong affinity of IAA in this region of the active site (Cruz et al., 2019).

The figure 5 shows a possible action mechanism of peruvianin-I, based on compositional similarity of the residues present in its active site and papain-like peptidases (Rzychon et al., 2015). Initially the catalytic process involves formation of a reactive thiolate/imidazolium ion pair (Cys-S^{-}/His-Im^{+}), which results from a proton transfer between CYS77 and HIS135. ASN90 that ensures an orientation of the HIS imidazole ring is essential

for successive stages of hydrolysis. After, the carbonyl carbon of the scissile peptide bond is attacked by thiolate anion and the oxygen converts into a single one (Step 1) and then the oxygen assumes a negative net charge allowing formation of the first tetrahedral transition state (step 2). Thus, the rotation of histidine residue allows the transfer of protons from the imidazolium cation to the nitrogen of the peptide bond allowing hydrolysis and subsequent cleavage of the substrate. Consequently, this new formation involves a hydrogen bond of amine substrate to HIS135, whereas the substrate carboxylic region is linked to CYS77 via a thioester bond, forming acyl enzyme (step 3). The next step involves dissociation of the amino part of the substrate and its replacement with a water molecule. The imidazole nitrogen contributes to polarization of the water molecule which allows it to attack the carbonyl carbon of acyl enzyme (step 4). This reaction allows the formation of the second tetrahedral intermediate (step 5). Finally, there is a deacylation via thioester which leads to reconstruction of the carboxyl group in the hydrolyzed substrate, which is associated with the release of an active enzyme (step 6).

Conclusion

Our findings include the first crystal structure of a GLP with proteolytic activity, evidencing the functional diversity of proteins belonging to the germin family. Peruvianin-I presents the same structural arrangement of germin or GLPs and the structural data confirm the absence of OXO and SOD activities. Additionally, with detailed investigation of the active site residues was possible to suggest an action mechanism for cysteine peptidase activity of peruvianin-I.

REFERENCES

Adams, P. D., P. V. Afonine, G. Bunkoczi, V. B. Chen, I. W. Davis, N. Echols, J. J. Headd, L. W. Hung, G. J. Kapral, R. W. Grosse-Kunstleve, A. J. McCoy, N. W. Moriarty, R. Oeffner, R. J. Read, D. C. Richardson, J. S. Richardson, T. C. Terwilliger and P. H. Zwart (2010). "PHENIX: a comprehensive Python-based system for macromolecular structure solution." Acta Crystallogr D Biol Crystallogr 66(Pt 2): 213-221.

Afonine, P. V., R. W. Grosse-Kunstleve, N. Echols, J. J. Headd, N. W. Moriarty, M. Mustyakimov, T. C. Terwilliger, A. Urzhumtsev, P. H. Zwart and P. D. Adams (2012). "Towards automated crystallographic structure refinement with phenix.refine." Acta Crystallogr D Biol Crystallogr 68(Pt 4): 352-367.

Barman, A.R., Banerjee, J., 2015. Versatility of germin-like proteins in their sequences, expressions, and functions. Funct. Integr. Genomics. https://doi.org/10.1007/s10142-015-0454-z

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–54.

Chruszcz, M., Maleki, S.J., Majorek, K.A., Demas, M., Bublin, M., Solberg, R., Hurlburt, B.K., Ruan, S., Mattisohn, C.P., Breiteneder, H., Minor, W., 2011. Structural and Immunologic Characterization of Ara h 1 , a Major Peanut Allergen * □ 286, 39318–39327. https://doi.org/10.1074/jbc.M111.270132

Chen, V. B., W. B. Arendall, 3rd, J. J. Headd, D. A. Keedy, R. M. Immormino, G. J. Kapral, L. W. Murray, J. S. Richardson and D. C. Richardson (2010). "MolProbity: all-atom structure validation for macromolecular crystallography." Acta Crystallogr D Biol Crystallogr 66(Pt 1): 12-21.

Collaborative Computational Project, N. (1994). "The CCP4 suite: programs for protein crystallography." Acta Crystallogr D Biol Crystallogr 50(Pt 5): 760-763.

Cruz, W.T., Bezerra, E.H.S., Grangeiro, T.B., Lopes, J.L.S., Silva, M.Z.R., Ramos, M. V, Rocha, B.A.M., Oliveira, J.S., Freitas, D.C., Freitas, C.D.T., 2019. Structural and enzymatic characterization of Peruvianin - I , the fi rst germin-like protein with proteolytic activity. Int. J. Biol. Macromol. 126, 1167–1176. https://doi.org/10.1016/j.ijbiomac.2019.01.023

DeLano, W. L. and J. W. Lam (2005). "PyMOL: A communications tool for computational models." Abstracts of Papers of the American Chemical Society 230: U1371-U1372.

Domsalla, A., Melzig, M.F., 2008. Occurrence and properties of proteases in plant latices. Planta Med. 74, 699–711. https://doi.org/10.1055/s-2008-1074530

Dunwell, J.M., Gibbings, J.G., Mahmood, T., Naqvi, S.M.S., 2008. Germin and germin-like proteins: Evolution, structure, and function.

Echols, N., R. W. Grosse-Kunstleve, P. V. Afonine, G. Bunkoczi, V. B. Chen, J. J. Headd, A. J. McCoy, N. W. Moriarty, R. J. Read, D. C. Richardson, J. S. Richardson, T. C. Terwilliger and P. D. Adams (2012). "Graphical tools for macromolecular crystallography in PHENIX." J Appl Crystallogr 45(Pt 3): 581-586.

Emsley, P. and K. Cowtan (2004). "Coot: model-building tools for molecular graphics." Acta Crystallogr D Biol Crystallogr 60(Pt 12 Pt 1): 2126-2132.

Freitas, C.D.T. De, Wallace, T., Maria, C., 2016a. Proteomic analysis and purification of an unusual germin-like protein with proteolytic activity in the latex of Thevetia peruviana 1115–1128. https://doi.org/10.1007/s00425-016-2468-8

Freitas, C.D.T. De, Wallace, T., Maria, C., 2016b. Proteomic analysis and purification of an unusual germin-like protein with proteolytic activity in the latex of Thevetia peruviana. https://doi.org/10.1007/s00425-016-2468-8

Fusetti, F., K. H. Schroter, R. A. Steiner, P. I. van Noort, T. Pijning, H. J. Rozeboom, K. H. Kalk, M. R. Egmond and B. W. Dijkstra (2002). "Crystal structure of the copper-containing quercetin 2,3-dioxygenase from Aspergillus japonicus." Structure **10**(2): 259-268.

Gomes, M.T.R., Ribeiro, H.A., Lopes, M.T.P., Guzman, F., Salas, C.E., 2010. Biochemical comparison of two proteolytic enzymes from Carica candamarcensis: Structural motifs underlying resistance to cystatin inhibition. Phytochemistry 71, 524–530. https://doi.org/10.1016/j.phytochem.2009.12.018

Hebert, D.N., Lamriben, L., Powers, E.T., Kelly, J.W., 2014. The intrinsic and extrinsic effects of N-linked glycans on glycoproteostasis. Nat. Chem. Biol. 10, 902.

Jardim, P., Santos, I.C. da S., Barbosa, J.A.R.G., de Freitas, S.M., Valadares, N.F., 2016. Crystal structure of VapC21 from Mycobacterium tuberculosis at 1.31 Å resolution. Biochem. Biophys. Res. Commun. 478, 1370–1375. https://doi.org/10.1016/j.bbrc.2016.08.130

Kabsch, W. (2010). "Xds." Acta Crystallogr D Biol Crystallogr 66(Pt 2): 125-132.

Lane, B. G. (1994). "Oxalate, germin, and the extracellular matrix of higher plants." FASEB J **8**(3): 294-301.

Lane, B. G., J. M. Dunwell, J. A. Ray, M. R. Schmitt and A. C. Cuming (1993). "Germin, a protein marker of early plant development, is an oxalate oxidase." J Biol Chem **268**(17): 12239-12242.

Mansilla, A.Y., Segarra, C.I., 2012. Structural and Functional Features of a Wheat Germin-Like Protein that Inhibits Trypsin 624–632. https://doi.org/10.1007/s11105-011-0372-8

Maruyama, N., Adachi, M., Takahashi, K., Yagasaki, K., Kohno, M., 2001. Crystal structures of recombinant and native soybean b -conglycinin b homotrimers 3604, 3595–3604.

Matthews, B. W. (1968). "Solvent content of protein crystals." J Mol Biol 33(2): 491-497.

McCoy, A. J., R. W. Grosse-Kunstleve, P. D. Adams, M. D. Winn, L. C. Storoni and R. J. Read (2007). "Phaser crystallographic software." J Appl Crystallogr 40(Pt 4): 658-674.

Morris, G. M., R. Huey and A. J. Olson (2008). "Using AutoDock for ligand-receptor docking." Curr Protoc Bioinformatics Chapter 8: Unit 8 14.

Mnif, I.H., Siala, R., Nasri, R., Mhamdi, S., Nasri, M., Kamoun, A.S., 2015. A cysteine protease isolated from the latex of Ficus microcarpa: purification and biochemical characterization. Appl. Biochem. Biotechnol. 175, 1732–44. https://doi.org/10.1007/s12010-014-1376-2

Novinec, M., Lenarcic, B., 2013. Papain-like peptidases : structure , function , and evolution. BioMol Concepts 4, 287–308. https://doi.org/10.1515/bmc-2012-0054

Opaleye, O., Rose, R.S., Whittaker, M.M., Woo, E.J., Whittaker, J.W., Pickersgill, R.W., 2006. Structural and spectroscopic studies shed light on the mechanism of oxalate oxidase. J. Biol. Chem. 281, 6428–6433. https://doi.org/10.1074/jbc.M510256200

Rajavel, M., Kulkarni, N.N., Gopal, B., 2008. Conformational Studies Suggest That the Double Stranded Helix Scaf- fold Provides an Optimal Balance Between Protein Stability and Function 244–249.

Ramos, M.V., Demarco, D., da Costa Souza, I.C., de Freitas, C.D.T., 2019. Laticifers, Latex, and Their Role in Plant Defense. Trends Plant Sci. xx, 1–15. https://doi.org/10.1016/j.tplants.2019.03.006

Ramos, M. V, Araújo, E.S., Jucá, T.L., Monteiro-Moreira, a C.O., Vasconcelos, I.M., Moreira, R. a, Viana, C. a, Beltramini, L.M., Pereira, D. a, Moreno, F.B., 2013. New insights into the complex mixture of latex cysteine peptidases in Calotropis procera. Int. J. Biol. Macromol. 58, 211–9. https://doi.org/10.1016/j.ijbiomac.2013.04.001

Rawlings, N.D., 2016. Biochimie Peptidase specificity from the substrate cleavage collection in the MEROPS database and a tool to measure cleavage site conservation. Biochimie

122, 5–30. https://doi.org/10.1016/j.biochi.2015.10.003

Rawlings, N.D., Barrett, A.J., Bateman, A., 2010. MEROPS: the peptidase database. Nucleic Acids Res. 38, 227–33. https://doi.org/10.1093/nar/gkp971

Rzychon, M., Commission, E., Selvita, S.A., 2015. Modes of inhibition of cysteine proteases Circled white star. Acta Biochimica Polonica. 51, 861-873.

Segarra, Â., Anahõ, C., Pinedo, M.L., Ronchi, V.P., Ine, C., Ciencias, F. De, Mar, U.N. De, 2003. RESEARCH PAPER A germin-like protein of wheat leaf apoplast inhibits serine proteases 54, 1335–1341. https://doi.org/10.1093/jxb/erg139

Strasser, R. (2016). "Plant protein glycosylation." Glycobiology **26**(9): 926-939.

Trott, O. and A. J. Olson (2010). "AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading." J Comput Chem 31(2): 455-461.

Uberto, R., Moomaw, E.W., 2013. Protein Similarity Networks Reveal Relationships among Sequence , Structure , and Function within the Cupin Superfamily 8. https://doi.org/10.1371/journal.pone.0074477

Winn, M.D., Ballard, C.C., Cowtan, K.D., Dodson, E.J., Emsley, P., Evans, P.R., Keegan, R.M., Krissinel, E.B., Leslie, A.G.W., McCoy, A., McNicholas, S.J., Murshudov, G.N., Pannu, N.S., Potterton, E.A., Powell, H.R., Read, R.J., Vagin, A., Wilson, K.S., 2011. Overview of the CCP4 suite and current developments. Acta Crystallogr. Sect. D Biol. Crystallogr. 67, 235–242. https://doi.org/10.1107/S0907444910045749

Woo, E., Dunwell, J.M., Goodenough, P.W., Marvier, A.C., Pickersgill, R.W., 2000. Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities. Nat. Struct. Biol. 7.

Woo, E., Marshall, J., Bauly, J., Chen, J., Venis, M., Napier, R.M., Pickersgill, R.W., 2002. Crystal structure of auxin-binding protein 1 in complex with auxin 21.

Figura 14. Crystals of Peruvianin-I obtained using 0.1 M sodium acetate trihydrate at pH 4.6 containing 2.0 M sodium formate at 298 K.

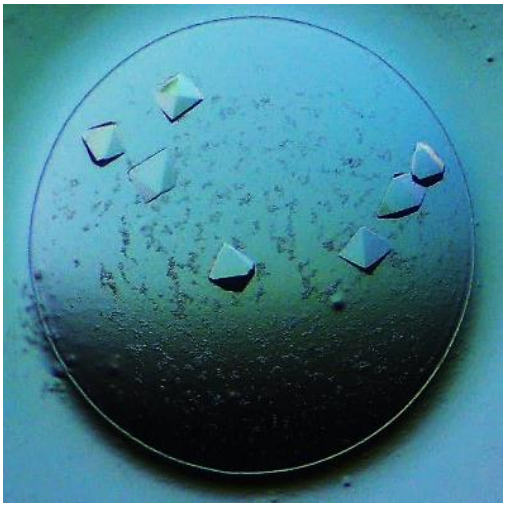


Figura 15. The biological assembly of Peruvianin-I hexamer and the structural similarity to the germin dimer. (A) The Peruvianin-I homohexamer biological assembly with a trimer of dimers. (B) Dimer assembly (green/orange) and N-glycans are shown in cartoon and stick representation. (C) The germin dimer (red) showing the close structural similarity with the Peruvianin-I dimer. It should be noted that the apparent Peruvianin-I missing C-terminal domain α-helical clasps due to the poor electron density of this region.

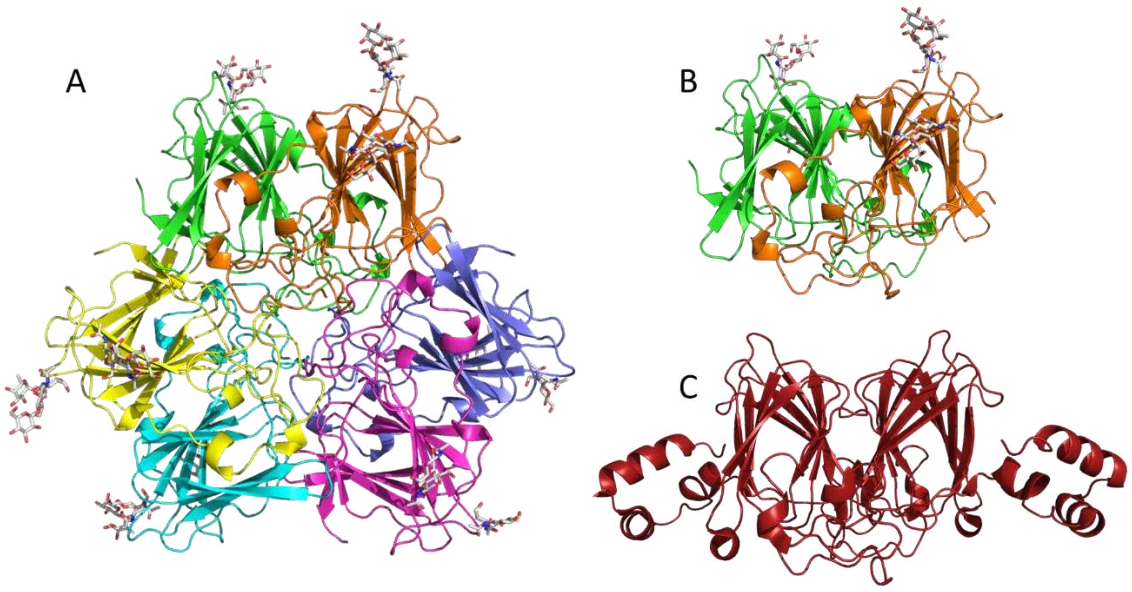


Figura 16. Overall monomer structure of glycosylated Peruvianin-I. (A) Glycosylation at ASN-55 and ANS-144 are shown with the stick model. The modeled N-glycans contain two N-acetyl-β-d-glucosamine (GlcNac) molecules, one β-d-mannopyranose (Man), and for ASN55 an additional α-d-fucopyranose (Fuc). (B, C). Electron density map 2Fo – Fc (contoured at 1σ) corresponding to the oligosaccharides N-linked to ASN-144 (B) an ASN-55 (C).

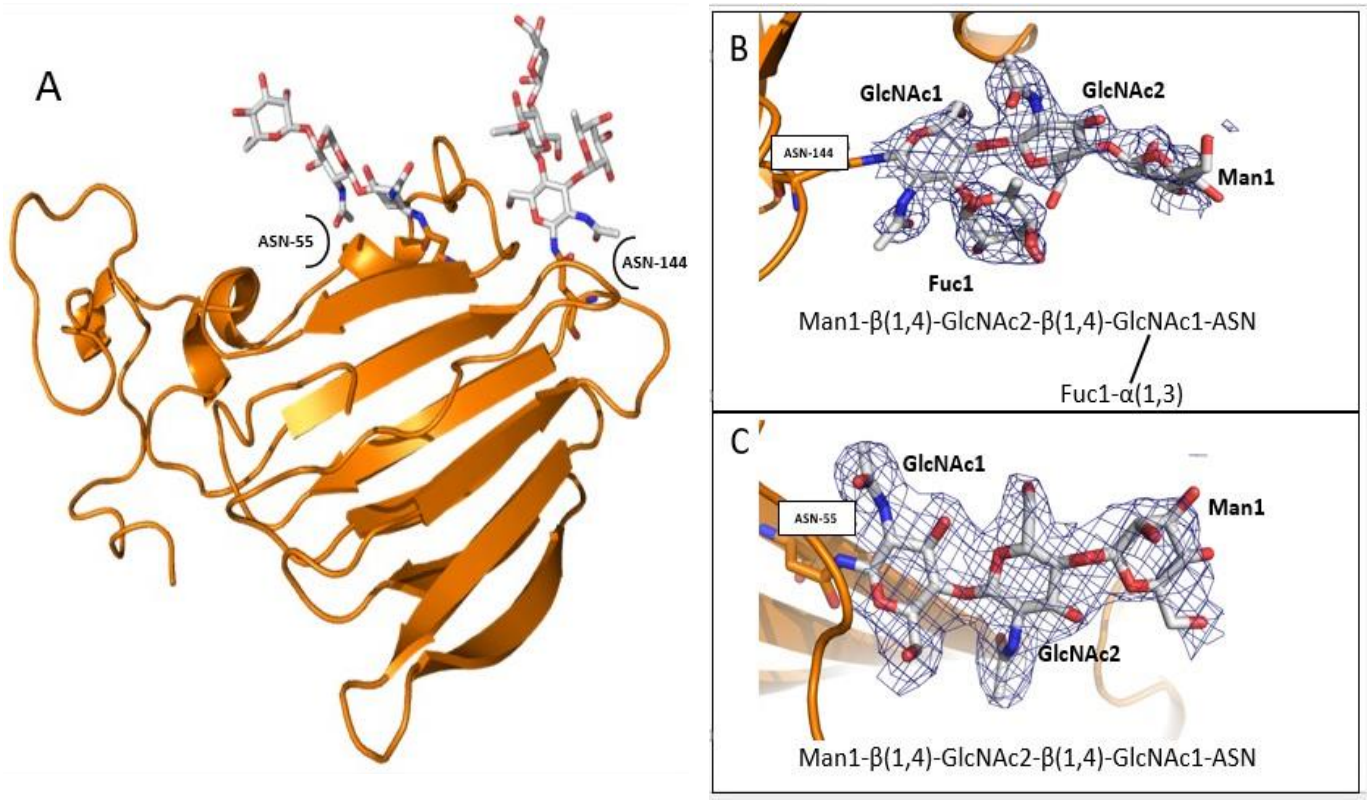


Figura 17. (A) Structure superimpose between Peruvianin-I (orange) and germin (cyan). (B) Four amino acids of catalytic site involved in the oxalate oxidase activity of the barley germin and their interaction with Mn^{2+} ion (green). (C) Modified catalytic site of Peruvianin-I: ARG 88, ASN 90, SER 95 and HIS135 residues absent of Mn^{2+} ion. There is a clear difference in the aminoacid composition between the catalytic sites of these enzymes.

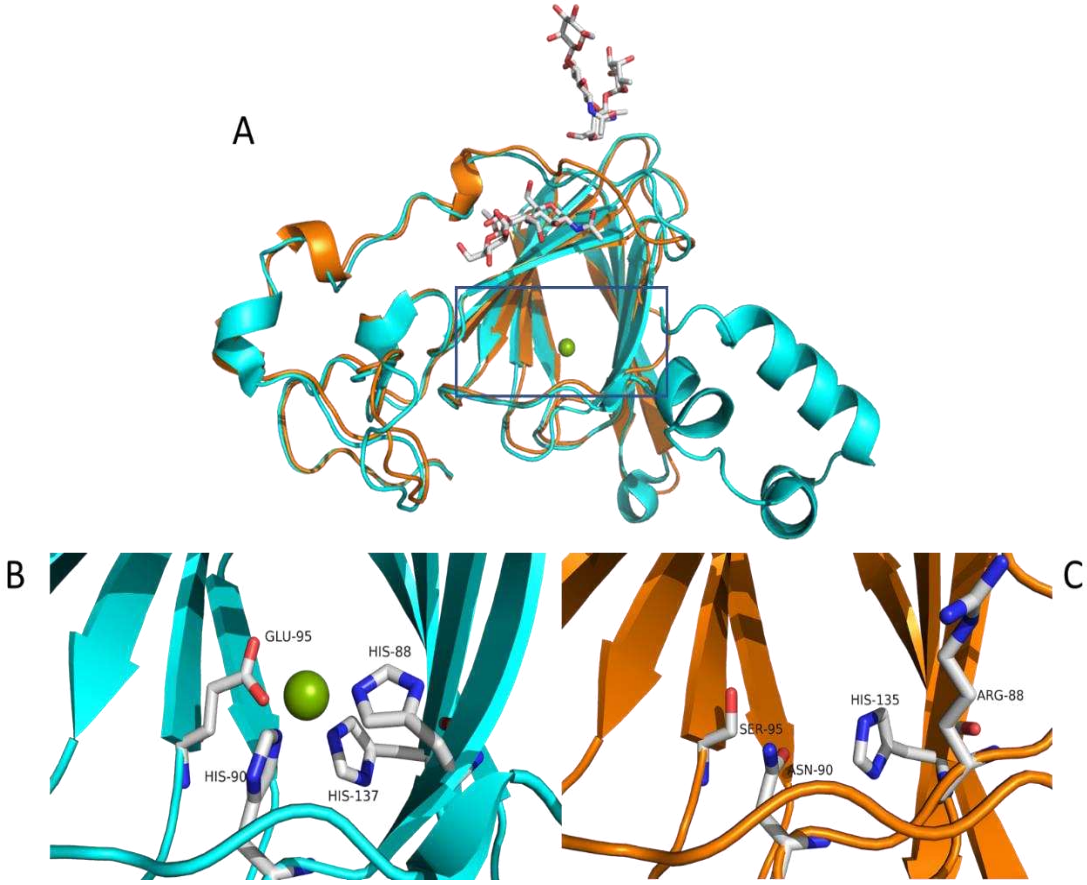


Figura 18. The catalytic mechanism suggested for the cysteine peptidase peruvianin-I. Description of all steps are presented in the text.

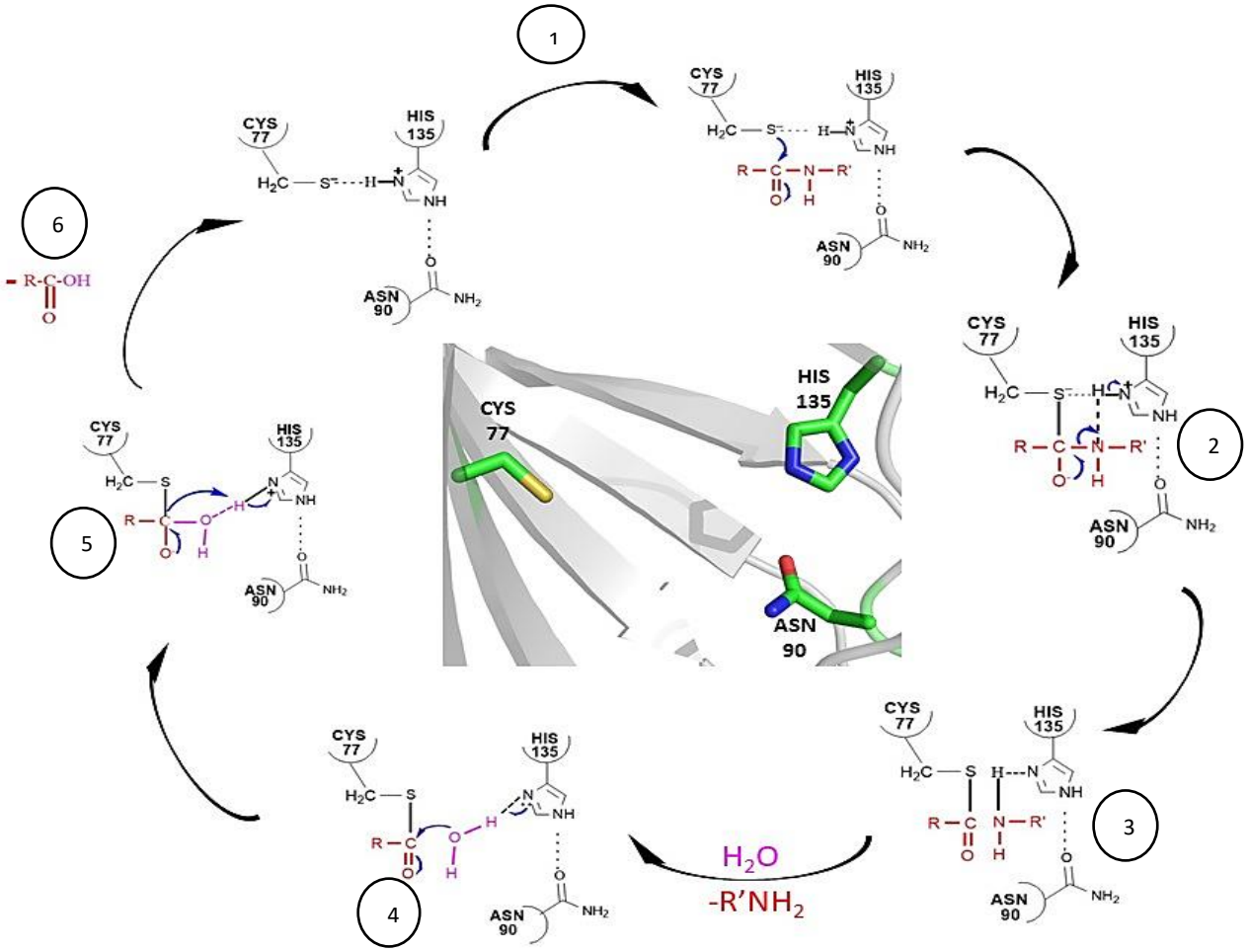


Figura 19. Molecular docking evidencing the amino acid residues involved in the cysteine proteolytic activity of Peruvianin-I, and their interaction site in the inset with the cysteine peptidase inhibitor iodoacetamide (IAA) in cyan. The arrangement of the oligosaccharides attached to the asn-144 and asn-55 residues are distal to the inner region of the hydrophobic center of the beta barrel motif which probably does not interfere in substrate insertion into the active site of the enzyme.

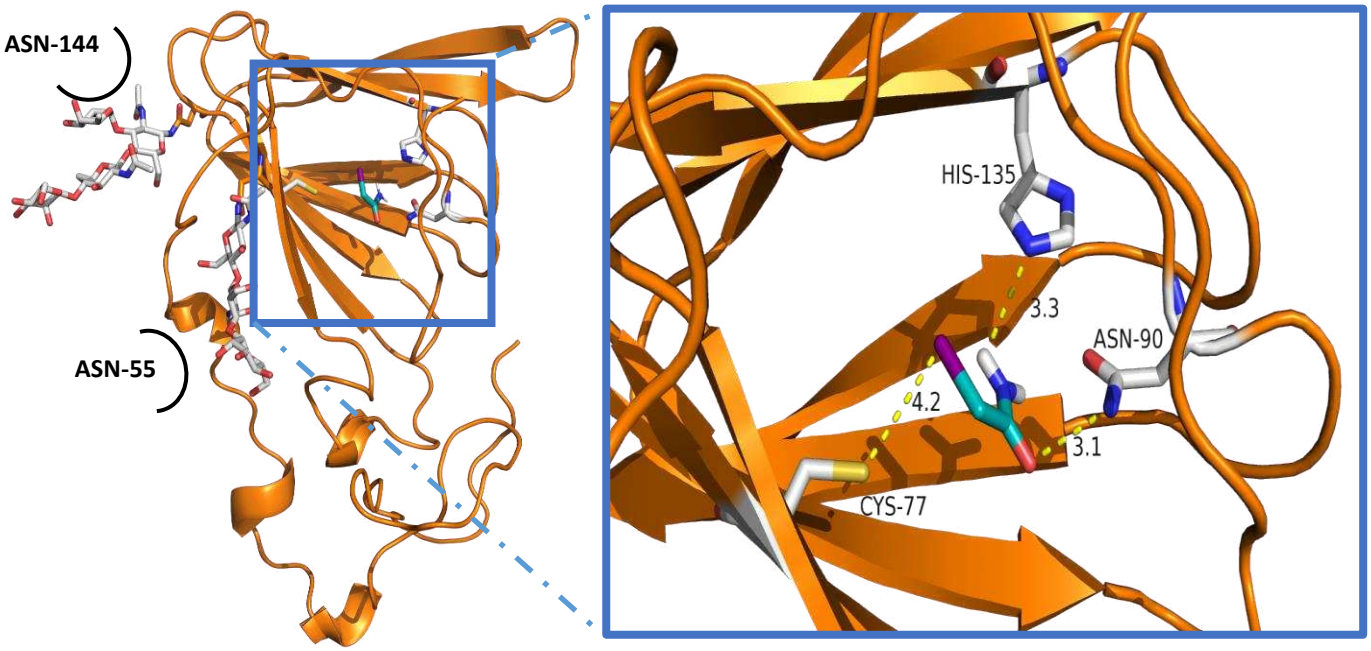


Tabela 4. Data collection and refinement statistics of Peruvianin-I.

DATA COLLECTION	
Source	LNLS
Wavelength (Å)	1.450
Resolution (Å)	44.32 – 2.15 (2.22 – 2.15)
Space group	$P4_12_12$
Unit cell (Å)	a = b = 88.64, c = 179.09
Molecules / a.u.	3
Unique reflections	39673 (3319)
Completeness (%)	99.8 (98.2)
R_{meas} [b]	0.058 (1.180)
R_{pim} [c]	0.016 (0.478)
R_{merge}	0.055 (1.071)
CC(1/2)	1.000 (0.735)
Multiplicity	22.9 (10.8)
I/sig(I)	39.7 (2.1)
B_{Wilson} (Å^2)	27.4
Software used for integration	XDS
Software used for scaling	Aimless
DATA REFINEMENT	
R_{cryst} [d] / R_{free} [e]	0.184/ 0.210
No. Reflections used for R_{free} (%)	5
R.m.s.d. bonds (Å)	0.009
R.m.s.d. angles (º)	1.045
Software used for refinement	PHENIX
Refinement method	ML/TLS

[a] Values in parentheses are for the highest resolution shell.

[b] $$R_{meas} = \frac{\sum_h \sqrt{\frac{n_h}{n_h - 1}} \sum_i^{n_h} \left| \hat{I}_h - I_{h,i} \right|}{\sum_h \sum_i^{n_h} I_{h,i}} \text{ with } \hat{I}_h = \frac{1}{n_h} \sum_i^{n_h} I_{h,i}$$

[c] $$R_{pim} = \sum_{hkl} \sqrt{\frac{1}{N-1}} \sum_i \left| I_i(hkl) - \overline{I(hkl)} \right| / \sum_{hkl} \sum_i I_i(hkl)$$

Where I (hkl) is the mean intensity of multiple I_i (*hkl*) observations of the symmetry-related reflections, N is the redundancy, n_h is the multiplicity, $\hat{I}_h$ is the average intensity and $I_{h,i}$ is the observed intensity.

[d] $R_{cryst} = \Sigma \, |F_o - F_c| \, / \, \Sigma \, F_o$

[e] R_{free} is the cross-validation R_{factor} computed for the test set of reflections (5 %) which are omitted in the refinement process.

5. CONSIDERAÇÕES FINAIS

Nossos achados incluem a primeira estrutura cristalina de um GLP com atividade proteolítica, evidenciando a diversidade funcional de proteínas pertencentes à família da germina.

A peruvianina-I apresenta o mesmo arranjo estrutural da germina ou dos GLPs e os dados estruturais confirmam a ausência de atividades de OXO e SOD.

Adicionalmente, com investigação detalhada dos resíduos do sítio ativo foi possível sugerir um mecanismo de ação para a atividade da cisteína peptidase da peruvianina-I.

REFERÊNCIAS

ADAMS PD; AFONINE PV; BUNKÓCZI G; CHEN VB; DAVIS IW; ECHOLS N, HEADD JJ; HUNG LW; KAPRAL GJ; GROSSE-KUNSTLEVE RW; MCCOY AJ; MORIARTY NW; OEFFNER R; READ RJ; RICHARDSON DC; RICHARDSON JS; TERWILLIGER TC; ZWART PH. **PHENIX: a comprehensive Python-based system for macromolecular structure solution.** [*s.l.*]: Acta Crystallogr D Biol Crystallogr. 2010 Feb;66(Pt 2):213-21. doi: 10.1107/S0907444909052925. Epub 2010 Jan 22. PMID: 20124702; PMCID: PMC2815670.

AGRAWAL, A.A.; KONNO, K.; 2009. **Latex: A Model for Understanding Mechanisms, Ecology, and Evolution of Plant Defense Against Herbivory**. [*s.l.*]: Annu. Rev. Ecol. Evol. Syst. 40, 311–331. https://doi.org/10.1146/annurev.ecolsys.110308.120307

ALTSCHUL SF; GISH W; MILLER W; MYERS EW; LIPMAN DJ; **Basic local alignment search tool.** [*s.l.*]: J Mol Biol. 1990 Oct 5;215(3):403-10. doi: 10.1016/S0022-2836(05)80360-2. PMID: 2231712.

ANTÃO, C.M.; MALCATA, F.X., 2005. **Plant serine proteases: biochemical, physiological and molecular features.** [*s.l.*]: Plant Physiol. Biochem. 43, 637–50. https://doi.org/10.1016/j.plaphy.2005.05.001

AZARKAN M; EL MOUSSAOUI A; VAN WUYTSWINKEL D; DEHON G; LOOZE Y. **Fractionation and purification of the enzymes stored in the latex of Carica papaya**. [*s.l.*]: J Chromatogr B Analyt Technol Biomed Life Sci. 2003 Jun 25;790(1-2):229-38. doi: 10.1016/s1570-0232(03)00084-9. PMID: 12767335.

BADGUJAR, S.B.; MAHAJAN, R.T.; 2014. **Identification and characterization of Euphorbia nivulia latex proteins**. [*s.l.*]:Int. J. Biol. Macromol. 64, 193–201. https://doi.org/10.1016/j.ijbiomac.2013.12.006

BADGUJAR, S.B.; PATEL, V. V; BANDIVDEKAR, A.H.; MAHAJAN, R.T.; 2014. **Traditional uses, phytochemistry and pharmacology of Ficus carica: a review.** [*s.l.*]: Pharm. Biol. 52, 1487–503. https://doi.org/10.3109/13880209.2014.892515

BAH, S.; PAULSEN, B.S.; DIALLO, D.; JOHANSEN, H.T.; 2006. **Characterization of cysteine proteases in Malian medicinal plants.** [*s.l.*]: J. Ethnopharmacol. 107, 189–98. https://doi.org/10.1016/j.jep.2006.03.008

BAN, N.; MCPHERSON, A.; 1995. **The structure of satellite panicum mosaic virus at 1.9 Å resolution.** [*s.l.*]: Nat. Struct. Biol. 2, 882–890. https://doi.org/10.1038/nsb1095-882

BANDARA, V.; WEINSTEIN, S. A; WHITE, J.; EDDLESTON, M.; 2010. **A review of the natural history, toxinology, diagnosis and clinical management of Nerium oleander (common**

oleander) and Thevetia peruviana (yellow oleander) poisoning. [*s.l.*]: Toxicon 56, 273–81. https://doi.org/10.1016/j.toxicon.2010.03.026

BAREL AO; GLAZER AN. **Spectroscopic studies on papain and some inactive derivatives**. [*s.l.*]: J Biol Chem. 1969 Jan 25;244(2):268-73. PMID: 5773296.

BARMAN, A.R.; BANERJEE, J.; 2015. **Versatility of germin-like proteins in their sequences, expressions, and functions**. [*s.l.*]: Funct. Integr. Genomics. https://doi.org/10.1007/s10142-015-0454-z

BARRETT, A.J.; FRED WOESSNER, J.; 2013. **Handbook of Proteolytic Enzymes, Handbook of Proteolytic Enzymes.** [*s.l.*]: Elsevier. https://doi.org/10.1016/B978-0-12-382219-2.00838-3

BAUER, G.; GORB, S.N.; KLEIN, M.-C.; NELLESEN, A.; VON TAPAVICZA, M.; SPECK, T.; 2014. **Comparative study on plant latex particles and latex coagulation in Ficus benjamina, Campanula glomerata and three Euphorbia species.** [*s.l.*]: PLoS One 9, e113336. https://doi.org/10.1371/journal.pone.0113336

BERACOCHEA VC; ALMASIA NI; PELUFFO L; NAHIRÑAK V; HOPP EH; PANIEGO N; HEINZ RA; VAZQUEZ-ROVERE C; LIA VV; **Sunflower germin-like protein HaGLP1 promotes ROS accumulation and enhances protection against fungal pathogens in transgenic Arabidopsis thaliana.** [*s.l.*]: Plant Cell Rep. 2015 Oct;34(10):1717-33. doi: 10.1007/s00299-015-1819-4. Epub 2015 Jun 13. PMID: 26070410.

BERNIER, F.; BERNA, A.; 2001. **Germins and germin-like proteins: Plant do-all proteins. But what do they do exactly?** [*s.l.*]: Plant Physiol. Biochem. https://doi.org/10.1016/S0981-9428(01)01285-2

BORDOLI L; KIEFER F; ARNOLD K; BENKERT P; BATTEY J; SCHWEDE T. **Protein structure homology modeling using SWISS-MODEL workspace.** [*s.l.*]: Nat Protoc. 2009;4(1):1-13. doi: 10.1038/nprot.2008.197. PMID: 19131951.

BREEN J; BELLGARD M. **Germin-like proteins (GLPs) in cereal genomes: gene clustering and dynamic roles in plant defence.** [*s.l.*]: Funct Integr Genomics. 2010 Nov;10(4):463-76. doi: 10.1007/s10142-010-0184-1. Epub 2010 Aug 4. PMID: 20683632.

BUTTS, C.T.; ZHANG, X.; KELLY, J.E.; ROSKAMP, K.W.; UNHELKAR, M.H.; FREITES, J.A.; TAHIR, S.; MARTIN, R.W.; 2016. **Sequence comparison, molecular modeling, and network analysis predict structural diversity in cysteine proteases from the Cape sundew, Drosera capensis**. [*s.l.*]: Comput. Struct. Biotechnol. J. 14, 271–282. https://doi.org/10.1016/j.csbj.2016.05.003

CALLAHAN, D.L.; ROESSNER, U.; DUMONTET, V.; PERRIER, N.; WEDD, A.G.; O'HAIR, R. A J.; BAKER, A.J.M.; KOLEV, S.D. 2008. **LC-MS and GC-MS metabolite profiling of nickel(II) complexes in the latex of the nickel-hyperaccumulating tree Sebertia acuminata and identification of methylated aldaric acid as a new nickel(II) ligand**. [*s.l.*]: Phytochemistry

69, 240–51. https://doi.org/10.1016/j.phytochem.2007.07.001

CARTER, C.; THORNBURG, R.W. **Germin-like proteins: structure, phylogeny, and function.** [*s.l.*]: *J. Plant Biol.* **42**, 97–108 (1999). https://doi.org/10.1007/BF03031017

CHATTOPADHYAY, **In silico analysis of the germin like protein multigene family members of tomato with predicted oxalate oxidase activity.** [*s.l.*]: Int. J. Agric. Environ. Biotechnol 7 (2014) 669–678, https://doi.org/10.5958/2230-732X.2014.01374.6.

CHENG X; HUANG X; LIU S; TANG M; HU W; PAN S. **Characterization of germin-like protein with polyphenol oxidase activity from Satsuma mandarine.** [*s.l.*]: Biochem Biophys Res Commun. 2014 Jul 4;449(3):313-8. doi: 10.1016/j.bbrc.2014.05.027. Epub 2014 May 17. PMID: 24845377.

CHRUSZCZ M; MALEKI SJ; MAJOREK KA; DEMAS M; BUBLIN M; SOLBERG R; HURLBURT BK; RUAN S; MATTISON CP; BREITENEDER H; MINOR W. **Structural and immunologic characterization of Ara h 1, a major peanut allergen**. [*s.l.*]: J Biol Chem. 2011 Nov 11;286(45):39318-27. doi: 10.1074/jbc.M111.270132.

CRUZ, W.T.; BEZERRA, E.H.S.; GRANGEIRO, T.B.; LOPES, J.L.S.; SILVA, M.Z.R.; RAMOS, M. V; ROCHA, B.A.M.; OLIVEIRA, J.S.; FREITAS, D.C.; FREITAS, C.D.T. 2019. **Structural and enzymatic characterization of Peruvianin - I , the fi rst germin-like protein with proteolytic activity**. [*s.l.*]: Int. J. Biol. Macromol. 126, 1167–1176. https://doi.org/10.1016/j.ijbiomac.2019.01.023

DÍAZ-MENDOZA M; VELASCO-ARROYO B; GONZÁLEZ-MELENDI P; MARTÍNEZ M; DÍAZ I. **C1A cysteine protease-cystatin interactions in leaf senescence**. [*s.l.*]: J Exp Bot. 2014 Jul;65(14):3825-33. doi: 10.1093/jxb/eru043. Epub 2014 Mar 5. PMID: 24600023.

DUMAS B; FREYSSINET G; PALLETT KE. **Tissue-Specific Expression of Germin-Like Oxalate Oxidase during Development and Fungal Infection of Barley Seedlings.** [*s.l.*]: Plant Physiol. 1995 Apr;107(4):1091-1096. doi: 10.1104/pp.107.4.1091. PMID: 12228419; PMCID: PMC157241.

DUNWELL, J.M., PURVIS, A., KHURI, S., 2004. **Cupins: the most functionally diverse protein superfamily?** [*s.l.*]: Phytochemistry 65, 7–17. https://doi.org/10.1016/j.phytochem.2003.08.016

DUNWELL, J.M.; CULHAM, A.; CARTER, C.E.; SOSA-AGUIRRE; C.R., GOODENOUGH, P.W.; 2001. **Evolution of functional diversity in the cupin superfamily**. [*s.l.*]: Trends Biochem. Sci. 26, 740–746. https://doi.org/10.1016/S0968-0004(01)01981-8

DUTTA, J.K; DATTAGUPTA, S; BISWAS, **Enhancement of proteolytic activity of a thermo-stable papain-like protease by structure-based rational design**. [*s.l.*]: PLoS One 8 (2013), e62619. https://doi.org/10.1371/journal.pone.0062619.

EKCHAWENG K; EVANGELISTI E; SCHORNACK S; TIAN M; CHURNGCHOW N; **The plant defense and pathogen counterdefense mediated by Hevea brasiliensis serine protease HbSPA and Phytophthora palmivora extracellular protease inhibitor PpEPI10**. [*s.l.*]: PLoS One. 2017 May 1;12(5):e0175795. doi: 10.1371/journal.pone.0175795. PMID: 28459807; PMCID: PMC5411025.

EVERT, R.F., 2006. **Esau's Plant Anatomy, Esau's Plant Anatomy: Meristems, Cells, and Tissues of the Plant Body: Their Structure, Function, and Development: Third Edition**. [*s.l.*]:John Wiley & Sons, Inc.

EWING B; HILLIER L; WENDL MC. **Green P. Base-calling of automated sequencer traces using phred. I. Accuracy assessment**. [*s.l.*]: Genome Res. 1998 Mar;8(3):175-85. doi: 10.1101/gr.8.3.175. PMID: 9521921.

FAYE, L.; CHRISPEELS, M.J.; 1988. **Common antigenic determinants in the glycoproteins of plants, molluscs and insects**. [*s.l.*]: Glycoconj. J. 5, 245–256. https://doi.org/10.1007/BF01049085

FEIJOO-SIOTA, L.; VILLA, T.G., 2010. **Native and Biotechnologically Engineered Plant Proteases with Industrial Applications**. [*s.l.*]: Food Bioprocess Technol. 4, 1066–1088. https://doi.org/10.1007/s11947-010-0431-4

FERNANDEZ-FUENTES N; MADRID-ALISTE CJ; RAI BK; FAJARDO JE; FISER A. **M4T: a comparative protein structure modeling server.** [*s.l.*]: Nucleic Acids Res. 2007 Jul;35(Web Server issue):W363-8. doi: 10.1093/nar/gkm341. Epub 2007 May 21. PMID: 17517764; PMCID: PMC1933164.

FERRÈ F; CLOTE P. **DiANNA: a web server for disulfide connectivity prediction.** [*s.l.*]: Nucleic Acids Res. 2005 Jul 1;33(Web Server issue):W230-2. doi: 10.1093/nar/gki412. PMID: 15980459; PMCID: PMC1160173.

FREITAS, C.D.T.; LEITE, H.B.; OLIVEIRA, J.P.B.; AMARAL, J.L.; EGITO, A.S.; VAIRO-CAVALLI, S.; LOBO, .D.P.; MONTEIRO-MOREIRA, A.C.O.; RAMOS, M. V. 2016. **Insights into milk-clotting activity of latex peptidases from Calotropis procera and Cryptostegia grandiflora**. [*s.l.*]: Food Res. Int. 87, 50–59. https://doi.org/10.1016/J.FOODRES.2016.06.020

FREITAS, C.D.T.; NOGUEIRA, F.C.S.; VASCONCELOS, I.M.; OLIVEIRA, J.T.A.; DOMONT, G.B., RAMOS, M.V., 2011. **Osmotin purified from the latex of Calotropis procera: biochemical characterization, biological activity and role in plant defense**. [*s.l.*]: Plant Physiol. Biochem. 49, 738–43. https://doi.org/10.1016/j.plaphy.2011.01.027

FREITAS, C.D.T.; OLIVEIRA, J.S.; MIRANDA, M.R. A; MACEDO, N.M.R.; SALES, M.P.; VILLAS-BOAS; L. A, RAMOS, M.V., 2007. **Enzymatic activities and protein profile of latex from Calotropis procera**. [*s.l.*]: Plant Physiol. Biochem. 45, 781–9. https://doi.org/10.1016/j.plaphy.2007.07.020

FREITAS, C.D.T; CRUZ, W.T.; SILVA, M.Z; VASCONCELOS, I.M.; MORENO, F.B.; MOREIRA, R.A.; MONTEIRO-MOREIRA, A.C.; ALENCAR, L.M.; SOUSA, J.S.; ROCHA, B.A.; RAMOS, M.V. **Proteomic analysis and purification of an unusual germin-like protein with proteolytic activity in the latex of Thevetia peruviana**. [*s.l.*]: Planta. 2016 May;243(5):1115-28. doi: 10.1007/s00425-016-2468-8.

FREITAS CDT; FREITAS DC; CRUZ WT; PORFÍRIO CTMN; SILVA MZR; OLIVEIRA JS; CARVALHO CPS; RAMOS MV. **Identification and characterization of two germin-like proteins with oxalate oxidase activity from Calotropis procera latex.** [*s.l.*]: Int J Biol Macromol. 2017 Dec;105(Pt 1):1051-1061. doi: 10.1016/j.ijbiomac.2017.07.133. Epub 2017 Jul 25. PMID: 28754622.

GHOSH R; CHAKRABORTY S; CHAKRABARTI C; DATTAGUPTA JK; BISWAS S. **Structural insights into the substrate specificity and activity of ervatamins, the papain-like cysteine proteases from a tropical plant, Ervatamia coronaria**. [*s.l.*]: FEBS J. 2008 Feb;275(3):421-34. doi: 10.1111/j.1742-4658.2007.06211.x. Epub 2007 Dec 19. PMID: 18167146.

GOMES, M.T.R.; RIBEIRO, H.A.; LOPES, M.T.P.; GUZMAN, F.; SALAS, C.E., 2010. **Biochemical comparison of two proteolytic enzymes from Carica candamarcensis: Structural motifs underlying resistance to cystatin inhibition.** [*s.l.*]: Phytochemistry 71, 524–530. https://doi.org/10.1016/j.phytochem.2009.12.018

GORDON D; ABAJIAN C; GREEN P. **Consed: a graphical tool for sequence finishing**. [*s.l.*]: Genome Res. 1998 Mar;8(3):195-202. doi: 10.1101/gr.8.3.195. PMID: 9521923.

GRANDITS, M.; OOSTENBRINK, C.; 2014. **Molecular dynamics simulations of the auxin-binding protein 1 in complex with indole-3-acetic acid and naphthalen-1-acetic acid**. [*s.l.*]: Proteins 82, 2744–2755. https://doi.org/10.1002/prot.24639

GRZELCZAK, Z.F.; LANE, B.G., 1984. **Signal resistance of a soluble protein to enzymic proteolysis. An unorthodox approach to the isolation and purification of germin, a rare growth-related protein.** [*s.l.*]: Can. J. Biochem. Cell Biol. 62, 1351–1353. https://doi.org/10.1139/o84-172

GRZONKA, Z.; JANKOWSKA, E.; KASPRZYKOWSKI, F.; KASPRZYKOWSKA, R.; ŁANKIEWICZ, L.; WICZK, W.; WIECZERZAK, E.; CIARKOWSKI, J.; DRABIK, P.; JANOWSKI, R.; KOZAK, M.; JASKÓLSKI, M.; GRUBB, A., 2001. **Structural studies of cysteine proteases and their inhibitors.** [*s.l.*]: Acta Biochim. Pol. 48, 1–20.

HALE, L.P.; GREER, P.K.; TRINH, C.T.; GOTTFRIED, M.R., 2005. **Treatment with oral bromelain decreases colonic inflammation in the IL-10-deficient murine model of inflammatory bowel disease.** [*s.l.*]: Clin. Immunol. 116, 135–42. https://doi.org/10.1016/j.clim.2005.04.011

HEBERT DN; LAMRIBEN L; POWERS ET; KELLY JW. **The intrinsic and extrinsic effects of N-linked glycans on glycoproteostasis.** [*s.l.*]: Nat Chem Biol. 2014 Nov;10(11):902-10. doi: 10.1038/nchembio.1651. Epub 2014 Oct 17. PMID: 25325701; PMCID: PMC4232232.

HO, C.C., 2014. **Chapter 4 - The Production of Natural Rubber from Hevea brasiliensis Latex: Colloidal Properties, Preservation, Purification and Processing, RSC Polymer Chemistry Series**. [*s.l.*]: Royal Society of Chemistry, Cambridge. https://doi.org/10.1039/9781849737647

HÖRDEGEN, P.; HERTZBERG, H.; HEILMANN, J.; LANGHANS, W.; MAURER, V.; 2003. **The anthelmintic efficacy of five plant products against gastrointestinal trichostrongylids in artificially infected lambs**. [*s.l.*]: Vet. Parasitol. 117, 51–60.

ILYAS M; RASHEED A; MAHMOOD T. **Functional characterization of germin and germin-like protein genes in various plant species using transgenic approaches.** [*s.l.*]: Biotechnol Lett. 2016 Sep;38(9):1405-21. doi: 10.1007/s10529-016-2129-9. Epub 2016 May 26. PMID: 27230937.

JAIKARAN AS; KENNEDY TD; DRATEWKA-KOS E; LANE BG. **Covalently bonded and adventitious glycans in germin.** [*s.l.*]:J Biol Chem. 1990 Jul 25;265(21):12503-12. PMID: 2115520.

JASHNI MK; MEHRABI R; COLLEMARE J; MESARICH CH; de WIT PJ. **The battle in the apoplast: further insights into the roles of proteases and their inhibitors in plant-pathogen interactions**. [*s.l.*]: Front Plant Sci. 2015 Aug 3;6:584. doi: 10.3389/fpls.2015.00584. PMID: 26284100; PMCID: PMC4522555.

JOSEPH, B.; RAJ, S.J. 2011. **Pharmacognostic and phytochemical properties of Ficus carica Linn – An overview**. [*s.l.*]: Int. J. PharmaTech Res. 3, 8–12.

KABAKOV AE; GABAI VL. **Protein aggregation as primary and characteristic cell reaction to various stresses**. [*s.l.*]: Experientia. 1993 Aug 15;49(8):706-13. doi: 10.1007/BF01923956. PMID: 8359277.

KARDOS, J.; BÓDI, A.; ZÁVODSZKY, P.; VENEKEI, I.; GRÁF, L., 1999. **Disulfide-linked propeptides stabilize the structure of zymogen and mature pancreatic serine proteases**. [*s.l.*]: Biochemistry 38, 12248–57.

KEKWICK, R.G. (2002). **Latex and Laticifers.** [*s.l.*]: In eLS, (Ed.). https://doi.org/10.1038/npg.els.0000913

KHOBRAGADE CN; BEEDKAR SD; BODADE RG; VINCHURKAR AS. **Comparative structural modeling and docking studies of oxalate oxidase: Possible implication in enzyme supplementation therapy for urolithiasis.** [*s.l.*]: Int J Biol Macromol. 2011 Apr 1;48(3):466-73. doi: 10.1016/j.ijbiomac.2011.01.007. Epub 2011 Jan 19. PMID: 21255608.

KONNO, K., 2011. **Plant latex and other exudates as plant defense systems: Roles of various defense chemicals and proteins contained therein.** [*s.l.*]: Phytochemistry. https://doi.org/10.1016/j.phytochem.2011.02.016

KO J; PARK H; HEO L; SEOK C. **GalaxyWEB server for protein structure prediction and refinement.** [*s.l.*]: Nucleic Acids Res. 2012 Jul;40(Web Server issue):W294-7. doi: 10.1093/nar/gks493. Epub 2012 May 30. PMID: 22649060; PMCID: PMC3394311.

KUMAGAI OS; DEMARCO R; LOPES JLS. **Advantages of synchrotron radiation circular dichroism spectroscopy to study intrinsically disordered proteins**. [*s.l.*]: Eur Biophys J. 2017 Oct;46(7):599-606. doi: 10.1007/s00249-017-1202-1. Epub 2017 Mar 3. PMID: 28258312.

KUNAKBAEVA, Z.; CARRASCO, R.; ROZAS, I., 2003. **An approximation to the mechanism of inhibition of cystein proteases: nucleophilic sulphur addition to Michael acceptors type compounds**. [*s.l.*]: J. Mol. Struct. THEOCHEM 626, 209–216. https://doi.org/10.1016/S0166-1280(03)00086-1

LANE, B.G. **Oxalate, germins, and higher-plant pathogens.** [*s.l.*]: IUBMB Life. 2002 Feb;53(2):67-75. doi: 10.1080/15216540211474. PMID: 12049198.

LANE, B.G. **Oxalate oxidases and differentiating surface structure in wheat: germins**. [*s.l.*]: Biochem J. 2000 Jul 1;349(Pt 1):309-21. doi: 10.1042/0264-6021:3490309. PMID: 10861243; PMCID: PMC1221152.

LANE, B.G.; BERNIERP, F.; DRATEWKA-KOS, E.; SHAFAI, R.; KENNEDY, D.; PYNE, C.; MUNRO, J.R.; VAUGHAN, T.; WALTERS, D.; ALTOMARE, F.; GENE, G., 1991. **Homologies between Members of the Germin Gene Family in Hexaploid Wheat and Similarities between These Wheat Germins and Certain**. [*s.l.*]: J. Biol. Chem. 266, 10461–10469.

LANGFORD, S.D.; BOOR, P.J., 1996. **Oleander toxicity: an examination of human and animal toxic exposures**. [*s.l.*]: Toxicology 109, 1–13. https://doi.org/10.1016/0300-483X(95)03296-R

LASKOWSKI RA; MACARTHUR MW; MOSS DS; THORNTON JM. **PROCHECK: a program to check the stereochemical quality of protein structures.** [*s.l.*]: Journal of Applied Crystallography. 1993 Apr;26(2):283-291. DOI: 10.1107/s0021889892009944.

LEES JG; SMITH BR; WIEN F; MILES AJ; WALLACE BA. **CDtool-an integrated software package for circular dichroism spectroscopic data processing, analysis, and archiving**. [*s.l.*]: Anal Biochem. 2004 Sep 15;332(2):285-9. doi: 10.1016/j.ab.2004.06.002. PMID: 15325297.

LEWINSOHN, T.M., 1991. **The geographical distribution of plant latex**. [*s.l.*]: Chemoecology 2, 64–68. https://doi.org/10.1007/BF01240668

LIMA, R.C. DE S.; SILVA, M.C.C.; AGUIAR, C.C.T.; CHAVES, E.M.C.; DIAS, K.C.F.; MACÊDO, D.S.; DE SOUSA, F.C.F.; CARVALHO, K. DE M.; RAMOS, M.V.;

VASCONCELOS, S.M.M.; 2012. **Anticonvulsant action of Calotropis procera latex proteins**. [*s.l.*]: Epilepsy Behav. 23, 123–6. https://doi.org/10.1016/j.yebeh.2011.11.008

LÓPEZ, L.M.I.; VIANA, C.A.; ERRASTI, M.E.; GARRO, M.L.; MARTEGANI, J.E.; 2017. **Latex peptidases of Calotropis procera for dehairing of leather as an alternative to environmentally toxic sodium sulfide treatment**. [*s.l.*]: Bioprocess and Biosystems Engineering. https://doi.org/10.1007/s00449-017-1796-9

MAHAJAN, R.T.; BADGUJAR, S.B., 2010. **Biological aspects of proteolytic enzymes : A Review.** [*s.l.*]:**,** Journal of Pharmacy Research.

MANSILLA, A.Y.; SEGARRA, C.I., 2012. **Structural and Functional Features of a Wheat Germin-Like Protein that Inhibits Trypsin.** [*s.l.*]: 624–632. https://doi.org/10.1007/s11105-011-0372-8

MANSUR, F.; LUOGA, W.; BUTTLE, D.J.; DUCE, I.R.; LOWE, A.; BEHNKE, J.M.; 2014. **The anthelmintic efficacy of natural plant cysteine proteinases against two rodent cestodes Hymenolepis diminuta and Hymenolepis microstoma in vitro.** [*s.l.*]: Vet. Parasitol. 201, 48–58. https://doi.org/10.1016/j.vetpar.2013.12.018

MITHÖFER, A.; BOLAND, W. 2012. **Plant defense against herbivores: chemical aspects.** [*s.l.*]: Annu. Rev. Plant Biol. 63, 431–50. https://doi.org/10.1146/annurev-arplant-042110-103854

MNIF, I.H.; SIALA, R.; NASRI, R.; MHAMDI, S.; NASRI, M.; KAMOUN, A.S., 2015. **A cysteine protease isolated from the latex of Ficus microcarpa: purification and biochemical characterization.** [*s.l.*]: Appl. Biochem. Biotechnol. 175, 1732–44. https://doi.org/10.1007/s12010-014-1376-2

MONTI, R.; BASILIO, C.A.; TREVISAN, H.C.; CONTIERO, J., 2000. **Purification of Papain from Fresh Latex of Carica papaya.** [*s.l.*]: Brazilian Arch. Biol. Technol. 43, 501–507.

MORCELLE, S.R.; CAFFINI, N.O.; PRIOLO, N., 2004. **Proteolytic properties of Funastrum clausum latex**. [*s.l.*]: Fitoterapia 75, 480–93. https://doi.org/10.1016/j.fitote.2004.04.006

NOVINEC, M.; LENARCIC, B.; 2013. **Papain-like peptidases : structure , function , and evolution.** [*s.l.*]: BioMol Concepts 4, 287–308. https://doi.org/10.1515/bmc-2012-0054

OTTO, H.-H.; SCHIRMEISTER, T., 1997. **Cysteine Proteases and Their Inhibitors**. [*s.l.*]: Chem. Rev. 97, 133–172. https://doi.org/10.1021/cr950025u

PAN HY; WHITTAKER MM; BOUVERET R; BERNA A; BERNIER F; WHITTAKER JW. **Characterization of wheat germin (oxalate oxidase) expressed by Pichia pastoris**. [*s.l.*]: Biochem Biophys Res Commun. 2007 May 18;356(4):925-9. doi: 10.1016/j.bbrc.2007.03.097. Epub 2007 Mar 26. PMID: 17399681; PMCID: PMC1987392.

PATEL, B.K.; JAGANNADHAM, M. V, 2003. **A high cysteine containing thiol proteinase**

from the latex of Ervatamia heyneana: purification and comparison with ervatamin B and C from Ervatamia coronaria. [*s.l.*]: J. Agric. Food Chem. 51, 6326–34. https://doi.org/10.1021/jf026184d

PATNAIK D; KHURANA P. **Germins and germin like proteins: an overview**. [*s.l.*]: Indian J Exp Biol. 2001 Mar;39(3):191-200. PMID: 11495276.

PELTON JT; MCLEAN LR. **Spectroscopic methods for analysis of protein secondary structure.** [*s.l.*]: Anal Biochem. 2000 Jan 15;277(2):167-76. doi: 10.1006/abio.1999.4320. PMID: 10625503.

PIROVANI CP; MACÊDO JN; CONTIM LA; MATRANGOLO FS; LOUREIRO ME; FONTES EP. **A sucrose-binding protein homologue from soybean exhibits GTP-binding activity that functions independently of sucrose transport activity.** [*s.l.*]: Eur J Biochem. 2002 Aug;269(16):3998-4008. doi: 10.1046/j.1432-1033.2002.03089.x. PMID: 12180976.

RAJAVEL M; KULKARNI NN; GOPAL B. **Conformational studies suggest that the double stranded beta helix scaffold provides an optimal balance between protein stability and function.** [*s.l.*]: Protein Pept Lett. 2008;15(3):244-9. doi: 10.2174/092986608783744289. PMID: 18336351.

RAMOS-SILVA, A.; TAVARES-CARREÓN; F., FIGUEROA M.; DE LA TORRE-ZAVALA S.; GASTELUM-ARELLANEZ, A.; RODRÍGUEZ-GARCÍA, A.; GALÁN-WONG, L.J.; AVILÉS-ARNAUT, H., 2017. **Anticancer potential of Thevetia peruviana fruit methanolic extract.** [*s.l.*]: BMC Complement. Altern. Med. 17, 1–11. https://doi.org/10.1186/s12906-017-1727-y

RAMOS, M.V.; DEMARCO, D.; DA COSTA SOUZA, I.C.; DE FREITAS, C.D.T.; 2019. **Laticifers, Latex, and Their Role in Plant Defense.** [*s.l.*]: Trends Plant Sci. xx, 1–15. https://doi.org/10.1016/j.tplants.2019.03.006

RAMOS, M. V.; FREITAS, C.D.T.; STANISÇUASKI, F.; MACEDO, L.L.P.; SALES, M.P.; SOUSA, D.P.; CARLINI, C.R., 2007. **Performance of distinct crop pests reared on diets enriched with latex proteins from Calotropis procera: Role of laticifer proteins in plant defense**. [*s.l.*]:Plant Sci. 173, 349–357. https://doi.org/10.1016/j.plantsci.2007.06.008

RAMOS, M. V; ARAÚJO, E.S.; JUCÁ, T.L.; MONTEIRO-MOREIRA, A C.O.; VASCONCELOS, I.M.; MOREIRA, R. A; VIANA, C. A; BELTRAMINI, L.M.; PEREIRA, D. A; MORENO, F.B., 2013. **New insights into the complex mixture of latex cysteine peptidases in Calotropis procera**. [*s.l.*]: Int. J. Biol. Macromol. 58, 211–9. https://doi.org/10.1016/j.ijbiomac.2013.04.001

RAMOS, M. V; SOUZA, D.P.; GOMES, M.T.R.; FREITAS, C.D.T.; CARVALHO, C.P.S.; JÚNIOR, P. A V.R.; SALAS, C.E., 2014. **A phytopathogenic cysteine peptidase from latex of wild rubber vine Cryptostegia grandiflora**. [*s.l.*]: Protein J. 33, 199–209. https://doi.org/10.1007/s10930-014-9551-4

RAWLINGS, N.D. 2016. **Biochimie Peptidase specificity from the substrate cleavage collection in the MEROPS database and a tool to measure cleavage site conservation**. [*s.l.*]:Biochimie 122, 5–30. https://doi.org/10.1016/j.biochi.2015.10.003

RAWLINGS, N.D.; BARRETT, A.J.; BATEMAN, A., 2010. **MEROPS: the peptidase database**. [*s.l.*]: Nucleic Acids Res. 38, 227–33. https://doi.org/10.1093/nar/gkp971

RODRÍGUEZ-LÓPEZ M; BAROJA-FERNÁNDEZ E; ZANDUETA-CRIADO A; MORENO-BRUNA B; MUÑOZ FJ; AKAZAWA T; POZUETA-ROMERO J. **Two isoforms of a nucleotide-sugar pyrophosphatase/phosphodiesterase from barley leaves (Hordeum vulgare L.) are distinct oligomers of HvGLP1, a germin-like protein.** [*s.l.*]:FEBS Lett. 2001 Feb 9;490(1-2):44-8. doi: 10.1016/s0014-5793(01)02135-4. PMID: 11172808.

RZYCHON, M.; COMMISSION, E.; SELVITA, S.A., 2015. **Modes of inhibition of cysteine proteases Circled white star**. [*s.l.*]: Acta Biochim. Pol. 51, 861–873.

SAKAMOTO A; NISHIMURA T; MIYAKI YI; WATANABE S; TAKAGI H; IZUMI S; SHIMADA H. **In vitro and in vivo evidence for oxalate oxidase activity of a germin-like protein from azalea.** [*s.l.*]:Biochem Biophys Res Commun. 2015 Mar 13;458(3):536-542. doi: 10.1016/j.bbrc.2015.02.002. Epub 2015 Feb 10. PMID: 25677624.

SCHALLER A. **A cut above the rest: the regulatory function of plant proteases**. [*s.l.*]:Planta. 2004 Dec;220(2):183-97. doi: 10.1007/s00425-004-1407-2. Epub 2004 Oct 29. PMID: 15517349.

SCHWEIZER, P.; CHRISTOFFEL, A.; DUDLER, R., 1999. **Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance.** [*s.l.*]:Plant J. 20, 541–52.

SEGARRA CI; CASALONGUÉ CA; PINEDO ML; RONCHI VP; CONDE RD. **A germin-like protein of wheat leaf apoplast inhibits serine proteases.** [*s.l.*]: J Exp Bot. 2003 May;54(386):1335-41. doi: 10.1093/jxb/erg139. PMID: 12709479.

SETHI, A.; MCAUSLANE, H.J.; RATHINASABAPATHI, B.; NUESSLY, G.S.; NAGATA, R.T., 2009. **Enzyme induction as a possible mechanism for latex-mediated insect resistance in romaine lettuce.** [*s.l.*]: J. Chem. Ecol. 35, 190–200. https://doi.org/10.1007/s10886-009-9596-6

SHEPHERD, ROSAMOND C. H. (Rosamond Charmian Hollis). (2004). **Pretty but poisonous : plants poisonous to people : an illustrated guide for Australia** */ by R. C. H. Shepherd*. Melbourne : R. G. and F. J. Richardson

SHIVAPRASAD, H. V; RIYAZ, M.; VENKATESH KUMAR, R.; DHARMAPPA, K.K.;

TARANNUM, S.; SIDDESHA, J.M.; RAJESH, R.; VISHWANATH, B.S., 2009. **Cysteine proteases from the Asclepiadaceae plants latex exhibited thrombin and plasmin like activities**. [*s.l.*]: J. Thromb. Thrombolysis 28, 304–8. https://doi.org/10.1007/s11239-008-0290-2

SILVA, L.G.; GARCIA, O.; LOPES, M.T.; SALAS, C.E., 1997. **Changes in protein profile during coagulation of latex from Carica papaya.** [*s.l.*]: Braz. J. Med. Biol. Res. 30, 615–9.

SIMS, A.H.; DUNN-COLEMAN, N.S.; ROBSON, G.D.; OLIVER, S.G., 2004. **Glutamic protease distribution is limited to filamentous fungi**. [*s.l.*]: FEMS Microbiol. Lett. 239, 95–101. https://doi.org/10.1016/j.femsle.2004.08.023

SKERN, T.; & SKERN, T. (2018). **Investigating Proteins with β-Sheets.** [*s.l.*]:*Exploring Protein Structure: Principles and Practice*, 197-226.

SOUZA, D.P.; FREITAS, C.D.T.; PEREIRA, D. A; NOGUEIRA, F.C.; SILVA, F.D. A; SALAS, C.E.; RAMOS, M. V, 2011. **Laticifer proteins play a defensive role against hemibiotrophic and necrotrophic phytopathogens.** [*s.l.*]:Planta 234, 183–93. https://doi.org/10.1007/s00425-011-1392-1

STRASSER R. **Plant protein glycosylation.** Glycobiology. 2016 Sep;26(9):926-939. doi: 10.1093/glycob/cww023. Epub 2016 Feb 23. PMID: 26911286; PMCID: PMC5045529.

SWIEZEWSKA, E.; DANIKIEWICZ, W. 2005. **Polyisoprenoids: structure, biosynthesis and function.** [*s.l.*]:Prog. Lipid Res. 44, 235–58. https://doi.org/10.1016/j.plipres.2005.05.002

TAMURA K; STECHER G; KUMAR S. MEGA11: **Molecular Evolutionary Genetics Analysis Version 11.** [*s.l.*]:Mol Biol Evol. 2021 Jun 25;38(7):3022-3027. doi: 10.1093/molbev/msab120. PMID: 33892491; PMCID: PMC8233496.

THOMPSON, E.W.; LANE, B.G., 1980. **Relation of protein synthesis in imbibing wheat embryos to the cell-free translational capacities of bulk mRNA from dry and imbibing embryos**. [*s.l.*]:J. Biol. Chem. 255, 5965–70.

THOMPSON JD; HIGGINS DG; GIBSON TJ. **CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.** [*s.l.*]:Nucleic Acids Res. 1994 Nov 11;22(22):4673-80. doi: 10.1093/nar/22.22.4673. PMID: 7984417; PMCID: PMC308517.

THOMSON, A.B.; KEELAN, M.; THIESEN, A.; CLANDININ, M.T.; ROPELESKI, M.; WILD, G.E., 2001. **Small bowel review: normal physiology part 1**. [*s.l.*]:Dig. Dis. Sci. 46, 2567–87.

TIAN, D.M.; CHENG, H.Y.; JIANG, M.M.; SHEN, W.Z.; TANG, J.S.; YAO, X.S.; 2016. **Cardiac Glycosides from the Seeds of Thevetia peruviana.** [*s.l.*]: J. Nat. Prod. 79, 38–50. https://doi.org/10.1021/acs.jnatprod.5b00611

TROTT O; OLSON AJ. **AutoDock Vina: improving the speed and accuracy of docking with a**

new scoring function, efficient optimization, and multithreading. [*s.l.*]:J Comput Chem. 2010 Jan 30;31(2):455-61. doi: 10.1002/jcc.21334. PMID: 19499576; PMCID: PMC3041641.

UBERTO R; MOOMAW EW. **Protein similarity networks reveal relationships among sequence, structure, and function within the Cupin superfamily**. [*s.l.*]: PLoS One. 2013 Sep 6;8(9):e74477. doi: 10.1371/journal.pone.0074477. PMID: 24040257; PMCID: PMC3765361.

VIANA, C.A.; OLIVEIRA, J.S.; FREITAS, C.D.T.; ALENCAR, N.M.N.; CARVALHO, C.P.S.; NISHI, B.C.; RAMOS, M. V, 2013. **Thrombin and plasmin-like activities in the latices of Cryptostegia grandiflora and Plumeria rubra**. [*s.l.*]:Blood Coagul. Fibrinolysis 24, 386–92. https://doi.org/10.1097/MBC.0b013e32835d540b

VIEILLE, C.; ZEIKUS, G.J., 2001. **Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol.** [*s.l.*]:Mol. Biol. Rev. 65, 1–43. https://doi.org/10.1128/MMBR.65.1.1-43.2001

VIERSTRA, R.D., 1996. **Proteolysis in plants: mechanisms and functions.** [*s.l.*]:Plant Mol. Biol. 32, 275–302. https://doi.org/10.1007/BF00039386

VRIEND G. **WHAT IF: a molecular modeling and drug design program**. [*s.l.*]:J Mol Graph. 1990 Mar;8(1):52-6, 29. doi: 10.1016/0263-7855(90)80070-v. PMID: 2268628.

WILKINS MR; GASTEIGER E; BAIROCH A; SANCHEZ JC; WILLIAMS KL; APPEL RD; HOCHSTRASSER DF. **Protein identification and analysis tools in the ExPASy server**. [*s.l.*]: Methods Mol Biol. 1999;112:531-52. doi: 10.1385/1-59259-584-7:531. PMID: 10027275.

WOO, E.J.; DUNWELL, J.M.; GOODENOUGH, P.W.; PICKERSGILL, R.W. 1998. **Barley oxalate oxidase is a hexameric protein related to seed storage proteins: evidence from X-ray crystallography**. [*s.l.*]:FEBS Lett. 437, 87–90. https://doi.org/10.1016/S0014-5793(98)01203-4

WOO, E.J, DUNWELL, J.M., GOODENOUGH, P.W., MARVIER, A.C., PICKERSGILL, R.W., 2000. **Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities**. [*s.l.*]:Nat. Struct. Biol. 7.

WOO, EJ, MARSHALL J, BAULY J, CHEN JG, VENIS M, NAPIER RM, PICKERSGILL RW. **Crystal structure of auxin-binding protein 1 in complex with auxin**. [*s.l.*]: EMBO J. 2002 Jun 17;21(12):2877-85. doi: 10.1093/emboj/cdf291. PMID: 12065401; PMCID: PMC126050.

WHITMORE L; WALLACE BA. **Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases**. [*s.l.*]:Biopolymers. 2008 May;89(5):392-400. doi: 10.1002/bip.20853. PMID: 17896349.

ZARE H; MOOSAVI-MOVAHEDI AA; SALAMI M; SHEIBANI N; KHAJEH K; HABIBI-REZAEI M. **Autolysis control and structural changes of purified ficin from Iranian fig latex with synthetic inhibitors**. [*s.l.*]: Int J Biol Macromol. 2016 Mar;84:464-71. doi: 10.1016/j.ijbiomac.2015.12.009. Epub 2015 Dec 21. PMID: 26718871; PMCID: PMC5223272.

ZHU-SALZMAN, K.; LUTHE, D.S.; FELTON, G.W. 2008. **Arthropod-inducible proteins: broad spectrum defenses against multiple herbivores**. [*s.l.*]:Plant Physiol. 146, 852–8. https://doi.org/10.1104/pp.107.112177